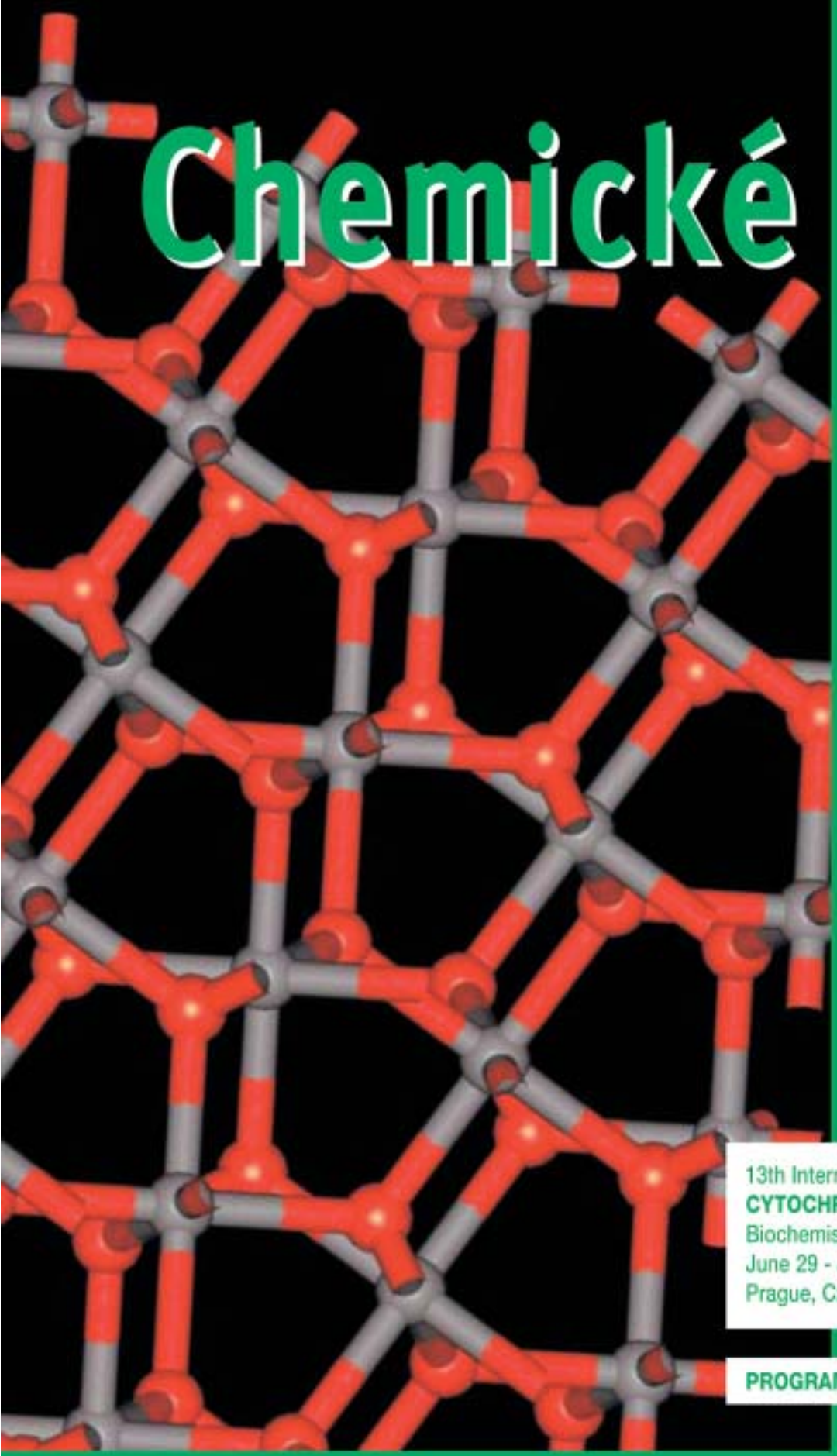

Chemické listy

S

P450 PRAGUE
29.6.–3.7.2003

13th International Conference on
CYTOCHROMES P450
Biochemistry, Biophysics and Drug Metabolism
June 29 - July 3, 2003
Prague, Czech Republic

PROGRAM/BOOK OF ABSTRACTS

Ročník 97
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combining innovation and experience

In the laboratories of Johnson & Johnson Pharmaceutical Research and Development (J&JPRD), a few thousands of researchers are continually searching for innovative molecules - molecules that can make the difference in a world where there is still a huge need for novel and affordable breakthrough drugs.

The largest site of Johnson & Johnson Pharmaceutical Research and Development is located in Beerse in the North of Belgium. The site was created on the 50 s by Dr. Paul Janssen, one of the most successful drug hunters of all time. Other major J&JPRD sites are located in the USA, viz. in Raritan, New Jersey, in Spring House, Pennsylvania, and in La Jolla, California. Besides, pharmaceutical R&D is also conducted by other Johnson & Johnson Pharma companies such as Centocor, Alza and Tibotec-Virco, in the USA as well as in Europe.

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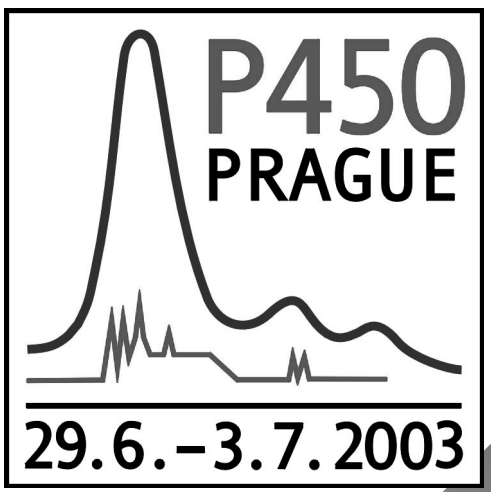
a career in science



13TH INTERNATIONAL CONFERENCE ON

CYTOCHROMES P450

Biochemistry, Biophysics and Drug Metabolism

Supported by the
European Commission, High-Level Scientific Conferences,
HPCF-CT-2002-00150
and by the
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PROGRAM / BOOK OF ABSTRACTS

June 29 - July 3, 2003

www.cyp2003.cz

P450 PRAGUE
29.6.–3.7.2003

Organized by	**Czech Chemical Society**
Co-organizers	**Palacký University, Olomouc**
	Czech Society for Experimental and Clinical Pharmacology and Toxicology
Under the auspices of	**Ministry of Education, Youths and Sports of Czech Republic**
	Ministry of Industry and Trade, Czech Republic

CONTENTS

WELCOME IN PRAGUE

Dear Colleagues:

We have a great pleasure to welcome you as participants of the 13th International Conference on Cytochromes P450 in Prague. The P450 community meets in this city for the second time - after fifteen years. In 1988, Prague hosted the International Congress of Biochemistry and Molecular Biology, where symposia dedicated to cytochromes P450 attracted many of you.

Since that time, many things have changed and happened. Our beloved P450 however remains to be one of the most interesting, intriguing and beautiful topics of contemporary biochemistry, biophysics and biochemical pharmacology. We see the rapid development of molecular biology, genomics and proteomics also in the field of P450. There is a growing interest in the medical community in the practical applications of P450 science - for example, the drug interactions based on P450-mediated metabolism can elucidate many unwanted drug effects. Traditionally, the core of our Conferences remains the same - fundamental questions of P450 biochemistry and biophysics.

We sincerely hope that you will find the scientific program interesting and that you will enjoy also the social events, accompanying the Conference. Prague is now an attractive city offering also a wide variety of pleasant trips and tourist attractions.

On behalf of the Organizing Committee,

Jiří Hudeček
Secretary of the Organizing Committee

Pavel Anzenbacher
Chairman of the Organizing Committee

INVITATION TO DALLAS

The next, 14th International Conference on Cytochromes P450, will be held in Dallas, Texas, USA, May 31 - June 4, 2005.
For more information, please visit www.p450dallas2005.us

COMMITTEES

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IMPORTANT CONTACTS

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The Organizing Committee is pleased to acknowledge the generous support of the following sponsors and exhibitors:

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SCIENTIFIC PROGRAM

LANGUAGE

The official Conference language will be English without simultaneous translation.

ORAL COMMUNICATIONS

Oral communications will consist of Invited Lectures, Forum of Young Scientists and Round Table Discussion on topics which deserve to be discussed in more detail. Overhead and data projection (IBM PC compatible, MS PowerPoint) will be available. The speakers are kindly requested to consult their presentations in the Preview Room at least 30 minutes before their presentation is scheduled, but preferably before the start of the respective session.

Round Table discussion is supposed to attract participants wishing to discuss their (innovative) opinion and views. The organizers welcome the proposals for a 10 min. talks. Please contact the Conference Secretary Dr. Jiří Hudeček.

PROGRAM OF THE CONFERENCE

Monday, June 30

8:00 - 8:15		**Conference Opening**
Hall A:	Plenary lectures:	**Structural Aspects (Chair: P. R. Ortiz de Montellano, J. R. Halpert)**
8:15 - 8:45	JR Halpert	**ML01** Structural basis of P450 2B specificity
8:45 - 9:15	E Scott	**ML02** A1.6 Å crystal structure of P450 2B4: novel features and implications
9:15 - 9:45	J Cosme	**ML03** Insights on the topology of the active site of human cytochrome 2C9 determined by X-ray crystallography
9:45 - 10:15	EF Johnson	**ML04** Structural studies of substrate binding to CYP2C drug metabolising P450s
10:15 - 10:30	**Coffee break**	
10:30 - 11:00	PR Ortiz de Montellano	**ML05** Radicals and stressed bacterial P450 systems
11:00 - 11:25	TL Poulos	**ML06** Structures of P450epoK, putidaredoxin, and putidaredoxin reductase
11:25 - 11:50	LL Wong	**ML07** Crystal structure - based engineering of substrate recognition in P450cam catalysis
11:50 - 12:15	I Schlichting	**ML08** Structural aspects of ligand binding to procaryotic and fungal P450
12:15 - 14:00	**LUNCH, POSTERS**	

Hall A:		**Nitric Oxide Synthase (Chair: B. S. S. Masters, D. Mansuy)**
14:00 - 14:30	BS Masters	**ML09** Revelations of nitric oxide synthase modulation: Hidden messages in sequence and structure
14:30 - 15:00	D Mansuy	**ML10** Formation of NO from xenobiotics
15:00 - 15:30	R Lange	**ML11** Tracking reactive intermediate states of NO synthase under extreme conditions of temperature and pressure
15:30 - 15:45	**Coffee break**	
15:45 - 16:15	I Sagami	**ML12** Regulation of electron transfer in neuronal NO synthase
16:15 - 16:45	CS Raman	**ML13** Structural and functional evidence for the evaluation of prokaryotic NO synthases from an ancestral cytochrome P450

Hall B:		**Bioinformatics (Chair: A. Archakov, J. A. Peterson)**
14:00 - 14:30	A Archakov	**ML14** Bioinformatic insight into unity and diversity of cytochromes P450
14:30 - 15:00	JA Peterson	**ML15** From sequence to structure and then function: How much of a leap is warranted?
15:00 - 15:30	AS Ivanov	**ML16** General trends in 3D modeling of cytochromes P450
15:30 - 15:45	**Coffee break**	
15:45 - 16:15	R Wade	**ML17** Cytochrome P450 friends and foes: Complex relationships under computational analysis
16:15 - 16:45	S Kelly	**ML18** New insights into microbial CYP biodiversity
16:45 - 17:15	AV Lisitsa	**ML19** Balance sheet for cytochrome P450 knowledgebase: 2000 protein sequences, 10 000 chemical substances
20:00	**CONCERT**	

Tuesday, July 1

Hall A:	Plenary lectures:	**Enzymology and Mechanisms 1 (Chair: F. P. Guengerich, J. H. Dawson)**
8:30 - 9:00	FP Guengerich	**TL01** Rate-limiting steps in cytochrome P450 reactions
9:00 - 9:30	S Shaik	**TL02** The protein, the active species and the chameleon: Reactivity patterns of oxygen transfer by cytochrome P450
9:30 - 10:00	SG Sligar	**TL03** P450's peccant proton pathways: Periclesian control of active oxygen
10:00 - 10:15	**Coffee break**	

Hall A:		**Enzymology and Mechanisms 2 (Chair: F. P. Guengerich, J. H. Dawson)**
10:15 - 10:45	JH Dawson	**TL04** Mechanistic studies of cytochromes P450
10:45 - 11:15	I Morishima	**TL05** Molecular mechanism of P450cam catalyzed oxygenation reaction regulated by the association with putidaredoxin
11:15 - 11:45	WD Woggon	**TL06** Design and synthesis of a new cytochrome P450 mimics
11:45 - 12:15	A Chougnet	**TL07** Design and synthesis of new fluorescent probes for evaluation of CYP3A4 drug-drug interactions

Hall B:		**Regulation of P450 Expression (Chair: P. Maurel, Y. Fujii-Kuriyama)**
10:15 - 10:45	P Maurel	**TL08** Overview of CYP gene regulation within a tangle of regulatory networks
10:45 - 11:15	Y Fujii-Kuriyama	**TL09** Molecular mechanisms of transactivations of Ah receptor in target gene expression
11:15 - 11:45	JM Pascussi	**TL10** Physiopathological factors affecting PXR and CAR gene expression
11:45 - 12:15	A Anderson	**TL11** Induction of rat CYP2B genes by phenobarbital-like inducers: Something old and something new

12:15 - 14:15 LUNCH, POSTERS

16:00 STEAMBOAT PARTY

Wednesday, July 2

Hall A:	Plenary lectures:	**P450 System: Component Interactions 1 (Chair: R. Bernhardt, P. M. Champion)**
8:15 - 8:45	R Bernhardt	**WL01** Mitochondrial cytochrome P450 systems: New insights into structure and function
8:45 - 9:15	PM Champion	**WL02** Resonance Raman and femtosecond coherence spectroscopy of P450: Interactions with reductase, oxygen and nitric oxide
9:15 - 9:45	WL Backes	**WL03** Interactions among reductase and multiple P450 enzymes in mixed reconstituted systems

9:45 - 10:00 Coffee break

Hall A:		**Applications 1 (Chair: P. Anzenbacher, A.Guillouzo)**
10:00 - 10:30	P Anzenbacher	**WL04** Experimental in vitro models of drug metabolism
10:30 - 11:00	U Fuhr	**WL05** Should we personalize the drug therapy according to cytochrome P450 genotype and/or phenotype?
11:00 - 11:30	O. Pelkonen	**WL06** In vitro to In vivo correlations - a critical assessment of various approaches to measure drug metabolism and metabolic interactions
11.30 - 12:00	DJ Waxman	**WL07** P450, anti-cancer drugs and gene therapies

Hall B:		P450: In Situ Function 1 (Chair: M. Waterman, R. Tyndale)
10:00 - 10:30	MR Waterman	**WL08** CYP51: Structure, function, evolution
10:30 - 11:00	D Rozman	**WL09** Lanosterol 14alpha- demethylase CYP51, cholesterol and reproduction
11:00 - 11:30	FJ Gonzalez	**WL10** P450 humanized mice: Novel tools for the study of drug and carcinogen metabolism and the search for endogenous human P450 substrates and functions
11:30 - 12:00	F Mitani	**WL11** Mechanism of the formation and maintenance of the functional zonation in the adrenal cortex
12:00 - 14:00	**LUNCH, POSTERS**	
Hall A:		**Applications 2 (Chair: P. Anzenbacher, A. Guillouzo)**
14:00 - 14:30	M Stiborová	**WL11** Utilization of cytochromes P450 to explain of a new mode of action of anti-cancer drug ellipticine
14:30 - 15:00	A Guillouzo	**WL12** Dual effects of the chemoprotective agent oltipraz on cytochromes P450
15:00 - 15:30	F Oesch	**WL13** Trans-species regenerating or stem cell derived hepatocyte-like cells: A perspective to solve the availability problem for human hepatocytes?
15:30 - 15:45	**Coffee break**	
15:45 - 16:15	S Coecke	**WL14** Metabolism: a bottle-neck for in vitro regulatory toxicity tests and testing strategies
16:15 - 16:45	CJ Henderson	**WL15** Applications of transgenic models in drug metabolism
Hall B: P450:		**P450: In Situ Function 2 (Chair: M. Waterman, R. Tyndale)**
14:00 - 14:30	HW Strobel	**WL16** Cytochrome P450 4F5: Response and role following brain trauma
14:30 - 15:00	RF Tyndale	**WL17** Commonly used drugs regulate CYP enzymes within the brain
15:00 - 15:15	**Coffee break**	
15:15 - 15:45	JH Capdevila	**WL18** The arachidonic acid monooxygenases: P450-eikosanoids as endogenous regulators of organ and body physiology
15:45 - 16:15	NG Avadhani	**WL19** Chimeric signals of xenobiotic-inducible CYPs: Mechanism of mitochondrial targeting and functional implications
19:00	**FAREWELL DINNER**	

Thursday, July 3

Hall A:		**P450 system: component interactions 2 (Chair: R. Bernhardt, P. M. Champion)**
8:30 - 9:00	DC Lamb	**CL02** Regulation and interaction of redox pertners with actinomycete CYPs
9:00 - 9:30	AW Munro	**CL03** Mechanisms and regulation of electron transfer in cytochrome P450 redox systems
10:00 - 10:15	**Coffee break**	
Hall B:		**P450 Genomics/Proteomics (Chair: M. Ingelman-Sundberg, U. M. Zanger)**
8:30 - 9:00	M Ingelman -Sundberg	**CL04** Mechanisms and consequences of interindividual variation in cytochrome P450 functional genomics
9:00 - 9:30	UM Zanger	**CL05** Role of genetic polymorphism, drug exposure and sex for expression and function of hepatic cytochromes P450
9:30 - 10:00	H Ohkawa	**CL06** Environmental genomics on plant P450 species metabolizing herbicides
10:00 - 10:15	**Coffee break**	
10:15- 12.30	**Forum of Young Scientists** – Moderators: J Wojcikowski, L. Antonović	
Hall A:	**Round Table Discussion** – Moderators: R. Lange, P. Hodek	
10:15 - 10:40	S Narashimhulu	**CL07** Differential behavior of the two subsites of P450 active site in binding of substrate and product
10:40 - 12:30	Speakers will be announced	
Hall A :	**Summary, closing**	
12:45 - 13:15	Summary	
13:15 - 13:30	**Closing Ceremony**	

The Organizing Committee gratefully acknowledges the sponsoring of following lectures:
TL08 (JOHNSON & JOHNSON PHARMACEUTICAL RESEARCH & DEVELOPMENT)
WL05 (ABBOTT Laboratories, s. r. o.),
WL06 (IN VITRO TECHNOLOGIES, Inc.),
ML05 (WYETH WHITEHALL CZECH, s.r.o.)

FORUM OF YOUNG SCIENTISTS

Oral communications of:
W. E. Straub, A. R. Dunn, C. de Graaf, Z. Guan, O. Roitel, P. Meinhold, T. S. Wong, M. Fink, L. M. Podust, L. B. von Weymarn, M. Duisken, A. L. Sukhodub, K. J. McLean, S. Pronko, N. Pons.

In addition to the authors listed above, the following EU Grant recipients are expected to present a short outline of their results:
A. Bonifacio, S. Catania, E. O'Donnell, J. Hodis, M. Tesařová, D. Herman, M. Štěrba, O. Georgescu.

POSTERS

POSTER PRESENTATIONS
Each day, space and time will be assigned to poster presentations. **Posters are located at the balcony and in the corridors (follow the arrow signs).** The authors are kindly requested to be available at their poster presentation for questions and discussion for at least 30 minutes on the announced day **between 12.15 and 14.00**. Pins will be provided, poster size 2 m (height) x 1 m. The authors are requested to set up their posters in the morning (starting from 8.00 on the announced day) and remove them after the end of the session (before 17.00).

POSTER SCHEDULE

Monday, June 30

MP01
Axial ligation for enhancing selectivity in catalytic epoxidation by cytochrome P450 models
C. K. Chang, T. S. Lai, and L-L Yeung

MP02
Functional analysis of CYP2D6.31 - Arg440His substitution diminishes enzyme activity by disrupting redox partner interaction
D. Allorge, D. Breant, J. Harlow, J. Chowdry, J.-M. Lo-Guidice, D. Chevalier, C. Cauffiez, M. Lhermitte, F. E. Blaney, G. T. Tucker, F. Broly, and S. W. Ellis

MP03
Covalent heme binding to CYP4B1 via Glu310 and a carbocation porphyrin intermediate
B. R. Baer, Y.-M. Zheng, M. J. Cheesman, N. Moguilevsky, K. L. Kunze, and A. E. Rettie

MP04
α-Naphthoflavone as a model ligand of the active site of CYP3A
L. Bořek-Dohalská, P. Hodek, and M. Stiborová

MP05
Structure-function relationship in cytochrome P450 CYP 2C11
C. Celier, C. Biagini, R. C. Maroun, and R. Philpot

MP06
Camphor hydroxylation by cytochrome P450cam: A theoretical study by hybrid Quantum Mechanical/Molecular Mechanical (QM/MM) method
S. Cohen, S. Shaik, J. C. Schöneboom, H. Lin, and W. Thiel

MP07
Cytochrome P450 3A4 allosteric mechanism studied by high-pressure spectroscopy
D. R. Davydov, and J. R. Halpert

MP08
A charge-pairing bundle around cys-154 is pivotal for the allosteric mechanism in cytochrome P450eryF
D. R. Davydov, A. Bochkareva, S. Kumar, and J. R. Halpert.

MP09
Molecular modeling of alkoxyresorufin oxidation by P450 1A1, 1A2 and their mutants
S. S. Ericksen, and G. D. Szklarz

MP10
Can P450 BM3 turn over substrates typically metabolised by class II human P450s? Evidence on the functional relationship of P450 BM3 with the human enzymes
A. Fantuzzi, and G. Gilardi

MP11
The role of Phe120 in CYP2D6 substrate binding
J. U. Flanagan, C. Kemp, M. Sutcliffe, M. J. I. Paine, G. C. K. Roberts, and C. R. Wolf

MP12
Resonance Raman spectra reveal subtle differences in heme structure of P450 enzymes
J. Hudeček, P. Anzenbacher, T. Shimizu, A.W. Munro, and T. Kitagawa

MP13
Fluorescence correlation spectroscopy study of interaction between CYP 3A4 and coumarin 6
M. Beneš, J. Pekárek, M. Hof, P. Anzenbacher, J. Hudeček

MP14
Probing the alterations in the active site of P420cam: high pressure studies on the P450cam-ligands and -inhibitors complexes
G. Hui Bon Hoa

MP15
CYP enzymes: moving towards virtual screening
N. Jourdan, and A. Mancy

MP16
Heme pocket compressibility in P450cam-CO: high pressure FTIR studies on the CO ligand stretch vibration
C. Jung, B. Canny, J. C. Chervin, and G. Hui Bon Hoa

MP17
Intermediate radical formation by the iron-oxo

complex produced in the reaction of cytochrome P450cam with oxidants
C. Jung , V. Schünemann, F. Lendzian, J. Contzen, A.-L. Barra, and A. X. Trautwein

MP18
Microliter scale surface enhanced resonance Raman scattering on cytochrome P450 BM3
P. H. J. Keizers, A. Bonifacio, J. N. M. Commandeur, G. van der Zwan, C. Gooijer, S. M. van der Vies, and N. P. E. Vermeulen

MP19
Membrane topology of P45017α studied by chemical modifications and mass-spectrometry
H. Kaneko, S. Izumi, T. Yamazaki, T. Hirata, and S. Kominami

MP20
The AFM study of complex formation within the cytochrome P450scc-containing system
V. Kuznetsov, Y. Ivanov, S. Usanov, and A. Archakov

MP21
The role of ferric-peroxo reactivity at the crossroads of P450 catalysis
T. M. Makris, I. G. Denisov, I. Schlichting, and S. G. Sligar

MP22
CYP2D6: role of Glu-216 and Asp-301 in quinidine binding
L.A. McLaughlin, M. J. Sutcliffe, M. J. I. Paine, G. C. K. Roberts, and C. R. Wolf

MP23
Crystallographic studies of perdeuterated P450cam
F. Meilleur, M.-T. Dauvergne, M. Haertlein, I. Schlichting, and D. Myles

MP24
Crystal structures of epothilone-D bound, epothilone-B bound, and substrate-free forms of cytochrome P450epoK
S. Nagano, H. Li, H. Shimizu, C. Nishida, H. Ogura, P. R. Ortiz de Montellano, and T. L. Poulos

MP25
Identification of the substrate-contact residues in cytochrome P450 27A1
D. Murtazina, S. Graham, J. A. Peterson, I. Bjorkhem, and I. Pikuleva

MP26
Metabolism prediction for cytochrome P450
I. Zamora, M. Ridderström, R. Vianello, G. Cruciani, and T. B. Andersson

MP27
Alteration of CYP 4A4 regiospecificity by site-directed mutagenesis of residues in the substrate binding channel
L. J. Roman, M. de la Garza, M. Mock, D. Harris, and B. S. Masters

MP28
Crystal structure of putidaredoxin
I. Sevrioukova, C. Garcia, H. Li, B. Bhaskar, and T. L. Poulos

MP29
Thiols prevent P420 formation and aggregation in 25-hydroxyvitamin D3 24-hydroxylase
M. J. Theisen, A. Annalora, S. A. Beretta, A. Pastuszyn, E. D. Matayoshi, J. L. Omdahl, M. L. Chiu

MP30
The selective inhibition of cytochrome P450 1 enzymes by the derivatives of rutaecarpine
Y.-F. Ueng, M.-J. Don, D. F. V. Lewis, S.-Y. Wang, and M.-W. Tsai

MP31
The protein stabilization of coumarin 7-hydroxylase by its own inhibitors
P. Viitala, O. Pelkonen, and R. Juvonen

MP32
Validation of a 3D-rebuilt P450 3A4 structure with cyclopeptide metabolism: implementation of new techniques involving soft-restrained dynamics docking simulations gives new insights on the multiple substrate specificity
F. André, N. Loiseau, M. Delaforge

MP33
Substrate dynamics in cytochrome P450-BM3 - dynamics of substrate binding by automated docking and molecular dynamics simulation
K. A. Feenstra, C. de Graaf, J. Starikow, J. N. M. Commandeur, and N. P. E. Vermeulen

MP34
A QM/MM study of cytochrome P450: the influence of the enzyme environment on specific steps in the catalytic cycle
A. R. Groenhof, M. Swart, A. W. Ehlers, and K. Lammertsma

MP35
Aspects on evolution of the CYP51 family
T. Režen, N. Debeljak, D. Kordiš, and D. Rozman

MP36
Identification of novel human cytochrome P450s, CYP4Z1 and the transcribed pseudogene CYP4Z2P
M. Rieger, R. Ebner, A. Kiessling, J. Rohayem, M. Schmitz, A. Temme, E. P. Rieber, and B. Weigle

MP37
Characterization of Arabidopsis thaliana CYP711A1
M. Shimoji, F. Durst, I. Benveniste, and R. Morgenstern

MP38
Inducible cytochrome P 450 (CYP2E1) by combined administration of acetaminophen and caffeine
V. Kovalenko, and A. Voronina

MP39
Molecular modeling and site-directed mutageneses defining catalytic sites in insect P450s metabolizing allelochemicals and insecticides
J. Baudry, X. Li, Z. Wen, L. Pan, M. R. Berenbaum, and M. A. Schuler

MP40
Transcript profiling of Arabidopsis thaliana P450s
S. Ali, H. Duan, Y. Ferhatoglu, M. Band, D. Werck-Reichhart, and M. A. Schuler

MP41
P450-dependent alkane monooxygenase of Acinetobacter sp. EB104 is encoded in a composite transposon of the plasmid pAC450
M. Ludewig, C. Schwarz, O. Asperger, S. Pääbo, and U. Hahn

MP42
CYP2C19 do not contribute to blood pressure
C. Bertrand-Thiebault, B. Marie, S. Droech, L. Ferrari, A. Thompson, D. Froenzler, S. Visvikis, and A. M. Batt

MP43
Comparison of NADPH-, NAD^+- and UDP-glycoside-dependent biotransformation of bohemine and roscovitine in vitro
K. Červenková, Z. Chmela, M. Belejová, L. Uherková, M. Rypka, and D. Riegrová

MP44
Biotransformation of olomoucine-type cyclin-dependent kinase inhibitors by precission-cut tissue slices from different animal species
K. Červenková, Z. Chmela, and M. Rypka

MP45
Significant role of CYP2A and CYP3A in biotransformation of bohemine by mouse liver microsomes in vitro
M. Rypka, Z. Chmela, K. Červenková, K. Lemr, and D. Riegrová

MP46
Carboxylic acid is the main product of roscovitine biotranformation in mice in vivo. Comparison with bohemine
Z. Chmela, M. Rypka, K. Červenková, K. Lemr, D. Riegrová, and J. Veselý

MP47
CYP2D is involved in the neurosteroidogenesis in the brain
W. Kishimoto, T. Hiroi, M. Osada, S. Imaoka, T. Igarashi, and Y. Funae

MP48
Analysis of cytochrome P450s in a pyrethroid resistant strain of Helicoverpa armigera (cotton bollworm)
V. Grubor, and D. G. Heckel

MP49
Influence of recipient gender on cytochrome P450 isoforms expression and activity in intrasplenic fetal liver tissue transplants in comparison to liver in rats
A. Lupp, S. Hugenschmidt, and D. Müller

MP50
High-throughput LC-MS/MS method for CYP2D6 phenotyping with dextromethorphan

T. E. Mürdter, G. Schänzle, G. Heinkele, K. Endrizzi, C. Marx , M. Schwab, M. Eichelbaum, and U. Zanger

MP51
Influence of methamphetamine on activity of CYP 2D1 in the isolated perfused Wistar albino rat livers
J. Slíva

MP52
Expression of aryl hydrocarbon receptor repressor in normal human tissues and inducibility by polycyclic aromatic hydrocarbons in human tumor-derived cell lines
Y. Tsuchiya, M. Nakajima, S. Itoh, M. Iwanari, and T. Yokoi

MP53
Regulation of mutation process induced by ionizing radiation in myelocariocytes of mice by peroxidase and extract from Armoracia rusticana L.
R. Agabeyli, T. Gasimova, and U. Alekperov

MP54
Mouse cytochromes P450 4F
L. Antonovic, X. Cui, H. Kawashima, and H. W. Strobel

MP55
TCDD induces CYP2A5 enzyme in mouse primary hepatocytes
S. Arpiainen, O. Pelkonen, and J. Hakkola

MP56
CYP2C8, CYP2C9, CYP3A7, PXR and CAR mRNA level increase in hepatoblastoma cell line HepG2, after HMG-CoA reductase inhibitors treatment
C. Bertrand-Thiebault, L. Ferrari, C. Masson, S. Visvikis, and A. M. Batt

MP57
Characterization of cytochrome P450 expression and induction profiles in the WIF-B9 cell line
F. Borde, V. E. Bender, S. Chevalier, C. Charuel, D. Cassio, and C. P. Biagini

MP58
The decrease of rat liver microsomal CYP3A2 catalytic activity during aging may be the consequence of an enhanced protein instability
V. Wauthier, R. K. Verbeeck, and P. B. Calderon

MP59
Cocaine, but not metamphetamine causes a concomittant decrease of CYP2C and constitutive androstane receptor (CAR) expressions in human astrocytoma cell line U373 MG
C. Malaplate-Armand, L. Ferrari, C. Masson, G. Siest, H. Lambert, S. Visvikis, and A.M. Batt

MP60
Functional characterization of a CYP3A7 variant with a longer C-terminal
C. Rodríguez-Antona, M. Hidestrand, A. Rane, and M. Ingelman-Sundberg

MP61
Effects of weaning to diets containing rice protein isolate (RPI) on growth, plasma IGF1 and expression of CYP2C11 and CYP4A1 in rat liver
M. J. J. Ronis, M. Reeves, H. Hardy, J. Badeaux, C. Dahl, D. Harisson, R. Haley, L. Humphrey, M. Ferguson, and T. M. Badger

MP62
Effects of weaning to diets containing soy protein isolate (SPI) or isoflavones on growth, plasma IGF1 and expression of CYP2C11 and CYP4A1 in rat liver
M. J. J. Ronis, M. Reeves, H. Hardy, J. Badeaux, C. Dahl, D. Harisson, R. Haley, L. Humphrey, M. Ferguson, and T. M. Badger

MP63
Human cryopreserved hepatocytes as an in vitro model to study cytochrome P450 induction
D. Roymans, P. Annaert, J. Van Houdt, A. Weygers, D. Jacobs, J. Hendrickx, G. Mannens, and W. Meuldermans

MP64
CYP1A regulation by α-tocopherol
Y. A. Sidorova, and A. Y. Grishanova

MP65
The role of cytochromes P450 in biotransformation of new antileukotrienic drug quinlukast in rat
B. Szotáková, L. Skálová, L. Šišpera, and V. Wsól

MP66
Effect of repeated albendazole administration to mouflons on albendazole metabolism in vivo and in vitro

J. Velík, L. Skálová, V. Baliharová, B. Szotáková, V. Wsól, and J. Lamka

MP67
Age related changes in the protein and mRNA levels of CYP2E1, CYP3A1, CYP3A2 as well as in their hepatic activities in Wistar rats
V. Wauthier, N. Maton, V. Allaeys, R. K. Verbeeck; and P. B. Calderon

MP68
Administration of CYP19 modulators to Gallus domesticus
T. Koblas, P. Trefil, M. Stiborová, and P. Hodek

Tuesday, July 1

TP01
Non-dissociative sequential metabolism of an 8-alkyl xanthine by human P450 1A2
Jason Boer, Kyle E. Allen, and Kent L. K. Kunze

TP02
Caffeine oxidation during prolonged exposure of rats to phenothiazine neuroleptics
W. A. Daniel, M. Kot, and J. Wójcikowski

TP03
Activity of NADPH-cytochrome P-450 reductase in the human heart, liver and lungs in the presence of padma 28 - in vitro study
J. Dudka, M. Murias, F. Burdan, J. Szumiłło, R. Klepacz , and J. Jodynis-Liebert

TP04
Ethanol influence on methanol oxidation via ADH and MEOS
K. Dawidek-Pietryka, J. Dudka, and K. Domańska

TP05
Activation of NADPH-cytochrome P-450 reductase in the human heart, liver and lungs by Ukrain - in vitro study
J. Dudka, M. Murias, F. Burdan, J. Szumiłło, R. Klepacz , and J. Jodynis-Liebert

TP06
The interaction between the anticancer drug ellipticine and cytochromes P450 dictates its pharmacological efficiencies
M. Stiborová, J. Sejbal, D. Aimová, T. Dlouhá, L. Bořek-Dohalská, K. Forsterová, J. Poljaková, M. Zachová, M. Stiborová-Rupertová, K. Kukačková, A. Březinová, J. Kučka, and E. Frei

TP07
Non-aromatic hydroxylation of bupivacaine in man
A. Kumar, B. Hofte, R. Ledger, and U. Tjaden

TP08
Inhibition of P450 enzymes participating in p-nitrophenol hydroxylation by drugs known as CYP2E1 inhibitors
K. Monostory, E. Hazai, and L. Vereczkey

TP09
CYP19 activity modulation: small change of flavonoid structure results in inhibitor-stimulator transition
D. Provazníková, P. Hodek, S. Smrček, B. Ištvánková, and M. Stiborová

TP10
Potent mechanism-based inhibition of human CYP2B6 by clopidogrel and ticlopidine
T. Richter, K. Klein, T. E. Mürdter, M. Schwab, M. Eichelbaum, and U. M. Zanger

TP11
CYP17 catalytic iron-oxygen species dictated by cytochrome b_5
P. Lee-Robichaud, Q. Sheikh, and M. Akhtar

TP12
Faradaic bioelectorchemistry for the drug metabolism assay
V. V. Shumyantseva, T. V. Bulko, and A. I. Archakov

TP13
Structural and functional characterizations of putidaredoxin-induced structural changes in cytochrome P450cam
T. Tosha, S. Nagano, J. K. Yano, S. Yoshioka, H. Harada, S. Takahashi, K. Ishimori, T. L. Poulos, and I. Morishima

TP14
Chiral aspects of reduction metabolism of flobufen in guinea pigs in vivo

F. Trejtnar, R. Král, V. Wsól, P. Pávek, and E.Kvasničková

TP15
Multiple P450 substrates in single run: rapid, comprehensive and clinically relevant in vitro metabolism test
M. Turpeinen, J. Uusitalo, J. Jalonen, and O. Pelkonen

TP16
Immobilisation of P450 BM-3 and an $NADP^+$ cofactor recycling system: towards a technical application
S. C. Maurer, R. D. Schmid, and V. Urlacher

TP17
Heterologous expression of full-length NADH-cytochrome b_5 reductase and its fusion protein with cytochrome b_5
A. A. Gilep, V. V. Kokhanovski, and S. A. Usanov

TP18
Overexpression of full-length outer mitochondrial cytochrome b5 in E. coli
A. A. Gilep, G. V. Sergeev, A. Ito, and S. A. Usanov

TP19
Engineering of proteolytically stable NADPH-cytochrome P450 reductase
A. A. Gilep, T. A. Bonina, R. W. Estabrook, and S. A. Usanov

TP20
C-terminal residues of cytochrome P450scc are required for correct folding, heme binding and catalysis
G. I. Lepesheva, N. V. Strushkevich, T. N. Azeva, and S. A. Usanov

TP21
Spectroscopic studies of cytochrome P4503A4 interaction with flavonoids
M. V. Chudaev, E. M. Tcherviakovsky, V. P. Kurchenko, and S. A. Usanov

TP22
Human cytochrome P450 2D6-catalyzed reactions: The kinetic interaction between two substrates
R. W. Wang, X. Cai, V. Didolkar, R. W. Edom, R. Evers, and D. C. Evans

TP23
Reduction of oxyferrous cytochrome P450 2B4 by cytochrome P450 reductase
H. Zhang, L. Gruenke, D. Arscott, A. Shen, C. Kasper, and L. Waskell

TP24
Human hepatic oxidation of dothiepin mediated by CYP2D6
H. Yin, P. Tran, and V. Fischer

TP25
Selective dehydrogenated intermediates are mechanism-based inactivators of CYP3A4, CYP2E1, AND CYP2F1
K. W. Skordos, S. J. Smeal, C. A. Reilly, D. L. Lanza, and G. S. Yost

TP26
Human and minipig cytochrome P450 2E1
E. Anzenbacherová, J. Baranová, R. Zuber, P. Anzenbacher, and P. Souček

TP27
Two novel human P450 enzymes, CYP4Z1 and CYP4X1
N. J. Horley, T. Jiang, and D. R. Bell

TP28
Polymorphism of P450 in Drosophila melanogaster
A. Brun-Barale, D. Crochard, S. Tares, L. Arthaud, J.-M. Bride, and M. Amichot

TP29
Bacterial monooxygenases in biotransformation and biodegradation of aliphatic and aromatic compounds
V. Leont'ev, T. Achramovitch, I. Burak, and O. Ignatovets

TP30
Rabbit as an animal model to study CYP3A-mediated drug-drug interactions
N. Chauret, L. Sleno, J. Silva, R. Houle, S. Day, and D. Nicoll-Griffith

TP31
CYP2D6 polymorphism and extrapyramidal symptoms in patients on long term antipsychotic treatment

V. Dolžan, B. Kores-Plesničar, B. Zalar, and K. Breskvar

TP32
CYP2U1, a thymus and cerebellum expressed cytochrome P450 converts arachidonic acid into bioactive derivatives 19- and 20-HETE
C. Helvig, S. Chuang, M. Taimi, G. Jones, M. Petkovich, J. A. White, and B. Korczak

TP33
A novel CYP26 family member metabolizes 9-cis-retinoic acid
M. Taimi, C. Helvig, M. Petkovich, and B. Korczak

TP34
cDNA cloning and biochemical characterization of CYP4F11, a novel human cytochrome P450
C. Helvig, and B. Korczak

TP35
CYP2C9 polymorphism and warfarin dose requirement
D. Herman, T. Globokar, P. Peternel, M. Stegnar, K. Breskvar,V. Dolžan

TP36
A streptomyces CYP105D1 exported in Escherichia coli induces periplasmic accumulation of porphyrin
M. K. Akhtar, N. N. Kaderbhai, and M. A. Kaderbhai

TP37
Novel SNP's in the CYP2B6 gene and their functional consequences
K. Klein, T. Lang, T. Richter, M. Eichelbaum, M. Schwab, and U. M. Zanger

TP38
Comparative analysis of CYP3A expression in human liver suggests only a minor role for CYP3A5 in drug metabolism
S. Malmebo, A. Westlind-Johnsson, A. Johansson, C. Otter, T. B. Andersson, I. Johansson, R. J. Edwards, A. R. Boobis, and M. Ingelman-Sundberg

TP39
Searching for the gene encoding steroid 11β-hydroxylase of the filamentous fungus Cochliobolus lunatus
E. Kuščer, L. Lah, and R. Komel

TP40
Transcript analysis and azole inhibition of mycobacterial CYPs
J. Parker, C. Jackson, T. Marczylo, S. Perally, D. Lamb, S. Kelly, and D. Kelly

TP41
CYP 2D6, intermediate metabolizer' phenotype as a consequence of genetically determined alternative splicing
C. Cesaro, S. Raimundo, K. Klein, E. Schaeffeler, M. Eichelbaum, M. Schwab, and U. M. Zanger

TP42
Identification of CYP2D6 genotype via a new sequencing protocol
J. K. Coller, H. M. James, D. Gillis, B. C. Sallustio, and A. A. Somogyi

TP43
Use of 2D-electrophoresis and MALDI-TOF in the identification of hepatic CYP1A isoforms of Prochilodus scrofa, a Brazilian freshwater fish
M.E.F. Da Silva, J. A. Silva, D. Martins, S. Marangoni, J.C. Novello, and N. C. Meirelles

TP44
NF-I regulates CYP2A5 transcription in mouse primary hepatocytes
J. Ulvila, O. Pelkonen, M. Negishi, and J. Hakkola

TP45
CYP2C9 phenotyping using low-dose tolbutamide
A. Jetter, M. Kinzig-Schippers, A. Skott, A. Lazar, D. Tomalik-Scharte, M. Walchner-Bonjean, U. Hering, H. Lück, W. Jabrane, D. Kasel, U. Fuhr, and F. Sörgel

TP46
A novel thymus-specific cytochrome P450, CYP2U1
M. Karlgren, and M. Ingelman-Sundberg

TP47
Rapid translocation of CYP11A1 into yeast mitochondria hampers its normal sorting and folding
I. E. Kovaleva, L. A. Novikova, P. A. Nazarov, S. I. Grivennikov, and V. N. Luzikov

TP48
Synthesis and properties of fused proteins compo-

sed of components of the cholesterol side-chain cleavage enzyme system
P. A. Nazarov, V. L. Drutsa, W. L. Miller, V. M. Shkumatov, V. N. Luzikov, and L. A. Novikova

TP49
P450 allele and haplotype frequency study in different populations using the CODELINK™ SNP assay
R. Bonner, B. Chui, M. Gaskin, P. Gwynne, A. Ledesma, T. Peck, T. Peters, A. Silbergleit, M. Amjadi, R. Feldman, and C.Yamashiro

TP50
Association of genetic polymorphisms in cytochrome P450 2E1 and other biotransformation enzymes with progression of lymphomas
P. Souček, J. Šarmanová, S. Šůsová, and I. Gut

TP51
Genome-wide expression profiling and xenobiotic inducibility of P450 monooxygenase genes in the white rot fungus Phanerochaete chrysosporium
J. S. Yadav, and H. Doddapaneni

TP52
Haplotype analysis of cytochrome P450 2B6 - linkage between promoter- and exonic mutations
J. Zukunft, T. Lang, K. Klein, T. Richter, M. Schwab, M. Eichelbaum, and U. M. Zanger

TP53
High level expression of porcine P450 reductase solubilized doman in Escherichia coli by modulating the local secondary structure of mRNA
Shigenobu Kimura, and Takashi Iyanagi

TP54
Interaction of constitutive androstane receptor-retinoic x receptor (CAR-RXR) heterodimers with elements of the CYP2B2 phenobarbital response unit
M.-J. Beaudet, A. Lachaud, and A. Anderson

TP55
The anticancer drug ellipticine acts as an inducer of cytochromes P450 1A1/2 and potentiates its own pharmacological efficiency
D. Aimová, T. Dlouhá, E. Frei, and M. Stiborová

TP56
Newly-identified transcription factor binding sites in the rat CYP2B2 phenobarbital response unit: HNF4 and Pbx-Prep1
M.-J. Beaudet, and A. Anderson

TP57
Rabbit CYP4B1 engineered for high-level expression in E. coli: ligand stabilization and processing of the N-terminus and heme prosthetic group
M. J. Cheesman, B. R. Baer, E. M. J. Gillam, and A. E. Rettie

TP58
Transcriptional regulation of the cholesterol biosynthetic pathway in the male gonad
K. Fon Tacer, D. Rozman

TP59
Constitutive androstane receptor-retinoic x receptor (CAR-RXR)-dependent transcriptional activation by the CYP2B2 phenobarbital response unit varies dramatically with different reporter constructs
M. Gravel, M. Desrochers, É. Trottier, and A. Anderson

TP60
Expression of mammalian microsomal P450s in plants using plant virus vectors
A. A. Jgoun, M. A. Eldarov, N. A. Petushkova, V. K Novikov, Y. L. Dorokhov, I. G. Atabekov, and K. G. Skryabin,

TP61
Expression of leukotriene B4 ω-hydroxylase in human leukocyte
H. Kunishi, Y. Masumoto, M. Eya, J. Ohta, S. Nishimoto, K. Nakata, and Y. Kikuta

TP62
NR3 and ER-7 nuclear receptor sites of the CYP2B2 phenobarbital response unit: mutational analysis of their importance for PB-dependent transcriptional activation
M. Laizier, Y. Paquet, A. Anderson

TP63
Characterization of cytochrome P-450 26 (CYP26) enzymatic activity and comparison with other CYPs involved in all-trans-retinoic acid (ATRA) metabolism
J. Marill , A. Picard-Cloutier, and G. G. Chabot

TP64
Distribution of glutathione S-transferases in mullet (Liza saliens)
A. Kirikbakan, and A. Sen

TP65
Biochemical, molecular genetic and environmental aspects of mullet (Liza saliens) CYP1A1
A. Sen, A. Kirikbakan, D. R. Buhler, and E. Arinc

TP66
Albendazole induced CYP1A activities in mouflons (Ovis musimon)
L. Skálová, V. Baliharová, J. Velík, B. Szotáková, V. Wsól, and J. Lamka

TP67
Cytochrome P450 3A4 expression is reduced in the presence of cancer-associated inflammation in a humanised CYP3A4 transgenic mouse model
K. Slaviero, A. Lehnert, S. Bierach, C. Liddle, S. Clarke, L. Rivory, G. Robertson

TP68
Activity of cytochromes P450 2C in rat cardiomyocytes
L. Kousalová, J. Baranová, J. Psotová, E. Anzenbacherová, and P. Anzenbacher

TP69
Metabolism of alpha-naphthoflavone and its thioanalogue by microsomal system
P. Valentová, P. Hodek, L. Bořek-Dohalská, M. Šulc, and M. Stiborová

TP70
Application of α-naphthoflavone during the growth and development of broilers
A. Mičáková, P. Hodek, M. Poplštein and P. Trefil

Wednesday, July 2

WP01
Molecular breeding of cytochrome P450 enzymes involved in xenobiotic metabolism
N. Rosic, T.G.A. Lonhienne, J.J. DeVoss, and E. M. J.Gillam

WP02
Aflatoxin B1 binding to mutant Phe209Ala CYP2A5 expressing Saccharomyces cerevisae yeast cells
P. Bartakova, H. El-Nezami, H. Raunio, and R. O. Juvonen

WP03
Engineering substrate recognition in catalysis by cytochrome P450cam
S. G. Bell, X. Chen, F. Xu, Z. Rao, and L.-L. Wong

WP04
Validation of commercially supplied cryopreserved hepatocytes from a range of species for use in metabolic profiling studies
C. Dilworth, R. Shannly-Henderson, V. Eagling, P. D. Fenn, and G. R. Murray

WP05
P450 2E1/BMR: studies of a human/bacterial P450 fusion protein
M. J. Fairhead, and G. Gilardi

WP06
O_2 metabolism by soluble cytochrome P450 reductase and its chimeras with C-termini of nitric oxide synthases assayed by diacetyldeuteroheme HRP
Y. Ishimura, S. P. Panda, M. Jáchymová, P. Martásek, T. Kitazume, J. A. Peterson, L. J. Roman, and B. S. S. Masters

WP07
An homogeneous assay for screening CYP2D6 inhibitors using scintillation proximity assay technology
E. Beynon, M. Price-Jones, J. Berry, and K. T. Hughes

WP08
Exploration of natural and artificial P450 sequence spaces
P. Urban, V. Abécassis, G. Truan, and D. Pompon

WP09
Cytochrome P4502E1 activity and free radical oxidation in the liver of hypothyroid rats with acute alcohol intoxication
N. Kaloshyna, T. Khomich, and P. Pronko

WP10
Assignment of omeprazole-sensitivity gene locus,

whose product mediates CYP1A1 induction, on human chromosome 10
H. Kikuchi, S. Fukushige, M. Shibazaki, A. Sohel, and T. Takeuchi

WP11
Study on organ specificity of CYP1A1 induction by 1-phenylazo-2-hydroxynaphthalene (Sudan I) in rats
V. Marková, V. Martínek, P. Hodek, and M. Stiborová

WP12
The use of bacterial membranes in the study of interactions of human cytochromes P450 and xenobiotics
E. Kondrová, and P. Souček

WP13
Azole antifungal drugs act upon cytochrome P450 mono-oxygenases to efficiently inhibit growth in Mycobacteria and Streptomycetes
K. R. Marshall, K. J. McLean, D. Leys, and A. W. Munro

WP14
Sudan I metabolism by human cytochrome P450 1A1 is stimulated by microsomal cytochrome b_5
V. Martínek, and M. Stiborová

WP15
P450 aromatase inhibition assay based on an ELISA system
Kazuhiro Matsui, Shigeaki Nishii, Takuya Ishibashi, and Masanori Oka

WP16
Cytochrome P450-mediated activation of carcinogenic 2-anisidine leads to formation of DNA adducts in vitro and in vivo
M. Mikšanová, L. Vondrášková, M. Šulc, H. H. Schmeiser, E. Frei, and M. Stiborová

WP17
Possible role of CYP450s in the nabumetone metabolism
M.Nobilis, E.Anzenbacherová, M.Holčapek, L. Kolářová, J. Květina, and P. Anzenbacher

WP18
Cytochrome P450 1A2 and N-acetyltransferase (NAT2) phenotypes as markers of cancer susceptibility by workers exposed to aromatic amines
J.. Pastera, P. Anzenbacher, Z. Fiala, R. Nádvorníková, J. Šalandová, and J. Květina

WP19
Method of preliminary selection of substances specific in relation to P450 isoforms
A. A. Pogrebnoy, V. E. Ryabinin, V. A. Potemkin, E. V. Bartashevich, and M. A. Grishina

WP20
Peroxidase and CYP-mediated ellipticine-DNA adduct formation explains the selective efficiency of this anticancer drug against breast cancer and leukemia
J. Poljaková, K. Forsterová, M. Šulc, E. Frei, and M. Stiborová

WP21
Distinct inhibition of human CYP2A6 and mouse CYP2A5 by derivatives of lactone, phenylethylamine and benzaldehyde
M. Rahnasto, H. Raunio, A. Poso, and R. O. Juvonen

WP22
Characterization of human P450 involved in oxidation of manidipine, a 1,4 dihydropyridine calcium channel blocker
B. Riccardi, P. Puccini, D. Spinabelli, and D. Acerbi

WP23
Human cytochromes P450 1A1/2 and NADPH: cytochrome P450 reductase activate carcinogenic aristolochic acid to form DNA adducts found in patients with Chinese herbs nephropathy
M. Stiborová, E. Frei, B. Sopko, K. Sopková, V. Marková, M. Laňková, T. Kumstýřová, M. Wiessler, and H. H. Schmeiser

WP24
Interaction of omeprazole and caffeine on cytochrome P450 1A2 evaluated using ^{13}C-caffeine breath test in rats
Z. Svoboda, J. Květina, J. Bureš, and V. Voříšek

WP25
Microsomal transformation of organophosphorus pesticides by white rot fungi
R. Vazquez-Duhalt, and J. Jauregui

WP26
Development of an in vitro reporter system to assess the transcriptional regulation of the human CYP1A2 gene
F. Amin, M. Town, A. R. Boobis, and D. S. Davies

WP27
SAR-by-NMR with CYP450eryF: implications for homotrophic/heterotrophic cooperativity
J. N. Lampe, J. T. Pearson, J. S. Grinstead, M. J. Dabrowski, A. P. Campbell, and W. M. Atkins

WP28
Improving the affinity of adrenodoxin for its partner proteins by directed evolution
A. Bichet, F. Hannemann, and R. Bernhardt

WP29
Novel reversible adduction of the P450 heme: inactivation of cytochrome P450 2E1 T303A by tert-butyl acetylene
A. L. Blobaum, U. M. Kent, and P. F. Hollenberg

WP30
Protein engineering of human cytochrome P450s for structure determination
V. Dodhia, R. Bazin, and G. Gilardi

WP31
Studies on interaction between fibrates and statins
H. Fujino, S. Shimada, I. Yamada, M. Hirano, Y. Tsunenari, and J. Kojima

WP32
The effects of chronic treatment with antidepressant drugs on the level and activity of CYP2C6, CYP2C11 and CYP3A1/2 in the rat liver
A. Haduch, and W. A. Daniel

WP33
The optical biosensor study of redox partners' interactions in the P4502B4 system in hydroxylation conditions
Yu.D. Ivanov, V.Yu. Kuznetsov, N.A. Petushkova, I.P. Kanaeva, and A.I Archakov

WP34
Atomic force microscopy (AFM) and optical biosensor studies of cytochrome P450's component interactions
Yu. D. Ivanov, I. P. Kanaeva, I. I. Karuzina, V.Yu. Kuznetsov, S. A. Usanov, G. Hui Bon Hoa, S. G. Sligar, and A. I. Archakov

WP35
Metabolism of N-benzyl-1-aminobenzotriazole by P450 2B1 results in a modified enzyme capable of N-demethylation but with abolished hydroxylation activity
U. M. Kent, R. A. Roof, L. Pascual, D. P. Ballou, and P. F. Hollenberg

WP36
A proton-shuttle mechanism mediated by the porphyrin in benzene hydroxylation by cytochrome P450 enzymes
S. P. de Visser, and S. Shaik

WP37
Effect of memantine on in vitro cytochrome P450-mediated metabolic activities in human liver microsomes
S. Micuda, L. Mundlova, E. Anzenbacherova, P. Anzenbacher, J. Chladek, J. Martinkova

WP38
21-fluorosteroids used in kinetic and 19F-NMR studies to probe CYP17 catalysis
E. O'Donnell, J. N. Wright, and P. Lee-Robichaud

WP39
Effects of thalidomide on the CYP-mediated metabolism of cyclophosphamide in the mouse
J. Paxton, P. Kestell, Q. Ding, L.-M. Ching, B. D. Palmer, S. Zhou, and B. C. Baguley

WP40
Reduction of the nitroaromatic antiandrogen nilutamide by NO synthases. Implications for the adverse effects of this antiandrogen drug
S. Dijols, K. Ask, C. Giroud, L. Casse, Y. Frapart, M.-A. Sari, P. Camus, D. Stuehr, D. Mansuy, and J.-L. Boucher

WP41
Dynamics of CYP2D6 activity and clinical response to paroxetine, alprazolam and their combination in patients with depressive disorder
E. Hadašová, A. Žourková, J. Tomandl, and B. Ravčuková

WP42
Inhibition of spontaneously produced nitric oxide in conventional and perfused hepatocyte culture improves cell functionality
N. Canová, E. Kmoníčková, and H. Farghali

WP43
Differences and comparisons of the active species (Cpd I) of cytochrome P450 and horse radish peroxidase as revealed by density functional theoretical studies
S. P. de Visser, P. K. Sharma, D. Kumar, and S. Shaik

WP44
Inhibition of inducible nitric oxide synthase activity by oltipraz in vitro and in activated microglia cells
L. L. Furge, P. R. Fields, W. E. Goode, M. C. Tressler, and R. S.-Truss

WP45
Endothelial nitric oxide synthase reductase domain is activated by calmodulin and phosphorylation
Y. Nishino, K. Yamamoto, Z.-W. Guan, S. Kimura, and T. Iyanagi

WP46
Recruitment of governing elements for electron transfer in nitric oxide synthase family
M. Jáchymová, P. Martásek, S. Panda, T. M. Shea, L. J. Roman, and B. S. S. Masters

WP47
Modulation of electron transfer in nitric oxide synthase using non amino acid guanidines and hydroxyguanidines
M. Moreau, J.-L. Boucher, S. Dijols, Y. Frapart, D. Lefevre, D. Stuehr, and D. Mansuy

WP48
On the role of the cysteine ligand in cytochromes P450
P. Rydberg, E. Sigfridsson, and U. Ryde

WP49
Is NOS a NOx-Synthase?
J. Santolini, Z.-Q. Wang, D. Konas, and D. J. Stuehr

WP50
Induction and modulation of inducible nitric oxide synthase expression following keratoplasty in mice
P. Střeštíková, J. Plšková, J. Martínek, M. Filipec, and H. Farghali

WP51
Mechanistic studies on the intramolecular one-electron transfer between the two flavins in iNOS flavin domain and NADPH-cytochrome P450 reductase
K. Yamamoto, Z.-W. Guan, Y. Nishino, S. Kimura, and T. Iyanagi

WP52
Xanthates (dithiocarbonates) metabolism by some monooxygenases
S. Yanev, B. Pandova, T. A. Vannelli, V. Todorova, and P. R. Ortiz de Montellano

WP53
The metabolic ratio carbamazepine/carbamazepine-epoxide in children and adult patients on antiepileptic polytherapy
B. Brzakovic, M. Pokrajac, Ž. Martinovic, B. Miljkovic, R. Velickovic

WP54
New in vitro metabolites of paclitaxel in humans, rats, minipigs and regular pigs and CYP involved in their formation
R. Václavíková, S. Horský, and I. Gut

WP55
Quantitative evaluation of relative contribution of human cytochrome P-450 isoenzymes (CYPs) to the metabolism of promazine
J. Wójcikowski, W.A. Daniel, and L. Pichard-Garcia, P. Maurel

WP56
Stimulation of human cytochrome P450 3A4 activity by anionic phospholipids and their micro -domains
K.-H. Kim, T. Ahn, and C.-H. Yun

WP57
Potent inhibition of human cytochrome P450 -catalyzed reactions by hemin
E.-Y. Kim, J.-S. Kim, M.-Y. Kim, W.-S. Koh, F. P. Guengerich, and C.-H.Yun

WP58
Genetic polymorphisms in human CYP2A6 gene and interindividual differences in nicotine metabolism
M. Nakajima, R. Yoshida, K. Nishimura, J.-T. Kwon, and T. Yokoi

WP59
Genetic polymorphism of canine cytochrome P450 2D15 gene
I. S. Pappas, and D. S. Katsiabas

WP60
The involvement of cytochrome P450 in the metabolism of chloroquine on human liver microsomes
K.-A. Kim, J.-Y. Park, J.-S. Lee, and S. Lim

WP61
Inhibition of cytochrome P450 activities by oleanolic acid and ursolic acid in human liver microsomes
K.-A. Kim, J.-S. Lee, H.-J. Park, C.-J. Kim, I.-S. Shim, S.-M. Han, J.-W. Kim, and S. Lim

WP62
Cloning, sequencing and heterologous expression of the genes coding for the P450mor system from Mycobacterium sp. strain HE5
B. Sielaff, and J. R. Andreesen

WP63
Coumarin 7-hydroxylase activity in liver and brain microsomes of mice
E. D. Ozdamar, M. Iscan, and B. C. Eice

WP64
CYP4F8 and CYP4F12: tissue distribution and catalytic properties
K. Stark, L. Schauer, J. Bylund, and E. H. Oliw

WP65
Baboon CYP11B1: the expression and structural analysis of two catalytically active isoforms
A. C. Swart, S. Graham, N. Brown, and P. Swart

WP66
An interspecies comparison of the metabolism of the anticancer agent Yondelis™ (trabectedin, ET-743)
M. Vermeir, J. Hendrickx, R. Hurkmans, J. Van Houdt, C. Zwijsen, N. Bode, P. Aviles, I. Manzanares, G. Mannens, W. Meuldermans, and R. De Coster

WP67
Differences in biotransformation of testosterone by brain microsomes of rat and rabbit: a preliminary study
L. Marvasi, P. Anfossi, and A. Zaghini

WP68
Polymorphism in cytochrome P450 2D6-mediated activation of malathion in rats
V. Silvari, S. Catania. A. Palamara, L. B. Martino, K. Zahlsen, and C. Costa

WP69
In vitro studies on the activation of the organophosphate pesticide malathion by cytochrome P450 2D6 in rat liver microsomes
C. Costa, S. Catania, V. D'Angelo, L. B. Martino, K. Zahlsen, and V. Silvari

WP70
Photoaffinity labeling of cytochrome P450 2B4
M. Karabec, M. Šulc, and P. Hodek

GENERAL INFORMATION

CONFERENCE VENUE

Národní dům na Vinohradech (National House of Vinohrady)
Náměstí Míru 9
120 53 Prague 2
Phone: +420-221 596 311, 221 596 111
Fax: +420-221 596 234
(Metro station „Náměstí Míru“ - line A)

All events of the Scientific Program are realized in this building. As the halls and rooms are mostly located at the same floor, there is no need to present a layout scheme of the building here. The lecture halls as well as the space allocated for posters and for exhibition are indicated by arrows and signs.

BADGES
Participants will be given a badge upon registration. The badge must be worn at all times; only registered participants will be allowed to enter the Conference area.

REGISTRATION DESK
The registration desk, located at the National House of Vinohrady (Conference Venue), will be opened for the duration of the Conference as follows:

Sunday, June 29, 2003	**12.00 - 20.00**
Monday, June 30, 2003	**7.30 - 17.00**
Tuesday, July 1, 2003	**8.00 - 14.00**
Wednesday, July 2 , 2003	**8.00 - 16.00**
Thursday, July 3, 2003	**8.00 - 13.00**

REGISTRATION FEES

Full participants
***On-site registration / paid after* APRIL 30, 2003**
560 USD

Full registration fee covers:
- Admission to all scientific sessions and exhibition area
- Conference documents (program, book of abstracts, list of participants)
- Certificate of participation
- Coffee/tea service
- Get-Together Party (Sunday, June 29, 2003)
- Opening Ceremony (Monday, June 30, 2003)
- Concert (Monday, June 30, 2003)
- 3 lunches
- Free ticket for Prague metropolitan transport valid for 5 days

Accompanying persons
***On-site registration / paid after* APRIL 30, 2003**
140 USD

Registration fee covers:
- Admission to exhibition area
- Get-Together Party (Sunday, June 29, 2003)
- Opening Ceremony (Monday, June 30, 2003)
- Concert (Monday, June 30, 2003)
- City Tour of Prague
- Free ticket for Prague metropolitan transport valid for 5 days

CERTIFICATE OF PARTICIPATION
The certificate of participation will be issued upon request to properly registered participants. The Certificate will be available at the registration desk on the last day of the Conference.

LUNCHES AND REFRESHMENT
Coffee or tea will be provided during the coffee breaks to all registered participants in the Exhibi-

tion/Refreshment Hall. Packed lunches will be served to all registered participants in exchange for the respective ticket at distribution points.

EXHIBITION
The exhibition is located at the Exhibition/Refreshment Hall just in front of the main lecture hall (Mayakoffsky Hall).

CURRENCY
The official currency is the Czech Crown (CZK = Kč). Exchange rate fluctuates daily, but at the time of printing this Program one USD was equivalent to approximately 26 CZK.

INSURANCE, SECURITY
The Conference Secretariat will not accept any liability for personal injuries, loss or damage to property of participants and accompanying persons. Kindly take your personal insurance policy. The site of the Conference is under security control. As Prague is attracting not only many tourists, but also dishonest people, please, take care of your personal belongings.

CLIMATE AND DRESS
The weather at the end of June and beginning of July is usually pleasant with an average day temperature of 20-25 °C. Informal dress will be suitable for the Conference and social events.

TRANSPORTATION
Metropolitan transportation in Prague is quite reliable and consists of trams, buses and metro (subway).

Metro is the most reliable and the most popular way of transport in Prague. Metro system consists of three lines - A, B, C, connecting suburban areas with the city center. On each metro train there is a map of lines with detailed description.

At the Registration Desk each participant and accompanying person will obtain a free ticket to Prague metropolitan transport (metro, buses, trams). The ticket is valid for 5 days (incl. the first day). Please stamp the ticket in the stamping machine when entering either the tram, the bus or at the entrance to the subway (Metro).

SOCIAL PROGRAM

The following social program will be prepared for the participants of the Conference. The additional tickets for Get-Together Party and Concert can be bought by the registration desk.

1) GET-TOGETHER PARTY
One ticket included in the registration fee
Sunday, June 29, 19.00-21.00
Additional ticket: 300 CZK

National House of Vinohrady
- Conference Venue (Náměstí Míru 9, Prague 2)
On Sunday, June 29, 2003, the participants are invited to the Mayakoffsky Hall, the most representative space of the National House. Welcome drink and small refreshment will be available, transfer will not be provided.

2) CONCERT
One ticket included in the registration fee
Monday, June 30, 20.00-21.00
Additional ticket: 300 CZK

St. Simon and Jude Church (Dušní 1, Prague 1)
Connection from the Conference Venue: by metro A from „Náměstí Míru“ to „Můstek“, 2 stops and by walk (approximately 30 minutes). The concert of ensemble „ Musica Gaudeans“ (Joyful Music) will take place at the concert hall situated at the baroque Church of St. Simon and Jude. The ensemble consisting of flute, oboe, guitar and cello will present the Conference participants an interesting program (Bach, Vivaldi, Paganini, Lobos). Transfer will not be provided.

3) STEAMBOAT PARTY
Tuesday, July 1, 16.00 -19.00 35 USD

Meeting point: steamer landing-place (between Palackého and Jiráskův bridges) at 15.45 hrs.
Connection to the meeting place from the

Conference Venue: by tram No. 4 or No. 16 from „Náměstí Míru“ to „Palackého náměstí“, 5 stops (approximately 20 minutes). During this afternoon trip on board of the biggest Prague steamer you will have an opportunity to see the most attractive sights in Prague. Enjoy a romantic afternoon with a buffet dinner and live swing music. Transfer to and from the steamer landing-place will be realized by public transportation.

4) FAREWELL DINNER
Wednesday, July 2, 19.00 - 24.00 65 USD

Trip to the Mělník Castle with wine tasting, dinner and program

Meeting point: at the Theatre **Vinohradske divadlo** just near by the Conference Venue.
Time: 18.40, meeting in front of the theatre, kindly note that the buses will depart from the theatre in 10 min. intervals. **The last one will depart at 19.00! Please, do not be late!**

Enjoy the atmosphere of the Renaissance castle near Prague. You will visit the historical interior and taste the wine from the cellars of the Lobkowicz noble family. Afterwards you will take part in a festival with knight tournament, Gothic and Renaissance dances while tasting the rich buffet dinner and drink the castle wines and famous Czech beer.
Departure from Mělník Castle: 23.00
Arrival to Prague: 24.00

TOURIST PROGRAM

Meeting point for all trips is at the registration desk at the Conference venue. At the trip counter you will receive your prepaid tickets and you may buy tickets for all tours if the place is available.

1) CITY TOUR

Sunday, June 29	**13.00 - 17.00**	**USD 28**
Monday, June 30	**10.00 - 14.00**	
Wednesday, July 2	***cancelled due to small number of participants***	

Sightseeing tour will take you to Prague Castle and some parts of Old Town and other historical city quarters. The bus will depart from the Conference venue. It will stop at the Castle area, which you will explore on foot. This is the biggest castle area in the world, still in use as a seat of the Presidents of the Czech Republic. The price covers the admission fee to St. Vitus Cathedral, Old Royal Palace and Golden Lane.

For registered accompanying persons one City tour is free of charge. It is advisable to book a seat in advance and choose the date of tour. Without the preliminary booking we cannot guarantee your seat.

2) JEWISH GHETTO + OLD TOWN SQUARE
Tuesday, July 1, 10.00 - 14.30 USD 42

The bus will take you from the Conference Venue to the Old Town Jewish quarter. You will visit its amazing synagogues incl. the oldest preserved synagogue in Europe and the Jewish Cemetery from XV century with its 1200 fascinating tombstones and mystic atmosphere. The famous Rabbi Löw, creator of the legendary Golem is resting here as well as numerous other personalities. At the end you will walk to the nearby Old Town Square with the popular Horologe presenting a moving show of 12 apostles. The bus will take you back to the Conference Venue or you can stay at the Old Town and enjoy its beauties on your own.

3) TRIP TO CASTLE KARLŠTEJN & NIŽBOR GLASS FACTORY
Wednesday, July 2, 9.00 - 17.30 65 USD

You will visit the family glass factory producing famous Czech crystal glass. A small glass souvenir is included, you will have also an opportunity to shop in the factory shop. From the factory the route follows through the romantic forested countryside to the Karlštejn Castle, founded in XIV century as a treasury for safeguarding the coronation jewels and holy relics. The castle is the most popular one in the whole country, except for Prague Castle.

Lunch will be served in the village below the castle in a typical restaurant.

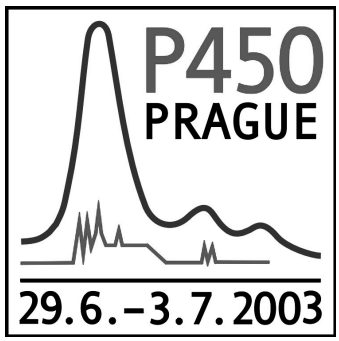
4) TRIP TO KONOPIŠTĚ CHATEAU
Thursday, July 3, 9.00 - 14.00 48 USD

This excursion will show you one of the most beautiful chateaux in Bohemia. The chateau has been once home of the successor of the Austrian throne, Franz Ferdinand d'Este, whose assassination in Sarajevo sparked off the World War I. The chateau offers a large display of historical weapons, hunting trophies, etc. You will see that Mr. d'Este was really a keen hunter and lover of fine arts. Beautifully furnished and decorated rooms will surely attract your eyes. A large English park surrounds the chateau.
Lunch is not included.

ACCOMMODATION

The participants who used the services of Conference Secretariat (CBT) for hotel booking and who need any further assistance regarding the room reservation are asked to kindly contact the Accommodation desk in the Registration office during the announced registration hours.

For an official invoice or receipt kindly contact the Accommodation desk, the relevant document will be issued on request.



13TH INTERNATIONAL CONFERENCE ON

CYTOCHROMES P450

Biochemistry, Biophysics and Drug Metabolism

ABSTRACTS

Prepared for print by
Jiří Hudeček

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1 LECTURES – MONDAY – ML

ML01 STRUCTURAL BASIS OF P450 2B SPECIFICITY

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Introduction

The long-term objective of our research is to elucidate the structural basis for the substrate specificities of cytochromes P450 of the 2B subfamily. P450 2B enzymes are very versatile catalysts with a broad range of substrates, including drugs, environmental carcinogens, and steroids. Along with members of the 2A and 2C subfamilies, P450 2B enzymes are thought to exhibit the least degree of catalytic preservation across mammalian species. This observation suggests a strong role for steric constraints imposed by the enzymes, as opposed to the inherent chemical reactivity of the compounds, in determining substrate specificity. Through studies during the past decade the key amino acid residues that dictate ligand binding orientation within the interior of the active site and contribute to substrate and inhibitor specificity of several P450 2B enzymes have been identified[1]. All these residues have counterparts in the active site of P450 2C5 inferred from the x-ray crystal structure[2]. Such information has allowed us to explain many of the functional differences among P450 2B enzymes and to begin to alter their activities in a rational manner. However, the promise of engineering novel or more efficient catalysts of specific oxidative reactions remains largely unmet, and the pathways by which different substrates gain access to the buried active site remain to be clarified. Our central hypothesis is that substrate specificity reflects the interplay between amino acid residues in the interior of the active site and those that line the substrate access channel.

A Rational Approach to Re-engineer Cytochrome P450 2B1 Regioselectivity Based on the Crystal Structure of P450 2C5. Interest in structure-function studies across P450 subfamilies was sparked by the considerable effort in a number of laboratories to use the P450 2C5 structure to model other mammalian P450 enzymes and predict their substrate specificities and stereo- and regioselectivity. An implicit assumption in all such models based on a single template is that the backbones of the enzymes are essentially invariant and that active site differences alone are responsible for specificity differences. Therefore, we sought to confer the progesterone hydroxylation specificity of P450 2C5 on 2B1. P450 2B1 is a high K_m progesterone 16-hydroxylase, whereas 2C5 is a low K_m progesterone 21-hydroxylase. Initially, nine individual P450 2B1 active site residues were changed to the corresponding 2C5 residues, and the mutants were purified from an E. coli expression system and assayed for progesterone hydroxylation. Based on the results, a quadruple mutant I114A/F206V/F297G/V363L, termed Q, was constructed that showed 60% of 2C5 progesterone 21-hydroxylase activity and 57% regioselectivity. Based on their 2C5-like testosterone hydroxylation profiles, S294D and I477F alone and in combination were added to the quadruple mutant. All three mutants showed enhanced regioselectivity (70%) for progesterone 21-hydroxylation, while only Q-I477F had a higher k_{cat}. Finally, V103I was added to Q-I477F and Q-S294D/I477F. Q-V103I/S294D/I477F showed a 3-fold higher k_{cat} than 2C5 and 80% regioselectivity for progesterone 21-hydroxylation. Docking of progesterone into a 3D model of this mutant indicates that 21-hydroxylation is favored. In conclusion, a systematic approach to convert P450 regioselectivity across subfamilies suggests that active site residues are mainly responsible for regioselectivity differences between 2B1 and 2C5, and validates the reliability of 2B1 models based on the crystal structure of 2C5[3].

Substrate access channel

Substrate interactions with cytochromes P450 have been proposed to occur in three stages:

1) recognition of the substrate by surface residues;

2) entry into the buried active site through a hydrophobic access channel;

3) substrate orientation in the active site to allow catalysis. Until recently, all known structures of bacterial cytochromes P450 suggested that substrate access to the buried active site occurred via the F-G region, a surface loop distal to the heme cavity. However, the structure of P450 51 indicates a large opening from the protein surface along the I helix N-terminus, at right angles to the F-G channel. The single available microsomal P450 structure (2C5) does not obviously favor one potential access route over the other. To determine whether the F-G region forms part of the substrate access channel in cytochrome P450 2B1, eleven residues between positions 208 and 230 were substituted with smaller and larger side chains in a highly expressed truncated form of the enzyme. Steady-state kinetic parameters were determined with the substrates testosterone, 7-ethoxy-4-trifluoromethylcoumarin (7-EFC), and 7-benzyoxyresorufin (7-BR). The largest changes, 2 to 6-fold increases in k_{cat} with testosterone and 7-EFC, were observed for L209A, which also exhibits an altered testosterone metabolite profile and probably forms part of the active site roof. F219W demonstrated little or no activity with any of the three substrates examined, although the K_s value for benzphetamine binding was unaltered. S221F showed little activity with 7-BR. No significant changes were observed in K_m (testosterone) or S50 (7-EFC) values for any of the mutants, in stark contrast to the ten-fold and hundred-fold changes in K_m observed for mutants in this region of other cytochromes P450[4]. The minimal changes in 2B1 did not support access via the F-G region of 2B1 and suggested the alternate

access route identified in P450 51. More recent unpublished studies suggest a role for residues in the N-terminal part of the I helix in regulating the efficiency of substrate hydroxylation, consistent with a role in substrate access.

Acknowledgment

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ML02 A 1.6 Å CRYSTAL STRUCTURE OF P450 2B4: NOVEL FEATURES AND IMPLICATIONS

EMILY E. SCOTT[a], YOU AI HE[a], MICHAEL R. WESTER[b], MARK A. WHITE[c], JAMES R. HALPERT[a], ERIC F. JOHNSON[b], and CHARLES D. STOUT[d]

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Introduction

Cytochromes P450 from families 1, 2, and 3 are among the most versatile biological catalysts known. Each enzyme is responsible for the biotransformation of an array of structurally variable xenobiotics and endogenous compounds, with overlapping specificities among enzymes. Understanding the structure-function relationships of these monooxygenases has been significantly enhanced by the structure of the first membrane P450, 2C5[1]. We have proposed that the versatility of substrate oxidation among the xenobiotic-metabolizing P450 enzymes is likely to be reflected in their structural diversity. We present here the first structure of a P450 from the 2B subfamily. Due to its availability as a highly purified enzyme, P450 2B4 has long served as a model for biophysical studies of mammalian cytochromes P450. Thus, structures of P450 2B4 have the potential to integrate decades of functional and mechanistic analysis.

Methods

Cytochrome P450 2B4 was engineered to remove the N-terminal transmembrane helix, substitute a number of charged residues at the new N-terminus, and add four histidine residues to the C-terminus. The resultant E. *coli*-expressed protein (2B4dH) was bound to membranes, but could be liberated by high salt conditions. Although the protein could be purified in the absence of detergents, the crystallized protein was extracted from membranes and stabilized during Ni^{2+}-affinity and CM-Sepharose chromatography with detergent. Analytical ultracentrifugation indicated that protein purified in the presence of sodium cholate was a monodisperse monomer, while purification in the presence of Cymal-5 yielded two species, presumably a monomer and a dimer.

Crystals were initially grown by equilibrating a concentrated protein solution including 30 mg/ml P450 and 4.8 mM Cymal-5 with 15% ethanol and 0.1 M sodium citrate, pH 5.5 using sitting drop vapor diffusion at 18° C. Macroseeding allowed the growth of single crystals with an increase in size and thickness, resulting in individual crystals up to 1 mm in length. Crystals were briefly flooded with mother liquor containing 30% glycercol and immediately frozen in liquid N_2 at 100 K.

Data analyzed for the final structure were collected at the Stanford Synchrotron Radiation Laboratory from a single crystal. Crystals diffracted to 1.6 Å. The space group is $C222_1$ with cell dimensions a=57.09 Å, b=114.20 Å, c=133.95 Å and a single molecule in the asymmetric unit (solvent content ~40%). The structure of 2B4 was solved by molecular replacement using a model of 2C5/3LVdH (1N6B) with the non-identical residues mutated to Ala. CNS was used for early rounds of refinement and SHELX for later rounds of refinement.

Structure of P450 2B4.

The secondary structure and general tertiary structure of P450 2B4 conform to the known P450 architecture, consisting of a beta sheet domain and an alpha helix domain. As expected, the structure of P450 2B4 shares more structural similarity with P450 2C5 than with the bacterial enzymes, but significant conformational differences were observed in the helix F-helix G region, the helix B-helix C region, and helix I relative to the 2C5 structure.

The F-G region is composed of helix F, two short helical segments (F' and G'), and helix G. The placement and structure of helix F is similar to that in 2C5 except that the C-terminal turn is unwound. The conformation of helix G is largely conserved, but it rises at a sharper angle above the active site than in 2C5. The F' and G' helices and the N-terminus of helix G extend away from the core of the protein, resulting in the formation of a large open cleft between the F-G region and the B'-C loop. This cleft extends

directly from the protein surface to the heme iron. The intermolecular packing is such that the extended F'-G' structural motif of one molecule fills the open cleft of a symmetry-related molecule. In the crystal, a dimer is formed by a bond between His226 at the extreme terminus of the F'-G' extension in one molecule and the heme iron of a molecule related by the crystallographic 2-fold axis. Spectral data indicate that this dimer forms reversibly in solution.

The opposing side of the cleft is formed primarily by the loop between helices B' and C and the C helix, the positions of which are altered relative to 2C5. The flexible loop between the helices B' and C demonstrates some of the highest B values in the structure. Corresponding to these conformational differences the propionate side chain of the heme ring D interacts with Arg98, found before the helix B', and with Arg133, found in the C terminus of helix C, instead of Trp120 and Arg124, both in the N terminus of helix C, in 2C5.

Finally, while helix I of 2C5 is relatively straight, 2B4 has a distinct bend in helix I. When the two structures are overlapped using the conserved tertiary structure, the N-termini of the I helices differ by 2.8 Å.

Although the possibility cannot be excluded that intermolecular contacts influence the structure of the F-G region in P450 2B4dH, crystallization of flexible protein regions generally appears to select an individual conformation from the continuum present in solution. The differences between the structure of P450 2C5, and the present structure of P450 2B4 suggest the possibility that the F-G and B'-C regions of family 2 enzymes may adopt a substantial range of energetically accessible conformations.

Acknowledgment

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ML03 INSIGHTS ON THE TOPOLOGY OF THE ACTIVE SITE OF HUMAN CYTOCHROME P450 2C9 DETERMINED BY X-RAY CRYSTALLOGRAPHY

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Cytochrome P450 2C9 (CYP2C9) ranks amongst the most important drug-metabolizing enzyme in humans. CYP2C9 is expressed at high level in human liver and is the enzyme responsible for the metabolism of more than 10% of the drugs currently in clinical use. Functional polymorphism of CYP2C9 also has clinical implications in drug-drug interactions and in variation in individual response to therapeutic drugs. To date, information on the topology of the CYP2C9 active site has derived from predictive models based on the bacterial and the rabbit CYP2C5 crystal structures, combined with site directed mutagenesis studies. However, the low amino acid sequence identity (20-30%) shared with the bacterial templates and the medium resolution in the CYP2C5 structure of regions that control the topology of the active site and the substrate recognition, has yielded unsatisfactory models for the prediction of the mode of binding of compounds in the active site. We have determined a 2.6 Å x-ray structure of a soluble form of CYP2C9. The crystal structure of CYP2C9 provides a basis for the accurate identification and localization of the key residues that constitute the active site and better understanding of the mechanisms that lead to differences in substrate specificities.

ML04 STRUCTURAL STUDIES OF SUBSTRATE BINDING TO CYP2C DRUG METABOLIZING P450S

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Comparative analysis of the mouse and human genomes indicates that genes encoding family 2 P450s exhibit some of the highest rates of non-synonymous nucleotide substitutions in these genomes.[1] This genetic diversity underlies a functional diversity that is particularly striking for family 2C P450s within and between species and is amplified by the capacity of these enzymes to recognize and metabolize structurally diverse substrates. Human 2C8, 2C9 and 2C19 contribute significantly to the metabolism of drugs used therapeutically, and genetic polymorphisms and metabolic drug-drug interactions involving these enzymes can lead to adverse drug effects. The structures of CYP 2C5, 2C8, and 2C9 illustrate a number of factors that contribute to the substrate selectivity and the catalytic diversity of these enzymes.

The structures of the CYP 2C proteins exhibit general features seen for soluble, bacterial P450s. The most signi-

ficant differences from soluble P450s are the presence of a longer polypeptide chain between helix F and helix G that contains two short helices, F' and G' and an extended N-terminal amino acid sequence that includes a hydrophobic transmembrane helix (residues 1-20) that is connected by a polar linker to a proline rich motif (residues 30-37) at the beginning of the catalytic domain. The N-terminal domain has been deleted in all three proteins to facilitate crystallization, and a 4 histidine tag was added to the C-terminus to facilitate purification. In addition, CYP 2C5 is mutated at 5 residues on helix F and in the turn preceding helix F' to facilitate crystallization.[2]

The structures of the three CYP 2C proteins are highly similar, but differ in features that contribute to substrate binding. Although these include differences in the amino acid side chains that can potentially contact substrates in the substrate-binding cavity, differences are also evident in the positioning of the peptide backbone. Differences in the conformation of the peptide backbone are greatest for helix B to helix C region and from helix E through the N-terminal portion of helix I. The flexibility of these regions also allows some degree of expansion or contraction to adapt to substrates of different sizes. As a result of differences in conformation and amino acid side-chains, the active site volume of 2C8 is roughly twice that of 2C5.

Several factors are likely to contribute to substrate binding. These include steric fit, van der Waals and hydrogen bonding interactions and the hydrophobic effect. The active site cavities are significantly larger than the space occupied by some substrates. This can give rise to multiple binding orientations for the substrate that can lead to alternative products. In general, both the substrates and the active site cavities are largely hydrophobic, and elimination of water from the hydrophobic surfaces of the substrate and the active site cavity will contribute significantly to the free energy of substrate binding. Residual water molecules that are not displaced by the substrate contribute to hydrogen bonding interactions between the polar moieties of the substrate and the protein, and these waters can mask unfavorable electrostatic interactions. Polar interactions contribute significantly to substrate binding and the regioselectivity of metabolism. This is clearly illustrated by the structures of CYP 2C5 and 2C9 with nonsteroidal anti-inflammatory drugs bound in their active sites.

Acknowledgment

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ML 05 RADICALS AND STRESSED BACTERIAL P450 ENZYMES

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Replacement of the heme iron atom has been achieved with many hemoproteins, but only limited success has been reported in this area for cytochrome P450 enzymes. Cytochrome P450cam has been reconstituted with other metal porphyrins, notably Co and Mn, but reconstitution requires fairly harsh conditions that can perturb the native metal environment.[1] We report here a biosynthetic approach to the assembly of metal substituted cytochrome P450 enzymes based on heterologous expression of the proteins in E. *coli* under controlled metal conditions. The approach thus far has been used to prepare cobalt-substituted cytochrome P450cam (CYP101) and CYP119 with no more than trace amounts of the iron porphyrin. Both of these proteins possess a lower affinity for the Co-PPIX prosthetic group than for heme and are less thermally stable. X-ray crystal structures of the two proteins suggest that these properties are due to a longer metal-sulfur bond. Earlier crystal structures of CYP119 with imidazole and 1-phenylimidazole coordinated to the iron atom demonstrated the presence of two distinct protein conformers.[2] 2D NMR studies of cobalt CYP119 without added ligands confirm the existence of at least two equilibrating protein conformers whose populations are altered upon ligand binding. Cobalt CYP119 has been found to catalyze the hydroxylation of lauric acid in the presence of NADPH and a surrogate electron donor system, but not in the presence of H_2O_2. The different reactivity profiles of the iron and cobalt proteins may reflect subtle differences in their hydroxylation mechanisms. In contrast, cobalt CYP101 reacts quantitatively with peroxides to yield novel porphyrin adducts that decompose to stable cobalt hydroxyporphyrins. The ability to prepare otherwise unperturbed metal-substituted P450 enzymes promises to open a new area of catalytic chemistry involving thiolate-ligated metalloprotein active sites. This work was supported by NIH Grant GM25515.

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ML06 STRUCTURES OF P450EPOK, PUTIDAREDOXIN, AND PUTIDAREDOXIN REDUCTASE

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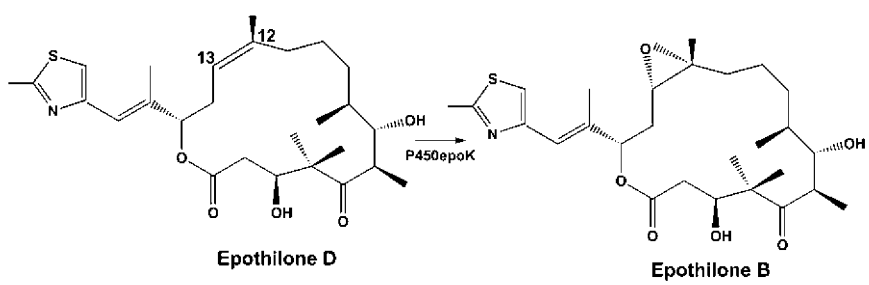
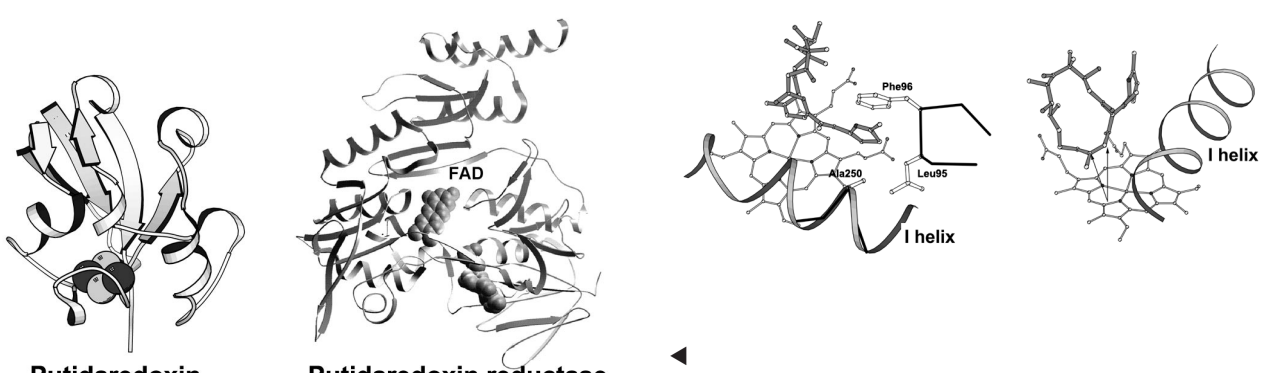
Epothilones (*Fig.1*) are naturally produced by the cellulose-degrading myxobacterium *Sorangium cellulosum* strain So ce90. Like the well-known anti-cancer agent, Taxol, epothilones bind to and stabilize microtubles leading to mitotic arrest of the cycle at G2-M phase and subsequently induces apoptosis in several cell lines. Epothilones offer some advantages since epothilones are effective against P-glycoprotein-expressing multi-drug resistant cell lines, are active in a cell line with taxol resistance, and epothilones water solubility is significantly greater than Taxol. Another advantage is that epothilones can be produced in large quantities using a heterologous expression system. One step in the biosynthesis of epothilones involves a 12-13 epoxidation (*Fig. 1*) by a cytochrome P450, P450epoK. The structures of P450epoK in the substrate-free, substrate-bound, and product bound complexes have been solved to a resolution of 2.65 Å, 1.93 Å, and 2.10 Å, respectively. As expected, P450epoK most closely resembles P450eryF, which also participates in the biosynthesis of a complex macrolide. The active site region of the substrate complex is shown in *Fig. 2*. Similar to other P450s, the atoms that undergo the epoxidation reaction are close to the heme iron and in a position to directly contact the Fe-linked O atom. The thiazole portion of the substrate provides key contact points which very likely are critical in substrate specificity. Interestingly, there are strong parallels to the binding of epothilones to P450epoK and Taxol to tubulin. An unexpected outcome of these structures is that there is little difference in the substrate-bound and -free structures. We had anticipated a more open conformation of the substrate access channel in the substrate-free structure but there is only a slight closure upon substrate binding. Nevertheless, there must be a fairly large open/close motion in order to accommodate such a large substrate.



◀ ***Fig. 1.*** *Reaction catalyzed by P450epoK.*

▼ ***Fig. 2.*** *Two views of the P450epok-substrate complex. Ala250 in the I helix and Phe96 provide key contact points with the thiazole part of the substrate. The arrows indicate the carbon atoms which form the epoxide.*



◀ ***Fig. 3.*** *Crystal structures of Pdx and Pdr.*

Structures of the electron transfer components of the P450cam monooxygenase system, putidaredoxin (Pdx) and putidaredoxin reductase (Pdr), have been solved to a resolution of 1.75 Å and 1.9 Å, respectively. A comparison with mammalian counterparts, adrenodoxin (Adx) and adrenodoxin reductase (Adr), reveals both similarities and

differences. The Pdx and Adx (1) structures clearly are very similar. However, Pdr and Adr (2) exhibit large differences. The FAD site is less exposed in Pdr owing to an additional β-pair situated near the cofactor. In addition, the C-terminal region of Pdr is positioned very differently than in Adr. Therefore, if Pdx and Pdr form a complex similar to the Adr-Adx complex, then the C-terminal domain in Pdr must move. This seems unlikely since the C-terminal region that would be required to move is composed of β-sheets that tie this region to the main body of the protein. Therefore, Pdx forms a complex with Pdr that must be quite different than the Adx-Adr complex. The implications of these structures for protein-protein recognition and electron transfer will be described.

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ML07 CRYSTAL STRUCTURE-BASED ENGINEERING OF SUBSTRATE RECOGNITION IN P450CAM CATALYSIS

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The C-H bond oxidation activity, under ambient conditions, of P450 enzymes has no equivalent in classical synthetic methodology and thus has great potential in applications such as fine chemical synthesis. For such processes to be viable the enzymes must have high activity and selectivity for the oxidation of unnatural substrates, and ideally it should be possible to alter the selectivity for the synthesis of isomeric compounds, including enantiomers. At the heart of this lies the challenge of understanding and ultimately manipulating the enzyme-substrate molecular recognition.

The terpenoid hydrocarbons $(C_5H_8)_n$ are biosynthesised by the coupling of isoprenyl units. The monoterpenes (n=2) are derived from geranyl pyrophosphate. Terpene synthases catalyse the dissociation of the pyrophosphate group, and the carbonium ion thus generated undergoes a series of rearrangements before deprotonation to form many different cyclic and acyclic skeletal structures. These parent hydrocarbons, and their oxygenated derivatives such as the alcohols, epoxides, aldehydes and ketones, are the major fragrance and flavouring components in plant essential oils. For example, carveol, perillyl alchol and isopiperitenol derived from the oxidation of limonene,[1,2] and verbenol, verbenone and myrtenol obtained from α-pinene oxidation, are fine chemicals.

We have investigated the oxidation of limonene and pinene by cytochrome $P450_{cam}$ (CYP101) from *Pseudomonas putida*.[3-5] The bicyclic (+)-α-pinene is structurally related to camphor, the natural substrate of $P450_{cam}$. The enzyme-substrate contacts in $P450_{cam}$ has been investigated extensively by biophysical methods as well as crystallography.[4,6] Based on this wealth of information and comparison of the structure of camphor and pinene, we introduced a number of active site mutations to compensate for the structural differences. We have shown that a small number of mutations can enhance the monoterpene oxidation activity of $P450_{cam}$. The effect of mutations on selectivity is complicated and sometimes non-additive. The crystal structure of a key mutant (F87W/Y96F/V247L) with (+)-α-pinene bound within the active site was determined to provide insights into the enzyme-substrate interactions.[7] The (+)-α-pinene substrate is bound in two different orientations in the enzyme. One of these is closely related to that of camphor in the wild type enzyme, while the other orientation can be generated by a simple rotation from the first orientation. The structure allows new mutations to be introduced to alter the selectivity of product formation.[7]

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ML08 STRUCTURAL ASPECTS OF LIGAND BINDING TO PROCARYOTIC AND FUNGAL P450S

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P450 enzymes are involved in numerous biological processes including the biosynthesis of lipids, steroids, antibiotics, and the degradation of xenobiotics. In line with the variety of reactions catalyzed, the size of the substrate varies significantly. Some P450s have open active sites (e.g. BM3), some have shielded active sites that open only transiently (e.g. P450cam), whereas others bind the substrate only when attached to carrier proteins (e.g. oxy proteins). In addition to the organic substrate, oxygen has to bind. Structural aspects of both organic and gaseous ligand binding are described and discussed.

ML09 REVELATIONS OF NITRIC OXIDE SYNTHASE MODULATION: HIDDEN MESSAGES IN SEQUENCE AND STRUCTURE

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This laboratory entered into the nitric oxide synthase (NOS) field as a result of the high sequence homology between NADPH-cytochrome P450 oxidoreductase (CYPOR) and the 641 C-terminal residues of neuronal NOS[1] and the finding that this enzyme was a flavoprotein containing one mol each of FAD and FMN per monomer[2,3]. Subsequent sequence comparisons have led to the conclusion that the differences between the simpler CYPOR structure and the more complex NOS isoforms have resulted from the evolution of these proteins to become modulated in order to participate in cell signaling processes. The formation of NO in cellular systems, by necessity, must be carefully regulated and localized.

Comparison of the NOS and CYPOR sequences identified autoregulatory inserts in the FMN-binding domains of both constitutive isoforms, neuronal (nNOS) and endothelial NOS (eNOS), absent in CYPOR and the inducible NOS isoform, that inhibit NOS activity until Ca^{+2}/calmodulin (CaM) is bound[4]. Furthermore, all three NOS isoforms contain C-terminal sequences that extend beyond the C-terminus of CYPOR at the identical residue in each isoform. This laboratory[5,6] demonstrated, by truncating the three isoforms to resemble CYPOR, that the additional C-terminal sequences act as inhibitory modulators of NOS activity. In all three isoforms, this truncation resulted in 7- to 10-fold increases in flavoprotein-mediated cytochrome *c* reduction that were reduced to the wild type activity upon the addition of Ca^{2+}/calmodulin. NO production by these truncated mutants is either only slightly affected in the case of inducible NOS, which contains tightly bound CaM, or is decreased by 45% and 33% in nNOS and eNOS, respectively, upon the addition of CaM. These results indicate an interactive relationship between the autoregulatory inserts and CaM binding in the activation of nNOS and eNOS.

Recent experiments (Roman *et al.* (2003) *in preparation*) have determined which structural domains are involved in the regulation of catalysis of both electron transfer from the flavoprotein domain **and** the production of NO through the heme domain by neuronal NOS. Chimeric constructs were expressed in which the FAD- and/or FMN-binding domains of CYPOR were swapped with the respective nNOS domain to determine the role of the flavin-binding subdomains in catalyzing these activities. Constructs of nNOS containing the FAD-binding domain of CYPOR catalyzed high rates of cytochrome c reduction (>7000 min^{-1} *vs*. 2500 min^{-1} for wild type) **in the absence of** CaM. Cytochrome c reduction was inhibited by the addition of CaM to the construct in which the FMN-binding domain was derived from nNOS and the FAD-binding domain was from CYPOR, similar to the C-terminal tail-less mutant of nNOS. The chimera, containing both flavin-binding domains of CYPOR, catalyzed a high rate of cytochrome c reduction that was unaffected by the addition of CaM. Ferricyanide reduction was **not affected** by the addition of CaM in any of the constructs. These results indicate the effects of CaM are primarily mediated through the FMN-binding domain of nNOS, since ferricyanide reduction occurs

through the FAD-binding domain of both CYPOR and NOS. The only chimera catalyzing NO formation contained the heme- and FMN-binding domains of nNOS and the FAD-binding domain of CYPOR and was capable of producing higher amounts of NO than the wild type nNOS- a *superenzyme*.

Chimeric constructs were also made by attaching the C-terminal tails of each of the three NOS isoforms to soluble CYPOR. In recent experiments (Jachymova *et al*. (2003) *in preparation*), chimeric proteins of CYPOR containing each of these C-termini were spectrally identical but showed decreasing cytochrome c and DCIP reductase activities corresponding to the length of the C-terminus added but revealed little effect on ferricyanide reduction. All three chimeras exhibited significantly increased (1.8-2-fold) NADPH oxidase activity. Also, the rate of reoxidation to the air-stable semiquinone state was substantially increased. These experiments corroborate the role of the C-terminus in regulating NOS activity and show the degree to which this regulation is mediated by the length of the C-terminal tail explaining, in part, the sluggish rate of eNOS catalysis, since it bears the longest C-terminus.

Site-directed mutants of the constitutive NOS isoforms (Panda *et al*. (2003) *in preparation*) in which the serine residue homologous to S457 of CYPOR is mutated to a threonine or alanine residue have been produced. These mutations, which have profound effects in CYPOR[7], have also been shown to affect electron transfer in NOS isoforms. The catalysis of cytochrome c reduction by the Ser to Ala mutants is inhibited to a large degree in both isoforms and both mutants unexpectedly showed measurable activity that was increased upon the addition of CaM. The S942T eNOS mutant, on the other hand, catalyzed cytochrome c reduction to the same extent as wild type in the absence of CaM but was stimulated to approximately half the activity of the wild type by CaM. The nNOS mutants, S1176A and S1176T, were both inhibited to a large extent but the addition of CaM produced very low, but measurable, cytochrome c reductase activities. Interestingly, the catalysis of NO production by nNOS S1176T was **unaffected** and that of eNOS S942T was **stimulated**, while the alanine mutants were both inhibited by 60-70%. As the hydroxyl group of the serine residue lies within H-bonding distance of the O4 and N5 positions of the isoalloxazine ring of FAD in the nNOS structure[8], it is interesting to speculate on the effects of these mutations on overall catalysis.

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ML10 FORMATION OF NITRIC OXIDE FROM XENOBIOTICS

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Several physiopathological situations correspond to a deficit in the biosynthesis of nitric oxide (NO) in some tissues or organs. In such circumstances, it is important to increase the level of NO locally. This is usually performed by administration of NO donors such as trinitroglycerin, which are unstable compounds with very low half-lives *in vivo*, and which have a very low selectivity for their targets. Another possible approach towards more selective compounds, that would lead to an increase of NO formation in a given tissue or organ, is to act on the two main enzymes involved in the metabolism of L-arginine, the NO-synthases and the arginases. In that context, a first strategy was to find exogenous molecules, xenobiotics, that would act as substrates of NOS with formation of NO. The administration of such molecules should lead to a selective increase of NO formation via a given NOS. A second strategy was to inhibit the arginases that catalyze the consumption of L-arginine with the formation of ormithine and urea, with the hope that the use of arginase inhibitors would locally increase NO formation by increasing the pool of arginine available for NOS.

As far as the first strategy is concerned, it was important to determine the structural factors that are required for a compound to be recognized by NOS and to be oxidized by these enzymes with formation of NO. For that purpose, we have synthesized a great number of N-substituted guanidines and N-hydroxyguanidines, and we have compared their modes of binding and affinities for NOS I, II and III, as well as their oxidation by these enzymes[1,2]. Most guanidines and N-hydroxyguanidines are well recognized by the NOS active site, with binding constants that may be similar to those of the NOS natural substrates, arginine and N-hydroxyarginine, provided that their substituent is small enough. However, only few of them are efficiently oxidi-

zed by NOS with formation of NO. This is particularly true for N-substituted guanidines, whose oxidation is more difficult than that of the corresponding N-hydroxyguanidines, as we only found so far very few exogenous guanidines acting as good substrates for NOS II. The best one, a fluorinated guanidine, is oxidized by NOS with formation of NO with a Vm value only 3-times lower than arginine itself [2]. This reaction not only occurs with recombinant NOS II but also in activated macrophages. The most active NOS substrates exhibit two characteristics : (i) they are well recognized by NOS active sites, and (ii) they markedly increase the rate of NADPH consumption by NOS. These properties are necessary but not sufficient, and supplementary properties appear to be required for obtaining a good NOS substrate.

Arginases, that catalyze the hydrolysis of L-arginine to ornithine and urea, are proteins involving a binuclear Mn (II) active site. We have synthesized powerful inhibitors of arginases, starting from the 3D structure of their active site. The most active inhibitors involved a N-hydroxyguanidine or amidoxine function and an (-aminoacid function separated by two or three atoms[3]. X-ray studies of several arginase-inhibitor complexes showed that the terminal (N)-OH group of these compounds acts as a bridging ligand of the Mn (II) atoms[4], after displacement of the H2O bridging ligand that is present in free arginase. Preliminary experiments indicate that such arginase inhibitors are able to increase NO formation in physiopathological situations involving a deficit of NO.

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ML11 TRACKING REACTIVE INTERMEDIATE STATES OF NO SYNTHASE UNDER EXTREME CONDITIONS OF TEMPERATURE AND PRESSURE

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The catalytic step of nitric oxide synthase (NOS), i.e., activation of oxygen and product formation, shows similarities, but also striking differences to the analogous P450 reaction. Especially, it involves the participation of an additional electron donor, tetrahydrobiopterin (BH4). The reaction is complex and fast, comprising elusive intermediates. Subzero temperature in the liquid state, and high pressure can be used as perturbing parameters to study this complex reaction step by step, and to characterize structurally intermediate states of the reaction. We used these techniques to study both NOS reaction cycles and to study specifically the second electron transfer from BH4 to the oxygen complex of NOS. Working under different experimental conditions with BH4 analogs, allowed to determine consecutive elementary steps. Intermediate species were analysed by UV/Vis absorbance and epr. Depending on the conditions, the ultimate intermediates were identified as ferrous and ferric NO complexes. CO binding studies under pressure revealed a kinetic control exerted by BH4.

ML12 REGULATION OF ELECTRON TRANSFER IN NEURONAL NITRIC-OXIDE SYNTHASE

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NOS consists of two functional domains: one is an amino-terminal oxygenase domain with a cytochrome-P450 (P450) like heme active site, the other is a carboxy-terminal reductase domain that contains the FAD, FMN, and NADPH binding sites. The two domains are connected by a calmodulin (CaM) binding site. CaM binding activates the interdomain electron transfers required for NO formation as well as

intradomain electron transfers in the reductase domain. Analysis of heterodimers consisting of one full-length nNOS subunit and one oxygenase-domain subunit support an inter-subunit electron-transfer mechanism for the wild-type nNOS, in that electrons for catalysis transfer in a Ca^{2+}/CaM dependent way from the reductase domain of one subunit to the heme of the other subunit, as proposed for iNOS. CaM-dependent regulation of nNOS is also controlled by an autoinhibitory domain identified within the FMN binding subdomain. An autoinhibitory domain deletion mutant (delta40) showed the activity of the wild type enzyme without Ca^{2+}/CaM and electrons in the mutant transfer from the reductase domain of one subunit to both of the oxygenase domains in a Ca^{2+}/CaM independent way, indicating that the electrons can be transferred via both inter-subunit and intra-subunit mechanisms. However, NO formation activity was exclusively linked to inter-subunit electron transfer and was observed only in the presence of Ca^{2+}/CaM.

We also studied NO formation activity in reconstituted systems consisting of the isolated oxygenase and reductase domains of nNOS with and without the CaM binding site. N^G-hydroxy-L-Arg (NHA), an intermediate in the physiological NO synthesis reaction, was found to be a viable substrate. Surprisingly, the NO formation activities with CaM binding sites on either reductase or oxygenase domains were decreased dramatically on addition of Ca^{2+}/CaM.

Taken together, these results suggest that the mechanism of regulation by CaM is not solely dependent on the activation of electron transfer to the nNOS hemes, but may involve additional structural factors linked to the catalytic action of the heme domain.

In addition to Ca^{2+}/CaM-dependent regulation, caveolin is known to down-regulate nNOS. To elucidate the mechanism of regulation by caveolin, we examined the effects of a scaffolding domain peptide of caveolin-1 (82-101: CaV1p1) on the catalytic activities of several nNOS mutants. CaV1 p1 inhibited Ca^{2+}/CaM-dependent but not independent-NO formation activity and the inhibition was partially recovered in the presence of excess Ca^{2+}/CaM. The results also indicate that (1) CaV1p1 can interact with the isolated reductase domain itself, (2) the presence of CaM is not essential for CaV1p1 inhibition, (3) the autoinhibitory domains of nNOS assist the inhibition of interdomain electron transfer from the reductase domain to the oxygenase domain by CaV1p1.

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ML13 STRUCTURAL AND FUNCTIONAL EVIDENCE FOR THE EVOLUTION OF PROKARYOTIC NO SYNTHASES FROM AN ANCESTRAL CYTOCHROME P450

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This talk will utilize nitric oxide synthase (NOS) as a model system to address the question of how novel functions evolve from preexisting protein templates. In mammals NOS is a self-sufficient enzyme in which a heme-thiolate containing oxygenase domain is fused to a di-flavin reductase domain. NOS catalyzes the five-electron oxidation of L-arginine to NO and L-citrulline. The cofactor tetrahydrobiopterin (H4B) and a high-affinity protein-protein interaction involving calmodulin (CaM) are a *sine qua non* for this reaction to occur. When we discovered NOS-like proteins in human pathogens, *Bacillus anthracis* and *Staphylococcus aureus*, we were surprised to find the catalytic heme domain in isolation. Although catalytically self-sufficient P450s like BM_3 are present in these organisms, a reductase domain is not attached to the C-terminus of the NOS-like protein. In addition, bacteria that encode a NOS-like protein do not have the capacity to biosynthesize H4B or CaM. Given these observations, we asked: (a) do prokaryotic NOS-like proteins function by generating NO? and (b) what reaction(s) do they catalyze? By combining high-resolution X-ray crystallography, molecular biology, proteomics, chemical genetics, and spectroscopy we have determined that NOS-like proteins have novel enzymatic activities that share similarities with co-factor independent

ML14 BIOINFORMATIC INSIGHT INTO THE UNITY AND DIVERSITY OF CYTOCHROMES P450

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During the past few decades the cytochromes P450 (CYPs) were the subject of extensive research owing to the ability of these enzymes to serve as drug targets and to their active participation in the drug metabolism. Mean-

while other functions and varieties of cytochromes P450 were discovered and currently this superfamily comprises over 2000 different protein species. In the present study the protein sequences of cytochromes P450 were submitted to the computer analysis for elucidation of the structural basics of their pronounced functional diversity. The basic local alignment search tool (BLAST) was used to demonstrate that CYP protein sequences share a certain general similarity; at the same time, it was shown that the CYP superfamily may be split into a number of groups of intimately related proteins. These groups, the families, were revealed by means of cluster analysis which demonstrated strong hierarchy among the animal, bacterial and fungal P450s, and the lack of such hierarchy for the plant enzymes.

Multiple alignment and consensus sequence analysis were the approaches taken to find out which structural peculiarities of P450s are responsible for the deviations from the random picture. Proteins within each family were aligned and collapsed to the corresponding consensus sequences, the alignment of which produced the consensus for the whole superfamily.

By the statistical methods it has been shown, that the distribution of the conserved positions along family consensus sequences is not even. In particular the conserved sites tend to make the compact clusters. Due to their non-random character it can be expected, that these clusters constitute the structural-functional motifs.

The superfamily consensus yielded a number of unity motifs (which are common to many P450 species, and which are mostly related to the heme-fixing assembly); whereas the cross-family comparison of consensus sequences enabled to retrieve some of the *diversity* motifs. Three consensus sequences (for the CYP51 and CYP2 families and for the superfamily) were compared to line up the unity and diversity motifs with the appropriate X-ray data.

Table 1.

Model quality	(Template) Cytochromes P450
	(CYP2C5)
good	2B11-2B12; 2B16; 2B19; 2C1-2C4; 2C6-2C20; 2C22-2C30; 2E1-2E2; 2F4; 2H1-2H2
average	2A1-2A11; 2A13-2A16; 2B1-2B6; 2B9-2B10; 2B13; 2B15; 2C31-2C32; 2C34-2C36; 2C42; 2D1-2D6; 2D9-2D11; 2D14; 2D16-2D18; 2D25; 2E1; 2F1-2F3; 2G1; 2J1-2J6; 2K4
satisfactory	1A1-1A7; 1B1; 2B7; 2C33; 2L1; 14A1-14A3; 17A1; 18A1; 21A1-21A3; 21B1; 23A1; 33A1; 33B1; 33E1-33E2; 34A5; 34A10; 35C1; 36A1; 71A20; 71D7; 76C2; 76C4; 98A1
	(CYP51A)
average	51A from plants
satisfactory	51A from mammals and yeast
	(CYP55A1)
good	55A1-55A3
average	105A1; 105A3; 105B1; 105D1
satisfactory	105A2; 105C1; 105E1; 107A1; 107B1; 107C1; 107D1; 107E1; 107G1; 107H1; 107J1; 109B1; 112A; 141A
	(CYP101A)
good	101A
average	127A
satisfactory	107E1
	(CYP102A)
good	102A; 102A3
satisfactory	3A2; 3A27; 13A6; 72A1; 72A14; 72C1
	(CYP107A)
good	107A01
average	105E1; 107B1; 107C1; 107G1; 107H1; 107J1
satisfactory	55A1-55A2; 105A1; 105A3; 105B1; 105C1; 105D01; 107D1; 107E1; 107F1; 109A; 109B1; 112A; 112A2; 113A1; 113B1; 114A; 121A; 121A01; 123A; 126A; 130A; 140A; 146A; 154C1
	(CYP108A)
satisfactory	107G1; 111A; 124A; 125A; 126A; 130A; 142A
	(CYP119A)
satisfactory	106A1-106A2; 107B1; 107H1; 107J1; 109A; 109B1; 113B1; 124A; 142A
	(CYP121A1)
satisfactory	55A01; 105A1; 105B1; 105C1; 105D1; 105E1; 107C1; 107D1; 107E1; 107F1; 107G1; 107H1; 112A
	(CYP154C1)
average	107B1
satisfactory	55A3; 105A1; 105D1; 107A1; 107C1; 107D1; 107E1; 107G1; 107H1; 107J1; 111A; 112A2; 123A; 129A; 141A
	(CYP165B)
average	105A; 105E1; 146A
satisfactory	55A1-55A3; 105A; 105A1; 105A3; 105B1; 105C1; 105D1; 107A1; 107B1; 107D1; 107E1; 107G1; 112A; 112A2; 122A1; 140A; 141A
	(CYP175A1)
-	-

ML16 GENERAL TRENDS IN 3D MODELLING OF CYTOCHROMES P450

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Currently >1000 P450s sequences are known and only 12 crystal structures (~ 1%) are available from PDB. Apparent deficiency of known 3D structures forces researchers to use the computer modelling. The main method suitable for creation of P450 models is homology modelling based on sequence and structural similarity between model and homologues with known 3D structures (templates). However this approach meets significant difficulties stipu-

lated by low identity of model and template sequences. CASP[1] experiments have shown that the accuracy of homology modelling depends on sequence identity. The model is almost good if identity > 40%. In case of P450s - all 12 known templates belong to different families. At the same time the classification of P450s is founded on sequences similarity and the mean value of identity for families' demarcation is 35-40%. Thus some common conclusions can be done: (i) each known structure of P450 can be used as template only for modelling of P450s from the same family as template; (ii) mismatching between 1D and 3D alignments of templates does not allow to utilize full set of known templates for comparative homology modelling; (iii) any attempts to model P450s from other than template's family will give the models with considerably low accuracy especially in the areas of surface loops. In case of rather low identity a special procedure must be done to determine the statistical significance of the alignment - comparing the actual alignment score with the average alignment score of random sequences derived from the original ones. The universal criterion for quality assessment of probable models is alignment significance score[2] (S). We calculated the values of significance score for all known P450s sequences on the basis of 12 available templates. The obtained results are presented in *Table 1* and can be considered as the prognosis on current state for P450s modelling. The quality of probable models was estimated by S values: good (50-80), average (30-50), satisfactory (20-30). These prospects of P450s modelling are increased practically with each new 3D structure. As the announced structures of human CYP2C9 and CYP3A4 solved by Astex[3] were not published in PDB and thus not available for P450 community, the best template for modelling of several eukariotic P450s nowadays is CYP2C5 (identity up to 40-60%) with CYP2 family members.

Designed model requires deep optimization by long time molecular dynamics simulation and energy minimization. However, there is no uniform optimal protocol for these procedures. Moreover, the problem, what model optimized with or without molecular dynamics simulation is more adequate to natural protein, is still discussed. So, in any case all results of molecular modelling must be validated (by structural, topological, molecular dynamics, statistic and experiment verification).

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ML17 CYTOCHROME P450'S FRIENDS AND FOES: COMPLEX RELATIONSHIPS UNDER COMPUTATIONAL ANALYSIS

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Cytochromes P450 are dependent on other proteins for their in vivo function. Redox proteins act as helper proteins providing electrons for the reaction catalysed by cytochrome P450s. On the other hand, proteasome-dependent degradation following ubiquitination at specific domains has been observed for cytochrome P450s[1,2]. Both types of P450-protein interaction may influence and be influenced by substrate binding and turnover as well as post-translational modifications such as phosphorylation. We are applying a range of computational approaches to gain insights into the determinants of these inter-related contributions to cytochrome P450 function and xenobiotic regulated turnover. In this presentation, insights from comparative modeling of mammalian cytochrome P450s into binding recognition determinants will first be discussed. Then results of a combined quantum-mechanical/continuum electrostatics procedure that we have developed for computing absolute redox potentials will be shown for two-iron-sulfur cluster ferredoxins related to those involved in electron transfer for class I cytochromes P450. Redox potentials can be computed with good accuracy and the calculations provide insights into the determinants of the redox potential value and should aid the design of mutant proteins with altered redox properties.

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ML18 NEW INSIGHTS INTO MICROBIAL CYP BIODIVERSITY

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The post-genomic evaluation of microbes has revealed unexpected diversity with some possessing a CYP complement even exceeding the numbers found in animals, such as in the higher basidiomycete fungus *Phanerochaete chrysosporium*. Most fungi and protists contain CYPs but the simple eukaryotes *Gamblia lamblia* and the malarial parasite *Plasmodium falciparum* did not and are presumably therefore sterol auxotrophs as at least CYP51 is essential for sterol synthesis.

Many bacterial genomes have now been sequenced and an absence of CYP genes is common in the proteobacteria and archaebacteria where about two-thirds of species fall into this category. CYP51 has been found in gram positive and gram negative bacteria and current thinking on the origin will be discussed together with novel CYP forms and classes recently revealed. The actinomycetes have revealed unexpected diversity with 1% of all genes being CYPs in *Mycobacterium smegmatis* and *Streptomyces avermitilis* containing 33 CYP genes involved in many cases in aspects of secondary metabolism. Functional genomic studies on the superfamily in microbes began with yeast CYP61 and will assist traditional approaches to revealing the roles of these genes and proteins.

ML19 BALANCE SHEET FOR CYTOCHROME P450 KNOWLEDGEBASE: 2000 PROTEIN SEQUENCES, 10000 CHEMICAL SUBSTANCES

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Introduction

The overall growth of the amount of information on cytochromes P450 requires the automatic means for parsing and annotation of the data. Relying on the previously proposed formalism for describing each P450 through its structure (i.e. protein sequence) and function (substrate, reaction, inhibitor and other) we describe the rules used to populate this data fields. These rules appear as a result of incorporation of the expert's opinion to the bioinformatic algorithms and thus we refer the developed system as a knowledgebase.

Materials and Methods

The data model for the knowledgebase was proposed earlier[1] in the frameworks of Cytochrome P450 Database. This Web-based application provides the input and display for the nomenclature, structure and function of cytochromes P450. To the end of 2001 there were 1200 different enzymatic species in the database.

Updating the structural data.

The cross-database comparison has shown that there is a definite threshold for the results of local alignment searches for new P450s in the global database. These were: 10^{-4} for the expectation value and 70 for the score. Guided by these figures we mined the global databases making each of the available P450s a query in a turn. Best hits were submitted to global alignment to declare whether the hit is identical to the existing entry, or it is a fragment, allelic variant, or homologue, which joins the existing (sub)family. The latter case required the cluster analysis to confirm the classification[2].

Obtaining the facts related to P450 functioning.

The sample of all P450-related PubMed abstracts (42 000 texts) was compared to the training set of the abstracts that describe the interaction between a P450 form and a chemical substance. From the comparison we have captured the words, whose frequency of occurrence assists in distinguishing the topic of the abstracts. Basing on this data and on the data from existing database the vocabularies of special terms were produced under the expert-control (e.g. vocabulary of systematic names of P450, of species names, names of low-molecular weight compounds, reactions, and etc.). Besides, the abstracts' sentences, which provide the fact of interaction (or non-intercation) between P450 and a ligand, were aligned to produce the stable linguistic constructs - the patterns. Vocabularies and patterns were used to develop the core of automatic extraction of information from the P450-related texts. The approach has generated the questionnaires for the experts, and upon the expert-provided correction the data entered the database.

Results

We have downloaded more then 500 new P450 sequences to the database. The current distribution of sequences among families and kingdoms is reported. Peculiarities of P450-oriented BLAST searches are discussed.

From the natural language processing we have obtained the vocabulary of over 10000 names of chemical

substances that were mentioned in relation to cytochromes P450. It was shown that the patterns are an effective tool to unravel the special terms from the biomedical text, especially when the results undergo extra filtering. The filtering included the comparison of the compound's name to MeSH (Medical Subject Headings) collection of terms. In 75% of cases the expert confirmed that the automatically selected abstract contains the information related to the interaction between the P450 from and drug/chemical substance. Parsing of the abstract demanded the correction in 68% of cases, but even in this case the correction required the „yes/no" answer and thus the efficiency of data input was high. The number of facts (P450 name - ligand) stored during the 2 months of work exceeded twice the manual input of the last five years. Currently the knowledgeable incorporates in total ~1900 facts, each in the relation to the corresponding MedLine record.

Conclusions

P450-related research provides a field, where the automatic means of data mining and annotation can be extremely effective. Their further development and application should enable the accumulation of enough facts, and these facts, combined with the standard QSAR and 3D bioinformatics will enable the real steering of the experimental work.

Acknowledgements

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2 LECTURES – TUESDAY – TL

TL01 RATE-LIMITING STEPS IN CYTOCHROME P450 REACTIONS

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Cytochrome P450 (P450) enzymes use a complex catalytic cycle, usually described with a minimum of 9 steps even without including any conformational changes. A general perception often imparted in the literature is that most P450 reactions are limited by the rate of transfer of either the first or second electron to the P450 heme, and another is that catalytic activity towards a substrate is simply the result of good docking in an active site. We have examined intermolecular kinetic hydrogen isotopes on k_{cat} (^{D}V) and K_m (^{D}K) as a means of investigating the rate-limiting aspect of C-H bond cleavage in P450 reactions. Both ^{D}V and $^{D}(V/K)$, the isotope effect on the ratio k_{cat}/K_m, are considered in interpretations of P450 mechanisms.1

P450 2D6

^{D}V and $^{D}(V/K)$ for the O-demethylation of 3- and 4-methoxyphenethylamine were in the range of 3-4.[2] This particular case is complicated by an apparent substrate inhibition at high concentrations; evidence has been presented that this inhibition can be explained by the oxidation of substrate to an inhibitory nitroso product.[2] The mutation E216Q decreased k_{cat}/K_m 400-fold but did not change $^{D}(V/K)$.[3]

P450 1A2

We previously reported ^{D}V and $^{D}(V/K)$ values of ~ 3 for the O-dealkylation of phenacetin and >10 for acetol formation with human P450 1A2.[4, 5] The basis of the different isotope effects for the two different reactions with the same substrate is still under investigation.

Further work with (rabbit) P450 1A2 shows high ^{D}V values for the O-dealkylation of other 4-substituted anisoles, in the range of 7-9.

The values for the intermolecular kinetic isotope effects are still less than the intramolecular isotope effects measured for the demethylation reactions and are consistent with partially-limiting C-H breaking. However, k_{cat} still appears to be less than the rate of bond breaking, with a contribution of a step late in the oxygen activation process with both P450s 2D6 and 1A2.[2, 5]

P450s 2E1 and 2A6

Work on the oxidation of ethanol and acetaldehyde by P450 2E showed deuterium isotope effects on K_m but not k_{cat}, due to the burst kinetics in these reactions.[6, 7] In a simplified reaction with a slow kinetic step following product formation, the expressions for k_{cat} and K_m are complex and K_m is a function of k_3, the C-H bond-breaking step.

Studies on the oxidation of *N,N*-dimethylnitrosamine show isotope effects on K_m (^{D}K) of ~3 with human P450s 2E1 and 2A6. However, isotope effects approach unity for both enzymes in the oxidation of *N,N*-diethylnitrosamine. Studies with asymmetric *N*-methyl, *N*-ethylnitrosamine are in progress to understand the phenomenon, as well as more studies on the oxidation of HCHO and CH_3CHO.[7]

P450 3A4

Testosterone 6β-hydroxylation is one of the most used assays of P450 3A4 activity and has one of the highest rates. The 6-d_2 substrate has been prepared and initial studies show ^{D}V ~ 5. Although much of the modeling work on P450 3A4 has assumed that the enzyme has little specific bonding ability and does oxidations based largely on ease of bond breaking,[8] this is one of the few experiments to test this hypothesis. Whether isotope effects are seen with more slowly oxidized substrates is yet unknown. Current investigations are also directed to examination of the kinetic contribution to the cooperativity seen in some P450 3A4 reactions, including testosterone 6β-hydroxylation.[9,10]

Summary

High intermolecular kinetic deuterium isotope effects have been found in several reactions catalyzed by human P450 enzymes. The results suggest that C-H bond breaking can be partially rate-limiting but that several catalytic steps contribute to the observed k_{cat} values.[5, 11] The results also indicate that K_m is still a complex and poorly understood parameter in most P450 reactions.

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TL02 THE PROTEIN, THE ACTIVE SPECIES AND THE CHAMELEON: REACTIVITY PATTERNS OF OXYGEN TRANSFER BY CYTOCHROME P450

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Theoretical results on Compound I (Cpd I) and its reactivity patterns in hydroxylation and epoxidation will be reviewed.[1-9] I shall begin by showing that Cpd I behaves like a chameleon species that adopts its geometry and electronic structure to the protein environment in which it is accommodated.[1-4] I shall then proceed to show that the mechanisms of oxygen transfer by Cpd I involve two-state reactivity (TSR), and will argue that this mechanism leads to a resolution of the controversy[5-7] regarding the rebound mechanism of alkane hydroxylation.[8,9] Subsequently, I will discuss recent model calculations of C-H hydroxylation and C=C epoxidation pathways, which show that not only Cpd I is a chameleon state, it is also a chameleon oxidant that changes its reactivity and selectivity patterns under the influence of hydrogen bonding and polarization effects, which mimic the protein environment.[10,11] I will argue that using a chameleon species in many different protein environments is one of the ways by which the enzyme modulates its selectivity. *The Chameleon oxidant can serve as a new paradigm in enzyme catalysis.*

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TL03 P450'S PECCANT PROTON PATHWAYS: PERICLESIAN CONTROL OF ACTIVE OXYGEN

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The desire for a detailed understanding of the chemistry by which the cytochromes P450 catalyze substrate oxidation events has been paramount in the field since the discovery of these ubiquitous heme-thiolate oxygenases a half-century ago. Progress towards this goal has often been measured by the identification of the critical intermediate states of heme- iron and the staged reduction steps of atmospheric dioxygen during the catalytic cycle. Identification of the ferrous-O_2 complex in the early 1970s focused attention on the features of the cytochromes P450 which were distinct from that of the globins which, in their normal function, reversibly bound dioxygen without „activation" to a state chemically competent to transfer an oxygen atom to a recalcitrant carbon center. Yet various spectroscopic tools utilized in the 1970s and 1980s showed the oxygen complexes in these systems to have many shared features, suggesting that the critical differences in chemical reactivity were due to control by protein residues in the active site. A great breakthrough was provided by Poulos and colleagues in the early 1980s with the crystal structure of the ferric resting state of P450cam CYP101. The gene sequence of CYP101 and its heterologous expression opened the Pandora's box of mutagenesis to build on the road map provided by the Poulos structure. A wealth of modern bioorganic and bioinorganic chemical approaches with native and mutant enzymes followed and provided tantalizing hints as to the subtle control of electrons and protons in the processes of oxygen activation and substrate functionalization. However, despite these great advances, the close of the 20th century saw no new reactive intermediates of the

P450 reaction cycle cleanly defined by structural or spectroscopic tools.

Beginning in the new millennium, however, x-ray structure of the ferrous dioxygen bound state of CYP101 was determined. This structure revealed subtle but critical conformational changes of P450 upon dioxygen binding and pointed to a dynamic active site where water and side chain motion contribute to the critical hydrogen bonding network responsible for delivery of protons for productive oxygenation and/or uncoupling events. More recently, the use of cryoradiology and thermal annealing together with state-of-the art resonance and optical spectroscopies defined two new sequential intermediates of P450 catalysis, the peroxo- and hydroperoxo- states. In my presentation, I will describe our current understanding of the intermediates and proton / electron transfer events that describe the monoxygenase process. I will also describe very recent unpublished results. For instance, with further structural analysis, spectroscopic investigations and additional active site mutations in the cytochrome P450s we can, for the first time, separate the defining processes of proton delivery which lead to (1) the interconversion of peroxo anion and hydroperoxo states, (2) the protonation leading to dioxygen bond scission and (3) the very different pathway open in peroxide uncoupling. Similar intermediate states are observed in other heme-oxygen systems, which leads to a unifying picture of the commonality of the peroxo-state and the linkage of protein dynamics to control water and side-chain positioning to define the „proton wires“ of dioxygen activation by the P450 monoxygenases.

TL04 OXYGEN ACTIVATION BY CYTOCHROME P450 AND NITRIC OXIDE SYNTHASE. MECHANISTIC EVIDENCE FROM STUDIES WITH P450-CAM AS A MODEL SYSTEM

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Cytochrome P450 is a versatile heme-containing oxygenase that transfers oxygen atoms from dioxygen to a wide range of organic substrates. Nitric oxide synthase is also an oxygenase; it first hydroxylates arginine to an N-hydroxyimine intermediate and then, in a second O-atom transfer step, forms citrulline and NO. Hydroxylation involves substrate binding to the ferric enzyme followed by reduction and oxygen binding to give the oxyferrous state. Addition of a second electron yields a peroxo-ferric intermediate, protonation of which generates a hydroperoxoferric state; lose of water then forms an oxo-ferryl porphyrin radical (Compound I). Although the latter is thought to be the ultimate oxidant, the peroxo- and hydroperoxoferric species have been proposed as secondary oxidants. The T252A mutant of P450-CAM was first prepared by Sligar and Ishimura and their co-workers. Because this mutant does not hydroxylate camphor, it must not form P450 Compound I. However, it still accepts electrons from NADH to give hydrogen peroxide, presumably via the peroxo- and hydroperoxoferric intermediates. Thus, T252A P450-CAM is the ideal mutant to test whether one and/or the other of these two species are capable of O-atom transfer. We have prepared a series of camphor analogues that contain reactive functional groups that are the site of O-atom transfer in order to investigate the mechanism of olefin epoxidation, ether O-dealkylation, amine N-dealkylation, thioether sulfoxidation and N-hydroxyimine denitrosation. With 5-methylenylcamphor, for example, we find that the T252A mutant is enzymatically active in olefin epoxidation, producing epoxide at 20% of the wild type rate. This clearly demonstrates that a second active oxidant is formed in the P450 reaction cycle. Results of our studies of oxygen activation by P450 with these model substrates, with emphasis on the involvement of a second oxidant in O-atom transfer, will be presented.

Funding from NIH (GM 26730). We thank Steve Sligar for providing the over expression system for T252A P450-CAM.

TL05 MOLECULAR MECHANISM OF P450CAM CATALYZED OXYGENATION REACTION REGULATED BY THE ASSOCIATION WITH PUTIDAREDOXIN

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In addition to being an electron shuttle to P450cam, Putidaredoxin (Pdx) is a conformational effector of P450cam. Low potential iron sulfur protein such as spinach ferredoxin and bovine adrenodoxin can transfer a first electron to P450cam but not a second electron, resulting in a very slow turnover of the P450cam monooxygenase reaction. This conformational change of P450cam induced by the binding of Pdx has remained to be solved. In order to characterize the Pdx-induced conformational changes in P450cam, we applied proton NMR to the P450cam-Pdx complex, and

also utilized the L358P mutant of P450cam as a model for the Pdx-bound P450cam, In the ring current shifted proton NMR spectra of carbon monoxy P450cam, the signals from the heme iron-bound Cys 357 protons, Thr 252 protons in P450cam, and 5-exo and 9-methyl protons of d-camphor were assigned. The Pdx-induced shifts for these proton signals suggest that the axial Cys moves closer to the heme iron, and that d-camphor moves toward the heme iron by 0.15~0.5 □. To gain further insight into the Pdx-induced structural changes in P450cam, we utilized L358P mutant as a model for Pdx bound P450cam, since the proton NMR spectrum of the L358P mutant was quite similar to that of Pdx bound P450cam. The X-ray crystallographic study of the CO form of L358P mutant showed that the heme plane tilts and Arg112, the site of binding with Pdx, is substantially dislocated in the mutant as compared with the wild type P450cam, in agreement with the NMR results. Moreover, the L358P mutant exhibited an enhanced association property with Pdx as compared with the wild type P450cam. This prompted us to examine the reactivities of oxy-L358P with the non-physiological electron donors such as dithionite and ascorbic acid. We observed enhancement of the cross reactivity with these non-physiological donors. Taken together, Pdx-induced structural changes in P450cam facilitate the monooxygenation reaction.

TL06 DESIGN AND SYNTHESIS OF NEW P450 ENZYME MIMICS

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P450 Enzyme models from our laboratory such as 1 and 2 (*Fig 1*), carrying aryl thiolate or alkyl thiolate ligands coordinating to Fe(III) displayed E_o ~ -600 and Ė700mV, that is far more negative than the corresponding values of the the resting state of $P450_{cam}$ (E_o = -300mV).[1], [2], [3]

Subsequently it was reported that the thiolate ligand in $P450_{cam}$ is hydrogen-bonded to amino acid residues of the protein backbone,[4] such that the negative charge at sulfur is distributed over a larger space segment than originally believed.[5] Since the electron donating character, i.e. the „point charge", at the fifth ligand coordinating to iron is the decisive factor to the redoxpotential of the heme-thiolate cofactor we decided to modify the proximal coordination site of **1** towards a more „diffuse" neagtive charge at/near sulfur in order to mimic more closely the natural ligand sphere.

We reasoned that such a „diffuse" negative charge could be created using a SO_3^- ligand coordinating to iron, we thus synthesized the cofactor models **3** and **4** conveniently from their correspending heme thiolate complexes, e.g. see **5** to **3**, *Fig. 2*.

By comparison of **3** and **5** it is obvious that by exchanging S^- for SO_3^- a very significant anodic shift of 285mV to E0 = -175mV can be observed which, within experimental error, coresponds exactly to the value of the high-spin E.S complex of cytochrome $P450_{cam}$. The complexes **3** and **4** not only resemble the native cofactor regarding electrochemistry but also with respct to reactivity. In this context regioselective epoxidation and halogenations of various substrates were catalyzed by **3** + PhIO and **4** + NBu_4OCl, respectively. Turnover approached 1800 at 0.1mol% catalyst concentration. The experimental results suggest that the active, oxidizing species generated in situ from e.g. **4** and PhIO is electronically similar to compound I, this conclusion is supported by DFT calculations.[6]

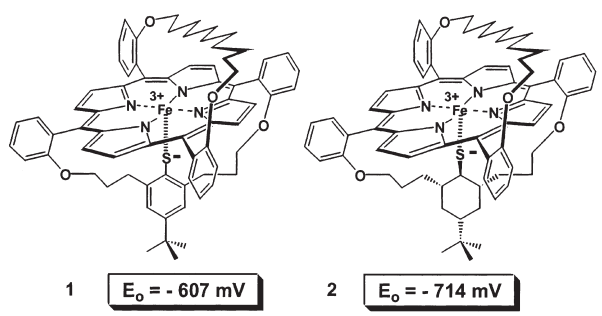

Fig. 1. *First generation P450 enzyme models of the resting state of* $P450_{cam}$

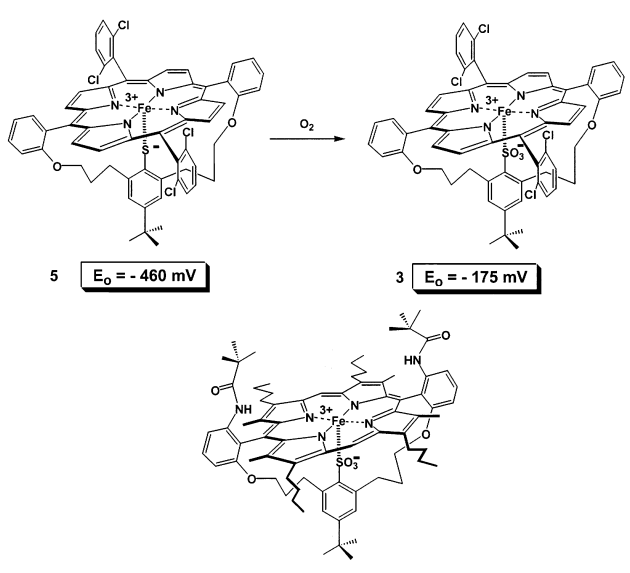

Fig. 2. *$P450_{cam}$ resting state models carrying a SO_3^- ccordinating to iron*

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TL07 DESIGN AND SYNTHESIS OF NEW FLUORESCENT PROBES FOR EVALUATION OF CYP3A4 DRUG-DRUG INTERACTIONS

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Cytochrome P450 3A4 (CYP3A4), the most abundant isoform in human liver and intestine, oxidizes many endogenous compounds as well as about half of the drugs on the market[1,2]. Although this enzyme shows broad substrate selectivity, it still exhibits high regio-and stereoselectivity. Furthermore the CYP3A4 catalyzed reactions present complex, non-Michaelis-Menten kinetics. The X-ray structure of the enzyme is still unknown despite decades of efforts but a large number of studies have been performed in order to better understand the mode of substrate/inhibitor/effector binding in the active site. These studies are of interest from a basic enzymology point of view, but also for practical applications. A large number of drug-drug interactions, leading to toxic or even lethal effects can indeed be explained by mutual inhibition at the CYP3A4 level of two co-administered compounds[3].

Recent studies based on UV-Vis spectral titrations[4] or fluorescence measurements[5] lead to the assumption that multiple substrates can bind within the active site, each having access to compound I. Models have been proposed to explain the behaviour of the different compounds tested but none of these models is able to predict the behaviour of a new ligand. This would be indeed of great importance for the screening of new drug candidates and the evaluation of their ability to produce drug- drug interactions at the CYP3A4 level.

We have designed and synthesized new CYP3A4 probes with the aim to rapidly screen the affinity of new compounds versus the CYP3A4 and to use these tools in the investigation of the CYP3A4 active site.

Favourably these compounds should be designed in such a way that their affinity to CYP3A4 could be measured directly and their displacement from the active site by potential drugs evaluated easily without performing the usual inhibition tests on a model substrate. We have therefore designed fluorescent derivatives whose binding to the CYP heme should be easily followed by direct spectral measurements such as fluorescence. Furthermore these inhibitors should display a Ki between 1-10 μM, which is considered to be a cut-off value to eliminate drugs binding too strongly to CYP 3A4 (i.e. able to displace the inhibitor from the CYP3A4 active site). We report here on the synthesis and the first tests performed with these new probes.

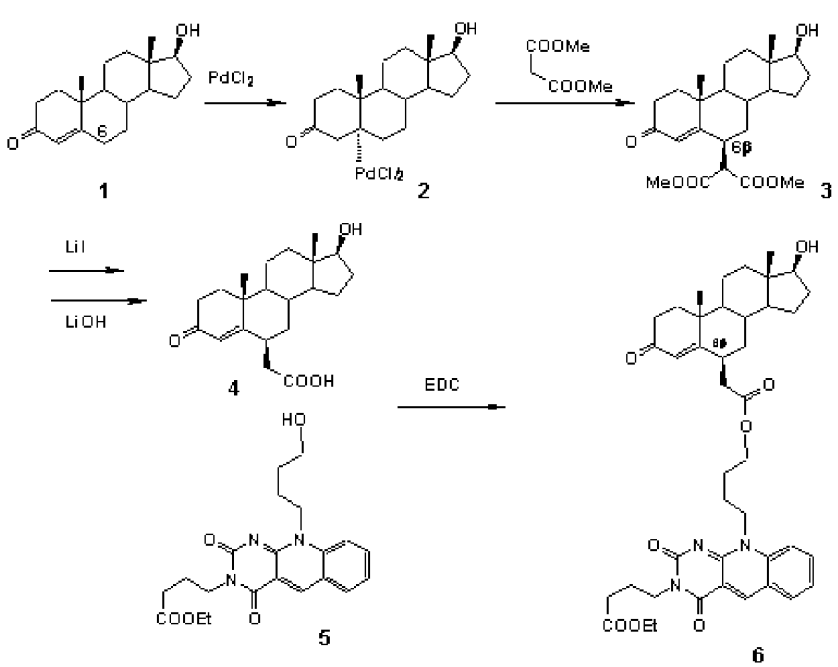

Fig. 1**. Synthesis of compound **6

The synthesis of one of the compounds synthesized as probe for CYP 3A4, the deazatestosterone derivative **6** is depicted in *Figure 1*. This derivative of testosterone should obviously bind to the „testosterone site“ and by ππ-stacking to the heme-thiolate of CYP 3A4 as indicated in *Figure 2*.

Compound **6** was used as inhibitor for the four model reactions generally used in inhibition tests, i.e. the metabolism of testosterone, midazolam, nifedipine and benzyloxycoumarine.

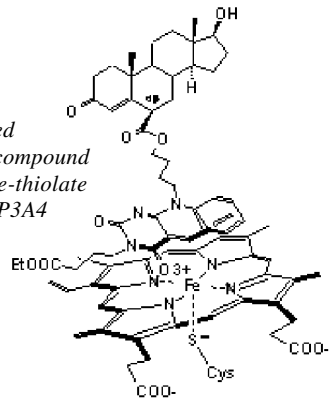

Fig. 2. *proposed interaction of compound* ***6*** *with the heme-thiolate cofactor of CYP3A4*

These four substrates are known to bind at different ligand binding sites in the CYP3A4 active site[4,6]. CYP3A4 selectively expressed in Baculovirus-insect-cells (Gentest-BD Biosciences) was used in these studies. The IC_{50} found with the four model substrates are all of the same range of magnitude (3, 0.5, 4.5 and 2 μM using testosterone, midazolam, nifedipine and benzyloxycoumarine, respectively) indicating that the probe compound **6** inhibits with the same potency the metabolism of different substrates, irrespectively of their binding in the active site.

It is easy to obtain the C-6α epimer of compound **6** by treating the testosterone derivative **4** under acidic conditions and coupling with the deazaflavine moiety **5** as indicated in *figure 1*. The IC_{50} obtained with the 6α-**6** are very similar to those obtained with the 6β isomer (2, 7 and 8 μM using midazolam, testosterone and nifedipin as substrates).

These results show that the two testosterone derivatives epimeric at C-6 may bind in the active site as indicated in *figure 2*.

Compound **6** and isomers are therefore good candidates for achieving a general binding of all sites present in CYP3A4. This is of great advantage in the evaluation of new drug candidates as ligands for CYP3A4 and offer new possibilities for testing their safety in co-medication with other drugs.

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TL08 OVERVIEW OF CYP GENE REGULATION WITHIN A TANGLE OF REGULATORY NETWORKS

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During species evolution, a complex signalling network, consisting of cytochromes P450 (CYPs) and other xenobiotic metabolizing and transporting systems (XMTS), and of specific nuclear xenoreceptors such as aryl hydrocarbon receptor (AhR)[1], pregnane X receptor (PXR)[2] and constitutive androstane receptor (CAR)[3], has been set up to cope with the risks presented by xenobiotics[4]. This network evolved in parallel with other signalling networks controlling endogenous metabolism. The consequence of this is that CYPs and XTMS may not be only regulated by xenobiotics, but also by physiopathological stimuli including glucocorticoids, sterols, biliary salts, pro-inflammatory cytokines and presumably others.

It has been known for many years that the AhR (member of the PAS family) controls CYP1A and other XMTS gene expression[1,4]. More recently, two xenoreceptors PXR (NR1I2) and CAR (NR1I3), activated by xenobiotics and drugs, have been identifed and characterised as members of the hormone nuclear receptor superfamily[2,3]. These receptors control the expression of CYP2 and CYP3A families, as well as of other XMTS, notably UDP glucuronyl- and glutathione-S-transferases, MDR1, and MRP2. Studying these regulations is important from a clinical point of view because they play a major part in the process of detoxication and AhR- as well as PXR/CAR-dependent enzyme induction is one of the major causes of drug interactions. Upon activation in response to xenobiotics such as TCDD, and rifampicin or phenobarbital, AhR and PXR or CAR transactivate their target genes as heterodimers with ARNT (AhR nuclear translocator) and RXR (retinoid X receptor), respectively. Human hepatocyte cultures represent the model of choice for investigating these receptors and their role on the CYP and XMTS gene induction. Indeed, the species-specificity that prevails in these processes precludes extrapolations from animals (rodents) to man.

We have developed specific tools (genomic clones, probes, antibodies) for PXR, CAR, glucocorticoid (GR) and vitamin D receptor (VDR, NR1I1). We investigated their regulation, functional interactions and role in the inducible expression of CYP2 and CYP3A genes. Our results showed that: i) expression of PXR and CAR is a limiting factor in

the detoxication process[5,6]; ii) expression and activity of these receptors is increased by glucocorticoids and decreased by pro-inflammatory cytokines (IL-1 and IL-6)[5-7]; iii) GR indirectly controls detoxication via the signal transmission cascade GR-PXR/CAR-CYP2/CYP3[8]; iv) CYP2C9 expression (one of major form present in adult human liver) is under the control of both GR and PXR/CAR, and we identified and characterised the 5'-response elements involved in this control[9]; v) VDR is able to bind to PXR- and CAR-"specific" response elements on CYP2B6, 2C9 and 3A4, and transactivate these genes[10]. This last observation is of critical importance. It shows that there are possible functional cross-talks between nuclear receptors whose specificity for their target genes is not as strict as previously thought. On important consequence of this is that xenobiotics and drugs might affect signalling pathways of endogenous metabolism including notably biliary salt and Vitamin D homeostasis. The aim of this lecture is to review: i) the recent developments on the mechanisms of induction, including the role of nuclear receptors such as AhR, PXR and CAR; ii) the possible interferences between xenobiotic-signalling pathways leading to CYP gene induction and other endogenous signalling pathways.

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TL09 MOLECULAR MECHANISMS OF TRANSACTIVATION OF AH RECEPTOR IN THE TARGET GENE EXPRESSION

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Arylhydrocarbon receptor was first identified as a binding factor to polycyclic aromatic hydrocarbons including 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) and 3-methylcholanthrene (3MC). The binding affinity of AhR was found to be extremely high toward TCDD and from genetic studies using mice, it was suggested that the AhR was involved in the inducible expression of xenobiotic metabolizing enzymes such as CYP1A1, 1A2 and UDP-glucuronosyltransferase. Molecular biological studies on the induction mechanism of the xenobiotic metabolizing enzymes led us to molecular cloning of AhR, revealing that AhR is a transcription factor with a charcteristic structural motif of bHLH-PAS which functions in association with a partner molecule, Arnt to activate the expression of the target genes in response to added inducers.

Under normal conditions, AhR exists in the cytoplasm in a complex with HSP90, XAP2 and P23. Upon binding with a ligand, the AhR complex translocates to the nuclei where AhR switches its partner molecule from the HSP90 complex to Arnt and then binds the XRE (xenobiotic response element) sequence in the promoter of the target genes to tansactivate their expression.

Although expression of CYP1A2 gene is also induced by dioxins through AhR and Arnt, its mode of expression is different from that of CYP1A1 gene whose expression is a prototype of the genes driven by the XRE sequence. While CYP1A1 is induced ubiquitously in various tissues by administration of inducers, both the constitutive and inducible expression of CYP1A2 gene is specifically limited to the liver. In AhR-KO mice, the constitutive expression of CYP1A2 gene was still retained, while the expression of CYP1A1 gene was totally abolished together with the loss of the inducible expression of CYP1A2 gene.

By DNA transfection experiments using the fusion gene consisting of the luciferase gene and the CYP1A2 gene upstream promoter sequence up to -9kb, we identified the inducible enhancer sequence of CYP1A2 gene which is

different from the XRE sequence of CYP1A1 gene. The enhancer sequence of CYP1A2 gene did not directly bind with the AhR and Arnt heterodimer, but interacted with it through factors directly binding to the sequence. Purification with affinity chromatography using CYP1A2 enhancer sequence and sequence analysis of the binding factor revealed that the binding factors were LBP-1a and 1c.

Transfection of the expression plasmid of LBP-1a or 1c enhanced the reporter gene expression driven by the CYP1A2 enhancer in response to the added inducer and this enhanced expression of the reporter gene were further strengthened by coexpression with AhR and Arnt. GST pull-down assay showed that LBP-1a and 1c physically interacted with AhR and Arnt. These results demonstrated that AhR and Arnt activate the expression of CYP1A2 gene as a coactivator in response to the inducers. The DNA binding site of the AhR/Arnt heterodimer was not necessary for the coactivator function.

This coactivator-like function of AhR/Arnt heterodimer was also observed with transactivation of ER (estrogen receptor) target genes such as cFOS and VEGF by interaction of non-liganded ER with the ligand-activated AhR/Arnt. The reporter gene consisting of the luciferase structural gene driven by the ERE (estrogen response element) sequence was activated by 3MC through AhR/Arnt in the presence of ER α or β without E_2. In this case, 3MC-activated AhR/Arnt interacted with ER bound on the ERE sequence to activate the expression of the reporter genes. The AF-1 region of ER was found to be the site for interaction with the heterodimer of AhR and Arnt, and the N-terminal DNA binding site of Arnt was not necessary for the coactivator function. These results indicate that the coactivator-like function is another common transcriptional role for AhR and Arnt.

We will also talk about functional roles of AhR in reproduction of female mice by using AhR knockout mice.

TL10 PATHOPHYSIOLOGICAL FACTORS AFFECTING CAR GENE EXPRESSION

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The drug-metabolizing cytochrome P450 (CYP) enzymes are down-regulated during inflammation. This suppression can result in increased clinical toxicity of drugs. Conversely, some drugs must be converted to their pharmacologically active or toxicologically active metabolites by these enzymes, and suppression of their metabolism can lead to a reduced therapeutic or toxic effect. Our resarch has focused on the negative regulation of the orphan nuclear receptor CAR (NR1I3) by proinflammatory cytokines such as interleukin-1β (IL-1β) and lipopolysaccharide (LPS) in human hepatocytes. CAR is a key transactivator of *CYP2B* and *CYP3A*, among others, upon phenobarbital administration. We found that IL-1β and LPS decrease CAR expression and suppress phenobarbital-mediated *CYP2B6* and *CYP3A4* induction in human hepatocytes. Moreover, our data suggest that activation of nuclear factor-kB (NF-kB) is a critical event in LPS- or IL-1β-mediated inhibition of CAR expression through the repression of ligand-activated glucocorticoid receptor action. Further, we observed that NFkB p65 interferes with the enhancer function of the distal glucocorticoid response element of the CAR promoter gene that we have identified recently. Finally, using chromatin immunoprecipitation, we demonstrated that the GR agonist dexamethasone induces histone H4 acetylation at the proximal CAR promoter region, whereas LPS and IL-1β inhibit this acetylation. These data suggest that GR/NF-kB interaction converges at the level of CAR transcription and involves chromatin remodeling. The results of this study may provide a mechanistic explanation for the long standing observation that sepsis provoke drug metabolism inhibition. Moreover, as CAR controls the expression of several genes involved in bilirubin elimination (the organic anion transporter SLC21A6, which facilitates the enter of free-bilirubin in the hepatocyte, the phase II UDP-glucuronosyltransferase 1A1 (UGT1A1) protein , the principal means to glucuronidate bilirubin, and the MRP2, which mediates the efflux of bilirubin diglucuronide across the apical membrane of the hepatocyte into the bile canaliculi), we hypothesized that NF-kB-induced CAR deficit may contribute to inflammation-induced hyperbilirubinaemia , as observed recently in CAR null mice.

TL11 INDUCTION OF RAT CYP2B GENES BY PHENOBARBITAL--LIKE INDUCERS : SOMETHING OLD AND SOMETHING NEW

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Hepatic CYPs play a critical role in the metabolism of hydrophobic xenobiotics[1,2]. The genes encoding these enzymes are either expressed constitutively or are induced by various chemicals[3]. Much progress has been made concerning the molecular mechanism whereby phenobarbital (PB) leads to the induction of the homologous rat *CYP2B2*

and *CYP2B1* and mouse *Cyp2b10* genes[4-9] and of the chicken *CYP2H1* gene[10]. Although the chicken *CYP2H1* gene is PB-inducible in a chicken hepatoma cell line[11], in the rodent system the only cultured cells in which *CYP2B* genes respond normally to PB treatment are primary hepatocytes[12,13]. The PB response unit (PBRU), a 163-bp *Sau*3AI fragment at coordinates -2317/-2155 in the *CYP2B2* 5'-flank, confers PB inducibility on heterologous promoters in primary rat hepatocytes and has the properties of a transcriptional enhancer[4,14,15]. The homologous region of the 5'-flank of the PB-inducible mouse *Cyp2b10* gene contains a 162-bp segment with similar properties[5]; it is 92% identical to the rat *CYP2B2* PBRU15.

The rat *CYP2B2* PBRU contains, among other putative transcription factor recognition sites, three direct repeats separated by 4 bp (DR-4), as well as an everted repeat separated by 7 bp (ER-7), of the nuclear receptor consensus hexamer half-site motif AGGTCA. Two of the DR-4 sites, NR1 and NR2, flank a nuclear factor 1 (NF1) site[16] and were recognized as putative nuclear receptor binding sites by Negishi and coworkers in the homologous mouse *Cyp2b10* fragment[16]. The third DR-4 site, NR3[17], is upstream of NR1 and NR2. Negishi and coworkers also defined a 51-bp PB responsive enhancer module (PBREM) within the mouse *Cyp2b10* PBRU sequence. The PBREM is limited to the NR1-NF1-NR2 elements and confers full PB responsiveness in primary mouse hepatocytes when placed directly adjacent to the heterologous tk promoter[16]. However, replacing the PBRU with the PBREM in the *CYP2B2* 5' flank, in the natural sequence context, reduces PB responsiveness in primary rat hepatocytes by at least four-fold6. This and other evidence6 indicates that sequences outside the PBREM are required for maximal PB responsiveness. Possible candidates for such sequences are the upstream NR3 site[17] and the overlapping ER-7 site. Indeed mutational inactivation of the ER-7A half site, which is the same sequence as the NR3B half site, reduces but does not abolish PB responsiveness; mutational inactivation of any NR1 or NR2 half site has a similar effect[6].

To determine which of NR3 or ER-7 are involved in conferring PB responsiveness, their unique half sites were mutated within the PBRU. The mutated plasmid reporter constructs were then tested in the natural sequence context for their ability to confer PB responsiveness in cultured primary rat hepatocytes. The results indicate that NR3 is the third element within the PBRU that is required to confer maximal PB responsiveness but the receptor that activates it remains to be discovered.

Negishi and coworkers[18,19] have shown that there is a PB-dependent nuclear accumulation of the constitutive androstane receptor (CAR) in mouse liver. CAR, in the form of a heterodimer with the retinoid X receptor (RXR) binds to the retinoic acid β2 response element (βRARE)[20,21] and to the NR1, NR2 and NR3 sites of the PBRU[17,22]. CAR-RXR heterodimers activate transcription of reporter genes driven by the PBREM and by oligomerized βRARE and NR1 sequences in cultured cell lines[9,18,19,22].

To assess the putative role of CAR-RXR binding to the PBRU NR sites in conferring PB responsiveness, individual base pairs of the NR sites were changed and the effects on CAR-RXR binding were determined. The same mutant sequences were incorporated into the NR sites within the PBRU and their effects on conferring PB responsiveness in luciferase reporter gene assays after transfection into primary rat hepatocytes were evaluated. The results suggest that something other than, or in addition to, CAR-RXR binding to the NR sites is required for maximal PB responsiveness.

Mutational analysis of a newly-identified HNF4 element present in the PBRU was undertaken, using a reporter construct with the PBRU in the natural sequence context transfected into primary rat hepatocytes. The results suggest that HNF4 acts as a negative regulator by competing for the binding to the NR1A half site thus preventing normal transcriptional activation via the NR1 element. At the NR2 site, mutation of the 4-bp spacer led to the loss of a prominent doublet in EMSA assays. The proteins responsible for this doublet were identified by supershift analysis. They were found to be Pbx1b-Prep1 and Pbx2-Prep1 heterodimers. The possible roles of HNF4 and Pbx-Prep1 as negative regulators of PB responsiveness are presently under investigation.

The glucocorticoid receptor interacting protein 1 (GRIP1) mediates PB-dependent nuclear translocation and activation of CAR *in vivo* but has been reported to have only modest effects *in vitro*[9]. Furthermore, normal PB responsiveness of *CYP2B* genes is not maintained in any known cell line. In an effort to understand the molecular mechanisms whereby PB induces *CYP2B* genes *in vivo* but not in cultured cells of hepatic origin, expression vectors for CAR, RXR and GRIP1 were cotransfected into HepG2 cells along with reporter constructs carrying the PBRU in different sequence contexts. CAR activated reporter gene transcription when the PBRU was juxtaposed to a basal promoter, but was essentially inactive when the PBRU was tested in the natural sequence context. GRIP1 dramatically increased CAR activation when tested with reporter constructs in which the PBRU was adjacent to the basal promoter and also, but to a much lesser degree, when the PBRU was in its natural sequence context. It may be that HepG2 and other cell lines lack as yet unidentified transcription factors that are necessary, in the natural sequence context, for the PBRU to confer PB responsiveness on the luciferase reporter.

Taken together, the results to be presented suggest that something other than, or in addition to, CAR-RXR binding to the NR sites is required for the rat *CYP2B2* PBRU to confer maximal PB responsiveness. Based on these results as well as others to be discussed, the following model is proposed to account for PB-dependent induction of the *CYP2B2* gene in primary rat hepatocytes. NR1 plus another site, herein identified as NR3, are principal binding sites for a PB-dependent transcription factor complex, presumably including CAR-RXR and as yet unknown factors.

NR2 is an accessory site. At least two of these three sites are required to confer a PB response. The maximal response is obtained when all three sites are functional and HNF4 and Pbx-Prep1 are not bound to their cognate sites.

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3 LECTURES – WEDNESDAY – WL

WL01 MITOCHONDRIAL CYTOCHROME P450 SYSTEMS: NEW INSIGHTS INTO STRUCTURE AND FUNCTION

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In the human adrenal cortex glucocorticoids, mineralocorticoids, and androgens are being synthesized. Three mitochondrial cytochromes P450 are involved into this reaction cascade. The initial step is the side-chain cleavage of cholesterol to form pregnenolone, catalyzed by CYP11A1. The 11β-hydroxylase (P45011B1) is responsible for the conversion of 11-deoxycortisol to cortisol. The aldosterone synthase (CYP11B2) catalyses the 11β-hydroxylation, 18-hydroxylation and 18-oxidation of deoxycorticosterone to form aldosterone.

Using site-directed mutagenesis in the C-terminal part of human CYP11B1 and CYP11B2 (putative I-helix region) we succeeded to change the regio- and stereoselectivity of steroid hydroxylation by very few point mutations[1,2]. Moreover, we were able to define the functional significance of amino acid 112 in the putative substrate access channel of human CYP11B2. Thus, we present the first example of an attribution of substrate recognition and conversion to the N-terminal part of human CYP11B2[3].

Mitochondrial cytochromes P450 get the electrons for oxygen activation and following substrate conversion from an iron-sulfur protein (adrenodoxin, Adx) of the [2Fe-2S] type and a FAD-containing reductase (AdR). To study the role of distinct amino acids of adrenodoxin in protein protein interaction and in electron transfer, mutants of adrenodoxin have been prepared by site-directed mutagenesis, expressed in E. coli, and their structural and functional properties have been characterised in detail over the past 10 years[4]. Different, but partially overlapping binding sites for the partner proteins have been found and mutants with improved electron transfer ability were obtained. A set of mutants, where residues Glu39, Glu41 and Thr49 were replaced led to the suggestion that Adx possesses a second, functionally relevant, binding site for the redox partners being apart from the negatively charged region between amino acids Glu65 and Asp79. This region is close to the one, which in the homologous ferredoxin of P. putida, putidaredoxin, is responsible for the interaction with CYP101. Moreover, calculation of the preferred electron transfer pathways using the program HARLEM revealed that this is the region with the highest conductivity for the electrons[5]. In addition, a mutant lacking the entire interaction domain was shown of still being able to accept electrons from AdR, whereas CYP11A1-dependent substrate conversion has been abolished. NMR studies of oxidised and reduced Adx together with biochemical data lead to the proposal of a new interaction mechanism among the three proteins[6]. The NMR technique was also used to characterise in detail the function of a conserved histidine for redox-dependent dynamics and the interaction region between Adx and cytochrome c.

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WL02 RESONANCE RAMAN AND FEMTOSECOND COHERENCE SPECTROSCOPY OF P450: INTERACTIONS WITH REDUCTASE, OXYGEN, AND NITRIC OXIDE

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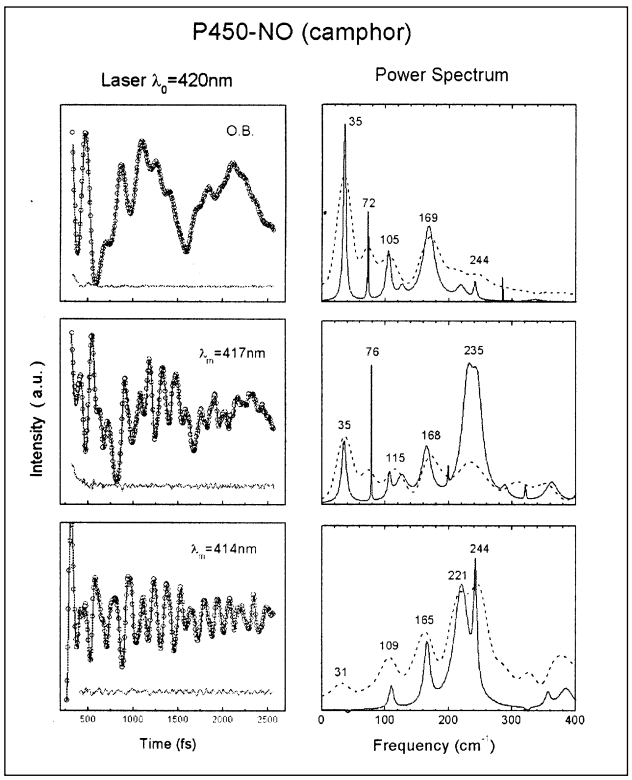
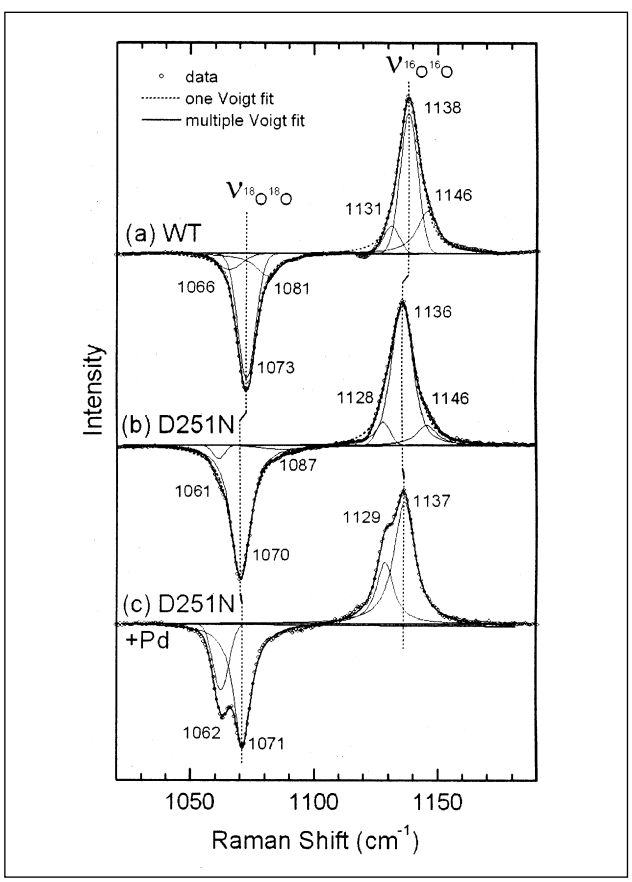
Resonance Raman and femtosecond coherence spectroscopies are complementary spectroscopic methods that operate in the frequency and time domains, respectively. These methods can be used to monitor structure and dynamics of the heme and its ligands in order to reveal conformational substates and to prepare and monitor coherent states of a sample under reaction conditions[1,2]. The experiments discribed here focus on the nuclear motions associated with the heme proteins such as cytochrome P450, myoglobin, hemoglobin, and cytochrome c. Subsequent to photolysis of a sixth axial heme ligand, the (initially planar) heme is left far from its final product state equilibrium geometry. This leads to coherent oscillations of those modes composing the reaction coordinate for ligand binding and dissociation. Analysis of the phase and amplitude of these oscillations can help to determine the details of the





reaction process. These studies, along with femtosecond continuum measurements of the spectral dynamics and ligand binding kinetics as a function of temperature suggest a general model for diatomic ligand binding in heme proteins.

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WL03 INTERACTIONS AMONG REDUCTASE AND MULTIPLE P450 ENZYMES IN MIXED RECONSTITUTED SYSTEMS

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Oxygenation reactions involving the cytochrome P450 system require interactions with other proteins to permit the transfer of necessary electrons to the heme protein. The flavoprotein NADPH-cytochrome P450 reductase (reductase) is essential for P450-dependent monooxygenase reactions, and has been shown to form a 1:1 molar complex with P450 proteins (Miwa and Lu, 1984;Miwa and Lu, 1984;Miwa *et al*., 1979). Each of these proteins is associated with the microsomal membrane. Interestingly, there is a large excess of P450 over the reductase, calling to question how the limiting concentrations of reductase can supply the necessary reducing equivalents to the excess of P450 molecules. Additionally, P450s exist in multiple forms, with each form having its own substrate selectivity and reductase binding characteristics. These characteristics raise the question: „How are the proteins of the P450 system organized within the microsomal membrane?“ In an effort to address this question, we have characterized the interactions of microsomal proteins in reconstituted systems containing reductase and multiple P450 enzymes.

We have previously examined the potential for one P450 to affect the catalytic behavior of another P450 isozyme. Interactions between P450s were demonstrated by comparing the catalytic behavior of CYP2B4 and CYP1A2 in simple and mixed reconstituted systems. Two substrates that are preferentially metabolized by CYP2B4 (benzphe-

tamine and 7-pentoxyresorufin (7PR)) were used to demonstrate the interaction. Whereas benzphetamine demethylation showed a small increase in catalytic activity when both CYP1A2 and CYP2B4 were present in the reconstituted system, CYP2B4-dependent 7-pentoxyresorufin-O-dealkylation (PROD) was found to be dramatically inhibited by the inclusion of CYP1A2 (Cawley *et al*., 1995). Interestingly, this inhibition of PROD was much larger when reductase levels were subsaturating. The results are consistent with the formation of a high affinity complex between CYP1A2 and reductase that prevents reductase binding and electron transfer to CYP2B4, particularly when reductase levels are subsaturating. Kinetic analysis of PROD activity at a series of reductase concentrations showed the system to be consistent with the formation of heteromeric CYP2B4-CYP1A2 complexes. In the presence of 7PR, the CYP1A2 moiety of the complex has an increased affinity for reductase, effectively drawing the reductase away from CYP2B4 and inhibiting this CYP2B4-dependent activity (Backes *et al.*, 1998). Similar interactions have also been observed in rabbit liver microsomes. Rabbits were treated with either phenobarbital (PB), β-naphthoflavone (βNF), or both inducers to enrich the microsomes with CYP2B4, CYP1A2, and both enzymes, respectively. The relative rates of benzphetamine demethylation and PROD were similar to those found in the corresponding reconstituted systems (i.e. benzphetamine metabolism was stimulated in rabbits treated with both inducers, whereas PROD was inhibited). These results suggest that the interactions observed in the reconstituted systems are not an artifact of the reconstitution process, but are also observed under the more natural conditions of the microsomal membrane (Cawley *et al*, 2001).

Several approaches have been used in an effort to characterize the interactions among these proteins. The effects of lipid concentration on reductase binding were examined in simple reconstituted systems containing only a single P450, and in mixed reconstituted systems containing both CYP1A2 and CYP2B4. Although the apparent affinity for reductase-CYP2B4 was not significantly affected, there was a 90% decrease in CYP2B4-dependent activities at saturating reductase when the lipid:P450 ratio was increased 10 fold. Interestingly, CYP1A2 under the same conditions showed only a modest decrease (~30%) in activity at high lipid concentrations. Again, the apparent affinity of reductase for the P450 was not substantially affected by lipid concentration.

We also examined the potential for the complex between CYP1A2 and CYP2B4 to be mediated by ionic interactions. The effect of ionic strength on PROD was examined using reconstituted systems containing reductase/CYP2B4, reductase/CYP1A2 and the mixed reconstituted system (CYP1A2/CYP2B4/reductase). PROD in each reconstituted system was affected by ionic strength, reaching a maximum at 50 mM sodium phosphate (pH 7.4). The activities continued to decrease at buffer concentrations of 75 mM, 200 mM and 500 mM. At low ionic strength, PROD activities in the mixed reconstituted systems were lower than observed for CYP2B4/reductase binary system, consistent with the interaction between CYP1A2 and CYP2B4. Interestingly, as the phosphate concentration was increased above 75 mM, inhibition of PROD in the mixed reconstituted system was no longer observed. In fact, at 500 mM phosphate, a synergistic response was obtained. These results are consistent with complex formation between CYP2B4 and CYP1A2 being mediated by ionic interactions.

The stability of the binary and mixed reconstituted systems was also examined. In these studies, reconstituted systems were prepared and examined after preincubation for up to 4 days. CYP1A2-dependent PROD was not diminished by preincubation, even exhibiting a small increase after preincubation for 4 days. In contrast, CYP2B4 activity showed a continual decline with each day of incubation. After preincubation of the reconstituted system for 4 days there was a 65% decrease in CYP2B4-dependent PROD. Interestingly, in the mixed reconstituted system, there was a continual increase in PROD activity with prolonged incubation times. When comparing CYP2B4/reductase and the CYP2B4/CYP1A2/reductase reconstituted systems, the characteristic inhibition of PROD in the mixed reconstituted system was no longer observed after preincubation for 4 days. These results are consistent with a slow reorganization of the proteins in the reconstituted system with time. (Supported in part by NIEHS 04344, and the Stanley S. Scott Cancer Center).

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WL04 EXPERIMENTAL *IN VITRO* MODELS OF DRUG METABOLISM

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The search for an ideal experimental model for *in vitro* studies on drug metabolism has continued since pharmacology was established as a respected discipline of medicine. Most recently, an increasing knowledge on properties of individual drug metabolizing enzymes in various species of experimental animals contributed significantly to understanding of the interspecies differences and to better choice of a species suitable for a given type of experiment. For example, the rat and the systems derived from rat are used now mostly as a first attempt to get the basic information on possible metabolic routes. Here, the experiments at the level of microsomal fraction are performed first. When an involvement of cytochromes P450 in metabolism of a drug is found, then the more reliable models should be used taking advantage from known properties of substrate specifity of cytochrome P450 forms in respective animal species[1]. Here, the more detailed study requires a model which can give answers on the role of a particular form of cytochrome P450 enzyme. The CYP3A29 form of pig or minipig is most probably the closest counterpart of the human CYP3A4 enzyme[2]. The studies with minipig or pig hepatocytes[3], microsomes[4] as well as with the reconstituted CYP systems[2] indicate their suitability in following the processes mediated by this CYP3A subfamily. For metabolic transformations dependent on CYP2D, the dog seems to give the results[5] close to those obtained with human CYP2D6. The CYP2C family of rat does not seem to be a good model of the human counterpart as it has different substrate specificities[6]. Hence, the results obtained with mouse models where CYP2C enzymes were found to participate should be taken with care. For CYP2C, the most suitable model seems to be the Rhesus monkey[7]. The CYP1A2 and CYP2E1 enzymes of man are very close in their properties to those of other species thus allowing their use in studies on drug metabolism.

Hence, it can be concluded that there is not an ideal single experimental animal model system available even when properties of many human CYP enzymes are close to those of the respective models. For studies on biotransformation of a compound, the results obtained with animal - based systems are of a preliminary character. An experiment with commercially available preparations consisting of a defined human enzyme or enzymes is inevitable. Although an ideal animal model apparently does not exist, the known similarities between P450s of animal and human origin may however be used in rationalizing the possible use of cells (hepatocytes) for construction of bioartificial liver-supporting devices.

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WL05 SHOULD WE PERSONALIZE DRUG THERAPY ACCORDING TO CYTOCHROME P450 GENOTYPE AND/OR PHENOTYPE?

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Large prospective randomized double-blind clinical trails are the gold standard to assess safety and efficacy of drug therapy. However, these studies are measuring average drug effects, and even if a clear benefit is observed based on clinical endpoints, there are individuals with a lack of therapeutic effect, and there are patients with an excessive drug response. More than 5 % of in-patients suffer from serious adverse drug reactions[1], and it has been estimated that at least half of these reactions could have been avoided by appropriate selection of drug and/or drug dose. Thus, a higher degree of personalization in drug therapy is expected to considerably improve the risk/benefit ratio.

Main reasons for the interindividual heterogeneity in drug response include heterogeneity in the disease(s) treated, in drug targets, in comedication, and/or in drug concentrations. Heterogeneity attributable to drug concentrations may be handled by a personalized dose adjustment. This may be done based on clinical response, on drug concentration (=therapeutic drug monitoring), or an factors known to predict clinical response and/or drug concentration.

Cytochrome P450 enzymes (CYPs) mediate rate-limiting steps of inactivation (or activation) of many important drugs and show pronounced interindividual differences in activity. It is reasonable to assume that individual activity of the respective CYP may have a major impact on the dose vs. response relationship of a drug. If this CYP is genetically polymorphic, the individual genotype may be the major factor determining enzyme activity. However, it is obvious that CYP polymorphisms and/or CYP activity is several levels of hierarchy away from drug response, and only if its impact is major on all subsequent levels, individual CYP activity is expected to be relevant for therapeutic outcome.

Several decades ago, it has been recognized that individual CYP activity (at this time not identified as such) may be related to differences in drug effects. During the last decade, extensive research has been carried out in order (i) to identify variant CYP genes related to aberrant activity, (ii) to identify enzymes involved in the metabolism of individual drugs, (iii) to assess frequencies of CYP alleles in different populations, (iv) to quantify the impact of CYP variants on pharmacokinetics of individual drugs, and (v) to develop methods for CYP phenotyping. As a result, e.g. today genotyping identifies >99 % of CYP2D6 poor metabolizers[2], individual elimination rates of tolbutamide may be derived from the contribution of individual CYP2C9 alleles[3], or 2/3 of the variation in docetaxel pharmacokinetics may be explained by individual CYP3A4 activity[4]. Furthermore, pharmaceutical companies incorporated CYP genotyping and CYP phenotyping in the drug development process.

Fewer studies have been conducted to relate CYP activities to drug effects in patients. Most of such studies are retrospective and have been done in small populations only. The few examples for prospective investigations include the assessment of the relationship between CYP2D6 genotype to efficacy and tolerability of haloperidol[5], or to the antiemetic effects of 5-hydroxytryptamine type 3 receptor antagonists[6]. A quantitative proposal for dose adjustment as it has been made for antidepressants based on CYP2D6 and CYP2C19 genotype is a rare exception[7]. No appropriate clinical studies clearly assessing the therapeutic benefit of dose personalization based on CYP activity are available to my knowledge. As a consequence, information on individual CYP activity currently is not being applied to personalize drug therapy. Because of the lack of data, despite its potential such procedures to date cannot be recommended outside clinical studies.

CYP genotyping has become increasingly reliable, simple and cheap, e.g. by light cycler and gene chip technologies. Likewise, CYP phenotyping made major progress, mainly by increased analytical sensitivity brought about by LC-MS/MS with the possibility to administer very low doses of marker drugs. The tools for assessment of individual activity of human CYPs with outstanding relevance for drug metabolism, i.e. CYP3A4, CYP2D6, CYP2C9, CYP1A2 and CYP2C19, are available. What is needed are large prospective randomized studies comparing traditional dose adjustment based on clinical effects only with additional dose personalization based on CYP activity. In these trials, both average efficacy / tolerability and the degree of interindividual variation in these aspects should be compared. From such studies, algorithms and software tools (expert systems) need to be derived to take CYP activity into account for dose calculation. Finally, the cost-effectiveness of the approach must be addressed. Only then, it will be possible to judge whether CYP genotyping and/or phenotyping is worthwhile to be considered in clinical medicine.

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WL06 *IN VITRO* TO *IN VIVO* CORRELATIONS - A CRITICAL ASSESSMENT OF VARIOUS APPROACHES TO MEASURE DRUG METABOLISM AND METABOLIC INTERACTIONS

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Elucidation of metabolic stability, metabolic routes, metabolising enzymes and their kinetics, and metabolic interactions is obviously important for any chemicals to which humans are exposed. It is even more important for pharmaceuticals, because this information is needed for selecting leads and candidate drugs during drug discovery and development. In short, metabolism determines to a great extent the pharmacokinetic properties of most drugs and is often behind interactions, metabolic idiosyncrasies and so on[2, 3].

Human-liver derived *in vitro* systems are thought to be useful in early studies of metabolic stability, in identifying enzymes capable of metabolising, and interacting with, drugs in use or under development (NCE, new chemical entity) and several of these systems are currently under validation as to their predictive power to the *in vivo* situation[4]. These approaches are expected to provide crucial information for more in-depth studies during the preclinical and clinical phases of drug development. Some examples of the approach have been published[1, 5].

The most important goals for using various human liver-derived in vitro systems include the following:

1) to elucidate and determine principal metabolic routes of an NCE and to tentatively identify principal metabolites;
2) to identify, with human liver homogenate or microsomal preparations, the CYP enzymes catalysing the principal oxidation routes (primary metabolites) and to gain some quantitative data on their significance for the overall metabolic fate of an NCE;
3) to provide useful background information for characterising potential interactions and physiological, genetic and pathological factors affecting the kinetics and variability of an NCE in in vivo situation.

Experimental methods available currently

Human liver samples obtained under proper ethical permission have been used as a golden standard. These preparations have usually been extensively characterised to be used for the primary screening (sufficient model activities, no known polymorphisms, expected effects of model inhibitors, quantitation of CYPs by Western blotting). Other experimental in vitro systems include liver slices, cultured hepatocytes, recombinant expressed enzymes, and permanent cell lines. Currently in silico models are being increasingly used for predicting metabolism and interactions.

During the development of an NCE, at least the following investigations are usually required, the earlier the better:

- Measurement of the disappearance of a chemical in human liver preparations; „metabolic stability“
- Identification of principal metabolites; „metabolite profile and proposed metabolic tree of a chemical“
- Development of an analytical „routine“ method for metabolites
- Identification of enzymes catalysing metabolic routes with the aid of diagnostic inhibitors and antibodies, recombinant enzymes and correlation studies
- Enzyme kinetic characterisation of principal metabolic reactions; for scaling up and predicting in vivo kinetics of an NCE
- Affinity of a chemical (and/or its principal metabolites) for CYP (or other) enzymes; for predicting potential metabolic interactions

Some typical experiments and their outcomes

Effects of various diagnostic CYP-selective inhibitors on NCE metabolism: an estimation of the role of each CYP enzyme to contribute to the metabolism of a chemical. On the basis of this information, it is possible to perform predictive calculations to the in vivo behaviour of a chemical.

Ability of recombinant enzymes to catalyse metabolite formation of an NCE: gives a direct indication of the potential of each recombinant CYP to catalyse the reaction. However, there is still somewhat uncertain of whether the results from recombinant enzymes could be extrapolated to the whole microsomes and to the in vivo situation.

Correlation of CYP-selective model activities with metabolite formation of an NCE: gives an indirect indication of the participation of each CYP enzyme to catalyse the reaction. The results are, however, confirmatory and should be evaluated in the context of other approaches.

After the above studies, it is possible to plan further preclinical, molecular, toxicological and clinicopharmacological studies, with focussed consideration of those CYP enzymes which are of importance for the metabolism and kinetics of an NCE.

Validation of various approaches

In vitro approaches for predicting in vivo behaviour of pharmaceuticals have been developing for quite some time, but still there are relatively few attempts to validate these approaches. The validation involve several steps: test development, pre-validation, validation, independent assessment and regulatory acceptance if appropriate. With respect to the methods described above, the selection of pre-validation and validation substances, in addition to proper substrates and inhibitors, is of utmost importance. Because ultimately cli-

nical significance is one of the more important goals, proper consideration should be given to the selection of selectivity, specificity and potency of various substances used, which should preferably be clinically used drugs, either currently or in the past, so that enough proper in vivo information is available for the assessment of prediction models. The prediction model itself should be adequately characterised with respect to conversion factors (e.g. what is the „standard" amount of microsomal protein in the unit of liver weight) and algorithms (e.g. what kind of values should be assumed for e.g. volume of distribution or renal clearance, if not known). These validation requirements mean that the process of validation is a rather cumbersome and time-consuming undertaking (and is regarded by some as a „dull" exercise) and not very often performed in this field.

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WL07 P450, ANTI-CANCER DRUGS AND GENE THERAPIES

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Several commonly used cancer chemotherapeutic prodrugs, including cyclophosphamide and ifosfamide, are activated via a liver cytochrome P450 (CYP)-catalyzed prodrug activation reaction. Rodent and human hepatic P450s display clear similarities in their catalytic specificities towards these anti-cancer prodrug substrates. Preclinical studies have shown that the chemosensitivity of tumors to P450 prodrugs can be dramatically increased by transfer of a rodent or human CYP gene, which confers on the target tumor tissue the capacity for localized prodrug activation and, in the case of cyclophosphamide, induces a mitochondrial/caspase 9-dependent pathway of apoptotic cell death. This P450 **g**ene-**d**irected **e**nzyme **p**rodrug **t**herapy (P450 GDEPT) greatly enhances the therapeutic effect of P450-activated anti-cancer prodrugs without increasing host toxicity associated with systemic distribution of active drug metabolites formed by the liver. The efficacy of P450 GDEPT can be augmented in several ways:

1) by further increasing the partition ratio for tumor:liver prodrug activation in favor of increased intratumoral metabolism, e.g., by co-expression of the flavoenzyme NADPH-P450 reductase, by localized prodrug delivery or by the selective pharmacologic inhibition of liver prodrug activation;

2) by anti-angiogenic scheduling of prodrug administration, which substantially magnifies the anti-tumor effect and leads to major regression of established tumors; and

3) by co-expression of an anti-apoptotic factor, which inhibits tumor cell apoptosis in a manner that prolongs the generation of soluble, bystander cytotoxic metabolites but does not ultimately block tumor cell death. P450 GDEPT prodrug substrates are diverse in their structure, mechanism of action and choice of optimal prodrug-activating P450 gene; they include both established and investigational anti-cancer prodrugs, as well as bioreductive drugs that can be activated by P450/P450 reductase in a hypoxic tumor environment. Several strategies have now been employed to achieve the tumor-selective gene delivery that is required for the success of P450 GDEPT; these include the use of tumor-targeted cellular vectors and oncolytic viruses that replicate in a tumor-selective manner. Overall, P450-based GDEPT presents several important, practical advantages over other GDEPT strategies that may facilitate the incorporation of P450 GDEPT into existing cancer treatment regimens. Recent reports of clinical efficacy in P450-based phase I/II gene therapy trials for breast and pancreatic cancer patients support this conclusion.

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WL08 CYP51: STRUCTURE, FUNCTION, EVOLUTION

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CYP51 (sterol 14α-demethylase) is an essential enzyme in sterol biosynthetic pathways and the only cytochrome P450 gene family having catalytically identical homologs in different phyla. It is required for conversion of lanosterol to cholesterol (animals), 24-methylenedihydrolanosterol to ergosterol (fungi/yeast) and obtusifoliol to phytosterols (plants). It is the most widely distributed member of the cytochrome P450 superfamily, also being found in lower eukaryotes and some bacteria and is perhaps the most ancient of P450s. All forms catalyze 14α-demethylation (14α-DM) of Δ8-sterols through a catalytic cycle involving three successive monooxygenation steps. Also, CYP51s are known to have high affinity for azole inhibitors and are primary targets for development of drugs for treatment of different fungus infections. Thus CYP51 is a particularly important member of the cytochrome P450 superfamily because of its potential for providing detailed understanding of structural requirements for substrate orientation *and* for its important role as a drug target in human infections. We have determined the high resolution X-ray structure of the soluble form of CYP51 from *Mycobacterium tuberculosis* (MT)[1,2]. This structure has allowed us to assign the location of secondary structural elements to other family members with high probability.

Across different phyla, CYP51 has relatively low sequence identity (22-32%). Yet all members metabolize only an extremely limited set of substrates, catalyzing the first step in sterol biosynthesis following cyclization. Alignment of the more than 50 known CYP51 sequences throughout biology reveals the presence of ~40 conserved amino acids. Three are the heme-ligated Cys and the conserved EXXR in the K-helix. We propose that included within these are the main structural requirements for 14α-DM, allowing the enzyme to catalyze the same reaction in organisms which are evolutionarily related over hundreds of millions of years. Many of these residues are located within the secondary structure elements designated as substrate recognition sequences (SRS) of P450s, supporting the notion of their essential role in 14α-DM. By site-directed mutagenesis of both MT and human CYP51 followed by detailed biochemical and biophysical analysis of the mutants we are identifying the role of conserved residues in substrate binding and enzymatic activity, protein-protein interactions and electron transfer, and maintenance of 3-D structure and protein folding. Connection between the effect of mutagenesis and the location of each residue will make it possible to predict the role of each in sterol 14α-DM and to assign the exact function to the CYP51 structural regions. Our preliminary studies have already established the essential role of conserved residues in of the three N-terminal SRSs of P450s (B'helix/BC loop, helix F and helix G) and led to the experimentally supported hypothesis that significant conformational rearrangements in MTCYP51 occur upon substrate binding (presumably closing BC loop, which brings it to the position similar to that found in substrate bound P450 structures for example, P450BM3[3] and movement of helices F and G towards the heme, resembling that shown in P450cam after interaction with Ru-linker substrates[4]).

As the studies progress new CYP51 sequences will appear from genomic sequencing which will prove useful in understanding CYP51 structure/function. Newly reported CYP51 sequences in the *Trypanosoma* family (*Trypanosoma brucei* and *Trypanosoma cruzi*) indicate that unlike the other reported CYP51 sequences, certain residues in the N-terminal SRSs are not conserved. This provides an additional opportunity to understand the role of the highly conserved residues in CYP51 function. This presentation will summarize our studies aimed at identifying the structural requirements for sterol 14α-demethylation.

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WL09 LANOSTEROL 14α-DEMETHYLASE, CHOLESTEROL AND REPRODUCTION

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Lanosterol 14α-demethylase (CYP51) is a microsomal enzyme that in the presence of NADPH cytochrome P450 reductase, NADPH and molecular oxygen removes the 14α-methyl group from lanosterol in a complex, three-step reaction. The final reaction product is 4,4,-dimethyl-5α-cholesta,8,14,24-dien-3β-ol, known also as folicular

fluid meiosis activating sterol (FF-MAS). FF-MAS and T-MAS (testis meiosis activating sterol), the product of the subsequent reaction in the cholesterol biosynthesis pathway, acumulate in female and male gonads, respectively. MAS stimulate the reinitiation of meiosis in mouse oocyte *in vitro* and are belived to have important roles in fertilisation [Byskov, 1995 #71]. In contrast to gonadal tissues, MAS are not easily detectable in the liver where they represent intermediates in cholesterol biosinthesis[1]. Biochemical mechanisms contributing to MAS accumulation in gonads are not yet understood. It is, however, believed that events at transcriptional and post-transcriptional levels contribute to dis-regulation of the cholesterol biosynthesis pathway, leading consequently to accumulation of MAS.

Transcription

Different transcription factors and coactivator proteins contribute to formation of transcriptional complexes at CYP51 promoter in liver and testis. In the liver, SREBP1a (immortal cell lines) or SREBP2 (tissue) are major transactivators that, however, require a cAMP-dependent transcription factor for optimal transactivation[2]. In the testis (male germ cells) CREMτ is the major transactivator but a germ cell-specific transcription factor SREBP-2gc might also be involved in CYP51 activation. Not only CYP51, also other cholesterogenic genes seem to be regulated in a different manner in liver compared to the testis. We have proposed earlier that a discordant regulation of the cholesterol biosynthesis pathway in the testis is the first regulatory level leading to accumulation of intermediates of cholesterol biosynthesis MAS[1].

Intracellular protein transport

Smooth endoplasmic reticulum of mouse liver contains a 53 kDa CYP51. Significant amount of this protein is detected also in cis Golgi but not in trans Golgi, suggesting retreival of CYP51 from Golgi back to the ER in the liver. A 53 kDa CYP51 is the major form also in mouse male germ cells where it resides in ER, Golgi and a male germ cell-specific Golgi-derived organelle acrosome. CYP51 remains detectable during all phases of acrosome development (15 days in the mouse), including most mature spermatids that lack ER and Golgi, indicating that in male germ cells CYP51 trafficks from ER through Golgi to its final destination acrosome. Detection of a 50 kDa CYP51 in acrosomal membranes of ram and bull ejaculated sperm suggests a conserved mechanism of CYP51 traffic in mammalian germ cells. The appearance of high molecular mass (HMM) CYP51-immunoreactive proteins is also a common feature. HMM CYP51s are detected in ram and bull acrosomal membranes and in Golgi-enriched fractions of mouse liver microsomes, suggesting that post-translational modifications leading to HMM compelxes arise in the Golgi. This is a first example of a cytochrome P450 protein with different intracellular trafficking and subcellular localization, depending on a cell type.

Enzymatic activity and synthesis of MAS

Beside on the ER, a complete lanosterol 14α-demethylase system (CYP51 and its redox partner NADPH-P450 reductase) is located also on acrosomal membranes and remains there stabilized for several days during sperm maturation phases. CYP51 enzymatic activity (conversion of lanosterol to FF-MAS) is detected in the liver microsomes of all tested mammals as well as in testis/isolated germ cells of rat, mouse and ram. Isolated acrosomal membranes of bull sperm as well as sperm homogenates of bull and ram also seem to harbor CYP51 enzymatic activity. FF-MAS, a direct product of the CYP51 enzymatic reaction, is not detectable in livers of adult (65-70 day-old) or pre-pubertal (21-23-day old) male mice, but is easily detectable in the tesits. Same applies to the testis meiosis activation sterol T-MAS. Besides in the testis, MAS are detectable also in brain, suggesting a similar regulatory mechanisms of cholesterol biosynthesis in both organs.

In conclusion, transcription of the mammalian CYP51 mRNA, CYP51 protein intracellular trafficking and likely also post-translational modification, differ between liver and testis (male germ cells). While CYP51 in the liver serves mainly to contribute to production of cholesterol, it is believed that CYP51 features in male egrm cells contribute majorly to production of MAS. MAS that were shown to re-initiate meiosis of mouse oocytes *in vitro* have yet not completely understood roles in mammalian reproduction *in vivo*.

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WL10 P450 HUMANIZED MICE: NOVEL TOOLS FOR THE STUDY OF DRUG AND CARCINOGEN METABOLISM, AND THE SEARCH FOR ENDOGENOUS HUMAN P450 SUBSTRATES AND FUNCTIONS

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There are marked species differences in the expression and catalytic activities of P450s that metabolize xenobio-

tics. This is especially notable between mice and rats, commonly used experimental models, and humans. P450 humanized mice were developed using and λ phage, BAC and PAC genomic clones as transgenes. Humanized lines expressing CYP1A1/CYP1A2, CYP1B1, CYP2D6, CYP2E1, CYP3A4 lines were characterized and found to accurately express human P450 catalytic activities at levels comparable to or higher than the corresponding activities found in human tissues. New highly sensitive LC-MS/MS-based assays were developed to perform pharmacokinetic studies using a single mouse and non-radiolabeled drugs. These novel mouse lines offer the opportunity to predict human drug and carcinogen metabolism and disposition, and to search for endogenous substrates for human P450s.

WL11 MECHANISM OF THE FORMATION AND MAINTENANCE OF THE FUNCTIONAL ZONATION IN THE ADRENAL CORTEX

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The adrenal gland consists of two ontogenetically and functionally distinct endocrine tissues, the cortex and the medulla. The cortex is mesodermal in origin and derived from the coelomic epithelium, while the medulla is ectodermal and derived from the neural crest. The cortex is subdivided further into three concentric zones, the zona glomerulosa (zG), the zona fasciculate (zF) and the zona reticularis (zR). These three zones are morphologically and functionally distinct from each other: zG cells secrete mineralocorticoids, zF cells give glucocorticoids, and zR cells produce adrenal androgens in many primates. Such specialization in the function is called „functional zonation" of the adrenal cortex.

Interestingly, all the enzymes involved in the biosyntheses of these corticosteroids from cholesterol are the members of cytochrome P450 family with an only exception of 3β-hydroxysteroid dehydrogenase/isomerase. The successful identification and purification of aldosterone-synthase cytochrome P450 (P450aldo) and glucocorticoid-synthesizing cytochrome P450 (P45011β) of rats in early 1990s[1], led us to investigate the molecular basis for such functional zonation of the adrenal cortex as well as mechanisms for the maintenance and development of rat adrenal glands.

The results so far accumulated indicate: (1) The functional zonation of the adrenal cortex is governed by the expression of each terminal steroidogenic enzyme such as P450aldo in zG and P45011β in zFR[2]. (2) Both functional and morphological zonation are recognized in the adrenal gland of adult rats, but not in that at fetal stages[3]. The zones were established in the third week after birth. (3) The adrenal gland after the enucleation can regenerate the entire adrenal cortex with both functional and morphological zonation[3], suggesting that the zonation is an inherent nature of the adrenocortical cells. (4) The fourth zone is present in the adult adrenal cortex as a thin cell layer between zG and zF[4]. The cells there do not express P450aldo, P45011β nor P45017α,lyase, an androgen-synthesizing enzyme. In other words, the zone lacks any differentiated function of adrenocortical zones. Accordingly it was named „the undifferentiated cell zone (zU)". (5) zU was shown to be the sites of cell proliferation under the non-growing (steady- state) conditions. The cells raised in the inner portion of zU migrate inward to zFR providing cell to these zones. We therefore concluded that zU is the stem cell zone of the adrenal cortex at least in non-growing rat[5].

We recently established adrenocortical cell-lines with properties of zU cells[6] from the adrenal corteces of mice carrying a temperature-sensitive SV 40T antigen gene. The cells secreted a hitherto unknown protein identified by subtractive hybridization between the cells without and with expressing P45011β[7]. The protein as well as its mRNA was detected in zG and zU, but not in zFR, and we designated it adrenocortical zonation factor-1(AZ-1). Its *in situ* expressions in the adrenocortical zones as well as in Y1 cells are inversely correlated with the expression of P45011β.

On the basis of these findings obtained by using cytochrome P450 enzymes as markers, mechanisms for the adrenocortical zone formation and its maintenance will be discussed.

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WL12 DUAL EFFECTS OF THE CHEMOPROTECTIVE AGENT OLTIPRAZ ON CYTOCHROMES P450

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A variety of natural products and synthetic chemicals are known to have chemopreventive properties; they include dithiolethiones, isothiocyanates, polyphenols, flavonoids and various other antioxidants. They may act by altering the balance between activation and inactivation of carcinogens through their action on phase 1 and phase 2 xenobiotic metabolizing enzymes. Oltipraz (OPZ), a synthetic derivative of 1,2-dithiole-3-thione, is one of the most promising chemoprotective agent, based on preclinical studies and a recent phase IIa clinical trial in China[1]. The chemical was first claimed to protect against carcinogenesis by inducing enzymes involved in their inactivation, such as glutathione transferases (GST), NADPH-quinone reductases and UDP-glucuronosyltransferases[2]. These results obtained from rodent studies were confirmed in humans by using primary human hepatocytes. However, the GST isoform pattern induced by OPZ was different[3].

More recent investigations showed that OPZ also exerted dual effects on cytochromes P450 (CYP). First, we showed that this agent was a rapid and potent inhibitor of CYP1A2 and CYP3A4 in human hepatocytes[4]. This observation was confirmed by measuring caffeine N^3-demethylation catalyzed by CYP1A2 in OPZ-treated rat liver and the use of human recombinant CYP1A2 expressed in *Escherichia coli* membranes[5, 6]. OPZ acts as a competitive and mechanism-based inhibitor of CYP1A2 with K_i of 1.5 μM and $k_{inactivation}$ of 0.19 min^{-1} [6]. It also inhibits in the following order CYP 3A4, 1A1, 1B1 and 2E1 and one of its metabolites, OPZ M3, had similar effects. This inhibitory effect was confirmed *in vivo* in humans by OPZ oral administration of 500 mg weekly or 125 mg daily and determination of aflatoxin B1 mercapturic acid formation and caffeine N-demethylation respectively[1, 7].

In addition, it was found that after 48hr of OPZ treatment, CYP1A1/2 and CYP2B1/2 were significantly increased in rat liver, at both mRNA, protein and activity levels. Interestingly, by using differentiated human Caco2 cells, derived from a colon adenocarcinoma, the induction of CYPA1 was demonstrated to occur at the transcriptional level and to be mediated by the aryl hydrocarbon receptor (AhR). An increased intracellular calcium concentration was required to obtain AhR activation by OPZ[8].

From the data of the literature it is obvious that OPZ effects are dependent upon the CYP enzymes, time and duration of treatment, dose, organ and species. Thus, to be effective on CYPs, OPZ treatment must be started before or be concomitant to exposure to the carcinogen. This compound is able to augment CYP1A expression and activity not only in rat liver but also in kidney, lung and intestine. Regarding CYP2B1 the transcripts were unchanged in the lung while the amount of corresponding apoprotein and activity was dramatically decreased, due to a rapid catabolism of the protein by the proteasome degradation pathway[9].

As a whole, these results from studies on OPZ demonstrate that evaluation of chemoprotective agents must consider their potential ability to modulate some CYP activities, that these effects can be inhibitory and/or inducing and suggest that these effects may have considerable consequences on the preventive capacity of such molecules *in vivo*.

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WL13 TRANS-SPECIES REGENERATING OR STEM-CELL DERIVED HEPATOCYTE-LIKE CELLS: A PERSPECTIVE TO SOLVE THE AVAILABILITY PROBLEM FOR HUMAN HEPATOCYTES?

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Hepatocytes represent a valuable tool in the evaluation of xenobiotics allowing for determination of (i) rate of disappearance of a compound, (ii) metabolic profiles, (iii) enzyme induction, (iv) drug-drug interaction, (v) cytotoxicity. We have established a technique that allows cryopreservation of hepatocytes in a way that guarantees similar enzyme activities in cryopreserved versus freshly isolated hepatocytes of the major drug metabolizing phase I and phase II pathways and allows examination of enzyme induction in cultures of cryopreserved hepatocytes. Cryopreserved human hepatocytes are available, but rare.

Stimulation of hepatocyte proliferation in vitro is difficult. We developed a technique that allows proliferation of human hepatocytes after transplanting into 2/3 hepatectomized nude rats. With this technique the yield of hepatocytes is rather low. We therefore sought conditions allowing for differentiation of human umbilical cord stem cells into the direction of hepatocytes. The cells were transplanted into livers of SCID-mice. Several hepatocyte markers such as human albumin were successfully brought to expression in these cells, whilst ß2-microglobulin was no longer detectable. This down regulation promises to represent an escape mechanism from killer T-cells of the host mice which may constitute an important prerequisite for growing human hepatocyte-like cells in animal hosts. So far the cells had not yet differentiated into perfect hepatocytes but clearly into hepatocyte-like cells.

WL14 METABOLISM: A BOTTLE-NECK FOR *IN VITRO* REGULATORY TOXICITY TESTS AND TESTING STRATEGIES

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The recent European Commission (EC) White Paper 'Strategy for a future chemicals policy' outlines a plan to up to 30 000 'existing chemicals' which are currently marketed in volumes greater than 1 ton per year, and for which essential human health and ecotoxicological data are lacking at a cost, predicted to be several billions. Assuming no increase in current capacities for chemical testing, the time scales for testing for chemical risk assessment for human health, proposed, might need to be extended by 36 years for the Base Set Testing, i.e. to 2048.

There is general consensus to develop alternative testing strategies using *in vitro* test systems, in order to reduce the time scale for the completion of the testing and to allow compliance with the new legal demands. This is exactly the challenging task of the European Centre for the validation of Alternative Methods (ECVAM) e.g. to make use of the recent technological advances made with regard to *in vitro* toxicological testing in order to validate relevant, reliable and robust high-thoughput alternative toxicological test systems targeting specifically the required regulatory toxicological studies for human health.

Approaches for identifying metabolism-mediated effects after chemical exposure and their inclusion in testing strategies are a high-priority in order to offer alternatives to in vivo animal testing. The major human CYPs involved in chemical metabolism are CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP1B1, CYP2C9, CYP2C19, CYP2D6, and CYP2E1. The key phase II enzymes include N-acetyl transferases, UDP-dependent glucuronyl transferases, phenol sulphotransferases, sulphotransferases, and glutathione S-transferases. Metabolism is identified as „the bottleneck" in *in vitro* test development. So far, no consensus has been reached on the optimal test systems to be used for obtaining the information required in the human health areas of high priority such as acute systemic toxicity or reproductive toxicity. However, the most widely-accepted approach is to progress from simple, inexpensive and less-specific *in vitro* or *in silico* models, such QSARs and human liver suspensions and subcellular fractions, to more demanding and more-complex models, such as cultures of primary human hepatocytes. In this way, the first tier acts as a preliminary screen for identifying any metabolism-mediated toxic effects followed by more specific tests, such as the induction of biotransformation enzymes or the occurrence of polymorphism-related effects.

WL15 APPLICATIONS OF TRANSGENIC MODELS IN DRUG METABOLISM

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Transgenic technology has the potential to revolutionise the field of drug metabolism; the ability to manipulate genetically the expression of individual drug metabolising enzymes in order to better understand their function(s) could lead to the development of new drugs for the treatment of diseases such as cancer, as well as the optimisation of treatment regimes with existing drugs.

Our Laboratory has been interested for several years in the expression and regulation of drug metabolising enzymes and the role of such enzymes in cellular processes such as carcinogenesis and drug resistance. Recently, we have developed several transgenic models to enable us to study how drug metabolising enzymes protect us from the chemically challenging environment in which we live.

The cytochrome P450 supergene family represents a group of Phase I drug metabolising enzymes catalysing the insertion of an atom of molecular oxygen into a huge range of diverse substrates[1]. A number of P450s have been 'knocked out' in the mouse; deletion of those P450s which carry out essentially 'house-keeping' reactions tend to result in embryonic lethality, or severe welfare problems, whereas deletion of P450s which carry out metabolism of xenobiotics tend to display little or no obvious phenotypic changes[2,3]. This is at least in part due to the overlapping substrate specificity demonstrated by P450s, and the ability of other isoenzymes to at least partially compensate for the absence of individual enzymes. In order to overcome this, we have deleted the sole electron donor to microsomal cytochrome P450s, cytochrome P450 reductase (CPR); as anticipated, this deletion was embryonic lethal[4,5]. However, our targeting strategy incorporated the loxP sites, allowing us to conditionally delete CPR when used in conjunction with a mouse line carrying Cre recombinase under the control of a tissue-specific promotor. We have now generated mice which lack hepatic CPR, and thus the P450 system in the liver is essentially inactivated[6]. Despite this, hepatic CPR null mice grow and develop normally, demonstrating that the hepatic P450 system is not essential for survival in the postnatal period, but that; however, they have enlarged, fatty livers, an almost complete lack of bile production, and a significantly lowered serum lipid profile. Work with these mice will be presented to demonstrate that hepatic CPR null mice will be a valuable model to study not only the role of hepatic and extrahepatic P450s in drug disposition, but also the role of Phase II enzymes and drug transporters.

We have also deleted a Phase II enzyme, glutathione S-transferase Pi (GstP), from mice. GstP has been found to be expressed at elevated levels in a number of animal and human tumours, and in cell lines made resistant to a variety of drugs[7,8]. Little, however, is known about the endogenous role of this enzyme. Mice lacking GstP are apparently phenotypically normal, with no change in morbidity or mortality. However, GstP null mice are found to have significantly higher levels of tumour formation, in the skin or lungs, when challenged chemically[9]. Furthermore, such mice also have altered sensitivity to the effects of drugs such as acetaminophen[10], involving a mechanism(s) which have not yet been fully elucidated.

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WL16 CYTOCHROME P450 4FS: RESPONSE AND ROLE FOLLOWING BRAIN TRAUMA

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Closed head injury resulting from traffic accidents or other blunt trauma often leads to a severe inflammatory

response in the brain and coma. The inflammatory cascade mediated in part by leukotriene and prostaglandin signals results in the migration of cells, ions and fluids into the site of injury. The production of leukotrienes and prostaglandins is catalyzed by lipoxygenase and cyclooxygenase 2. respectively, from arachidonic acid. The cytochrome P4504F subfamily has been implicated by Kikuta and others in metabolizing leukotriene and prostaglandin to products inactive as prompts of the inflammatory cascade and therefore playing a role in modulating the levels of leukotrienes and prostaglandins present. We report that the levels of lipoxygenase and cycloxygenase expression reach a maximum of 24 hours after controlled cortical impact in rats while CYP4F expression is suppresed at this time point. At later time points CYP4F levels rise while lipoxygenase and cyclooxygenase levels decrease, marking the recovery phase of the injury. Similar patterns are observed in the tissues especially in the lung, as the function of time after injury.

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WL17 COMMONLY USED DRUGS REGULATE CYP ENZYMES WITHIN THE BRAIN

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CYP enzymes in the brain are of interest due to their potential role in the (in)activation of centrally acting drugs, in the metabolism of endogenous compounds, and also due to their ability to generate toxic metabolites and/or oxygen stress that may cause neuronal damage.

Identification and Localization in the Brain

Many CYPs demonstrate constitutive expression within animal and human brains, are distributed heterogeneously among brain regions (e.g. members of CYP1, 2, 3 and 4 families), and are found in both neurons and glial cells (for review[1]). Many CYPs are located at the blood-brain interface and in circumventricular organs (regions unprotected by the blood-brain barrier) such as the choroid plexus and posterior pituitary. Brain CYPs are reported to occur at about 1% of hepatic levels, but most earlier studies treated the brain as a homogeneous organ. CYP levels can vary several fold among brain regions, and in specific cells (e.g. Purkinje neurons) levels can be as high as those found in hepatocytes. While hepatic CYPs are located primarily in the ER, in brain, the presence, inducibility and metabolic activity of many drug metabolizing CYPs have been observed in mitochondrial membranes, in neuronal processes, in the plasma membrane as well as in the ER.

Induction

Brain CYPs are inducible by many common hepatic inducers (e.g. CYP2B1 by phenobarbital). Many compounds affect liver and brain differently, e.g. ethanol induces CYP2B1 in rat liver but not brain, whereas nicotine induces CYP2B1 in rat brain but not liver (for review[2]). Induction is usually cell-type specific and tends to occur at low doses. Induction of brain CYPs may be detrimental, for example by 1) rerouting the metabolism of endogenous compounds, 2) increasing cellular oxidative stress, and 3) increasing production of toxic metabolites (e.g. procarcinogens, amphetamine metabolites).

Metabolism by Brain CYPs

CYP enzymatic activity is reported for both rodent and human brain. Brain membranes have been shown to metabolize the same probe substrates used to assess specific hepatic CYP activity, such as 7-pentoxyresorufin for CYP2B1/2, 7-ethoxyresorufin for CYP1A1/2, N-nitrosodimethylamine and p-nitrophenol for CYP2E1, dextromethorphan for CYP2D. Many other hepatic CYP substrates such as bufuralol, amytriptyline, nicotine, phencyclidine, amphetamines, and neurotoxins such as organophosphorous insecticides are metabolized by brain tissues. While it is unlikely that brain CYPs contribute to overall clearance of xenobiotics, their punctate, region- and cell-specific expression suggests that CNS CYP enzyme activity may create micro-environments in the brain with differing drug and metabolite levels (not detected or predicted by plasma drug monitoring). Coupled with the sensitivity of CNS CYPs to induction, this may in part account for interindividual variation in response to centrally acting drugs and neurotoxins, and may have implications for individual variation in receptor adaptation and cross-tolerance to different drugs.

CYP2D6 and Alcoholics

CYP2D6 is expressed in liver, brain and other extrahepatic tissues where it metabolizes a range of centrally acting drugs and toxins. Members of the CYP2D family have been localized in rat brain where they have a heterogeneous distribution[3,4]. As ethanol can induce CYP2D in rat brain, we hypothesized that CYP2D6 expression would be higher in brains of human alcoholics. We examined regional and cellular expression of CYP2D6 mRNA and protein by RT-PCR, southern blotting, slot blotting, immunoblotting and immunocytochemistry[5]. A significant correlation was found between mean mRNA and CYP2D6 protein levels across 13 human brain regions. Higher expression was detected in 13 brain regions of alcoholics (n=8) compared to non-alcoholics (n=5) (ANOVA $p<0.001$). In hippocam-

pus the higher levels were localized in CA1-3 pyramidal cells and dentate gyrus granular neurons. In cerebellum this was localized in Purkinje cells and their dendrites. Both of these brain regions, and these same cell-types, are susceptible to damage by alcoholism. A poor metabolizer (CYP2D6*4/*4) was identified and in this brain sample there was no detectable CY2D6 protein, confirming the specificity of the antibody used. This indicates that for those with genetically deficient variants of the enzyme, metabolism within the brain will differ from those with the active forms independently of the presence of inducers. Together these data suggest 1) CYP2D6 in brain is inducible and 2) that in alcoholics the higher levels of brain CYP2D6 expression may contribute to altered sensitivity to centrally acting drugs and to the mediation of neurotoxic and behavioral effects of alcohol.

CYP2E1, Alcohol and Nicotine

Ethanol and nicotine are commonly co-abused drugs. CYP2E1 metabolizes ethanol and bioactivates tobacco-derived procarcinogens. Ethanol and nicotine can induce hepatic CYP2E1[6] and we hypothesized that both centrally active drugs could also induce CYP2E1 within the brain[7]. Male rats were treated with saline, ethanol (3.0 g kg^{-1} by gavage) or nicotine (1.0 mg kg^{-1} s.c.) for 7 days. Ethanol treatment significantly increased CYP2E1 in olfactory bulbs, frontal cortex, hippocampus and cerebellum while nicotine induced CYP2E1 in olfactory bulbs, frontal cortex, olfactory tubercle, cerebellum and brainstem. Immunocytochemical analysis revealed that the induction was cell-type specific. Consistent with the increased CYP2E1 found in rat brain following drug treatments, brains from alcoholics and alcoholic smokers showed greater staining of granular cells of the dentate gyrus and the pyramidal cells of CA2 and CA3 hippocampal regions as well as of cerebellar Purkinje cells compared to nonalcoholic nonsmokers. Moreover, greater CYP2E1 immunoreactivity was observed in the frontal cortices in the alcoholic smokers in comparison to nonalcoholic nonsmokers and alcoholic nonsmokers. To investigate if nicotine could contribute to the increased CYP2E1 observed in alcoholic smokers, we treated human neuroblastoma IMR-32 cells in culture and found significantly higher CYP2E1 immunostaining in nicotine-treated cells (0.1-10 nM). CYP2E1 induction in the brain, by ethanol or nicotine, may influence the central effects of ethanol and the development of nervous tissue pathologies observed in alcoholics and smokers.

CYP2B6, Alcohol and Nicotine

CYP2B6 metabolizes drugs such as nicotine and bupropion, and many toxins and carcinogens. Nicotine induces CYP2B1 in rat brain[8] but not liver[9] and in humans polymorphic variation in CYP2B6 affects smoking cessation rates10. We compared CYP2B6 expression in brains of human smokers and nonsmokers and alcoholics and nonalcoholics (n=26). Human CYP2B6 expression was brain region-specific and was observed in both neurons and astrocytes. CYP2B6 levels were higher in brains of smokers and alcoholics, particularly in cerebellar Purkinje cells and hippocampal pyramidal neurons, cells known to be damaged in alcoholics. Significantly more ($p<0.05$) CYP2B6 protein was seen in 4 brain regions of smoking alcoholics compared with nonsmoking nonalcoholics: hippocampus, caudate nucleus, putamen and cerebellar hemisphere. The genetic variant C1459T (R487C) has been associated with reduced hepatic enzyme levels, stability and activity. Preliminary genotyping of this small sample (n=24) suggests that individuals with the CC genotype have higher brain CYP2B6 than those with the CT or TT genotype. Higher brain CYP2B6 activity in smokers and alcoholics may alter the sensitivity to centrally acting drugs, increase the susceptibility to neurotoxins and carcinogenic xenobiotics, and may contribute to central tolerance to nicotine.

Much remains to be understood before we can firmly establish relationships between the presence of CYPs in brain, their function in this highly heterogeneous and complex organ, and the resulting consequences on overall drug effect and brain function.

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WL18 THE ARACHIDONIC ACID MONOOXYGENASES: P450-EICOSANOIDS AS ENDOGENOUS REGULATORS OF ORGAN AND BODY PHYSIOLOGY

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CYP P450s of the 4A and 2C gene subfamilies are responsible for most of the arachidonic acid epoxidation and ω/ω-1 hydroxylation catalyzed by human and rodent kidney microsomal fractions[1]. The expression of these proteins is highly regulated and controlled by physiological and pathophysiological factors such as diet, salt intake, hormones, and diseases such as diabetes and hypertension[1,2]. By virtue of their vasoactive and ion transport properties, the epoxyeicosatrienoic and hydroxyeicosatetraenoic acids (EETs and HETEs, respectively), modulate kidney function and body fluid and salt homeostasis[2,3]. Experimental manipulation of Cyp 4A or 2C gene expression changes the activities of the renal arachidonic acid epoxygenases and ω/ω-1 hydroxylases and the kidney biosynthesis of EETs and/or HETEs. These changes CYP P450-eicosanoid biosynthesis are associated with alterations in renovascular tone, the capacity of the organ to excrete salt and water, and ultimately, with changes in systemic blood pressures[1-3]. Genetically induced alterations in renal Cyp 4a10 expression and activity, depresses the biosynthesis and urinary excretion of pro-natriuretic, anti-hypertensive EETs, and leads to the development of salt sensitive hypertension. Targeted disruption of the Cyp 4a14 gene causes the upregulation of the Cyp 4a12 gene, an increase in the biosynthesis of pro-hypertensive 20-HETE in the kidney, and renal vasoconstriction4. These Cyp 4a14 (-/-) genotype specific changes in Cyp 4a expression are androgen-mediated and result in the development of androgen-dependent, sexually dimorphic, hypertension[1,4]. The presence of androgen--regulated CYP 4A and 2C isoforms in microvessels dissected from rat kidney, suggests that CYP P450 gene transcription and hemodynamic factors are key components of the mechanisms by which the metabolites of the CYP P450 arachidonic acid monooxygenases regulate renal physiology and systemic blood pressures. Genomic cloning and sequence analysis shows the existence of two human 4A isoforms, CYPs 4a11 and 4A22. Only CYP 4A11 is expressed in kidney and catalyzes the hydroxylation of arachidonic acid. These results, as well as epidemiological evidence suggesting a role for sex-dependent mechanisms in the pathophysiology of human hypertension, suggest CYP 4A11 as a novel candidate gene for the analysis of the molecular mechanisms of human hypertension.

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WL19 CHIMERIC SIGNALS OF XENOBIOTIC INDUCIBLE CYPS: MECHANISM OF MITOCHONDRIAL TARGETING AND FUNCTIONAL IMPLICATIONS

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Xenobiotic inducible CYP1A1, 2B1, 2E1 and also a number of other CYPs contain „atypical“ N-terminal signals at their N-termini, termed „chimeric signals“, and bimodally targeted to both the ER and mitochondria under both *in vitro* and *in vivo* conditions. A positively charged domain spanning 12-23 amino acids, containing two or more evenly spaced positive residues, immediately past the N-terminal ER targeting and transmembrane domains functions as a cryptic mitochondrial targeting signal in these CYPs. Mitochondrial targeting of apoproteins appears to be a regulated process requiring the activation of cryptic signal either by endoproteolytic processing at the N-terminus as in the case of CYP1A1 (and also 1A2 and 1B1) or by PKA mediated phosphorylation at internal sites as in 2B1, 2E1 and 2D6. Mitochondrially targeted CYPs functionally interact with Adx through charge pairing and catalyze enzymatic activity for different substrates in an Adx+Adr supported system. Under chronic inducer treatment conditions (BNF) the mitochondrial targeted, N-terminal truncated CYP1A1 (termed P450MT2) accumulates with time to become a major fraction of cellular/tissue pool. Furthermore, P450MT2 exhibits high specificity for N-demethylation of erythromycin, and an array of antidepressants and neuroactive drugs that are not the marker substrates for the intact microsome associated CYP1A1. The presentation will focus on a) mechanisms of mitochondrial targeting of CYPs with chimeric signals, b) mode of interaction of mitochondrial targeted CYPs, c) molecular and biochemical characteristics of the cytoslic protease, which is induced/activated by chemical, and or, oxidative stress, and d) possible physiological and pathological implications of mitochondrial targeted CYPs.

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4 LECTURES – THURSDAY – CL

CL02 REGULATION AND INTERACTION OF REDOX PARTNERS WITH ACTINOMYCETE CYPS

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The genus *Streptomyces* produces more than two-thirds of naturally occurring antibiotics and a wide array of other secondary metabolites. These include numerous antibacterial agents (e.g. erythromycin, tetracycline), anticancer agents (doxorubicin, adriamycin), immunosuppressants (FK506, rapamcycin), antiparasitic agents (avermectin, nemadectin), antifungals (amphotericin, griseofulvin, nystatin), cardiovascular agents (lovastatin, compactin) and veterinary products (monensin, tylosine). Invariably, cytochromes P450 are involved in the biosynthetic pathways of many of these compounds. We report the pattern of mRNA expression for the cytochrome P450 (CYP)/ferredoxin (fdx)/ferredoxin reductase (fpr) complement of the model organism of this genus, *Streptomyces coelicolor* A3(2). While transcription for all of the 18 CYPs occurred from the onset of germination, throughout developmental growth and secondary metabolism, fdx/fpr expression did not, indicating that availability of reductase regulated CYP activity. Exposure of cells for 6h following germination to the polycyclic aromatic hydrocarbons dimethylbenzanthracene and benzo[a]pyrene, saw a loss of some specific CYP messages depending on the compound used. However, activation of transcription of a specific fpr gene, FR2 (SCO7117), was observed, indicating that it may interact with specific CYPs involved in the xenobiotic metabolism response. As a test of this hypothesis we expressed in *Escherichia coli* and purified to homogeneity five CYPs (CYP105D5, which is specifically involved in xenobiotic metabolism, and CYP154A1, CYP154C1, CYP158A1 and CYP158A2, which are putatively involved in secondary metabolism) and the two ferredoxin reductases (FR2 and FR3) proposed to shuttle electrons to CYP via iron-sulphur ferredoxins. Interestingly, FR2 preferentially reduced CYP105D5 (>90% reduction) compared to the other CYPs (<20% reduction) while FR3 preferentially reduced CYP154A1, CYP154C1, CYP158A1 and CYP158A2 (>85%) compared to CYP105D5 (<10%). We propose that control of CYP activity occurs through expression of specific ferredoxin reductases both during the normal life - cycle and following xenobiotic exposure. The results are extrapolated to other members of actinomycetes.

CL03 MECHANISM AND REGULATION OF ELECTRON TRANSFER IN CYTOCHROME P450 REDOX SYSTEMS

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The cytochromes P450 typically require the delivery of two electrons from redox partner enzymes to facilitate the reductive activation of bound molecular oxygen, leading to the oxygenation of substrate[1]. In eukaryotic microsomal systems, these are usually delivered by the diflavin (FAD- and FMN-containing) enzyme NADPH-cytochrome P450 reductase (CPR)[2]. CPR-like enzymes are also found in prokaryotes - the most notable example being the fatty acid hydroxylase cytochrome P450/CPR fusion enzyme flavocytochrome P450 BM3 from *Bacillus megaterium*[3]. In recent studies, we have determined the thermodynamic properties of a number of human and bacterial diflavin reductases, including P450 BM3 and human CPR[4, 5] (*Figure 1*). In both these cases, genetic dissection was used to produce

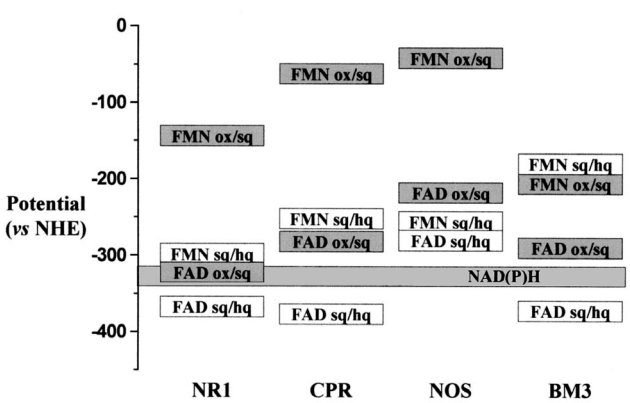

Fig. 1. *Scheme showing the respective reduction potentials for the FAD and FMN cofactors in various members of the diflavin reductase enzyme family. The midpoint reduction potentials for the oxidized/semiquinone (ox/sq) and semiquinone/hydroquinone (sq/hq) transitions of the FAD and FMN cofactors are shown for flavocytochrome P450 BM3 (BM3), neuronal nitric oxide synthase (NOS), human CPR (CPR) and human novel reductase 1 (NR1). NR1 is the most recently characterized member of the diflavin reductase family[14]. Potentials are shown relative to that of the common reductant for these reductases - NADPH (-320 mV).*

the separate FAD/NADPH-binding and FMN-binding domains of the enzymes, enabling the kinetic and potentiometric properties of the individual flavins to be studied in isolation. Thermodynamic properties of the isolated flavin domains were found to be identical, within error, to those for the intact CPR enzymes. These data support early theories of the evolutionary origin of CPR-like enzymes from fusions of genes encoding ferredoxin $NADP^+$-reductase-like and flavodoxin-like progenitors[6], and are also in agreement with structural data for the rat CPR isoform, which shows the flavin-binding domains to be clearly spatially separated[7]. Our recent kinetic studies on the BM3 and human CPR enzymes have revealed novel regulatory features; specifically as regards the mechanisms by which control is exerted over the processes of electron transfer between NADPH and FAD, and between FAD and FMN cofactors.

Stopped-flow kinetic studies of human CPR reveal the importance of Trp-676 in the control of the reductive reaction by NADPH[8]. In rat CPR, The aromatic ring of Trp-677 shields the isoalloxazine ring of the FAD flavin from solvent. The corresponding Trp-676 in human CPR should have the same structural configuration, and studies on W676H and W676A mutants confirm the importance of the residue in human CPR[8]. Trp-676 acts as a „trigger“ to release $NADP^+$, and release of NADP+ from the active site of the W676H mutant, post hydride transfer to the FAD, was retarded. This led to an accumulation of a stable EH_2-$NADP^+$ complex in stopped-flow mixing experiments with NADPH, due to slow dissociation of $NADP^+$. Analysis of the concentration dependence of FAD reduction in human CPR and its FAD/NADPH domain revealed that electron transfer was accelerated at low [NADPH], suggesting the presence of a second, inhibitory NADPH-binding site in the enzyme[8, 9]. The inhibitory site has lower affinity for NADPH, but its population results in retardation of release of $NADP^+$ from the catalytic site - and persistence of the charge transfer complex. We have recently observed a similar phenomenon in the adrenodoxin reductase homologue FprA from *Mycobacterium tuberculosis* (see accompanying paper from McLean *et al.*,[10]). In FprA there is a remarkable (up to 8-fold) acceleration in the NADPH-to-FAD electron transfer observed as [NADPH] is decreased below 100 μM.

The P450 BM3 CPR and FAD/NADPH domains did not exhibit this pattern of accelerated NADPH-to-FAD electron transfer at low [NADPH], instead showing a more „normal“ positive, hyperbolic dependence of electron transfer rate on [NADPH] (see accompanying paper from Roitel *et al.*,[1]1). However, studies of the ability of the 4-electron reduced CPR domain and 2-electron reduced FAD/NADPH domains to pass electrons to $NADP^+$ indicated that the rate of this reaction was reciprocally related to [$NADP^+$]. For P450 BM3 we envisage that the dual pyridine nucleotide binding site model remains viable, but that for this enzyme there may be discrimination of the second binding site towards $NADP^+$, perhaps reflecting a closer evolutionary relationship to the ferredoxin $NADP^+$ reductases (*Figure 2*,[11]). Studies of the „reverse“ ($NADP^+$ reduction) reaction with dithionite-reduced human FAD/NADPH domain indicate that the hydride transfer reaction occurs more rapidly in this direction (~ 8 s^{-1}) than in the physiologically relevant direction (~ 3 s^{-1}) under the experimental conditions[9]. This is in accordance both with the proposed evolutionary origin of the domain, and with the respective reduction potentials for the $NADP^+$/NADPH and human CPR FADH2/FAD redox couples (-320 mV and -333 mV, respectively,[5]).

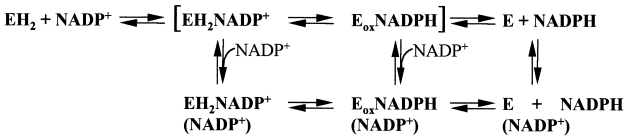

Fig. 2. *Reaction scheme for interaction of the CPR domain of P450 BM3 with oxidized and reduced nicotinamide nucleotide cofactors. Oxidized (E, E_{ox}) and reduced (EH_2) forms of the enzyme interact with either NADPH or $NADP^+$ cofactors, which may bind to either a catalytic (stronger) or non-catalytic (weaker) pyridine nucleotide binding site. Occupation of the non-catalytic site is indicated by the appearance of the $NADP^+$ in parentheses (although NADPH can also occupy the second site). The EH_2NADP^+ and $E_{ox}NADPH$ species are in dynamic equilibrium, and the reaction is reversible - yielding either EH_2 and $NADP^+$ (following dissociation of $NADP^+$ from reduced enzyme) or E_{ox} and NADPH (following dissociation of NADPH from oxidized enzyme). Both equilibrium species may bind a second molecule of $NADP^+$(H) to the second (non-catalytic site), and NADPH can also bind NADPH to the catalytic site after population of the non-catalytic site by $NADP^+$(H).*

To investigate the rates of internal (i.e. between FAD and FMN cofactors) electron transfer in human CPR, temperature jump relaxation spectroscopy was used. In key experiments, the enzyme was pre-reduced to a 2-electron level under anaerobic conditions using either dithionite or NADPH as the reductant. Following a rapid (4 μs) temperature jump (of approximately 7 °C), the rate of electronic re-equilibration in the systems was determined. In preceding potentiometric studies, the proximity of the FAD oxidised/semiquinone and FMN semiquinone/hydroquinone was established, suggesting that thermal perturbation of the 2-electron reduced enzyme would affect the proportions of enzyme existing in either FAD semiquinone/FMN semiquinone or FAD oxidised/FMN hydroquinone forms[5]. This proved to be the case, and a rate of 55 s^{-1} was determined for inter-flavin electron transfer in the NADPH-reduced enzyme[12]. The corresponding rate for dithionite-reduced enzyme was only 11 s^{-1}, demonstrating that nicotinamide coenzyme binding is of considerable importance in regulating the internal electron transfer rate. Increasing solution viscosity dramatically reduced inter-flavin electron transfer rate, suggesting conformational gating of the process[12]. More recent studies have examined the influence of the

binding of molecular „fragments“ of pyridine nucleotide cofactors on the electron transfer reaction in human CPR[13]. Collectively, these data have provided new insights into the mechanisms of control of reactivity in the diflavin reductase enzyme family.

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CL04 MECHANISMS AND CONSEQUENCES OF INTERINDIVIDUAL VARIATION IN CYTOCHROME P450 FUNCTIONAL GENOMICS

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The majority of human P450-dependent xenobiotic metabolism is carried out by polymorphic enzymes which can cause abolished, quantitatively or qualitatively altered or enhanced metabolism. The latter situation is due to stable duplication, multiduplication or amplification of active genes, most likely in response to dietary components that have resulted in a selection of alleles with multiple non-inducible genes. An updated list of variant CYP alleles is present at the Home Page of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/). Several examples exist where subjects carrying certain alleles suffer from a lack of drug efficacy due to ultrarapid metabolism or, alternatively, adverse effects from the drug treatment due to the presence of defective alleles. Dosage requirements for several commonly used drugs that have a narrow therapeutic range can differ more than 20-fold dependent on the genotype or the enzyme expression status. By contrast, carcinogen metabolising cytochrome P450s are less polymorphic and no firm relationships have been established linking increased risk for cancer with any specific P450 polymorphism. In the present overview recent aspects of cytochrome P450 polymorphism is discussed. A new cellular level for the polymorphic expression of functional cytochrome P450s of different specificities, namely trans-splicing, is presented. Furthermore, new polymorphic CYP alleles will be presented which affect the ability for induction of gene expression.

CL05 ROLE OF GENETIC POLYMORPHISM, DRUG EXPOSURE AND SEX FOR EXPRESSION AND FUNCTION OF HEPATIC CYTOCHROMES P450

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About one dozen P450 isozymes of families CYP1, CYP2 and CYP3 are collectively responsible for most phase I biotransformations in human liver. Variability in the expression and function of these isozymes is a major cause for unpredictable drug response. Principal mechanisms underlying inter- and intraindividual variability are genetic polymorphisms, regulation of expression by xenobiotic and endogenous substances, as well as physiological factors including sex, liver disease and others. To dissect the contributions of these different factors to P450 variability, we established a large human liver bank consisting of over 300 surgical tissue samples with extensive clinical documentation. Recent work concentrated on families 2 and 3, including CYPs 2D6, 2B6, and the 3A family.

CYP2D6 is involved in the metabolism of about one fourth of all drugs. Its expression is highly polymorphic with more than 70 known alleles which code for protein

products with variant or lacking function[1]. This leads to four phenotypes in the Caucasian population which are termed ultrarapid (UM), extensive (EM), intermediate (IM) and poor metabolizers (PM) depending on their drug oxidation capacity. Using liver samples of phenotyped patients, we have shown that these phenotypes are the result of different protein expression levels with UM, EM, IM, and PM expressing about 24, 8, 2.5 and 0 pmoles of CYP2D6 per mg of microsomal protein[2]. In about 20% of UMs, increased expression is known to be due to the presence of three or more gene copies (e.g., 2D6*2x2). The residual 80% of UMs are genetically less well predictable because the involved genotypes are also frequent among EMs. By contrast, the IM phenotype is a distinct subgroup of about 10-15% of the population most of whom have the genotype *IM / *PM, with *IM designating a functionally impaired allele (*41, *9, *10) and *PM designating any nullallele[3]. Recent work on the most frequent *41 allele, which was previously shown to be genetically distinct from *2 in a putative NFkappaB binding site at -1584 bp[3] suggests that this promoter polymorphism can not explain the large difference in phenotypic expression between *2 and *41. Mutation scanning revealed a novel intronic mutation that was found to be specific for *41. RT-PCR experiments on liver RNA from genotyped indicviduals demonstrated a close correspondence between its presence and a nonfunctional 2D6 splice variant. Together, these data provide molecular explanations for the differentiated genetic determination of CYP2D6 function, which has been known for over 25 years.

The CYP3A isozymes 3A4, 3A5, 3A7 and 3A43 are expressed at very different levels in human liver with CYP3A4, the major form[4], contributing critically to the metabolism of at least half of all drugs. Although a number of polymorphic CYP3A4 alleles were recently identified[1], these are rare and do not make a significant contribution to CYP3A4 expression variability, which was found to be 50-fold both at the level of protein (6 to 295 pmoles per mg of microsomal protein) and ~70-fold at the level of mRNA. More important is inducibility by endogenous and exogenous substances which bind as ligands to the orphan nuclear receptors PXR (pregnane X receptor) or CAR (constitutive androstane receptor), thereby causing up-regulation of gene transcription. By analyzing CYP3A4 expression in human liver samples in relation to pre-surgical drug exposure, it was observed that treatment with carbamazepine or St. John's wort lead to an approx. four-fold in vivo induction of 3A4-protein, but most drugs produced smaller or no effects. Because there is substantial evidence for sex-related differences in drug pharmacokinetics in humans, in particular for CYP3A4 substrates, we systematically analyzed a larger number of liver samples. By carefully controlling for confounding factors, we observed for the first time a sexual dimorphism in CYP3A4 expression with women expressing roughly twice the amount of 3A4 protein than men. In contrast to CYP3A4, expression of both 3A5 and 3A7 was found to be largely determined by genetic polymorphisms.

Human CYP2B6 recently gained more attention since it was shown to be involved in the metabolic activation and inactivation of a number of clinically important drugs, including cyclophosphamide and bupropion. CYP2B6 is inducible by a range of substances including phenobarbital, and the nuclear receptors CAR and PXR play a role in the induction process. In the first systematic polymorphism analysis of the *CYP2B6* gene, we could show that 2B6 is highly polymorphic with numerous alleles coding for variant proteins with different expression levels[5]. For example, a mutation in exon 9 (R487C) was associated with almost 8-fold decreased protein levels in human liver. Furthermore, we recently identified more than ten novel alleles with amino acid changes, some of which affect expression and function drastically, as shown by expression in insect cells. Thus, with respect to the balance between genetic and nongenetic factors controlling expression, CYP2B6 appears to take an intermediate position between CYP2D6 and CYP3A4.

These studies demonstrate the value of large human liver collections to dissect the various factors responsible for variability in P450 expression and function and they contribute to the understanding of interindividual variability in drug response.

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CL06 ENVIRONMENTAL GENOMICS ON PLANT P450 SPECIES METABOLIZING HERBICIDES

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Current genome projects revealed that there are 3 P450 genes *Saccharomyces cerevisiae*, 57 genes in *Homo sapiens*, 80 genes *Caenorhabditis elegans*, 88 genes in *Drosophila melanogaster*, approximately 150 genes in *Phanerochaete chrysosporium*, 273 genes in *Arabidopsis thaliana* and 458 genes in *Oryza sativa*.

Particularly, higher plants were found to have outstandingly large number of P450 genes. However, the enzyme function is known for around 60 P450 species in plants. We worked on systematic reverse genetics of the P450 superfamily in *Arabidopsis*. Based on the combination of cytochrome P450 databases, T-DNA mutant lines of *Arabidopsis*, in planta transformation of *Arabidopsis* and heterologous expression systems, the function of CYP71B11 and CYP86A1 was identified. CYP71B11 catalyzed hydroxylation of sulphonylurea herbicides and seemed to be involved in the herbicide selectivity and resistance. CYP86A1 catalyzed w-hydroxylation of fatty acids, and involved in production of cutin monomers. This P450 species also seemed to be related to tolerance to the herbicide pyributicarb.

Discussions include application of P450 species metabolizing herbicides for phytoremediation and monitoring of environmental contaminants in plants.

CL07 TRANSIENT DIFFERENCE SPECTROSCOPY OF SUBSTRATE (S) AND PRODUCT (P) BINDING TO CYP450: (A NOVEL INSIGHT INTO PRODUCT BINDING)

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Substrate-induced low- to high-spin state transition reflected as shift in the Soret band from ~417nm to ~390nm has been observed with many P450s (E). This is the basis for the well known S-induced Type I difference spectrum with absorption minimum at ~420nm and maximum at ~390nm and an isosbestic point at ~406nm, indicative of high spin complex. Insights into structural changes associated with these transitions come from crystallographic data on P450cam. The substrate-free low-spin enzyme is six-coordinated with a water covalently bound to iron at L_6 which is buried in the active site cavity. The substrate camphor binds to the protein moiety (will be referred to as Site 1), releasing the L_6 water converting the heme to five-coordinated high-spin state. The product 5-exo-OHCamphor (P) binds to Site 1 as well as to the iron (Site 2) through its -OH group forming Fe-O bond resulting in six-coordinated low-spin complex. Whereas five-coordinated Site 1 complexes are high spin the Site 2 complexes are low spin. Since the heme is buried in the active site cavity, the Site 2 would not be directly accessible to exogenous P therefore the P is not expected to bind to both sites at the same time. We investigated this by transient difference spectroscopy using $P450_{2B4}$, by the Temperature-jump relaxation technique. The static difference spectrum produced by the substrate benzphetamine (S) was normal Type I difference spectrum. The static spectrum produced by the product des-methyl benzphetamine (P) which forms low-spin complex was much smaller and distorted with little absorption increase at 390nm. However in the time-scale of the T-jump technique, the transient difference spectrum produced by the P was also normal Type I spectrum with the expected absorption increase at 390nm, indicative of high-spin complex,. This indicates that low-spin EP complex formation may proceed via. transient high-spin intermediate. It is possible that the time difference between product binding to Site 1 and Site 2 is such that appreciable amount of high spin EP complex could be observed in the T-jump time scale. This indicates that product binding to form low spin complex may be a two-step process.

5 POSTERS – MONDAY – MP

MP01 AXIAL LIGATION FOR ENHANCING SELECTIVITY IN CATALYTIC EPOXIDATION BY CYTOCHROME P450 MODELS

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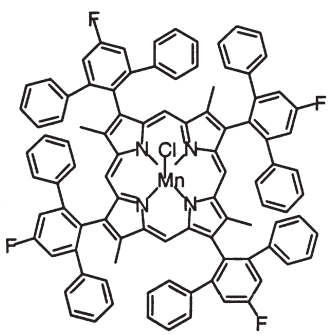
Shape selectivity of cytochrome P450 monooxygenase to a large extent comes from the narrow hydrophobic pocket formed by the arrangement of amino acid residues pointing toward the oxidation reaction site. To mimic such a pocket, heme model compouds have been developed to demonstrate shape selectivity in the oxidation of hydrocarbon substrates.[1,2] Typically, such compounds are difficult to synthesize and the selectivity is not particularly remarkable. The catalytic activity and selectivity of metalloporphyrins in biomimetic oxidation chemistry of cytochrome P450 can be affected by the axial coordination. We have observed a remarkable axial ligand effect for enhancing regioselectivity in diene epoxidation by a barrel-shaped porphyrin **1** as well as for altering enantionselectivity in methylstyrene epoxidation by a chiral porphyrin **2**.[3, 4]



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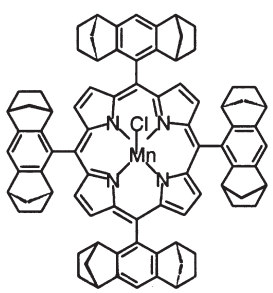

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Using metal-oxo species derived from manganese porphyrin **1** with PhIO or MCPBA, enhancements in regioselectivity towards less substituted C=C bond in diene epoxidations can be achieved by simply adding organic bases as axial ligand. For example, the selectivity to produce 8,9-limoene oxide can be elevated from 33% (ligand free) to 66% (with DMAP or 4-t-butylpyridine). We have examined the effect of various bases and ruled out the electronic effect to be solely responsible for this phenomenon. A plausible explanation is the presence of structural modulation that the binding of organic bases in the highly crowded environment would restrict the rotational freedom of the terphenyl rings against the porphyrin plane leading to comparatively more rigid pocket on the other side for improved selectivity. Similarly, enantioselectivity is also improved by pyridine bases with catalyst **2**. In this case, the electronic effect may play a major role. We have carried out epoxidation of *cis*-β-methylstyrene using **2** and pyridines of various electronic properties. A linear free energy relationship has been established for electron donating region. And the best enantioselectiivty is 81 % ee.

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MP02 FUNCTIONAL ANALYSIS OF CYP2D6.31 - ARG440HIS SUBSTITUTION DIMINISHES ENZYME ACTIVITY BY DISRUPTING REDOX PARTNER INTERACTION

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Cytochrome P450 2D6 is an important human drug-metabolizing enzyme that exhibits a marked genetic polymorphism. Numerous inactivating mutations of the *CYP2D6* gene have been characterized and homozygous or compound heterozygous individuals for such non-functional alleles exhibit a poor metabolizer phenotype. However, the consequences for expression and/or catalytic activity of a substantial number of other allelic variants of *CYP2D6*

that occur at very low frequencies remain to be determined. One such rare allele, *CYP2D6*31*, is characterized by mutations encoding three amino-acid substitutions: $Arg^{296}Cys$, $Arg^{440}His$ and $Ser^{486}Thr$. The identification of this allele in an individual with an in vivo PM phenotype prompted us to analyze the functional consequence of these substitutions on enzyme activity using yeast as a heterologous expression system.

We demonstrated that the $Arg^{440}His$ substitution, alone or in combination with $Arg^{296}Cys$ and/or $Ser^{486}Thr$, altered the kinetics of debrisoquine 4-hydroxylation and dextromethorphan *O*-demethylation such that their intrinsic clearances (Vmax/Km) were decreased by over 95% compared to those observed with the wild-type enzyme. The rate of oxidation of *rac*-metoprolol at single substrate concentrations was also markedly decreased with each mutant form of protein containing the $Arg^{440}His$ substitution. These *in vitro* data confirm that the *CYP2D6*31* allele encodes essentially a non-functional enzyme compatible with an *in vivo* PM phenotype, and that the $Arg^{440}His$ exchange is the inactivating mutation. A homology model of CYP2D6 based on the crystal structure of rabbit CYP2C5 locates Arg^{440} on the proximal surface of the protein. Docking the structure of the FMN domain of human cytochrome P450 reductase to the CYP2D6 model suggests that Arg^{440} is a key member of a cluster of basic amino acid residues important for binding between the two proteins and efficient electron transfer.

MP03 COVALENT HEME BINDING TO CYP4B1 *VIA* GLU310 AND A CARBOCATION PORPHYRIN INTERMEDIATE

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Cytochrome P450 4B1 (CYP4B1) is a principally extrahepatic P450 isoform that is found in mammalian lung and kidney tissues. Recent investigations have shown that CYP4B1, and other members of the CYP4 family, are covalently linked to their prosthetic heme group through an ester linkage with the protein. Sequence alignments of covalently linked CYP4 enzymes indicate a unique glutamic acid in the I-helix motif FEGHDT as the site of attachment. This residue was mutated to glycine, alanine, and aspartate to evaluate the role of E310 in covalent heme binding and catalysis. None of these mutants displayed covalently linked heme, demonstrating that E310 is required for covalent binding. All three mutants show decreased rates of lauric acid hydroxylation with the A310 and G310 mutants exhibiting altered regioselectivity favoring ω-1 and ω -2 hydroxylation of lauric acid respectively. Uniquely, the acid-dissociable heme obtained from the D310 mutant exhibited the same chromatographic behavior as a hydroxymethylheme species released upon base treatment of the covalently linked wild-type enzyme. Expression of D310 in $H_2{}^{18}O$ enriched media resulted in incorporation of the heavy isotope into the hydroxymethylheme at a molar ratio of ~0.8:1. Finally, the CYP4B1 hydroxymethylheme released from the wild-type enzyme upon base treatment was further fractionated to detect isomeric products. Comparison of HPLC UV/Vis traces of these hemes with hydroxymethylheme standards suggests a mixture of 5-hydroxymethylheme (85%) and 8-hydroxymethylheme (15%). These data (i) show that E310 serves as the site of covalent attachment of the heme to the protein backbone of rabbit CYP4B1; (ii) demonstrate the mechanism of covalent heme attachment most likely involves a heme carbocation species; and (iii) suggest that heme is bound to wild-type CYP4B1 in two distinct orientations.

This work was supported by GM49054 and GM07750.

MP04 α-NAPHTHOFLAVONE AS A MODEL LIGAND OF THE ACTIVE SITE OF CYP3A

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Determination of the structure of the binding sites of the CYP protein molecules and the structure-function relationships are in the center of interest. Understanding this area may increase our knowledge useful for development of new drugs as well as for decreasing activation of many carcinogens. From this point of view, the CYPs of a 3A subfamily are the most intensively studied. The activities of human CYP3A4 are regulated by binding of various ligands, which leads either to their stimulation or inhibition. The flavonoid α-naphthoflavone (α-NF) is one of such compounds (ligands). It is often employed as a selective inhibitor of CYP1A1/2. However, α-NF interacts also with other CYP enzymes, human CYP3A4 and rabbit CYP3A6, influencing their activities. We determined stimulation of 17β-estradiol 2-hydroxylation catalyzed by CYP3A, while other CYP3A activities tested in our study were inhibited; the inhibition of erythromycin and tamoxifen N-demethylation was reversible, while progesterone and testosterone 6β-hydroxylation was inhibited by α-NF

in an irreversible manner[1]. These results suggest that the effect of α-NF on CYP3A activities is strongly dependent on individual substrate. Moreover, here we show that stimulation and/or inhibition effects of α-NF are dictated by the complexity of the CYP3A enzymatic system. In the enzymatic systems, where oxidation of CYP3A substrates is very effective (microsomes and Supersomes™), α-NF irreversibly inhibited testosterone 6β-hydroxylation. In other systems (CYP3A reconstituted with NADPH: CYP reductase) the CYP3A activity is stimulated or not affected. The results obtained in our studies may be utilized for suggestion of a model for the protein domain of the CYP3A active site. Our results indicate that CYP3A contains at least two, or probably three, distinct binding sites for substrates. We found that α-NF is reversibly bound to the same binding site as erythromycin and tamoxifen, competing with them for binding to the active center of CYP3A. The second binding site, distinct from the first one, is the binding site for progesterone and testosterone. To this site, α-NF reactive species, generated by its oxidation with CYP3A, and causing the irreversible inactivation of the enzyme, are expected to be bound. Evidence for the third substrate-binding site arises from analysis of 17β-estradiol 2-hydroxylation in the presence of α-NF, cooperativity of α-NF binding to CYP3A and from the mode of the protection caused by 17β-estradiol against α-NF-mediated CYP3A inactivation.

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MP05 STRUCTURE-FUNCTION RELATIONSHIP IN CYTOCHROME P450 CYP 2C11

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Among cytochrome P450 family, CYP 2C11 (isolated from male rat liver) is characterized by its ability to hydroxylate testosterone at 2 positions : 2-alpha and 16-alpha. We have previously shown that the decrease in testosterone hydroxylating activity observed in an allelic variant (Gunn rat) is associated with 3 mutations (aminoacids 1, 116 and 187).

The aim of the present study was to evaluate the respective contribution of these residues to the catalytic efficiency and to the regio- and the strereospecificity of the CYP 2C11. We constructed single or double mutants, co-expressed them with NADPH cytochrome P450 reductase in baculovirus/insect cell system and determined the testosterone metabolism in microsomal fractions. P450 spectra from all the mutants were similar to that of wild type microsomal fractions.

Each of nine substitutions in the wild type allele diminished the catalytic activity without modifications in the regio- and the stereospecificity. None of ten subtitutions in the Gunn allele was able to restore the level of the wild type protein.

These results will be discussed by comparison with the X-ray crystal structure published for CYP 2C5 (1) which exhibits close similarity with CYP 2C11.

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MP06 CAMPHOR HYDROXYLATION BY CYTOCHROME P450CAM: A THEORETICAL STUDY BY HYBRID QUANTUM MECHANICAL/ MOLECULAR MECHANICAL (QM/MM) METHOD

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Cytochrome P450cam catalyzes the stereoselective and regioselective hydroxylation of camphor, which produce, with high efficiency, 5-exo-hydroxycamphor. The active species of P450cam, CpdI, is an oxo-iron-portoporphyrin complex, which is ligated to a cysteinato amino acid. Our recent theoretical study on CpdI[1] in the enzyme environment revealed that it behaves as a chameleon and adapts its electronic character and geometry to the environment. Due to the significant effect of the environment on CpdI, we examined the mechanism for camphor hydroxylation by CpdI within the native protein environment. We used hybrid QM/MM calculations with UB3LYP density functional QM Hamiltonian. In these calculations, a suitably trunca-

ted CpdI species is treated with the QM method, while the protein environment is treated with MM. The results show that the reaction proceeds in a stepwise manner via the rebound mechanism and exhibit a two-state reactivity scenario in agreement with gas-phase calculations[2,3]. The mechanistic and energetic features of the hydroxylation reaction as well as a comparison to the gas phase results will be presented[4].

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MP07 CYTOCHROME P450 3A4 ALLOSTERIC MECHANISM STUDIED BY HIGH-PRESSURE SPECTROSCOPY

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Hydrostatic pressure perturbation was used to study the allosteric mechanisms in human microsomal cytochrome P450 3A4 (3A4). This approach is especially valuable for studies of P450-substrate interactions due to the central role of heme pocket hydration in the mechanism of the substrate-induced spin shift in the P450 heme iron. The pressure-related behavior of 3A4 with the non-allosteric substrate bromocriptine (BCT, K_d = 0.3 μM) was quite similar to that reported earlier for other cytochromes P450. A pressure induced-spin shift in both substrate-free (ΔV^o = -29 ml/mol) and substrate bound (ΔV^o = -40 ml/mol) enzyme was detected at rather low pressures, so that the percentage of high-spin 3A4 was negligible above 2.5 kbar. At higher pressures this process was accompanied by a P450→ P420 transition (ΔV^o = -36 ml/mol, $P_{1/2}$ = 2.7 kbar) that involves about 70% of the total hemoprotein. However, the behavior of 3A4 with the allosteric substrate 1-pyrene butanol (1PB, S_{50} = 12 μM, n = 1.6) was in drastic contrast to that described above. In this case the pressure-induced high → low spin shift was observed only in substrate-free enzyme. At high concentrations of 1PB the amplitude of the spin shift was very low, showing that hydrostatic pressure induces no substrate dissociation nor increase in the heme pocket hydration in 1PB-3A4 complex. Moreover, increasing hydrostatic pressure results in a marked increase in the cooperativity of 3A4. In our interpretation these findings reveal a conformational transition, which results from binding of the first molecule of ligand (1PB) and is central to the allosteric mechanism. This transition apparently decreases the accessibility of the active site so that water flux into the heme pocket is impeded and the high-spin state of the heme iron is stabilized. This hypothesis is also supported by the results of stop-flow and pressure-jump experiments on the kinetics of 3A4 interactions with 1PB and BCT. The unusual stability of the high-spin state of 3A4 complex with 1-PB was observed only in 3A4 oligomers. Upon dissociation of 3A4 oligomers in the presence of detergents the difference in barotropic properties between 3A4-1PB and 3A4-BCT complexes disappears, suggesting involvement of subunit interactions in the allosteric mechanism. (This research was supported by grant GM54995 from the NIH).

MP08 A CHARGE-PAIRING BUNDLE AROUND CYS-154 IS PIVOTAL FOR THE ALLOSTERIC MECHANISM IN CYTOCHROME P450ERYF

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In order to explore the allosteric mechanism in cytochrome P450eryF we studied the effect of chemical modification and site-directed mutagenesis of the unique surface-exposed cysteine residue (Cys-154). Binding of 1-pyrenebutanol (1PB) to P450eryF exhibits distinct positive cooperativity (S_{50} = 13 μM, n = 2.6). The modification of Cys-154 with 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) results in complete disappearance of the allosteric phenomenon. The same result was observed when Cys-154 was replaced with isoleucine. We also found that an increase in ionic strength suppresses the cooperativity of the enzyme. The ionic strength dependence of the Hill coefficient exhibits two distinct minima at I = 0.25 M (n = 1.2) and I = 0.85 M (n = 1.5). Importantly, this decrease in cooperativity is associated with a profound shift of the spin equilibrium of the substrate-bound enzyme towards the high-spin state. CPM modification of Cys-154 as well as the C154I mutation completely prevents these effects.

Cys-154 is located in the loop between D and E α-helices and is surrounded by a network of salt bridges formed

by amino acid residues located in close proximity. We hypothesized that the binding of the first substrate molecule to the enzyme promotes the dissociation or rearrangement of these salt bridges that modulate the spin equilibrium in the enzyme-substrate complex. Modification of Cys-154 as well as its replacement with isoleucine impairs this mechanism and deprives the enzyme of its allosteric properties. These results indicate that the rearrangement of a charge-pairing bundle between the C, D and E α-helices in P450eryF is crucial for allostery and substrate-induced spin transitions in the enzyme. (This research was supported in part by grant H-1458 from the Robert A. Welch Foundation and grant GM54995 from the NIH).

MP09 MOLECULAR MODELING OF ALKOXYRESORUFIN OXIDATION BY P450 1A1, 1A2 AND THEIR MUTANTS

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The two members of P450 1A subfamily, 1A1 and 1A2, share 72% amino acid sequence identity, but display different substrate specificities and inhibitor susceptibilities. Some substrates are metabolized by both enzymes, but with different efficiencies. For example, 1A1 effectively oxidizes 7-ethoxyresorufin, while 1A2, although able to metabolize this substrate, exhibits preference towards the 7-methoxyresorufin. Substrate specificity, product profiles, and product formation rates may be attributed to a substrate's tendency to satisfy the catalytic requirement of site approach to the activated oxoheme. The substrate must assume an appropriate near attack configuration for the reaction to proceed and to yield product. Therefore, the replacement of residues present in the active site should alter substrate binding orientation and dynamics, and thus product formation rates and the extent of uncoupling.

The homology models of P450 1A1[1] and 1A2 were used to dock enzyme substrates into the active site and to identify key residues that might be important for activity. Several of these key residues are different between the two enzymes. Thus, we have constructed several reciprocal active site mutants of 1A1 and 1A2, evaluated their methoxyresorufin-O-dealkylase and ethoxyresorufin-O-dealkylase activities, and compared the results with those obtained from docking and molecular dynamics simulations of the substrates bound in models of P450 1A1 and 1A2 wild type and mutants. These methods have been successfully used to explain the effect of mutations at position 382 in P450 1A1[2].

Current results further demonstrate the utility of molecular models to explain differences in substrate selectivity between P450 1A1, 1A2 and their mutants. For example, the preference of P450 1A2 for methoxyresorufin and P450 1A1 for ethoxyresorufin may be rationalized by substrate dynamics within the active site. In P450 1A2 WT, methoxyresorufin binds with the methoxy group close to the ferryl oxygen and remains in its proximity during molecular dynamics. On the other hand, this substrate is highly mobile in P450 1A1 WT and the site of oxidation is much farther from the ferryl oxygen. This hinders methoxyresorufin-*O*-dealkylation, consistent with the experimental data. In contrast, ethoxyresorufin, which is an excellent substrate for P450 1A1 WT, binds with its ethoxy site of oxidation in constant proximity of the ferryl oxygen. Similar reasoning can be applied to explain changes of activity in mutant P450s. For example, the loss of methoxyresorufin-*O*-dealkylase and ethoxyresorufin-*O*-dealkylase activities in P450 1A2 L382A may be explained by the findings that sites of oxidation for both substrates did not approach the ferryl oxygen in simulations and substrate mobility was greatly increased. In general, the loss of activity observed in several P450 1A1 and 1A2 mutants may be linked to increased substrate mobility and binding orientations unfavorable to substrate oxidation.

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MP10 CAN P450 BM3 TURN OVER SUBSTRATES TYPICALLY METABOLISED BY CLASS II HUMAN P450S? EVIDENCE ON THE FUNCTIONAL RELATIONSHIP OF P450 BM3 WITH THE HUMAN ENZYMES

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Present in the three domains of life, cytochrome P450 monooxygenases constitute one of the most diverse enzyme superfamilies. In most cases, the cytochrome P450 redox partners are synthesised as separate polypeptides. However, in the fatty acid hydroxylase P450 BM3 of *Bacillus megaterium*, the haem domain is fused at the carboxyl terminus with a NADPH:cytochrome P450 reductase

module. As self-sufficient enzyme, it has the highest catalytic activity known for a P450 monooxygenase. The molecular phylogeny illustrates that the BM3 cluster, with a eukaryotic (fungal) and prokaryotic branch, is more related to eukaryotic P450s than to most of the prokaryotic P450s. By virtue of its similarity to microsomal P450s this soluble prokaryotic enzyme has served as a 3D model for the membrane-associated eukaryotic P450s. Inspired by the domain structure of P450 BM3, several soluble fusion proteins comprising class II P450s fused with NADPH:cytochrome P450 reductase have been expressed to facilitate their biochemical characterisation.

Can P450 BM3 turn over substrates typically metabolised by classII human P450s? To address this question 16 drugs, clustered in 4 different groups depending on the specificity for different human P450s, were screened using a high-throughput screening method[1]. 7 positives were identified and further characterised. The Km determined for all the drugs investigated is in the millimolar range, 1000 fold higher that the one determined for the human P450s. Nevertheless due to the high catalytic efficiency of P450 BM3, the observed Vmax is 10 to 100 times higher than the values reported in literature for the human enzymes. The metabolic products were analysed and characterised by HPLC.

These findings suggest that P450 BM3 can be used not only to derive 3D models for human P450s, but also as an experimental model for the human enzymes where in a common scaffold few alterations account for different functional characteristics.

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MP11 THE ROLE OF PHE120 IN CYP2D6 SUBSTRATE BINDING

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Although residues influencing CYP2D6 substrate specificity with respect to basic nitrogen containing compounds are well characterised, little is known about the contribution of other active site residues to substrate binding and orientation. We have recently described a structural model of CYP2D6 that identifies the aromatic residue Phe120 (located in the B'-C loop) as a likely major feature of the active site topology, aiding substrate binding. To examine the role of Phe120, substitutions to Ala, Leu, Tyr, Ser and His have been prepared in bacterial membranes coexpressing the human cytochrome P450 reductase. Subsequent characterization has been carried out using the prototypical CYP2D6 activities bufuralol 1' hydroxylation and dextromethorphan O- and N-demethylation. A peak representing a novel hydroxylated dextromethorphan metabolite was observed in the HPLC trace for the Phe120 Ala mutant, indicating a role for the aromatic side chain in substrate orientation. The larger effects on Km values determined for dextromethorphan O-demethylation compared with those for bufuralol 1' hydroxylation, indicate a substrate dependent role in binding for Phe120. The results presented here indicate that the aromatic character of the Phe120 side chain is important in aiding dextromethorphan binding, while it seems to have a non-specific space filling role in bufuralol binding.

MP12 RESONANCE RAMAN SPECTRA REVEAL SUBTLE DIFFERENCES IN HEME STRUCTURE OF P450 ENZYMES

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P450 enzymes are able to metabolize a wide variety of substrates. Obviously, the main responsibility for this variability lies with their apoprotein part, on the other hand, a question might arise, how much are the differences between various P450 isoforms (and P450-type proteins, such as NO synthase oxygenase domain) reflected in the structure of their active prosthetic group itself and in the interactions of the heme with the apoprotein part of the molecule.

Resonance Raman spectroscopy offers a possibility to study the structural details of the heme moiety and therefore might bring some insight into this question. Recently, we reported differences in heme vinyl orientation of various P450s, as judged from the vinyl stretching vibrations at around 1620 cm^{-1}.

Tab. 1: *Comparison of low-frequency resonance Raman spectra of P450 proteins and model hemeproteins (positions of Raman bands in cm-1, assignments according to[5,6])*

Protein	ν8	δ(CβCcCd)	δ(CβCaCb) + δ(CβMe)		Ref.
CYP3A4	342	376		420	TW
CYP1A2	346	377		421	TW
CYP51	348	377		418	TW
CYP2B4	347	380	(405)[a]	423	[1]
CYP101	347	379		424	[2]
CYP102	344	380	393 (397)[b]	423	TW ([3])
CYP119	345	380	390, 400	(430)	[4]
CYP11A1	344	379	391, 399	(415)[a], 420	[1]
NOSoxy	349	380	390, 405	430	TW
Mb Fe(III)	344	376	409	440	[5]
Cyt c (II)	347	376,382	394[c], 401[c]	413, 421	[6]

[a] band not apparent in the measured spectra, found by a peak-fitting procedure
[b] holoenzyme [3]
[c] $\delta(C_\beta C_a S)$

Unfortunately, these vinyl modes are to some extent obscured by the presence of strong skeletal vibration of porphyrin (ν_{10}) in the same spectral region. In this work, we therefore examined the vinyl modes in low-frequency spectral region several P450 proteins. (CYP 102 A1 from B. megaterium, both the flavocytochrome and isolated heme domain and several its mutants, namely F393H, Y51F and W96Y, CYP51 from Mycobacterium tuberculosis, and human recombinant CYP 1A2 and CYP 3A4 in presence and absence of substrate/inhibitor and in resting and reduced state. For a comparison, the heme (oxygenase) domain of neuronal nitric oxide synthase (nNOS) was also studied.

In the low frequency region, the pattern of vinyl bending modes is different between the P450 proteins studied so far. *(Tab. 1*) At least two different groups might be distinguished: the first one does not have any observable band between the propionate bending (at around 380 cm^{-1}) and the vinyl/methyl bending at approximately 420 cm^{-1} (CYP 3A4, 1A2, 51, 2B4, 101). The second group (CYP 102, 119, 11A1 and the heme domain of nNOS) has a doublet located around 390/400 cm^{-1} (or a single broad band above 400 cm^{-1}). This variability may reflect either a difference between vinyl substituents in 2- and 4-position, or some restriction in vinyl mobility, as suggested by the comparison with spectra of cytochrome *c*, where the corresponding groups are covalently linked to the apoprotein via thioether bonds.

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MP13 FLUORESCENCE CORRELATION SPECTROSCOPY STUDY OF INTERACTION BETWEEN CYP 3A4 AND COUMARIN 6

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Fluorescence correlation spectroscopy is a method capable to provide information about diffusion constants of fluorescent compounds in solution. We used this method to investigate the binding of coumarin 6 (3-(2-benzothiazoyl)-7-*N,N*-diethylaminocoumarin), resorufin, 7-*O*-ethylresorufin and hypericin to the human recombinant cytochrome P-450 3A4. From these compounds, the coumarin 6 is the best suitable bright fluorescent dye; the other compounds studied behave in a similar way to coumarin 6, but the data are more difficult to analyze.

Titration of coumarin 6 solution in buffer with cytochrome P-450 solution resulted in formation of a fluorescent complex coumarin-enzyme, manifested by appearance of a slower diffusing component (diffusion time τ = 0.64 ms) in addition to that of free coumarin (τ = 31 μs). This diffusion time is larger than expected for a spherical molecule of the size of CYP 3A4 (which should be about 0.15 ms), suggesting a significant deviation of the molecular shape from sphericity.

At concentrations of cytochrome P-450 exceeding 1 μmol/l, the diffusion time of the slowly diffusing component (representing the enzyme-coumarin complex) remarkably increased to about 1.1 ms. Most probably, this increase reflects an aggregation of P-450 molecule at concentrations higher than this limit. The size of aggregate might only be estimated (as it depends on the shape of the particle); most probably these are hexamers. (As reported previously for CYP 2B6 and 101 []).

The aggregation apparently prevents full binding of the fluorescent molecule (coumarin 6), which is demonstrated by the persistence of the fast diffusing component at even high P450 concentrations.

Whereas the increase of buffer ionic strength (up to 300 mM) did not change this aggregation behavior, addition of ethanol to final concentration 200 mM (< 1%) prevented the formation of aggregates and allowed protein titration to almost full coumarin 6 binding.

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MP14 PROBING THE ALTERATIONS IN THE ACTIVE SITE OF P420CAM: HIGH PRESSURE STUDIES ON THE P450CAM-LIGANDS AND -INHIBITORS COMPLEXES

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We have shown that cytochrome P450cam is a relatively compressible protein (pressure-induced shift of the Soret band: Jung, C., Hui Bon Hoa, G., Davydov, G.D., Gill, E., Heremans, K., Eur. J. Biochem. *233*, 600 (1995)) and that pressure as high as 250-300 MPa, leads to a deformation of the heme pocket (pressure-induced deformation of the cytochrome P450cam active site: R.A., Tschirret-Guth, G., Hui Bon Hoa, and P.R. Ortiz de Montellano, J. Am. Chem. Soc. *120*, 3590 (1998)). To get more insight into the structural alterations of the active site, we has undertaken the study of the effect of pressure on the complexes between different states of the heme iron with various exogenous ligands and inhibitors such as CN, imidazole, 4-phenyl-imidazole and metyrapone. Starting with metyrapone bound to the oxidized P450cam (Soret band at 420nm and two α-β bands at 568 and 536nm) and to the reduced P450cam (Soret band at 442nm and two α-β bands at 539 and 564nm plus one smaller peak at 524nm), increase pressure till 300 MPa red-shifted the spectra of the oxidized complex to 424nm in the Soret band and to 540nm in the α-β region and blue-shifted the spectra of reduced complex at 400MPa to a new spectra centered at 420nm in the Soret with a splitting of the α-β bands centered at 558nm (higher peak) and at 526nm (lower peak). Such presure-induced spectra transitions correspond respectively to the effect of pressure on the oxidized P450cam to yield the oxidized inactivated P420 and on the reduced P450cam (Soret band at 412nm) to yield a reduced P420 in which the Soret band is red-shifted to 427nm with a huge increase in the extinction coefficient and the α-β bands are splitted into a higher peak at 560nm and a lower peak at 530nm. Similar results are obtained by using the other large ligands and inhibitors. Compression of the active site of P450cam results in an expulsion of larger ligands and possibly an alteration of the substrate access channel in a way to prevent their rebinding. In addition the heme iron of the pressure-induced reduced cytochrome P420 is hexacoordinated the spectra of which ressembles that of reduced cytochrome b_5 or cytochrome c. Such results are discussed in term of a high pressure-induced huge alteration of the active site capable of bringing close to the heme iron an histidine residue forming a sixth ligand. However the strenght of binding and/or the dynamics of such potential sixth ligand are weak enough to allow the binding of carbon monoxide and cyanide to the heme iron.

MP15 CYP ENZYMES: MOVING TOWARDS VIRTUAL SCREENING

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Cytochrome P450 is the major enzyme system responsible for catalysing the oxidation of xenobiotics. Therefore, CYP enzymes do play a very important role in clearance and drug interactions. In silico models were built from a set of chemically diverse molecules from literature or in house data either on human liver microsomes or on recombinant human CYP assays. Those models are able to indicate if a new molecule will be a substrate or not of a CYP enzyme and it requires only the 3D structure of the molecule. Therefore, it identifies the physicochemical and structural features which drive the affinity for the CYP enzyme. This method can be used at a screening level to take into account an ADME property and to guide chemical synthesis.

For the CYP2C9 and CYP2D6 enzymes, the data were respectively divided into a training set of 65 and 70 molecules to build the QSPR model and a testing set of 30 and

40 compounds to validate the models. The descriptors used come from the VOLSURF program which produce molecular descriptors from GRID molecular Interaction Fields (MIF). The MIF are obtained with four probes: DRY probe which simulates hydrophobic interactions, N1 amide nitrogen probe which simulate H-bond donor interaction, O carbonyl oxygen probe simulating H-bond acceptor interaction and a positively charged probe.

For the CYP3A4 enzyme the data were divided into a training set of 90 molecules and a testing set of 40 molecules. Five probes are used: H2O, DRY, O, N:= and an amphiphilic probe.

Partial Least Square analysis was applied and the final models have 3 PLS components with $r^2_{training} = 0.74$, $Q^2_{training} = 0.65$ and $r^2_{testing} = 0.69$ for CYP2C9, $r^2_{training} = 0.8$, $Q^2_{training} = 0.77$ and $r^2_{testing} = 0.72$ for CYP2D6 and $r^2_{training} = 0.71$, $Q^2_{training} = 0.61$ and $r^2_{testing} = 0.65$ for CYP3A4.

MP16 HEME POCKET COMPRESSIBILITY IN P450CAM-CO: HIGH PRESSURE FTIR STUDIES ON THE CO LIGAND STRETCH VIBRATION

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The effect of hydrostatic pressure on the stretch vibration band of the CO ligand in P450cam-CO bound with various substrates is measured with a membrane driven sapphire anvil high-pressure cell mounted in the Fourier transform infrared spectrometer (FTIR). The *in-situ* pressure in the cell is determined with the ruby R_1 luminescence band. For different substrate complexes the frequency of the CO stretch vibration is linearily shifted to lower values with increasing pressure. The slope of the pressure-induced shift is interpreted as isothermal compressibility of the heme pocket and found to be related to the high-spin state content which is produced by the different substrates in the Fe(III)-form of the enzyme. The compressibilities determined by FTIR would suggest that the P450-substrate complex with 100% high-spin state content is more compressible than the substrate-free complex which is in contradiction to the previously reported results obtained using the visible absorption spectroscopy (pressure-induced shift of the Soret band: Jung, C., Hui Bon Hoa, Davydov, G.D., Gill, E. , Heremans, K.: Eur. J. Biochem. 233, 600-606 (1995)). It will be demonstrated that such contradictory conclusion is only apparent and can be explained by the dual role of heme pocket water molecules. Water molecules can both make polar interactions to the CO ligand, inducing a red-shift of the CO stretch mode, and compensate electrostatic potentials of the protein on the distal side, leading to the loss of originally existing contacts to the CO ligand which results in a blue shift of the CO stretch mode. With this model our data indicate that an increased heme pocket hydration should disturb ligand-distal side contacts which are needed for a specific proton transfer in oxygen activation resulting in an increased uncoupling H_2O_2 formation.

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MP17 INTERMEDIATE RADICAL FORMATION BY THE IRON-OXO COMPLEX PRODUCED IN THE REACTION OF CYTOCHROME P450CAM WITH OXIDANTS

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The hydroxylating key intermediate in the reaction cycle of P450 is generally assumed to be a ferryl iron (Fe(IV); $S_{Fe}=1$) and a porphyrin-π-cation radical (S=1/2) (compound I) in analogy to chloroperoxidase. For P450, however, this electron configuration has not unequivocally been proven experimentally. We have recently found for wild type P450cam (CYP101) that a freeze-quenched intermediate (8ms - 200ms) in the reaction of peroxy acetic acid with the substrate-free enzyme is characterized by a ferryl iron (Fe(IV); $S_{Fe}=1$) and a radical ($S_{rad}=1/2$) which is not located at the porphyrin. By high field EPR at 94 GHz and 285 GHz we have now unambiguously identified the radical site as a tyrosyl radical. The simulation of the hyperfine structure of the 94 GHz EPR spectrum of wild type P450cam yields the orientation of the tyrosyl radical side chain. When assuming no major structural change induced by radical generation as compared with the X-ray structure, the obtained data are compatible only with Y96. In mutant Y96F the 9.6 GHz as well as the 94 GHz EPR

spectra show significantly different hyperfine structures as compared with those of the wild type. The deduced tyrosyl side chain orientation agrees now perfectly with Y75. No significant radical signal could be detected in the Y96F/Y75F double mutant excluding the formation of tyrosine radicals but also of a porphyrin radical. For P450cam with tyrosines closely located to the heme we suggest that an initially formed, but up to now undetected, porphyrin cation radical is reductively quenched by intramolecular electron transfer from the tyrosines.

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MP18 MICROLITER SCALE SURFACE ENHANCED RESONANCE RAMAN SCATTERING ON CYTOCHROME P450 BM3

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Cytochrome P450s play a major role in the oxidative metabolism of a large variety of lipophilic endogenous and exogenous compounds. These enzymes contain a heme domain, needed to activate molecular oxygen that can subsequently be incorporated in the substrate. Cytochrome P450 BM3 (CYP102), from the prokaryote Bacillus Megaterium, is a cytosolic P450 that is often used as a model for the membrane bound human CYPs. Surface Enhanced Resonance Raman Scattering (SERRS) is a powerful tool to explore the active site of heme proteins like P450s[1]. Intensities of Raman active vibrations can be greatly enhanced when the interrogating laser beam has a wavelength that coincides with an electronic transition of the sample. Furthermore the adsorbance of the sample on a metal surface enhances sensitivity and selectivity to a certain part of the protein. In this study the heme domain of CYP102 was expressed, purified and substrate interactions were studied with SERRS. By using a Raman microscope, the sample volume could be minimised to microliters, opening possibilities for low level expressible CYPs as well. The SERR spectrum of CYP102 heme domain (0.2 mM) shows the same features as reported previously on milliliter scale[1]. Clearly the ferric state marker band is shown (ν_4 at 1372 cm^{-1}), several bands are found in the 1600 cm^{-1} region, as previously reported, and also there is a shoulder of the ν_{29} vibration at 1400 cm^{-1}. Addition of lauric acid to CYP102 before aggregation with the silver particles induced several changes in the SERR spectrum in the 1100 cm^{-1} region and also in the 1500 cm^{-1} region where the increase in the ratio of ν_{29}/ν_4 is pronouncedly found. With the current setup, the SERRS technique can be applied to study the CYP102 heme domain on a picomole scale, giving good spectral information with high resolution and seems promising for studying human CYPs as well.

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MP19 MEMBRANE TOPOLOGY OF P45017α STUDIED BY CHEMICAL MODIFICATIONS AND MASS-SPECTROMETRY

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P45017α locates on the membranes of endoplasmic reticulum of adrenal and gonadal glands and catalyzes 17α-hydroxylation and C17-20 bond cleavage reactions of C21-steroids. Since hydrophobic substrate steroids are concentrated in the membranes, it is a reasonable proposal that the substrate entrance in the enzyme is oriented towards the membrane and the substrate accesses to the binding site directly from the membrane. P450 accepts electrons from NADPH-P450-reductase through the interaction with the reductase on the membrane. The membranes play important roles in the reaction of P450s and the membrane topology of P450 has been investigated by various methods. We have developed a new method for investigation of the topology by combination of chemical modifications and mass-spectrometry.

Recombinant guinea pig P45017α (His)4 was purified and was incorporated into liposome membranes by the cholate dialysis method using egg york phosphatidylcholine. Acetylation of lysine residues of P45017α was carried out with acetic anhydride at pH 9. Glycinamidation of acidic

residues of P45017α was performed with EDC in 50mM glycinamide solution at pH 5. The positions of modified amino acid residues of P45017α were deduced from the tryptic peptide mappings using MALDI TOF mass-spectrometry (Brucker Bioflex).

More than 20 times repetition of the mass-spectrometric measurements confirmed that 16 lysine residues were acetylated in detergent solubilized P45017α but some of them could not be acetylated in the proteoliposomes. Those nonacetylated lysine residues were located near both N and C-terminals. We could identify 10 glycinamidated acidic residues in the detergent solubilized P45017α but a few of them were not modified in the proteoliposomes. The nonmodified acidic amino acid residues were located in or near the F-G loop.

The difference in the reactivities of amino acid residues of P45017α between in the detergent solubilized state and in the proteoliposomes must be depending on the accessibility of the water-soluble modification reagents to the residues. The nonmodified residues in the proteoliposomes must be at or near the membrane binding domains.

The present study revealed the existence of the membrane binding domains at N- and C-terminals and F-G loop. The combination of mass-spectrometry and chemical modification is a powerful and convenient method for the study of membrane topology of the proteins.

MP20 THE AFM STUDY OF COMPLEX FORMATION WITHIN THE CYTOCHROME P450SCC-CONTAINING SYSTEM

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An application of atomic force microscopy (AFM) for the revelation and identification of individual molecules and their complexes within the monooxygenase cytochrome P450scc system was demonstrated. The binary complexes were differentiated from the individual molecules based on the height parameter. The experiments were carried out using the direct adsorption method. As supports were used the hydrophobic surface of highly oriented pyrolytic graphite (HOPG) and the hydrophilic surface of mica. All AFM experiments were carried out in a tapping mode on a Solver P47H microscope (NT-MDT).

The AFM images of molecules constituting the P450scc system - i.e. those monomeric and aggregated cytochrome P450scc (P450scc), adrenodoxin (Ad), adrenodoxin-reductase (AdR) and of their complexes - were obtained on two surface types, graphite and mica. The image heights on graphite/mica were 2.2±0.3 nm and 2.6±0.3 nm/2.7±0.3 nm for the P450scc monomers and aggregates, respectively; 1.8±0.3 nm/1.4±0.3 nm and 2.1±0.3nm/2.2±0.3 nm for Ad and AdR, respectively. It was shown that AFM enables to reveal and visualize complexes formed between the water-soluble proteins (Ad/AdR) and, also, between the membrane-bound and membrane-soluble proteins (P450scc/Ad, P450scc/AdR). The heights of the binary AdR/P450scc, AdR/Ad and Ad/P450scc were found to be, respectively, 3.2-3.4 nm, 3.1-3.4 nm and 2.8-3.6 nm.

MP21 THE ROLE OF FERRIC-PEROXO REACTIVITY AT THE CROSSROADS OF P450 CATALYSIS

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The peroxo-ferric species serves as a critical junction in not only the P450 monoxygenation scheme, but in the oxidative transformations of a whole host of heme and non-heme metalloenzymes. The robustness of the ferric-peroxo intermediates, in both protonated and unprotonated forms, manifests itself in a veritable complexity of fates in the P450 catalytic cycle. Former studies have implicated the peroxo-species as (1) an intermediate on the pathway of Compound I formation (2) a branchpoint for productive and peroxide evolving chemistries, and (3) a direct oxidant in a number of electrophilic and nucleophilic oxidations. Our recent results utilize cryoradiolytic methodologies for the direct visualization of peroxo-iron evolution as a function of annealing temperature in native and a series of active site mutant P450s. This has allowed the direct separation of reaction pathways for the discrete heme-oxygen intermediate involved. Furthermore, in the combinatorial use of active-site mutants with discriminant reactivity patterns, one can control both the relative stoichiometry of each of these fates and ascertain the role of each intermediate in various P450 catalyzed oxidation reactions. This regulation of peroxo reactivity is directly linked to finely-tuned „proton wires“, elucidated by both structural and spectroscopic measurements. Supported in part by National Institutes of Health grants GM31756 and GM33775.

MP22 CYP2D6: ROLE OF GLU-216 AND ASP-301 IN QUINIDINE BINDING

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We have shown recently, directed by our structural modeling studies, that the CYP2D6 active site residues Asp-301 and Glu-216 play a key role in the recognition of basic nitrogen containing substrates. We have further examined the effect of mutations to these residues on interactions with the protypical 2D6 inhibitor, quinidine. Removal of the negative charge from either or both residues reduced inhibition of bufuralol 1' hydroxylation and dextromethorphan O-demethylation by at least 100 fold. Concomitantly, major alterations in binding were obvious from changes in optical binding spectra. Removing the negative charge from residue 216 and/or 301 led to a shift in the position of the A_{max} from 390 nm to ~404 nm and A_{min}, from 419 nm to ~426 nm. Apparent K_d s of wild type, E216D and D301E were in the range 0.25-0.50 μM, while single non-conservative substitutions resulted in 30 to 64-fold increases in K_d. The double E216Q/D301Q mutant exhibited the largest decrease in quinidine binding with an apparent K_d of 65 μM. Interestingly, the relaxation in binding affinity was associated with the appearance of new metabolites following incubations with quinidine. D301Q and D301N produced small amounts of 3-hydroxyquinidine, D301A and D301F mutants produced O-demethylated quinidine, and E216Q/D301Q produced both metabolites. The implications with respect to CYP2D6 structure and function are discussed.

MP23 CRYSTALLOGRAPHIC STUDIES OF PERDEUTERATED P450CAM

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Cytochrome P450cam from the soil bacteria *Pseudomonas Putida* is the monooxygenase responsible for the stereo- and regiospecific hydroxylation of camphor when used as sole carbon source. P450cam is the model system to study structure function relationships in P450s because of its ready availability, aqueous solubility and relative ease of purification.

The mechanism of activation of the heme bound dioxygen is still unclear, though a rather complicated proton delivery pathway is likely to be involved. Unfortunately, hydrogen atoms cannot be seen at all in the available X-ray crystal structures. Neutron crystallography would help determine the key hydrogen atom positions in the protein, but preliminary experiments show that the large hydrogen incoherent scattering background seriously reduces the signal to noise. This problem can be overcome by deuterating the sample, either partially, by soaking crystals in D_2O, or more fully, by preparing completely (per)deuterated protein samples.

We have developed protocols for over-expression of perdeuterated P450cam in high yield using fully deuterated minimal media and high cell density culture techniques. Mass spectrometry shows that deuteration of the protein is greater than 99%. X-ray quality crystals have been grown and the structure of perdeuterated P450cam determined in both $P4_32_12$ and $P2_12_12_1$ crystal forms at 1.7 and 2.0 Å resolution, respectively. No significant structural changes were detected between the hydrogenated and perdeuterated forms. We have now determined the X-ray structure of a perdeuterated P450cam-CN^- bound complex that mimics the P450cam-O_2 bound intermediary state. The cyanide complex is structurally identical to the oxy complex, in particular the two water molecules observed in the P450cam-O_2 complex are there. Neutron diffraction analysis of these crystals should now reveal their protonation state and the location of the hydrogens.

MP24 CRYSTAL STRUCTURES OF EPOTHILONE-D BOUND, EPOTHILONE-B BOUND, AND SUBSTRATE-FREE FORMS OF CYTOCHROME P450EPOK

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Epothilones[1] are potential anticancer drugs that stabilize microtubules by binding to tubulin in a manner similar to taxol. Epothilones are produced by a series of enzymes[2] including polyketide synthases (PKSs), a nonribosomal peptide synthethase (NRPS) and P450epoK heme containing monooxygenase in the myxobacterium *Soranigiun celluosum*. P450epoK catalyzes the epoxidation of epothilone C and D, which are synthesized by PKSs and NRPS, into epothilone A and B, respectively (*Fig. 1*). The 2.10-, 1.93, and 2.65-Å crystal structures reported here for epothilone D- and B-bound, and substrate-free forms are the first crystal structures of epothilone binding protein. The substrate-free P450epoK exhibits triangular P450 fold with a large vacant substrate-binding pocket just above the heme prosthetic group. The crystal structures of epothilone-bound forms reveal that minor but significant structural change has occurred upon epothilone binding. The epothilones are positioned with its macrolide ring roughly perpendicular to the heme plane and makes a number of hydrogen bonds, π–π stacking interaction between thiazole ring and Tyr96, and hydrophobic interactions. These new structures provide useful information to understand epothilone-tubulin interaction as well as substrate incorporation and recognition by P450 enzyme family.

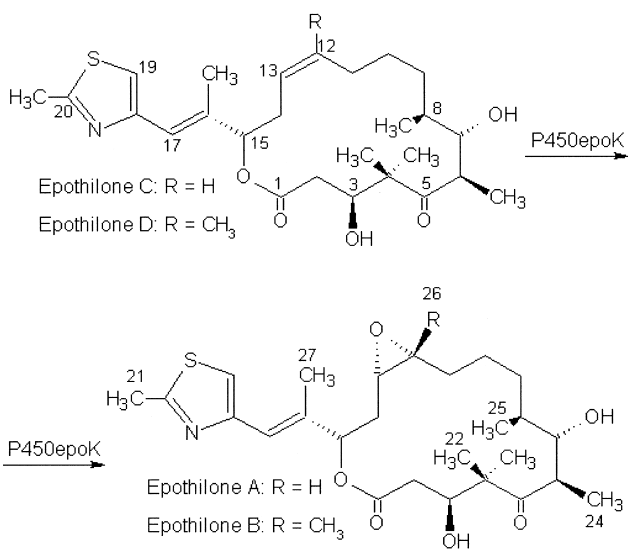

Fig. 1.

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MP25 IDENTIFICATION OF THE SUBSTRATE-CONTACT RESIDUES IN CYTOCHROME P450 27A1

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Cytochrome P450 27A1 (P450 27A1) plays an important role in cholesterol degradation to bile acids. This enzyme catalyzes multiple oxidation reactions at the C-27 atom of steroids in the classical (hepatic) bile acid biosynthetic pathway and cholesterol in the alternative (peripheral) pathway. Little is known about the mode of interaction of P450 27A1 with different substrates. The present study utilized heterologous expression in *E. coli* and site-directed mutagenesis to identify substrate-contact residues in P450 27A1. Targets for mutation were selected based on a substrate-bound computer model of P450 27A1 that was constructed based on the crystal structure of P450 BM-P. We also capitalized on the observation that determinants of substrate specificity in P450s whose structures have been solved by X-ray crystallography are located at similar alignment positions to hold substrate in place within the active site. Twenty alignment positions that encompass four secondary structural elements that form the sides of the active site in structurally characterized P450 were investigated. Generally two substitutions, one to a smaller-

-sized residue and the other one is to a lager-sized residue, were introduced at each alignment position under study. Totally more than 30 mutants were generated. Interaction of P450 27A1 wild type and mutants with one of the enzyme's substrates 5β-cholestane-3α, 7α, 12α-triol was evaluated by using spectral binding assay. The apparent binding constants of the H103F, T110V, M301C, V304L, V367L, N370L, V482A, L283I, L483I, and L483V mutants were increased 16-80-fold indicating that the mutated residues are likely involved in the interaction with this substrate. Investigation of the catalytic properties and product profiles of the mutants that showed significantly impaired binding of the substrate is in progress.

MP26 METABOLISM PREDICTION FOR CYTOCHROME P450

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The use of computational methods to predict the site of metabolism and the enzyme responsible of the reaction enables the possibility to optimize the drug metabolism properties of virtual compounds in the drug discovery process, reducing the lead optimization cycle time. The aim of this work is to present a method able to predict the site of metabolism based on a pharmacophoric representation of the GRID molecular interaction fields computed on the protein models, a pharmacophore fingerprint calculated on the substrates and considering the chemical reactivity. The technique is not limited to GRID molecular interaction fields, but any type of fields can be used. In this sense a field representing the selective interaction between the different cytochromes was used to describe the protein responsible for the metabolic reaction. The selective interactions were obtained from an analysis on CYP1A2, 2C9, 2C19, 2D6, and 3A4. The methodology was used on a set of 90 reactions catalyzed by CYP2C9, 50 for CYP2D6 and 180 for the CYP3A4 substrates. The rate of good prediction for the site of metabolism was over 75 % for all the cytochromes.

MP27 ALTERATION OF CYP 4A4 REGIOSPECIFICITY BY SITE-DIRECTED MUTAGENESIS OF RESIDUES IN THE SUBSTRATE BINDING CHANNEL

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In contrast to all other P450s which hydroxylate eicosanoids and/or fatty acids at the ω-1, ω-2, or ω-3 positions[1,2], P4504As very specifically catalyze hydroxylation at the more energetically unfavorable omega carbon[3,4]. It is possible that steric restriction of the substrate access channel near the heme of the CYP 4As is responsible for limiting hydroxylation to the omega carbon. Modeling studies of CYP4A11[5,6] identify several residues deep in the pocket that may be responsible for this characteristic. Residue E321 of CYP 4A4 appears to be positioned near the bottom of the channel and thus controls its depth. We mutated E321 to alanine and found that in PGE1 metabolism, the ratio of ω:(ω-1) products was decreased, *i.e.*, more (ω-1) PGE_1 was formed. Another interesting residue at the bottom of the substrate-binding channel of CYP 4A4 is L132; this residue has been mutated to phenylalanine (as in CYP102, which makes only (ω-1,2 or 3) products) and the resultant protein is being characterized. Additional residues potentially involved in substrate binding or in defining the shape of this channel are being mutated for future studies.

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MP28 CRYSTAL STRUCTURE OF PUTIDAREDOXIN

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Putidaredoxin (Pdx) from *Pseudomonas putida* shuttles as one-electron donor/acceptor between NADH-dependent FAD-containing putidaredoxin reductase and the terminal oxygenase cytochrome P450cam (P450cam, CYP101). Despite many efforts, Pdx has resisted crystallization. Pochapsky and co-workers have applied NMR methodology to obtain a partial structure of Pdx where the [2Fe-2S] cluster, which could not be defined due to the paramagnetic broadening effect, was modeled using the crystal structure of *Anabena* ferredoxin[1,2]. Later, the Pdx model was refined using a gallium-substituted apoprotein[3] and modeling based on the crystal structure of bovine Adx[4]. In addition, multidimensional NMR methods and NMR relaxation measurements were utilized to investigate structural changes that occur upon reduction of the metal center in Pdx[5,6].

This hybrid structure of Pdx has served as a guide in the structure/function studies on Pdx for many research groups. However, the accuracy of the model has to be evaluated and this could be achieved by determining the crystal structure of the protein. For this reason, we have prepared Cys73Ser and Cys73Ser/Cys85Ser mutants to improve Pdx stability and crystallized and solved the structures of the mutant proteins to 1.65 and 1.47Å, respectively. Our structural data revealed the precise environment of the [2Fe-2S] cluster in Pdx that differs from that modeled in the NMR structure. Dissimilarity between the NMR and x-ray structures is also observed in the (αhelical and C-terminal domains of the protein. We will discuss these structural differences and their implications for the Pdx-redox partner interactions and electron transfer.

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MP29 THIOLS PREVENT P420 FORMATION AND AGGREGATION IN 25-HYDROXYVITAMIN D_3 24-HYDROXYLASE

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Calcium homeostasis is affected by levels of the hormone 1,25-hydroxyvitamin D_3 (1,25OH-D_3).[1] The membrane-associated, mitochondrial cytochrome P450C24 (C24) deactivates 1,25OH-D_3 by hydroxylation at the 24-position, and the inactive hormone is subsequently further modified and excreted. Understanding C24 activity is important for therapeutic efforts to modulate calcium levels. *In vitro* experiments reveal that recombinant rat C24 forms large aggregates concomitant with the P450 to P420 transition. Both phenomena are prevented by the presence of thiols. Interestingly, thiols do not perturb the C24 spectrum, suggesting that they do not bind in the active site, unlike what is observed with P450cam.[2] Various biophysical characterizations demonstrate no major differences between the P450 and P420 states, nor any effect of thiols on the protein tertiary structure. It is suggested that one or more surface cysteines may cause nonspecific aggregation that predisposes C24 to convert to the P420 form. With P450cam, such a reactive surface cysteine was found to cause a mixed population of dimers and monomers, however there was no suggestion of larger aggregates or correlation with P420 formation.[3]

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MP30 THE SELECTIVE INHIBITION OF CYTOCHROME P450 1 ENZYMES BY THE DERIVATIVES OF RUTAECARPINE

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Three cytochrome P450 (CYP) members, CYP1A1, CYP1A2, and CYP1B1, have been identified in human liver microsomes and show importance in the activation and detoxication of a number of endogenous and exogenous compounds. Our previous report showed that the alkaloid rutaecarpine selectively inhibited CYP1A2 in mouse and human liver microsomes. To have potent and selective inhibition of human CYP1 members, methoxyl and halogenated derivatives of rutaecarpine were synthesized. Structural modelling shows a good fitting of rutaecarpine with the putative active site of human CYP1A2. Two hydrogen bonds can be formed between the keto- and N14-groups of rutaecarpine and the Thr^{208} and Thr^{473} residues of CYP1A2, respectively. The C-ring moiety of rutaecarpine forms π-π stacking interaction with the aromatic ring of Phe^{205} residue of CYP1A2. 7-Ethoxyresorufin *O*-deethylation activities of *Escherichia coli* membranes expressing bicistronic human CYP1 members were determined. 7,8-Dehydrorutaecarpine with C7-C8 double bond in the C-ring strongly inhibited CYP1A1, CYP1A2, and CYP1B1 catalytic activities but without selectivity. Among the derivatives, 10- and 11-methoxyrutaecarpine are the most selective CYP1B1 inhibitors. 1-Methoxyrutaecarpine and 1,2-dimethoxyrutaecarpine are the most selective CYP1A2 inhibitors. However, the selectivity was not improved by the introduction of halogen groups. (Supported by National Research Institute of Chinese Medicine and NSC91-2320-B-077-010)

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MP31 THE PROTEIN STABILIZATION OF COUMARIN 7-HYDROXYLASE BY ITS OWN INHIBITORS

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The murine CYP2A5 expression is regulated in a unique fashion compared with other CYP forms. It is inducible by phenobarbital (PB), but also several structurally unrelated chemicals induce it. As far as the mechanisms of CYP2A5 induction are concerned, receptor-mediated induction of CYP2A5 is essentially ruled out due to the wide variation in the structures of the chemicals that act as COH (coumarin 7-hydroxylase) inducers. In most induction studies the increases of CYP2A5 correlate well with the elevation of the corresponding mRNA levels which may be due to an increased transcription of *Cyp2a5* gene or stabilization of mRNA.

Some inhibitors of P450 enzymes can elevate its enzyme activity by stabilizing the protein. In this work we have studied effects of potent COH inhibitors such as γ-lactones, tranylcypromine, metyrapone and pilocarpine on COH enzyme activity in mouse primary hepatocytes. Lactone derivatives coumarin, γ-nonanoic lactone, γ-phenyl- γ-butyrolactone, 7-methylcoumarin and 7-amino-4-methylcoumarin did not cause any induction of COH, PROD or EROD activity. In contrast to lactones other COH inhibitors such as tranylcypromine, metyrapone and pilocarpine increased COH activity about 5-fold, but had no effect on CYP2A5 mRNA levels. Metyrapone induced PROD activity 3.5-fold. N-atom containing inhibitors induced COH activity but did not increase mRNA level suggesting that they could stabilize CYP2A5 protein. Nitrogen seems to act a significant role, because other COH inhibitors were not capable of affecting COH activity. Apparent induction of CYP2A5-associated COH activity on the basis of protein stabilization represents another mechanism of regulation of this enzyme.

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MP32 VALIDATION OF A 3D-REBUILT P450 3A4 STRUCTURE WITH CYCLOPEPTIDE METABOLISM : IMPLEMENTATION OF NEW TECHNIQUES INVOLVING SOFT-RESTRAINED DYNAMICS DOCKING SIMULATIONS GIVES NEW INSIGHTS ON THE MULTIPLE SUBSTRATE SPECIFICITY

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CYP3A4 is the most important family member of drug metabolizing enzymes in humans, as it affects as many as 50% of all known drugs interacting with this form of cytochrome P450. However, it is still one the most poorly understood member within the CYP family, with respect to its drug metabolizing action. The absence of any crystal structure available in the PDB or in the literature represents a major problem in drug development, which can be overcome by rebuilding methods such as comparative modeling techniques, based on robust distance geometry protocols for structure optimization. We propose, in this study, to validate the CYP3A4 isoform 3D model rebuilt at the laboratory using in-house modeling 3D alignment tools[1], by analyzing the drug pathway through different possible clefts in the structure and towards the heme active site. For this purpose, we implemented techniques issued from molecular dynamics simulations with a special attention to drag the substrate from the outer medium under soft-restrained conditions. This new approach allows to take into account the flexibility of the protein, a prevalent feature in the CYP3A4 structure, as well as the flexibility of the ligand, and the relationship between its size and chemical structure and the potential entrance site in the enzyme.

Rebuilding method:

To rebuild the structure of CYP3A4, six high-resolution X-ray structures of bacterial, fungal and mammalian CYPs were selected to form the initial multiple template. These structures displayed low identity (about 20 % in average), with significant parts in the N-terminal region that are not conserved and cannot be aligned by standard multiple primary sequence alignment methods. The interest of our method resides in the use of a local structural alignment tool (GOK ABI, Paris) in the internal coordinates space, based on phi/psi or alpha/tau analysis, that allows to determine common structural blocks (CSB) with no pivot structure choice. Conserved and variable zones in the structures of CYP templates can be identified. CSBs were then aligned against the CYP3A4 target sequence using in-house tools designed for multiple profile alignment, to generate about 58,000 distance constraints and 760 dihedral constraints for the whole structure rebuilding. This set of CSB and the corresponding constraints dataset can be used as a robust framework to rebuild the isoforms of the whole CYP 3A family in human and mammals.

Information drawn from the 3D rebuilt structure:

The CYP3A4 structural model was submitted to various molecular dynamics (MD) simulations, either for checking the stability of the structure without the building distance constraints, or to verify the consistency of the model with the various mutants of the literature. Biochemical data of substrate interaction and metabolism obtained at the laboratory were interpreted by submitting the 3D model to MD simulations in the presence of various substrates such as testosterone or cyclotetrapeptides. Simulations were performed with the substrate positioned initially out of the protein structure, and not within the heminic active site. with no initial guess in the orientation of the ligand, as it is free to evolve during the MD simulations. Soft distance constraints were applied to the substrate to guide it towards the heme, so that different entrances and pathways can be examined through the simulations. All the simulation results showed that:

1) two different gates in the enzyme structure proved to be accessible, depending on the substrate, with respect to its size or chemical structure,

2) several interacting helices play a crucial role in the gating and the protection of the active site after substrate entrance,

3) at least two substrate molecules can be channeled towards the active site(testosterone/α- naphtoflavone cooperativity has been studied in details),

4) energetic profiles can be estimated from the force field parameters and account for variations in the access to the active site, 5) the final positions of the ligand in the vicinity of the heme (distance Fe-substrate) were found in agreement with experimental metabolism data of the natural cyclotetrapeptide phytotoxin tentoxin and some of its natural derivatives[2].

Thus, the rebuilt structure seemed to account for the multi-specific features of CYP3A4 isoform, in terms of substrate channeling, and allosteric behavior. This is notable, as many drugs in the past have been withdrawn from the market or stopped in clinical trials due to adverse side effects from their interactions with these enzymes. This is, to our knowledge, the first model of CYP3A4 available with such predictive capability[3].

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MP33 SUBSTRATE DYNAMICS IN CYTOCHROME P450-BM3 - DYNAMICS OF SUBSTRATE BINDING BY AUTOMATED DOCKING AND MOLECULAR DYNAMICS SIMULATION

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Cytochrome P450 BM3 (Cyp102) from *Bacillus megaterium* catalyses the hydroxylation of fatty acids. Several mutants have been described that also hydroxylate alkanes, like octane. Hydroxylation takes place on sub-terminal positions (ω-1, ω-2, ω-3), but not on the terminal (ω) position. The interactions between enzyme and substrate are very subtle; there are few specific ionic or hydrogen bonding interactions, and no 'complementary fit' of the substrate in the binding cavity. Using a combination of computational methods, these interactions and differences between mutants are investigated. Binding modes are found from automated docking, additional dynamics and binding statistics come from Molecular Dynamics (MD) simulations. Additionally, as a step towards accurate prediction of product ratio's, intrinsic reactivities are obtained from Quantum Mechanical (QM) calculations of the substrate transition state complex.

Substrates were docked into the proteins using the Gold automated docking program[1]. MD simulations were started from several most dissimilar binding orientations, each for a period of 10ns, using the Gromacs MD package[2] and the Gromos forcefield[3]. Wild-type and mutant dynamical features are characterized using Essential Dynamics (ED) analysis[4].

Qualitative differences in dynamical behaviour, as seen in the ED analysis, between wild-type and some of the mutants were observed, both for the dynamics of the whole enzyme and for the active site region only. Binding of the substrates was found to be highly dynamical, the substrates have much freedom to move around the active site interior and bind transiently at different positions, sometimes for relatively long times of several nanoseconds. Statistically, the ω orientation of the substrates with respect to the heme iron atom was clearly preferential in the MD simulations. On the other hand, the activation barrier calculated for hydrogen atom abstraction on the ω position by a neutral hydroxyl radical[5] was higher than that for the other positions, which would shift the overall product formation away from ω hydroxylation. The calculated barrier heights are very sensitive to the electrostatic environment.

In summary, it is concluded that the combination of simulation and analysis of the dynamics, and quantum mechanical calculations is a valuable tool to investigate and understand substrate binding and product formation.

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MP34 A QM/MM STUDY OF CYTOCHROME P450: THE INFLUENCE OF THE ENZYME ENVIRONMENT ON SPECIFIC STEPS IN THE CATALYTIC CYCLE

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Cytochromes P450 catalyze the stereospecific addition of molecular oxygen to nonactivated hydrocarbons and therefore perform many vital bioregulatory functions. The mechanism of this monooxygenation reaction has been studied extensively, but remains a subject of discussion and debate. Also many quantum chemical studies have been devoted to this topic, but few take the enzyme environment explicitly into account.

In this contribution, we report a QM/MM study on the active site of P450cam in which its protein environment is

taken into account. The entire system is split into a QM part for the active site and a MM part for the enzyme environment. All interactions between the QM and MM parts are taken into account by using a protein-specific force field (AMBER95) that is also used for the interactions within the MM part. The QM part is described by Density Functional Theory, which enables the treatment of larger QM systems, in this case up to approximately 150 atoms. A recently developed link model (AddRemove) is used for treating the interactions at the QM/MM boundary.

Results will be presented for specific steps along the catalytic cycle, with emphasis on the spin states and properties that relate to experimental data. The QM/MM results will be compared with the results for the isolated active site, *i.e*. iron porphyrin with or without the axial iron ligands. This enables a separation of the intrinsic properties of the iron porphyrin ring from the properties induced by the presence of either the axial ligands or the full enzyme environment. Mainly the first steps of the catalytic cycle will be addressed. For these steps many experimental data are available to validate the computational setup. The predictive power of this approach will be explored for the subsequent steps of the catalytic cycle for which far less experimental data are available.

MP35 ASPECTS ON EVOLUTION OF THE CYP51 FAMILY

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Cholesterol is an essential component of eukaryotic membranes. Its metabolites (steroid hormones, bile acids, vitamin D and oxysterols) have many different phyisiological functions. A member of the cytochrome P450 superfamily CYP51 (lanosterol 14α-demethylase) participates in the postsqualene portion of cholesterol biosynthesis and catalyses the removal of the 14α-methyl group of sterols. Additional role of CYP51 is belived to be in overproduction of MAS (meiosis activating sterol), which are supposed to be signalling molecules with a role in mammalian reproduction. Enzymes with CYP51 activity are found in three eukaryotic phyla and also in some prokaryotes. The eukaryotic CYP51s use different substrates: lanosterol (mammals and fungi), 24-methylene-24,25-dihydrolanosterol (fungi) and obtusifoliol (plants). The presence of sterols in prokaryotes is limited to only some taxonomical groups. Up-to-date sterols were found and their synthesis shown for the *Methylococcaceae* family (*Methylococcus capsulatus*) and for the *Mycabacterium sp*. In both cases proteins with CYP51 activity have been isolated. This led to a conclusion that CYP51 has developed very early in the cytochrome P450 evolution and was transfered vertically from prokaryotes to eukaryotes. A putative CYP51 was found also in *Streptomyces coelicor*, but the sterol synthesis in this prokaryote has not yet been clearly demonstrated. Construction of a phylogenetic tree containing CYP51 genes from all so far available eukaryotic and prokaryotic sequences gave the topology ((bacteria,plant)(animals,fungi)), which differs from the previousy proposed topology (bacteria(plant(animals,fungi)))[1]. In order to evaluate the credibility of the proposed novel CYP51 filogeny, all available bacterial cytochrome P450 sequences have been analyzed. *Mycobacterium* and *Methylococcus capsulatus* CYP51s clustered together in the bacterial CYP dendrogram, however, the *Streptomyces coelicor* CYP51 locates in a distant branch, suggesting that *S. coelicor* might not originate from a CYP51 ancestor. The ((bacteria,plant)(animal,fungi)) topology of the CYP51 cluster has been preserved after the closest bacterial neighbours to the *Mycobacterium/Methylococcus* cluster have been added. This indicates again that the *Mycobacterium/Methylococcus CYP51s* are closer to plant CYP51s as compared to any other bacterial cytochrome P450. Available biochemical data support a close relation between bacterial and plant CYP51s. The *Mycobacterium tuberculosis* CYP51 enzyme is similar to plant CYP51, using obtusifoliol as a preferred substrate[2]. These data lead us to propose that *Mycobaterium sp*. and *Methylococcus capsulatus* CYP51 genes were gained through a lateral gene transfer from plants. We propose further that *Streptomyces coelicor CYP51* belongs to another bacterial CYP group and has at present an incorrect gene family annotation. Additional prove of our hypothesis comes from a recent independent investigation where the *Mycobacterium tuberculosis* proteins have been compared against all bacterial and eukaryotic proteins. This phylogenetic analysis proposed that eight Mycobacterial genes, one of them being CYP51, have been aquired by a lateral gene transfer from eukaryotes[3].

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MP36 IDENTIFICATION OF NOVEL HUMAN CYTOCHROME P450S, CYP4Z1 AND THE TRANSCRIBED PSEUDOGENE CYP4Z2P

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We identified the full length cDNA of a novel human cytochrome P450 (CYP), CYP4Z1, and of a closely related pseudogene CYP4Z2P from the breast carcinoma line SK-BR-3. They represent members of subfamily 4Z, that have already been predicted by the CYP450 Nomenclature Committee[1] based on genomic database research. Apart from expressed sequence tags (ESTs) and partial genomic sequences, experimental data revealing full length mRNA sequences were missing so far.

The cDNA of CYP4Z1 (1910 bp) contains an open reading frame of 1515 bp, that codes for a 505 aa protein. CYP4Z1 meets the characteristics of a functional CYP enzyme with its conserved heme-binding domain, substrate recognition sites and subcellular localisation signal. The sequence identity is higher to members of subfamilies 4A (51%) and 4B (45%) than to 4F (40%), but the highest identity (54%) was found to CYP4X1, a recently identified CYP[2]. Most of CYP4A, 4B and 4F family members have a conserved glutamic acid in their I-helix region, that enables them to bind heme covalently and represents a unique feature in the CYP 4 family[3]. In contrast, CYP4Z1 as well as CYP4X1 contain alanine instead of glutamic acid at this position. Therefore, 4Z1 and 4X1 might have an exceptional position within family 4. Functional data are required to elucidate the substrate spectrum of both enzymes.

In contrast to CYP4Z1, a single nucleotide change in exon 8 of the CYP4Z2P gene causes an early stop codon that leads to a truncated CYP4Z2 protein of 340 aa lacking the heme-binding site and the endoplasmic reticulum retention signal KKVC. Thus, it represents a transcribed CYP pseudogene, consequently named CYP4Z2P. The homology to CYP4Z1 in the first 340 aa is 96%.

The mapping of both new CYP4Z genes on chromosome 1p33 by bioinformatics elucidated the genomic organisation with highly conserved intron/exon boundaries. The genes for CYP4Z1 and CYP4Z2P consisting of 12 exons are localised in head-to-head orientation in closeness to the genes of CYP4A11 and CYP4B1. Functional analyses on CYP4Z1 recombinantly expressed in E.coli and generation of monoclonal antibodies are ongoing studies.

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MP37 CHARACTERIZATION OF ARABIDOPSIS THALIANA CYP711A1

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We cloned recently several fatty acid ω-hydroxylases from various plant species which formed the first members of the CYP86 and CYP94 families[1, 2]. Phylogenetic analysis of these P450s showed that they all clustered in one branch of the „non-A"[3] group of plant P450 and that they appeared closer to animal or fungal fatty acid hydroxylases than to other plant P450. One of these P450, CYP711A1 which is located at the base of this „fatty acid hydroxylase branch" is phylogenetically related to the CYP74 family which uses fatty acid hydroperoxides as substrates, and to mammalian CYP5, the thromboxane A synthase. These P450 do not require an electron transfer system for catalytic activity. We have now cloned CYP711A1 from mRNA of Arabidopsis thaliana seedlings induced with 1mM methyl jasmonate for 24h. The protein was expressed in WAT11 yeast strain carrying an A. thaliana NADPH-P450 reductase and another yeast strain expressing the original yeast reductases. The CO-difference spectra as evidence of functional P450 and the substrate specificity of CYP711A1 will be presented.

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MP38 INDUCIBLE CYTOCHROME P 450 (CYP2E1) BY COMBINED ADMINISTRATION OF ACETAMINOPHEN AND CAFFEINE

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CYP consist of a superfamily that play an important role in the metabolism of a wide variety of xenobiotics, including drugs, carcinogens and environmental agents, as well as endogenous compounds such as steroids and fatty acids. The individuals izoforms have different regulatory and functional characteristics includingselective substrate specificities. CYP2E1 is of particular interest because it is involved in the metabolism activation of many drugs associated with toxic effects. Mixed analgesic preparations comprising both acetaminophen and caffeine are widely used. Due to ability of the said substances to induce cytochrome P-450 2E1, the effect of combined administration of both acetaminophen and caffeine on *p*-nitrophenol hydroxylase (*p*-NPH) activity in liver microsomes (a marker of cytochrome P-450 activity) as well as alanine aminotransferase (AlAT) activity in blood serum was studied using unbred male mice.

It was shown that acetaminophen administered intragastrically to mice during 3 days once daily at a range of 100-350 mg/kg dose dependently elevated the activities of *p*-NPH in liver microsomes and AlAT in blood serum. Caffeine administered intragastrically during 3 days once daily at a range of 18-70 mg/kg didn't affect the activities of AlAT but *p*-NPH in liver microsomes dose dependently increased in fold. However, the combined administration of both caffeine and acetaminophen significantly increased the activites of *p*-NPH in liver microsomes in 4,3 fold compared of normal. An increase in AlAT of 4 fold the upper limit of normal (ULN) was recorded of pretreatment of both drugs. *p*-NPH and AlAT activities were more increased with combined administration of both caffeine and acetaminophen as compared to acetaminophen alone. The high correlation between both *p*-NPH and AlAT activities was observed. Co-administrered composition „MV" with acetaminophen and caffeine in doses 350 and 70 mg/kg accordingly decreased *p*-NPH and AlAT activities to normal levels.

Our experience suggests that the combined administration of caffeine and acetaminophen potentiates acetaminophen hepatotoxicity that should be taken into consideration in developing and using such preparations. As long as analgesic mixtures containing paracetamol and caffeine are available, analgesic hepatotoxicity will continue to be a problem also.

MP 39 MOLECULAR MODELING AND SITE-DIRECTED MUTAGENESES DEFINING CATALYTIC SITES IN INSECT P450S METABOLIZING ALLELOCHEMICALS AND INSECTICIDES

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P450-mediated detoxification strategies are encountered in insects in the evolution of resistances to toxic plant allelochemicals and synthetic insecticides. Examples of these include the *Papilio polyxenes* CYP6B1 protein that metabolizes toxic furanocoumarins present in its hostplants, the *Helicoverpa zea* CYP6B8 protein that metabolizes furanocoumarins in its hostplants and insecticides, the *Musca domestica* CYP6D1 protein that metabolizes pyrethroid insecticides and polycyclic aromatic hydrobarbons and several *Anopheles gambiae* P450s that metabolize pyrethroids and other insecticides. To elucidate catalytic sites in some of the *Papilio* and *Helicoverpa* CYP6B proteins, we have constructed substrate-docked models of the CYP6B1 and CYP6B8 proteins based on sequence alignments with bacterial CYP102. In the CYP6B1 model, multiple aromatic amino acids contribute to the aromatic-aromatic network that stabilizes this P450's catalytic site and allows for interactions with this P450's hydrophobic furanocoumarin substrates. Mutational analyses coupled with baculovirus expression studies support these roles in substrate recognition. In the CYP6B8 model, many of these aromatic residues are replaced with residues allowing for the formation of a more open catalytic pocket capable of accepting furanocoumarins as well as a range of plant allelochemicals and insecticides. Substrate binding analyses in a number of CYP6B1 catalytic site mutants have indicated that the altered activities for many of these mutants correlate with changes in heme spin state effected by alterations in the hydrogen bonding network surrounding the heme.

Additional modeling and mutagenesis in the CYP6B8 catalytic site is focused on identifying residues interacting with the broader range of substrates metabolized by this P450.

MP40 TRANSCRIPT PROFILING OF *ARABIDOPSIS THALIANA* P450S

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In plants, P450s function in the biosynthesis of lignins, pigments, defense compounds, fatty acids, hormones and growth regulators as well as in the metabolism of herbicides, insecticides and pollutants. Towards defining the function of the 272 P450 genes existing in *Arabidopsis*, we have analyzed P450 gene-specific microarrays for constitutive and inducible transcript profiles in seedlings exposed to a variety of chemicals and environmental stresses. Among several chemicals analyzed for their ability to induce transcription of sets of P450 genes are several plant signaling molecules (JA, SA), phenobarbital, herbicides (DICAMBA, atrazine) and fungal defense activators (BTH). Among the stresses analyzed so far are osmotic and cold stresses. Expression profiles for subsets of these P450s have been correlated with RT-PCR analyses using gene-specific probes and with oligonucleotide array profiles. These analyses indicate that some subsets of these P450s are expressed in response to specific chemical cues while other subsets of these P450s are expressed in response to overlapping sets of chemical cues. Heterologous expression of several *Arabidopsis* P450s in baculovirus expression systems coupled with substrate binding analyses is being used to define the ranges of chemicals hydroxylated by each of these sequences.

MP41 P450-DEPENDENT ALKANE MONOOXYGENASE OF *ACINETOBACTER SP.* EB104 IS ENCODED IN A COMPOSITE TRANSPOSON OF THE PLASMID PAC450

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In addition to the P450-independent alkane monooxygenase found in many strains of the genus *Acinetobacter*, the species EB104 – that is able to grow on the toxic middle-chain *n*-alkanes - contains an alkane monooxygenase consisting of the P450non (CYP153), the ferredoxin nonadoxin (Nd) and the NADH-dependent nonadoxin reductase[1]. With the aim to exploit this unusual bacterial alkane monooxygenase as a biocatalyst and model enzyme for the biotransformation of chemically highly inert and very hydrophobic compounds its genetics were studied in more detail. Southern hybridisation experiments[2] show that the three genes of the alkane monooxygenase which are organised in a single operon[3] are localised together with the gene of a putative transcription regulator on the native plasmid pAC450 of *Acinetobacter sp*. EB104. The regulator protein belongs to the family AraC/XylS. Sequencing of the entire plasmid revealed that the P450 regulon is encoded within a 17-kb composite class-I transposon suggesting the acquirement of this metabolic feature by means of horizontal gene transfer. Putative genes of further enzymes of alkane degradation could not be detected on the plasmid, neither within the transposon nor outside of it. The transposon is flanked by two identical IS elements each containing a transposase with 99% nucleotide sequence identity to the transposase in the transposon Tn5047 of plasmid pKLH201 from *Acinetobacter calcoaceticus* KHW14[4]. Tn5047 carries mercury resistance the genes of which have not been detected in plasmid pAC450 of *A. sp*. EB104. Comparable high similarities could not be found for any other putative ORFs detected on the plasmid. Further sequence analysis is in progress to get still more information on origin and relationship of the pAC450 components and its importance for the bacterium *A. sp*. EB104. Altogether, the results described here support the hypothesis that the acquirement of the P450non system by means of the plasmid pAC450 enables this bacterium to withstand toxic environment by conferring resi-

stance against middle-chain alkanes and other dangerous hydrocarbons, respectively.

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MP42 CYP2C19 DO NOT CONTRIBUTE TO BLOOD PRESSURE.

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The polymorphism of CYP2C19 is due to at least two major and several minor variant alleles of CYP2C19 among the 19 alleles reported (www.imm.ki.se/CYPalleles/cyp2c19.htm) (20.02.03). The principal defective allele CYP2C19*2 consist of an aberrant splice site in exon 5[1]. CYP2C19 is usually characterized by S-mephenitoin 4'-hydroxylation or omeprazole 5-hydroxylation. In poor metabolizer (PM), both activities are extremely low. PMs are representing 2-5% of caucasians. CYP2C19 is also able to oxygenate arachidonic acid to 19-HETE, 14,15-epoxyeicosatrienoic acid (EET) and 8,9-EET as main metabolites[2]. For this last reason, we investigated the possible linkage between blood pressure and the polymorphism of CYP2C19 among the subjects included in the Stanislas Cohort. This study observed 545 unrelated adult Caucasians enroled in the Stanislas Cohort. We used Kinetic Thermal Cycler method to determine CYP2C19 genotype. The relationship between genotype and continuous variables was assessed by one-way ANOVA. Multiple linear regression analysis was performed to evaluate the association between CYP2C9*2 polymorphism and blood pressure. The frequency of the genotype mutation for rare allele was 4.2% in position 681 and 3.8% in position 990. The allele frequency of CYP2C19*2 990 C/T was 17.7% ± 1.2 and the frequency of CYP2C19*2 681 G/A was 18.2% ± 1.2. The observed frequencies of the homozygotes (CC vs GG) and heterozygotes (CT vs GA) genotypes were in agreement with those predicted according to the Hardy-Weinberg rule. The allele frequencies observed for CYP2C19 were in accordance with the data in litterature. However, if it is reported that CYP2C19 oxygenates arachidonic acid to 19-HETE, 14,15-EET and 8,9-EET which are vasoactive compounds, the low catalytic activity of CYP2C19*2 do not contribute to the changes in blood pressure.

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MP43 COMPARISON OF NADPH-, NAD^+- AND UDP-GLYCOSIDE-DEPENDENT BIOTRANSFORMATION OF BOHEMINE AND ROSCOVITINE IN VITRO

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The purpose of the present study was to utilize subcellular fractions of mouse liver and kidney to compare biotransformation of 6-benzylamino-2-(3-hydroxypropylamino)-9-isopropylpurine (bohemine) and 6-benzylamino-2-(1-hydroxy-methylpropylamino)-9-isopropylpurine (roscovitine).

Methods

The compounds were ^3H-labeled on the C8 atom of purine ring. The following systems were used to evaluate the biotranformation: 1. Microsomal liver fraction + NADPH; 2. Microsomal liver or kidney fraction + UDP- glucose or UDP- glucuronic acid; and 3. Cytosolic liver or kidney fraction + NAD^+. Kinetic data were obtained by sampling the incubation mixtures in intervals followed by their denaturation, thin-layer chromatography, and autoradiography with densitometric evaluation.

Results

First, roscovitine was transformed, by mouse liver microsomal NADPH-dependent system, about twice as rapidly than bohemine. The main product resulting from roscovitine was its corresponding carboxylic acid while bohemine

was preferentially metabolized by dealkylation processes[1]. Second, in the microsomal-UDP-glycoside systems, bohemine glucosidation was catalyzed about fifty times (liver) and five times (kidney) faster than that of roscovitine. Furthermore, glucuronidation of bohemine was catalyzed, to a limited extend, by the liver and, even less, by kidney microsomes while glucuronidation of roscovitine was not detected. Third, in both liver and kidney cytosolic, NAD+-dependent systems, oxidation of bohemine into its corresponding carboxylic acid occured. Roscovitine was transformed about fifteen times more slowly than bohemine by liver cytosol and no reaction of roscovitine occured in the kidney cytosol.

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Conclusions

Roscovitine exerted higher degree of metabolic stability over bohemine when explored *in vitro*. This is in accord with the evidence of its slower clearance obtained in *in vivo* experiments in mice[2].

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MP44 BIOTRANSFORMATION OF OLOMOUCINE-TYPE CYCLIN-DEPENDENT KINASE INHIBITORS BY PRECISSION-CUT TISSUE SLICES FROM DIFFERENT ANIMAL SPECIES

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The aim of this study was to compare biotransformation of ^{3}H-labeled olomoucine, bohemine, roscovitine, compound A.2.J.36, and compound A.2.3.9.(R)* by liver and kidney slices of rodents and primates.

Methods

Liver and kidney slices (8 mm in diametr; thickness 200-250 μm) from mouse, rat and monkey (*Macaca mulatta*) were incubated in 95% O_2/5% CO_2 atmosphere. Incubation medium was sampled in regular intervals and metabolite production was followed up by TLC /^{3}H autoradiography with densitometric evaluation.

Results

All tested substances produced metabolites that began to appear within the first 30 minutes of incubation. Appropriate carboxylic acids that arose by oxidation of a -CH_2OH side-chain group situated on C2 atom of the purine ring were produced preferentially from the parent compounds. However, mouse kidney slices were unable to transform bohemine into the appropriate acid and monkey liver and kidney slices from all tested species were unable to transform roscovitine into the appropriate acid. A.2.J.36 and A.2.3.9.(R) were only slightly oxidized into their acids or not at all. Additionally, bohemine was glucosylated by mouse kidney slices, producing bohemine-O-βD-glucoside, above all the other compounds. Other glycosylated metabolites - glucuronides - were the main products created from A.2.J.36 and A.2.3.9 (R) by mouse, rat and monkey kidney slices. Except of mouse and rat kidney slices in the presence of A.2.J.36, the liver slices metabolized the tested substances more effectively than the kidney ones. Notably, roscovitine emerged as the most stable parent compound among all.

Conclusions

These observations are in concordance with those received in in vitro studies of bohemine and roscovitine metabolism exploiting mouse subcellular fractions supplemented with NADPH, NAD^+, and/or glycosyl donors[1], and with those of bohemine and roscovitine in in vivo experiments in mice[2]. The work demonstrates how the precission-cut tissue slices technique can complete data obtained using other model systems.

∗)bohemine: 6-benzylamino-2-(3-hydroxypropylamino)-9-isopropylpurine

roscovitine: 6-benzylamino-2-(1-hydroxymethylpropylamino)-9-isopropylpurine

olomoucin: 2-(hydroxyethylamino)-6-benzylamino-9-methylpurine

Compound A.2.J.36: 2-(2-hydroxy-1-isopropylethylamino)-6-(2-hydroxybenzylamino)-9-isopropylpurine

Compound A.2.3.9.(R): 2-(2-hydroxy-1-ethylamino)-6-(3-hydroxybenzylamino)-9-isopropylpurine

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MP45 SIGNIFICANT ROLE OF CYP2A AND CYP3A IN BIOTRANSFORMATION OF BOHEMINE BY MOUSE LIVER MICROSOMES IN VITRO

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The aim of this study was to characterize the biotransformation of selective cyclin-dependent kinase inhibitor 6-benzylamino-2-(3-hydroxy-propylamino)-9-isopropylpurine (bohemine) by mouse liver microsomes.

Methods

Metabolite profiles of 8-^{3}H-labelled bohemine were established by TLC/^{3}H-autoradiography, and enzymatic and MS analyses were utilized to elucidate chemical structures of the metabolites. Participation of individual CYPs was followed up by testing with enzyme selective CYP inhibitors[1].

Results

Scheme of primary NADPH-dependent pathways have been proposed involving N^2- and N9-dealkylation, N^6-debenzylation, aromatic hydroxylation, and C2-side chain oxidation of bohemine. Three of the primary metabolites detected, namely 6-benzylamino-2-(3-hydroxypropylamino)purine (M-4), 6-amino-2-(3-hydroxypropylamino)-9-isopropylpurine (M-5), and 6-(4-hydroxybenzylamino)-2-(3-hydroxypropylamino)-9-isopropylpurine (M-6), all of them retaining their parent primary hydroxyl group, were subsequently shown to be converted by a liver cytosolic, NAD^+-dependent system, into their corresponding carboxylic acids. Metabolite M-6 was subject to microsomal glycosidations requiring UDP-sugar donors. NADPH-dependent conversion of M-6 into M-5 by microsomes was also demonstrate substrate (8-methoxypsoralen, ketoconazole, troleandomycin and coumarin) were utilized to identify CYPs involved in bohemine biotransformation. The findings suggested that *CYP2a* and *CYP3a* substantially contribute to NADPH-dependent bohemine transformation *in vitro*.

Conclusions

The present findings will facilitate experiments designed to dissect enzymatic systems catalyzing clearence of C2,C6,N9-trisubstituted purine compounds from mammalian tissues.

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MP46 CARBOXYLIC ACID IS THE MAIN PRODUCT OF ROSCOVITINE BIOTRANFORMATION IN MICE *IN VIVO*. COMPARISON WITH BOHEMINE

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The purpose of the present study was to investigate the biotransformation of selective cyclin-dependent kinase inhibitor 6-benzylamino-2-(1-hydroxymethylpropylamino)-9-isopropylpurine (roscovitine) in mice *in vivo* and to compare it with that of 6-benzylamino-2-(3-hydroxypropylamino)-9-isopropylpurine (bohemine) *in vivo*[1].

Methods

^{3}H-labeled parent compounds were administered intravenously and ^{3}H-radioactivity in blood, liver, kidney, urine, and gut was monitored by liquid scintillation counting. Levels of the parent compounds and those of their metabolites were assessed by thin-layer chromatography and autoradiography[1, 2].

Results

The level of roscovitine in blood decreased from 5.9 % of the administered dose (2 min after the administration) to 0.7 % (30 min after the administration). The level of bohemine declined more rapidly (from 4.6 % in 2 min to 0.15 % in 30 min)[1]. The liver and kidney were the organs responsible for biotransformation and elimination of both com-

pounds. A carboxylic acid was produced from roscovitine by oxidation of its -CH_2OH side group. The carboxylic acid was the main metabolite of the parent compound. Based on the additional findings obtained *in vitro*, this oxidation mostly ocured in NADPH-dependent manner[3]. On the other hand, bohemine carboxylic acid was also identified. However, its formation was mainly mediated by cytosolic, NAD^+-dependent, 4-methylpyrazol sensitive alcohol dehydrogenase class I[1].

Conclusions

The clearance of roscovitine in mice is about four times slower than that of bohemine. The result is of direct practical importance considering a preclinical testing of these compounds as potential novel anticancer agents.

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MP47 CYP2D IS INVOLVED IN THE NEUROSTEROIDOGENESIS IN THE BRAIN

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We previously reported that the brain CYP2D4 and CYP2D6 are involved in progesterone 21-hydroxylation[1]. In the brain, the enzyme responsible for steroid 21-hydroxylation has not been well elucidated although very small amount of P450c21 mRNA was present. In this study, we investigated whether CYP2D functions as steroid 21-hydroxylase in the brain. We also focused our efforts on the regional CYP2D expression in the brain using RT-PCR and Western blot analysis.

Recombinant enzymes of rat CYP2D1, CYP2D2, CYP2D3 and CYP2D4, and human CYP2D6 were expressed in yeast[2]. Each microsomal fraction was incubated with progesterone and 17OH-progesterone, and those 21-hydroxylated metabolites were quantified by LC-MS/MS.

Only rat CYP2D4 and human CYP2D6, predominant CYP2D isoforms in the brain, possessed 21-hydroxylation activities for progesterone and 17OH-progesterone. These activities were also observed in rat brain microsomes and could be effectively inhibited by quinidine and anti-CYP2D antibodies. RT-PCR and Western blot analysis revealed that mRNA and protein of CYP2D4 distributed throughout the rat brain whereas those of P450c21 were solely detected. Furthermore, rat CYP2D4 and human CYP2D6 catalyzed 21-hydroxylation of allopregnanolone, one of the representative allosteric GABA modulator. These results strongly suggest that the brain CYP2D isoforms play an important role in the regulation of brain neurosteroids level as steroid 21-hydroxylase.

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MP48 ANALYSIS OF CYTOCHROME P450S IN A PYRETHROID RESISTANT STRAIN OF *HELICOVERPA ARMIGERA* (COTTON BOLLWORM)

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Helicoverpa armigera is a serious pest of cotton and other crops around the world. It has evolved resistance to most classes of insecticides. The AN02 strain from Australia exhibits 50 fold larval resistance and 100 fold adult resistance to the pyrethroid, fenvalerate. This resistance is suppressible by piperonyl butoxide (suggesting a detoxicative mechanism), and past work has focused on the Cyp6B family of cytochrome p450s. Linkage mapping with AFLPs was used to assign the fenvalerate resistance gene to Linkage Group 13 (LG13), while a Cyp6B cluster maps to LG14. This argues against Cyp6B cis-acting factors affecting resistance, but would be consistent with trans-acting factors on LG13 affecting Cyp6B expression levels. A larval midgut cDNA library was screened for

p450 genes and several members of the Cyp9A family were recovered, but not Cyp6B. The most abundant Cyp9A p450 was mapped to LG10.

Real-time RT-PCR was used on individuals from both resistant and susceptible strains to explore tissue specific and developmental expression of CYP6s and CYP9s. Preliminary results suggest there is individual variation in relative expression levels of Cyp6B2, Cyp6B6, and Cyp6B7 that may have a genetic basis.

MP49 INFLUENCE OF RECIPIENT GENDER ON CYTOCHROME P450 ISOFORMS EXPRESSION AND ACTIVITY IN INTRASPLENIC FETAL LIVER TISSUE TRANSPLANTS IN COMPARISON TO LIVER IN RATS

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Ectopic liver cell transplants can serve as a tool to study humoral, nerval and other topic influences on liver cell differentiation, multiplication, function and sensitivity to foreign compounds. Rat livers display a sex-specific cytochrome P450 (P450) isoforms expression pattern which is regulated by a differential profile of growth hormone (GH) secretion in male and female rats. The aim of the present study was to elucidate whether intrasplenic fetal liver tissue transplants of originally mixed sex are subject to the same regulatory influence of the GH secretion pattern as the cells within the orthotopic livers and thus will adapt to the gender of the recipients with respect to P450 isoforms expression.

Syngenic fetal liver tissue suspensions of mixed sex were transplanted into the spleens of adult male or female Fischer 344 rats. Four months after surgery transplant-recipients and age-matched control animals were treated either with (-naphthoflavone (BNF), phenobarbital (PB), dexamethasone (DEX) or the solvent and sacrificed 24 or 48 hours thereafter. Expression of the P450 isoforms 1A1, 2B1, 2E1, 3A2 and 4A1 in livers and transplant-containing spleens was assessed by immunohistochemistry. P450 dependent monooxygenase functions were measured biochemically using different model reactions for distinct P450 subtypes: ethoxyresorufin O-deethylation (EROD), ethoxycoumarin O-deethylation (ECOD), p-nitrophenol hydroxylation (PNPH), pentoxyresorufin O-depentylation (PROD) and testosterone hydroxylation (TH) at different positions.

The livers of both male and female rats displayed nearly no P450 1A1, but a distinct P450 2B1, 2E1, 3A2 and 4A1 expression. Whereas no gender differences were observed in the immunostaining for P450 1A1, the expression of P450 2B1, 3A2 and 4A1 was stronger in males and that for P450 2E1 in females. Like in the livers the immunostaining for P450 2B1, 3A2 and 4A1 was more pronounced in transplants of male recipients and that for P450 2E1 in those of female hosts. With some sex-specific differences both in livers and in transplants the P450 1A1 and 2E1 expression was increased by BNF, that of P450 2B1 by PB, and that of P450 3A2 by DEX. Spleens of control rats displayed nearly no P450 isoforms expression. Additionally, only very low monooxygenase activities were detectable in the spleens of male or female control rats. In contrast, with most model reactions distinct activities were seen in the transplant-containing organs. Like the respective livers, transplant-containing spleens from male rats displayed higher basal ECOD and 2α-, 2β-, 6β-, 14α-, 15α-, 15β-, 16α-, 16(- and 17-TH activities than those from females, whereas the opposite was the case for the PNPH and 6α- and 7α-TH activities. No gender dependence was seen with basal EROD, PROD and 15β-TH activities. With nearly all model reactions the sex-specific differences in the inducibility by BNF, PB or DEX seen in the livers could be observed also in the transplant-containing spleens. P450 activities, respective gender-differences and sex-specific inducibility correlated well to the corresponding P450 enzyme expression patterns.

In summary, the results of the present investigation indicate that the P450 system of the intrasplenically transplanted liver cells is influenced by the recipient gender like that of the livers.

MP50 HIGH-THROUGHPUT LC-MS/MS METHOD FOR CYP2D6 PHENOTYPING WITH DEXTROMETHORPHAN

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Background

In contrast to methods for genotyping, phenotyping usually still needs time consuming sample preparation and analytical procedures. On the other hand, the transformation of a probe drug gives a better understanding of the actual metabolic situation. Therefore, we developed a high-

through put method for the determination of dextromethorphan and its phase I and phase II metabolites.

Methods

Healthy volunteers (n=31) received 30 mg dextromethorphan once monthly followed by a 12 hours urine collection period over a time period of 7 months. Aliquots of the urine were spiked with a mix of stable isotope labelled internal standards and 10 µl were injected on the LC-MS/MS system with on line sample preparation (API 3000, Applied Biosystems). Total cycle time was 6 min. Genotype of CYP2D6 was determined by multiplex PCR.

Results

Dextromethorphan (DT), dextrorphan (DX), dextrorphan glucuronide (DXG), dextrorphan sulfate (DXS), methoxymorphinan (MM), 3-hydroxymorphinan (HM) and morphinan glucuronide (MG) could be quantified within a single chromatogram in less than 4 min. With limits of quantification ranging from 4 to 37nM MG could be quantified even in PMs. The metabolic ratio (MR) calculated from DX + DXG + DXS and DT correlated with the MR calculated from HM + MG and MM (r^2=0.89, $p<0.0001$). Both logMR showed a trimodal distribution with separate subgroups for PMs, extensive metabolizers (EMs) and intermediate metabolizers. In contrarst, both logMR accounting for glucuronidation and sulfation showed a normal distribution although PMs had lower glucuronidation and sulfation rates compared to EMs ($p<0.0001$).

Conclusion

The described method is suitable for phenotyping for CYP2D6 and can give additional information about glucuronidation and sulfation capacities.

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MP51 INFLUENCE OF METHAMPHETAMINE ON ACTIVITY OF CYP 2D1 IN THE ISOLATED PERFUSED WISTAR ALBINO RAT LIVERS

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It is known that methamphetamine influences the oxidative metabolism in the liver (in the pathway of cytochrome P450 2D1). The present study used the model of isolated perfused liver of Wistar albino rats to ascertain the influence of methamphetamine on cytochrome P450 2D1 based on analysis of the metabolites of dextromethorphane (DEM). DEM is a typical substrate for that cytochrome isoenzyme. For treatment twenty four rats were randomly divided into 3 groups: a) 2 ml of saline solution/kg/day for 10 days, intraperitoneally, b) methamphetamine 10mg/kg/day for 10 days, intraperitoneally c) methamphetamine 10 mg/l *in vitro*. The quantitative evaluation of the DEM metabolites was performed by HPLC (high pressure of liquide chromatography). For the statistical analysis of the results the Student t- test was used. The significant difference comparing to controls was found just in the concentration of the main DEM metabolite dextrorphane in the last period of perfusion (after 120 min) in the group of rats pretreated 10 days *in vivo* with methamphetamine, 10 mg/kg/ day. This finding indicates the stimulation of cytochrome 2D1. Furthermore, a small trend of insignificant increase in the amount of some other metabolites was recorded. Thus, the present results brought evidence on possible facilitative influence of MET on the activity of CYP 2D1.

MP52 EXPRESSION OF ARYL HYDROCARBON RECEPTOR REPRESSOR IN NORMAL HUMAN TISSUES AND INDUCIBILITY BY POLYCYCLIC AROMATIC HYDROCARBONS IN HUMAN TUMOR-DERIVED CELL LINES

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Aryl hydrocarbon receptor repressor (AhRR) has been recently identified as a negative factor that suppresses aryl hydrocarbon receptor (AhR)-mediated transcriptional gene expression. In the present study, the expression level of AhRR in normal human tissues was determined. AhRR mRNA was detected in liver, breast, colon, kidney, lung, bladder, uterus, testis, ovary, and adrenal gland. The expression level in the testis was prominently high. AhRR mRNA was also detected in various human tissue-derived cell lines, HepG2 (hepatocellular carcinoma), MCF-7 (breast carcinoma), LS-180 (colon carcinoma), ACHN (renal carcinoma), A549 (lung carcinoma), HT-1197 (bladder carcinoma), HeLa (cervix of uterus adenocarcinoma), NEC14 (testis embryonal carcinoma), and OMC-3 (ovarian carcinoma). Since the expression level of AhRR mRNA was prominently high in HeLa cells, it is suggested that the high expression level of AhRR might work as a negative factor for the low inducibility of the CYP1 family in HeLa cells. The expression of AhRR mRNA was induced by

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or 3-methylchoranthrene (3-MC) in HepG2, MCF-7, LS-180, and OMC-3 cells, but not in ACHN, A549, HT-1197, HeLa, and NEC14 cells. The responsiveness was similar to the cell-specific inducibility of the CYP1 family. The inducibility of AhRR mRNA by nitropolycyclic aromatic hydrocarbons (NPAHs) as well as their parent PAHs was compared in HepG2 and OMC-3 cells. The chemical-specific inducibility of AhRR was also similar to that of the CYP1 family determined in our previous study. These results indicated that AhRR is also induced in chemical- and cell-specific manners.

MP53 REGULATION OF MUTATION PROCESS INDUCED BY IONIZING RADIATION IN MYELOCARIOCYTES OF MICE BY PEROXIDASE AND EXTRACT FROM *ARMORACIA RUSTICANA* L.

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The participation of antioxidant enzymes in a regulation of mutation and aging processes induced by environmental genotoxicants has been shown in series of works[1-7]. The special actuality is acquired study of plant extracts as perspective agents for prevention both spontaneous and induced mutation process. The genetic activity of peroxidase - EC 1.11.1.7 (P) (Reanal) and extract (EHR) from a roots of a horse-radish (Armoracia rusticana L.) and their influence on frequency of spontaneous and induced by gamma rays aberrations of chromosomes (CA) in myelocariocytes of mice have been studied. The males of laboratory mice, 2-month age and with mass 18-20g have been used as object of experiments. Method - the analysis of CA frequency in mice marrow cells. The P and EHR administrated per os (30mg/kg) daily during 6 days before irradiation. Then, animals irradiated by gamma-rays (GR) in 1Gr dose, 20 rad/sek, and source is Co^{60} and mortified in 24 hours after irradiation. The mice divided into 6 groups, on 5 in each group: 1) control, standard ration (SR); 2) SR+P; 3) SR+EHR; 4) SR + GR; 5) P+GR; 6) EHR+GR. The administration to the SR of P and EHR has not increased the control level of CA in marrow cells of mice (*Fig. 1*). In experiments have been shown the tendency of the CA frequency decreasing in these variants. The addition to SR of P completely neutralizes genotoxic action of GR and decrease frequency the of CA from 10.68±1.68 % to 1.54±0.39 %, $P < 0.001$; EHR till 4.31±0.59, $P < 0.001$. The comparative assessment of the antimutagenic efficiency of EHR and P has revealed high efficiency of P (85 %), the efficiency of EHR has made 54 %. The experiments had shown the ability of P and EHR to prevent the mutation process, induced by GR in myelocariocytes of mice. It is obvious that P as an element of nonspecific system can play a stabilizing role at various pathological processes, including, connected with a damage of the genetic structures by ionizing radiations.

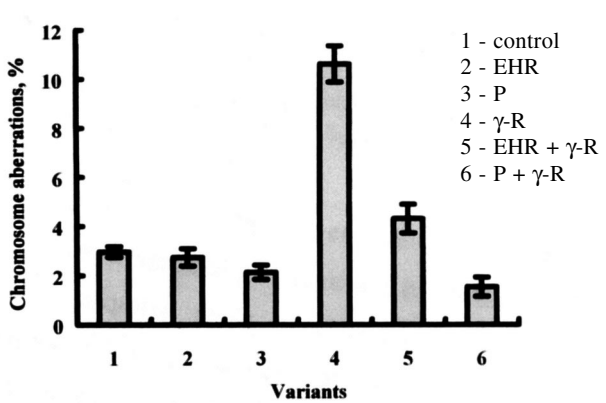

Fig. 1. *The influence of (P) and EHR on frequency of spontaneous and induced by (GR) chromosome aberrations in marrow cells of mice.*

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MP54 MOUSE CYTOCHROMES P450 4F

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Cytochromes P450 4F (CYP 4F) have been newly discovered and are a subfamily of cytochromes P450 that have hydroxylation activity toward many endogenous compounds, such as fatty acids, prostaglandins and leukotrienes, which are important mediators involved in the inflammatory response. These findings suggest that CYP 4Fs can modulate the level of these physiologically important substances and in that way regulate the level of inflammation. Also inflammation itself may regulate the level of CYP 4F expression and enzyme activity, which will further affect the metabolism by CYP 4Fs. To date six family members have been cloned in human and rodent. Four rat isoforms have been cloned in our laboratory. Five novel mouse cytochromes P450 4Fs genes, two from liver CYP 4F14 and CYP 4F15 and three from kidney CYP 4F13, CYP 4F16 and CYP 4F18 were cloned in our laboratory. The mouse isoforms share 90 percent or higher nucleotide sequence homology with their orthologues in rat. Unlike members of cytochrome P450 4A subfamily, expression of CYP 4F isoforms is repressed by clofibrate in the rat liver in contrast to CYP 4As, whose expression is induced by clofibrate treatment. Rat CYP 4F isoforms also metabolize exogenous drug substrates, the brain acting drugs, such as imipramine and chlorpromazine. This work is focusing on the study of mouse CYP 4Fs enzymatic activity, substrate specificity, molecular basis of their regulation and their role in inflammation.

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MP55 TCDD INDUCES CYP2A5 ENZYME IN MOUSE PRIMARY HEPATOCYTES

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2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes several biochemical and toxic effects, such as teratogenesis, immunosuppression and tumor promotion. It is, together with other polycyclic aromatic hydrocarbons (PAHs), a well-known inducer of xenobiotic metabolizing cytochrome P450 (CYP) enzymes of family 1, i.e. CYP1A1 and CYP1B1, and aryl hydrocarbon receptor (AHR) mediated mechanism is responsible for the induction[1].

Mouse CYP2A5 and its human orthologue CYP2A6 metabolize many toxic substrates, such as nicotine, nitrosamines and aflatoxins. CYP2A5 is induced by number of structurally diverse compounds including phenobarbital and pyrazol2. Interestingly, we have discovered that also TCDD induces CYP2A5. In the present study the mechanism of TCDD induction of CYP2A5 was investigated in mouse primary hepatocytes.

1 μM TCDD caused 3-fold elevation of coumarin hydroxylation (COH) catalysed by CYP2A5 after 24 h exposure. Further, this was accompanied by similar elevation of the CYP2A5 apoprotein level studied by immunoblotting. Also CYP2A5 mRNA level was induced 2-3 fold by TCDD treatment. -3033 to +10 of the Cyp2a5 5'-flanking region was cloned in front of a luciferase reporter gene and the construct was transfected into primary hepatocytes. TCDD treatment induced luciferase activity 3-5 fold suggesting involvement of transcriptional regulation.

Regulation of gene expression by TCDD is predominantly mediated by AHR and its dimerization partner aryl hydrocarbon nuclear translocator (ARNT). DBA/2 and C56BL/6 mouse strains show genetically-determined differential responses to AHR ligands3. The induction of Cyp2a5 by TCDD was studied in hepatocytes of these two mouse strains and was found to correlate with Cyp1a1 induction suggesting involvement of AHR also in the induction mechanism of Cyp2a5.

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MP56 CYP2C8, CYP2C9, CYP3A7, PXR AND CAR MRNA LEVEL INCREASE IN HEPATOBLASTOMA CELL LINE HEPG2, AFTER HMG-COA REDUCTASE INHIBITORS TREATMENT

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Statins which are 3-hydroxy-3-methylglutarylcoenzyme A (HMGCoA) reductase inhibitors are largely used to decrease plasma cholesterol level and reduce cardiovascular and cerebrovascular events in patients with established coronary artery disease. The phase I metabolism of most statins involves the P450 subfamilies CYP3A and CYP 2C. It was also demonstrated with lovastatin, that these drugs, except pravastatin, activate the Pregnane X Receptor (PXR)[1] and up-regulate CYP3A1/2 in primary cultured rat hepatocytes[2]. Fluvastatin and cerivastatin increase CYP2C protein and mRNA expression in porcine coronary artery endothelial cells[3]. CYP2C9 expression is regulated by both nuclear receptors PXR and CAR, an orphan receptor[4]. This study focused on the effects of five statins (atorvastatin, fluvastatin, lovastatin, pravastatin and cerivastatin) on CYP2C8, CYP2C9, CYP3A7, PXR and CAR mRNA expression in HepG2 hepatoblastoma cell line. After 72 h treatment, atorvastatin, fluvastatin, lovastatin (0.1 to 10 μM) and cerivastatin (0.1 to 10 nM) increased CYP3A7 mRNA level (2-4 fold.). CYP2C8 and CYP2C9 mRNA level increased 1.5-2.5 fold. After 96 h treatment, atorvastatin, fluvastatin, lovastatin (1mM) clearly increased CYP2C9 protein level. Pravastatin (hydrophilic) had no effect on any CYP mRNA level tested. After 48 h treatment, fluvastatin and cerivastatin increased CAR mRNA expression (2.5 and 1.5 fold, respectively). In the same conditions, fluvastatin increased PXR mRNA level. Atorvastatin increased PXR mRNA expression (3.5 fold) after 72 h treatment. These increases in both PXR and CAR nuclear receptors expression are related with the increase in CYP2C9 mRNA expression. Statins, at least lovastatin, are known to activate PXR receptor. Moreover, our data demonstrate that fluvastatin or cerivastatin not only activate PXR, but also enhance PXR mRNA expression, contributing in the increase in CYP3A7. All together, our data demonstrate that CYP2C8, CYP2C9 and CYP3A7 and nuclear receptors PXR and CAR mRNA expression are modulated by various lipophilic statins, suggesting that the increase in CYP3A and CYP2C mRNA and protein expressions after treatment with these lipophilic statins results from their effects on both expression level and activation of nuclear receptors PXR and CAR.

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MP57 CHARACTERIZATION OF CYTOCHROME P450 EXPRESSION AND INDUCTION PROFILES IN THE WIF-B9 CELL LINE

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The WIF-B9 model is a unique, highly differentiated and polarized hepatoma cell line, obtained by hybridization of rat hepatoma cells (Fao) and WI38 human fibroblasts. It presents morphological features, such as functional bile canaliculi[1], that are close to those of primary hepatocytes[2]. We previously demonstrated that WIF-B9 is a valuable model for *in vitro* hepatotoxicity investigations[3]. We further evaluated the metabolic capacity of WIF-B9 using competitive RT-PCR. Five rat isoforms (CYP 1A1, 2B1/2, 2E1 and 4A1) and four human isoforms (CYP 1A1, 2Cs, 2D6, 2E1) are constitutively expressed in this cell line. Human forms are expressed at 2-4 orders of magnitude lower (ranking from 4 10^2 to 2.5 10^4 copies/ng total RNA) than that of the rat forms (ranking from 4 10^5 to 6.4 10^6 copies/ng total RNA). The induction potential of these rat and human P450 forms was assessed using seven different reference inducers. Rat CYP 1A1/2, 3A1/2 and 4A1 were inducible by 20 to 48 h treatment with β-naphthoflavone (32μM), dexamethasone (8μM), and clofibrate (500μM), respectively. Rat CYP 1A1, 1A2 and 3A1 were the most inducible forms (>100 fold). Human CYP 1A1 and 2Cs were induced 3 fold by 48 h treatment with phenobarbital (430μM). These results indicate that the rat and human cytochrome P450s are differentially inducible in this cell line

and that it should then represent a useful tool for evaluating metabolism-toxicity relationships. We are currently investigating the protein profile of cytochrome P450 induction.

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MP58 THE DECREASE OF RAT LIVER MICROSOMAL CYP3A2 CATALYTIC ACTIVITY DURING AGING MAY BE THE CONSEQUENCE OF AN ENHANCED PROTEIN INSTABILITY

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CYP3A is one of the most important P450 enzymes that metabolises approximately half the drugs in use today[1]. Since CYP3A2 is the predominant expressed form in adult male rats[2], the aim of this work was to study the microsomal catalytic activity of CYP3A2 in rats of different ages and to compare these results to levels of protein oxidation, proteasome activity and antioxidant enzyme activities. Microsomes and cytosol were prepared from livers of male Wistar rats (HAN) with ages ranging from 3 to 24 months. The activity of CYP3A2 was assayed by monitoring the formation of hydroxylated metabolites of midazolam by HPLC. The amount of CYP3A2 apoprotein was estimated by Western Blot and the mRNA levels by RT-PCR. The proteasome activity (chymotrypsin-like) was measured by following the release of the fluorogenic AMC group after proteolytic cleavage (excitation: 365 nm; emission: 450 nm). The protein carbonyl content was determined by immunoblotting after derivation with DNPH (diphenylhydrazine). Catalase was measured with the TiSO4 method and GSH peroxidase by recording the NADPH oxidation. Midazolam hydroxylation increased from 3 to 9 months, remained constant between 9 and 18 months and then progressively decreased until 24 months. At 24 months, the CYP3A2 protein was decreased but levels of mRNA remained unchanged. The levels of carbonyl protein were increased in microsomes, while a decrease was observed in cytosolic proteins. Neither catalase nor the proteasome activity changed over the whole period, but GSH peroxidase activity decreased. Taken together, these results suggest that loss in enzyme activity may be explained by a decreased amount of CYP3A2 protein. Since levels of mRNA are not modified during aging, the loss in proteins may be due to either a decreased protein translation or an enhanced degradation. The former possibility is unlikely because protein synthesis rates (incorporation of radiolabeled leucine into proteins) remained unchanged. We hypothesise that CYP3A2 is modified during aging (protein carbonylation ?) thus rendering the protein highly instable and subject to degradation by the proteasome. Indeed, we have recently reported that CYP3A2 is highly sensitive to changes in cellular environment[3].

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MP59 COCAINE, BUT NOT METAMPHETAMINE CAUSES A CONCOMITTANT DECREASE OF CYP2C AND CONSTITUTIVE ANDROSTANE RECEPTOR (CAR) EXPRESSIONS IN HUMAN ASTROCYTOMA CELL LINE U373 MG

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Cocaine and metamphetamine are psychostimulant abused drugs associated with a strong risk for intracranial ischemic and hemorrhage stroke. Several unclear mechanisms may be responsible for this cerebrovascular complication including vasoconstrictive processes followed by

blood flow alterations[1]. Cytochromes P450, especially astroglial CYP2C may play role in modulating cerebrovascular functions, participating to vasodilatators metabolites generation from arachidonic acid[2]. Mechanisms of CYP2C mRNA regulation implicates two specific receptors: the constitutive androstane receptor (CAR) thought to be inlvoved in phenobarbital induction and pregnane X receptor (PXR) thought to be involved in rifampicin induction[3,4,5]. In this work, we examined CYP2C regulation in response to cocaine and metamphetamine in an astroglial cell line model, by RT-PCR and western-blotting analysis. U373 MG, a human astrocytoma cell line, expressed at least two isoforms of CYP2C family namely CYP2C8 and CYP2C9. A 48 hrs treatment with cocaine (1, 10 and 100 μM) caused a significative total CYP2C, CYP2C8 and CYP2C9 mRNA decrease and a CYP2C9 protein level decrease, whereas metamphetamine had no effect on CYP2C mRNA expression. These effects of cocaine were accompanied by an obvious repression of CAR mRNA reaching - 90 % with 10 μM cocaine. Such data suggest that this receptor is involved in CYP2C repression by cocaine, in this astroglial cell line. These findings represent one possible molecular mechanism involved in cocaine cerebrovascular effects and neurotoxicity.

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MP60 FUNCTIONAL CHARACTERIZATION OF A SPLICED CYP3A7 VARIANT WITH A LONGER C-TERMINAL, EXPRESSED IN HUMAN FETAL LIVER

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The human cytochrome P4503A (*CYP3A*) locus contains three pseudogenes, *CYP3AP1, CYP3AP2* and *CYP3AP3* and four genes *CYP3A4, CYP3A5, CYP3A7* and *CYP3A43*. The different CYP3A enzymes are expressed in a developmental and tissue-specific manner. In the fetal liver, CYP3A7 is the most abundant CYP, playing an important role in the metabolism of steroids, in particular dehydroepiandrosterone (DHEA), and drugs. Recently, a CYP3A7 mRNA variant, consisting of the 13 exons of *CYP3A7* and two additional exons at the 3'end from the *CYP3AP1*, was detected in human liver[1]. This transcript would encode a CYP3A7 protein where the last 4 amino acids in the C-terminal have been replaced by a unique sequence of 36 amino acids from the two exons of the pseudogene. We found this variant incidentally when screening fetal liver and further examined the expression using semi-quantitative RT-PCR. The mRNA expression of the CYP3A7-variant was higher in fetal livers than in adult livers and was not detected in human hepatoma cell lines. There was a high interindividual difference in fetal hepatic expression and the variant overall represented 1% of CYP3A7 mRNA in the fetal livers. The CYP3A7 and CYP3A7-variant proteins were heterologously expressed in yeast and in the mammalian HEK293 cells, and the activities of both enzymes were assayed using [^{3}H]DHEA. Unexpectedly the CYP3A7-variant was catalytically very active and hydroxylated DHEA in the 7α-position in contrast to CYP3A7 which mainly formed the 16α-OH product. Similar results were obtained both in yeast and in HEK293 microsomes. It is concluded that a trans-spliced product of the *CYP3A7* gene is expressed in fetal liver and that the corresponding enzyme exhibits a different specificity as compared to CYP3A7.

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MP61 EFFECTS OF WEANING TO DIETS CONTAINING RICE PROTEIN ISOLATE (RPI) ON GROWTH, PLASMA IGF_1 AND EXPRESSION OF CYP2C11 AND CYP4A1 IN RAT LIVER

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Rice contains a number of phytochemicals including vitamin E derivatives, tocotrienols and oryzanols which have been suggested to contribute to health beneficial effects of rice consumption including reductions in cholesterol[1,2]. In addition, protein isolates from rice are in widespread use in the food industry; have been shown to produce dietary protection against DMBA-induced mammary tumorigenesis in rats[3,4]; and have many associated phytochemical constituents[5]. We fed groups of time-impregnated female Sprague-Dawley rats pelleted AIN93G diets with casein as the sole protein source *ad libitum* from gestational d. 4 until their pups weaned at post-natal d.21. Litters were culled to 5 male and 5 female pups and N = 8-10 male or female pups were weaned onto amino acid matched pelleted, diets, containing either casein (CAS) or rice protein isolate (RPI) as the sole protein source, *ad libitum* beginning on post-natal d. 15 until sacrifice at post-natal d. 34. Body weight gain was measured and serum IGF_1 concentrations were assessed by radioimmunoassay. In addition liver microsomes were prepared. Testosterone 16α-hydroxylase was measured in male pups as an indicator of CYP2C11-dependent monooxygenase activity, CYP2C11 apoprotein was immunoquantified by Western blot and CYP2C11 mRNA was quantitated by real time RT-PCR. In addition, CYP4A1 apoprotein and mRNA were measured in liver microsomes from male and female pups by Western blot and real time PCR. Feeding RPI reduced body weight gain in both male and female pups ($p < 0.05$). This was accompanied by reductions in plasma IGF_1 concentration ($p < 0.05$) indicating a suppression or delay in development of the GH/IGF_1 axis in these animals. CYP2C11 mRNA and apoprotein expression and testosterone 16α-hydroxylase were also inhibited in male pups ($p < 0.05$). Since food intake was not measured it is unclear if this effect was due to reduced intake, reduced dietary energy availability or a direct effect of RPI on GH secretion. CYP4A1 mRNA and apoprotein were increased 4-5-fold in pups of both sex suggesting a possible peroxisomal proliferator response to components of RPI. Supported in part by ARS CRIS #6251-51000-003-065 and Riceland Foods, Inc.

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MP62 EFFECTS OF WEANING TO DIETS CONTAINING SOY PROTEIN ISOLATE (SPI) OR ISOFLAVONES ON GROWTH, PLASMA IGF1 AND EXPRESSION OF CYP2C11 AND CYP4A1 IN RAT LIVER

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Soy protein isolate (SPI^+) is fed to over 1 million children each year in the U.S. as part of soy-infant formula. Soy has been shown to have a number of health beneficial effects including reductions in cholesterol[1] and has been suggested to increase lean body mass[2,3]. The health effects have appear to be associated with both the soy protein component itself and to isoflavone phytoestrogens bound to SPI^+ [1]. In the current study we fed groups of time-impregnated female Sprague-Dawley rats pelleted AIN93G diets with casein as the sole protein source *ad libitum* from gestational d. 4 until their pups weaned at post-natal d.21. Litters were culled to 5 male and 5 female pups and N = 8-10 male or female pups were weaned onto amino acid matched, pelleted, diets containing either casein (CAS) or SPI^+, *ad libitum* beginning on post-natal d. 15 until sacrifice at post-natal d. 34. Other groups were fed SPI stripped of phytochemicals by ethanol washes (SPI^-) or CAS diets supplemented with the soy-isoflavones genistein or daidzein at a level

of 1 mg/kg Body weight gain and serum IGF_1 concentrations were measured. CYP2C11-dependent testosterone 16_-hydroxylase was measured in liver microsomes from male pups, CYP2C11 apoprotein was quantified by Western blot and CYP2C11 mRNA by real time RT-PCR. In addition, CYP4A1 apoprotein and mRNA were measured in male and female pups and microsomal CYP4A1-dependent lauric acid 12-hydroxylase was assessed. Feeding SPI^+ or SPI^- reduced body weight gain in both male and female pups ($p < 0.05$). This was accompanied by reductions in plasma IGF1 indicating a suppression or delay in development of the GH/IGF_1 axis in these animals. CYP2C11 mRNA and apoprotein expression and testosterone 16α-hydroxylase were also inhibited in male pups fed SPI^+ or SPI^- ($p < 0.05$). Since food intake was not measured it is unclear if this effect was due to reduced intake, reduced dietary energy availability or a direct effect of SPI on GH secretion. No such effects were observed in pups fed CAS + gesistein or daidzein. Lauric acid 12-hydroxylase; CYP4A1 apoprotein and mRNA expression were inhibited ($p < 0.05$) in pups of both sex fed SPI^+ but not SPI- or isoflavones suggesting a possible inhibition of peroxisomal proliferation response by non-isoflavone phytochemical components bound to SPI. Supported in Part by ARS CRIS #6251-51000-003-065 and DuPont Protein Technologies.

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MP63 HUMAN CRYOPRESERVED HEPATOCYTES AS AN IN VITRO MODEL TO STUDY CYTOCHROME P450 INDUCTION

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Major restrictions are connected to current in vitro models for cytochrome P450 (CYP) induction screening during drug development. Although fresh human hepatocytes are still considered as 'the golden standard' to study CYP-induction in preclinical development, their supply is often erratic and/or their condition is in many cases poor. In addition, assay standardization is rendered more difficult due to a high interindividual variation of CYP expression in hepatocytes. Improvement of cryopreservation methods and optimalization of culturing conditions have led to the possibility to culture viable and plateable hepatocytes after cryopreservation. A major advantage for the pharmaceutical industry is that long-term induction experiments can be planned precisely and that the hepatocyte populations used in these studies can be standardized easily. Human cryopreserved hepatocytes (HCH's) from four different donors were plated, the cells were allowed to recover for 48h and then incubated with 50 μM omeprazole, 25 μM rifampicin, 10 μM dexamethasone, 100 μM phenobarbital, 100 μM clofibric acid or 150 mM ethanol for another 48h and compared to non-treated cells. CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4 induction was determined by measuring 7-ethoxyresorufin-O-demethylation, [^{14}C]s-mephenytoin-N-demethylation, [^{14}C]tolbutamide-methyl-hydroxylation, [^{14}C]s-mephenytoin-4'-hydroxylation, chlorzoxazone-6-hydroxylation and [^{14}C]testosterone-6(-hydroxylation respectively. In addition, mRNA and protein expression was analysed by TaqMan QRT-PCR and immunodetection. In general, HCH's responded well to the respective typical inucers. For example, activity, protein and mRNA expression of batch 1 was increased 13-, 25-, and 79-fold respectively upon omeprazole treatment. A 2- to 3-fold induction was observed of CYP2C9 mRNA after rifampicin and phenobarbital treatment, while maximally a 17-, 6-, and 24-fold induction was measured of CYP3A4 upon treatment with the same typical inducers. Induction profiles of the different HCH-batches will be discussed in detail. In conclusion, HCH's may be a good alternative to fresh hepatocytes to study CYP induction.

MP64 CYP1A REGULATION BY α-TOCOPHEROL

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CYP1A play dominant role in the bioactivation of polycyclic aromatic hydrocarbons (PAH) and heterocyclic amines, widespread chemical carcinogens, to genotoxic derivatives. Until the recent times only planar aromatic chemicals considered among CYP1A1 inducers. At the last 2 decades was shown that compounds with other structure might induce CYP1A1. CYP1A1 induction by PAH is mediated by Ah receptor, transcription factor regulated *CYP1A1* gene expression. In the present study effects of α-tocopherol on rat hepatic CYP1A were investigated on male Wistar rats

weighting 100 - 120 g. Oil solution of α-tocopherol was administered *per os* in dose 150 mg/kg body weight daily, once or during 12 days. CYP1A1-dependent 7-ethoxyresorufin O-deethylase (EROD), CYP1A2-dependent 7-methoxyresorufin O-demethylase (MROD) and NADPH-cytochrome P450 reductase activities were measured in rat hepatic microsomes. *CYP1A1* and *CYP1A2* mRNA levels in the liver of control and experimental animals were measured by semi-quantitative RT-PCR technique. Formation of Ah receptor complex with oligonucleotide XRE3, containing 3 repeats of DNA sequence, which specifically binds with AhR, in the liver nuclear extracts was determined by gel-retardation technique. The single α-tocopherol administration led to 3,5-fold enhancement of EROD activity; 3,3-fold of MROD activity and had no effect on NADPH-cytochrome P450 reductase activity. 12 days long administration of α-tocopherol led to 2-fold enchancement of EROD activity and had no effect on MROD and NADPH-cytochrome P450 reductase activities. *CYP1A1* mRNA level in the liver of experimental animals was significantly higher than in control after 12 days long administration of α-tocopherol, though this treatment has no effect on CYP1A2 mRNA level. Ah receptor complex with XRE3 was detected only in rat liver nuclear extracts after single α-tocopherol administration. No complex formation was observed in control nuclear extracts or experimental after 12 days long administration of α-tocopherol. Thus, enhancement of CYP1A1 activity and mRNA level under α-tocopherol action is mediated by Ah receptor.

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MP65 THE ROLE OF CYTOCHROMES P450 IN BIOTRANSFORMATION OF NEW ANTILEUKOTRIENIC DRUG QUINLUKAST IN RAT

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Anti-leukotrienes drugs have become available for the clinical management of asthma. They are functioning either by blocking the interaction of leukotrienes with receptors or by inhibiting leukotriene synthesis. The new promising drug belonging to anti-leukotrienes is quinlukast[1] (Q), 4-(4-(quinoline-2'-yl-methoxy)phenylsulphanyl)benzoic acid (VÚFB 19363), *Fig. 1*. It is now under investigation for its anti-inflammatory and anti-asthmatic effects. In comparison to the specific effect of montelukast and zafirlukast, Q displays multiple antileukotrienic effects[2]. Phase I bio-

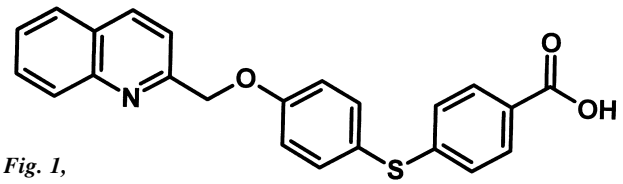

Fig. 1,

transformation study of Q in male rats *in vitro* had revealed that Q sulfoxide is the main metabolite identified in microsomal fraction and in isolated hepatocytes. Also other metabolites of Q were found, which structure is still not known (M3, M5). The present study was conducted to characterise the enzymes involved in Q biotransformation in isolated rat hepatocytes.

The primary cultures of rat hepatocytes were 48 hours treated by inducers of different CYPs (rifampicin, dexamethasone, phenobarbital, ethanol, β-naphthoflavone). Then Q (50 μM) was incubated 24 hours in primary culture of induced or control hepatocytes. The effects of CYPs inhibitors ketoconazole, methylpyrazole, metyrapone and α-naphthoflavone (2, 10, 50 μM) on Q metabolism were tested in induced and control hepatocytes.

Significant induction of Q S-oxide formation (6 times) by dexamethasone and strong concentration-dependent inhibition by ketoconazole was observed. Dexamethasone also induced formation of metabolite M5 (twice) and ketoconazole strongly inhibited this metabolite. CYP1A inducer, β-naphthoflavone, induced 10 times production of metabolite M3 and strong inhibition effect of α-naphthoflavone on its formation was observed. These results indicate that CYP3A catalyse sulfoxidation of Q and formation of its metabolite M5 and CYP1A catalyse formation of metabolite M3 in primary culture of rat hepatocytes.

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MP66 EFFECT OF REPEATED ALBENDAZOLE ADMINISTRATION TO MOUFLONS ON ALBENDAZOLE METABOLISM IN VIVO AND IN VITRO

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Albendazole (ABZ) is a benzimidazole derivate with a broad-spectrum activity against animal helminth parasite. The effective treatment of polygastric animals can be often reached by a single dose administration of ABZ but treatment of some resistant parasitic diseases (e.g. small lungworms and small liver flukes) require repeated drug administration. After oral administration in animals, ABZ is metabolised to biologically active albendazole sulfoxide (ABZSO) in two enantiomeric forms: (–)-ABZSO and (+)-ABZSO. It is known that the CYPs (mainly CYP3A) system generates (–)-ABZSO, while the FMO system selectively produces (+)-ABZSO. CYP1A mediate ABZSO oxidation to inactive albendazole sulfone ($ABZSO_2$)[1]. Inter-species differences in quantitative aspects as well as stereospecificity of ABZ metabolism was described[2,3]. In laboratory animals and human cells ABZ administrations caused induction of CYPs, mainly CYP1A[4,5].

The aim of this project was to study effect of repeated ABZ administration on its oxidative metabolism.

Adult mouflon ewes (8 animals) from game enclosure Vlkov (Czech Republic) were divided into two groups: first group was treated repeatedly by therapeutic doses of ABZ (p.o. 5 (7.5 mg/kg of body weigh), second group represented control. Repeatedly, 24 h after each dose, blood samples were collected and metabolites were quantified by HPLC. Animals were culled 24 h after the last dose. The bile from gall bladder, blood, and ruminal fluid were sampled for HPLC analyses. Whole liver and small intestine were removed immediately and stored in liquid nitrogen during transport to laboratory. Microsomes were prepared from homogenate of both tissues of individual animals. In vitro biotransformation of ABZ and ABZSO were tested in liver and intestinal microsomes.

Plasma level of total ABZSO did not significantly change during whole experiment (5 days), but the relative proportion of (+)-ABZSO enantiomer significantly increased. Increasing $ABZSO_2$ plasma concentration in dependence on number of ABZ doses were also observed. In bile, $ABZSO_2$ and both ABZSO enantiomers were found in concentration similar to their concentration in plasma. Parent drug was detected only in abomasal fluid. The repeated administration of ABZ to mouflons did not affected ABZ sulfoxidation in microsomes but oxidation of ABZSO was significantly faster in microsomes from ABZ treated animals.

We can conclude that administration of repeated doses of ABZ led to acceleration of its own metabolism probably due to CYP1A induction. Increasing deactivation of ABZ could result in the failure of therapy.

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MP67 AGE RELATED CHANGES IN THE PROTEIN AND MRNA LEVELS OF CYP2E1, CYP3A1, CYP3A2 AS WELL AS IN THEIR HEPATIC ACTIVITIES IN WISTAR RATS

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The biotransformation of a drug and, therefore its therapeutic effect, may be modified during ageing. Among different causative factors, the impairment of normal cellular functions by free radicals has been evoked as playing a critical role. The aim of our study was to evaluate the effect of age on the expression and activity of CYP2E1, CYP3A1 and CYP3A2. Total cytochrome P450, protein and mRNA levels and activities for each isoform were investigated. In addition, we determined some markers of oxidative stress like TBARS (thiobarbituric acid reactive

substances), histochemical detection of aldehydes, carbonyl proteins and GSH content. Liver microsomes were prepared from male Wistar rats of 3, 8, 11 and 18 months old. Chlorzoxazone was used as a probe to assess CYP2E1 activity and midazolam for CYP3A1 and CYP3A2 activities. Metabolite formation, 6-hydroxy-chlorzoxazone for chlorzoxazone and α-OH midazolam and 4-OH midazolam for midazolam was quantified by HPLC. Protein isoform contents were determined by Western Blot and mRNA levels by RT-PCR. Lipid peroxidation was assessed following the formation of TBARS and by histochemical detection of aldehydes, the direct Schiff's reaction. Protein carbonyl content was determined by measuring at 355 nm the formation of hydrazone after reaction with DNPH (diphenylhydrazine). GSH level was estimated fluorimetrically by determination of the thiol content by reaction with OPT (o-phtaldialdehyde). Results revealed that total cytochrome P450, the expression and the activity of CYP3A1 and CYP3A2 did not change until 18 months. Nevertheless, chlorzoxazone hydroxylation increased from 3 to 8 months, remained constant between 8 and 11 months and then progressively decreased until 18 months. Interestingly, microsomal protein and mRNA level of CYP2E1 did not change over the whole period. While the amount of proteins did not change, their functionality may be affected by oxidative stress (increase in TBARS and histochemical aldehyde detection, decrease in GSH level). However, we did not observe any change in carbonyl protein content. The decrease in chlorzoxazone biotransformation in rats after 11 months is most probably due to post-translational modifications of CYP2E1 proteins and well correlated with the accumulation of damage due to oxidative stress. Since no change in the midazolam α- and 4- hydroxylation activities as well as protein and mRNA content was observed for the CYP3A1 and CYP3A2 until 18 months, it seems that such isoforms are less affected by the oxidative environment.

MP68 ADMINISTRATION OF CYP19 MODULATORS TO *GALLUS DOMESTICUS*

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Estrone, one of female sex hormones, is formed by a unique cytochrome P450 CYP19 which builds a phenolic ring A of all estrogens. This enzyme is present in ovarian tissue of birds. The modulation of CYP19 activity might significantly change the estrone/testosterne ratio and consequently is expected to change the poultry sex phenotype. For example, in ovo administration of CYP19 inhibitor, Fadrazole, elicited testes formation in genotype females. Female masculinisation resulting in lowered body fat and increased volume of muscles might be important in the respect of the chicken meat production. Natural plant compounds, flavonoids, were selected as potential CYP19 inhibitors. Their inhibitory capacity towards CYP activities has been extensively studied because of their potential use as agents blocking the initiation stage of carcinogenesis. Synthetic and naturally occurring flavonoids are effective inhibitors of mammalian CYP1A1, 1A2, 1B1 and 3A4 as well as CYP19. The presence of C4 oxo-group of the flavonoid skeleton, which is approaching the heme iron (based on models), seems to be crucial for inhibition. One can expect that the substitution of oxygen for sulphur in this position will result in a higher binding capability. Hence, synthetic flavonoids, 7,8-benzoflavone (ANF) and its thioanalogue (SANF), were tested in vivo as food additive for hatched animals. Resulting changes of CYP expression, steroid hormone levels, body composition, and chicken behavior were monitored. Short-time i.p. administration of ANF, SANF and 5,6-benzoflavone (BNF), used for comparison, caused large induction of chicken CYP crossreacting with antibodies against rat CYP1A1. CYP recognized by anti-rabbit CYP2B4 antibody was elevated, too, while CYPs detected by antibodies against rabbit CYP3A6 and 2E1 were not significantly affected. These results were confirmed with metabolic data: 7-ethoxyresorufin deethylase activity was increased 10-30 times by application of flavonoids, showing BNF to be most efficient one. Effect of long-time p.o. treatment of animals with ANF and SANF in respect to CYP expression in various organs is being currently examined. In case of female chicken both, ANF and SANF, caused induction of CYP1A4, while SANF induced CYP1A5 in male chicken. There were no significant changes detected by antibodies against rabbit CYP3A6 and 2E1.

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6 POSTERS – TUESDAY – TP

TP01 NON-DISSOCIATIVE SEQUENTIAL METABOLISM OF AN 8-ALKYL XANTHINE BY HUMAN P450 1A2[1]

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Cytochrome P450 enzymes are versatile catalysts with broad substrate specificities. It is not unusual to find that a product of a P450-catalyzed reaction may undergo further metabolism by P450 enzymes. Examples where the sequence of oxidation reactions is catalyzed by the same enzyme and where the initial substrate undergoes multiple oxidation events before dissociation from the enzyme are found among the steroid metabolizing P450. For instance, $P450_{arom}$ and $P450_{scc}$ are known to catalyze three successive oxidation reactions on the same substrate molecule prior to releasing the final product. Examples of non-dissociative sequential metabolism amongst the xenobiotic metabolizing P450 enzymes are relatively rare. An interesting example is found in the conversion of ethanol to acetic acid by CYP2E1[2].

We have recently discovered a unique example of sequential metabolism where CYP1A2 catalyzes a sequence of three oxidative reactions directed towards the isopropyl sidechain of an 8-alkyl xanthine (IPCH) (*Figure 1*). Isotope dilution methodologies[3] were employed in order to determine the true branching ratios for each reaction in the sequence. This analysis revealed that substantial fractions of the olefin (77%) and diol (32%) that are formed in the enzyme active site are sequentially converted to the next metabolite (diol and ketone respectively) before they can be released from the enzyme. Overall, 25% of the first-formed primary olefin metabolite is converted to the tertiary ketone metabolite within the confines of the enzyme active site. Branching ratios (k_{cat}/k_{off}) were found to be highly sensitive to rates of electron transfer. Apparent K_m and k_{cat} values for substrates IPCH, olefin and diol were separately determined. The analytic solution of the scheme using the steady state assumption was transformed using the Cleland nomenclature into a form that accepted the measured apparent Michaelis-Menton rate constants. The results of this effort were estimates of the rate constants for all binding and catalytic steps. Branching ratios determined from our estimates of the true internal rate constants compared favorably with the branching ratios from the isotope dilution experiments. These results suggest a role for CYP1A2 active site water in the conversion of olefin to diol via the putative epoxide intermediate. A similar role for active site water in the deactivation of reactive imidazo-alkide intermediates produced by the metabolism of other 8-alkylxanthines such as furafylline by CYP1A2 has been proposed[4].

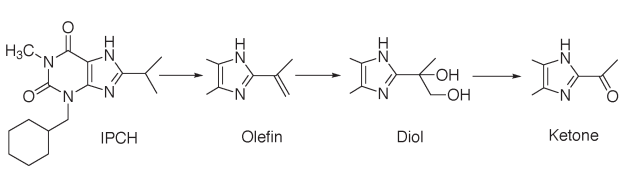

Fig. 1.

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TP02 CAFFEINE OXIDATION DURING PROLONGED EXPOSURE OF RATS TO PHENOTHIAZINE NEUROLEPTICS

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Because of its natural character, caffeine is used as a test substance for estimation of metabolic phenotype with regard to the activity of NAT2 and CYP1A2 in humans and rats. The primary metabolic pathways of caffeine are 3-N-demethylation to paraxanthine (CYP1A2), 1-N-demethylation to theobromine and 7-N-demethylation to theophylline (CYP1A2 and other enzymes), and C-8-hydroxylation to 1,3,7-trimethyluric acid (mainly CYP3A). There are some suggestions that 1-N-demethylation and 7-N-demethylation engage probably also isoenzymes CYP2B and/or CYP2E1. Our previous studies indicated that phenothiazine neuroleptics given *in vitro* to rat liver microsomes inhibited the above-described oxidation pathways of caffeine metabolism. The aim of our present work was to study the effect of 24 h-exposure and chronic treatment with pharmacological doses of classic neuroleptics (phenothiazines with different side chains and aromatic ring substituents)

on the activity of rat cytochrome P-450 measured by caffeine oxidation. The investigated neuroleptics were administered intraperitoneally (ip.), twice a day for one day or two weeks at the following pharmacological doses (mg/kg i.p.): promazine, levomepromazine, thioridazine and perazine 10, chlorpromazine 3. The rats were sacrificed at 12 h (one-day treatment) or 24 h (two-week treatment) after the drug withdrawal, and liver microsomes were prepared by differential centrifugation in 20 mM Tris/KCl buffer (pH = 7.4), including washing with 0.15 M KCl, according to a conventional method. The *in vitro* metabolic studies were carried out at linear dependence of the metabolite formation on time, and protein and substrate concentrations. The rates of 3-N-, 1-N- and 7-N-demethylation and C-8-hydroxylation of caffeine were assessed at a substrate concentration of 800 μM. Caffeine and its four oxidized metabolites were assessed using the HPLC method (Daniel et al. 2001) adapted from Rasmussen and Brøsen (1996). One-day (i.e. 24 h) exposure to phenothiazine neuroleptics resulted in a significant increase in the rates of the four oxidation pathways of caffeine by promazine and perazine. Moreover, thioridazine significantly increased the rates of 1-N- and 3-N-demethylation, while chlorpromazine enhanced that of C-8-hydroxylation of caffeine. Levomepromazine did not produce any significant changes in the rate of caffeine oxidation. After two-week treatment with the investigated phenothiazines, the increased rates of caffeine oxidation pathways, which appeared after one-day exposure, were still maintained, with an exception of the thioridazine effect on 1-N- and 3-N-demethylation. The obtained results indicate that, apart from a direct effect of phenothiazine neuroleptics on the activity of cytochrome P-450 (competitive or mixed inhibition), prolonged exposure of rats to those drugs may stimulate the activity of its different isoforms, i.e. of CYP1A2, CYP3A, and probably of other ones. Thus, the effect of the phenothiazine neuroleptics on the activity of cytochrome P-450 *in vivo* will be a resultant of their direct inhibitory effect on the enzyme (dependent on a current drug concentration) and the induction level (time-dependent). Adequate studies are in progress to identify CYP isoforms responsible for the catalysis of particular pathways of caffeine oxidation, which should allow for a full interpretation of the changes in CYP activity observed after neuroleptic treatment. It seems also interesting to find out whether similar effects develop during long neuroleptic therapy of schizophrenic patients.

TP03 ACTIVITY OF NADPH-CYTOCHROME P-450 REDUCTASE IN THE HUMAN HEART, LIVER AND LUNGS IN THE PRESENCE OF PADMA 28 – *IN VITRO* STUDY

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Padma 28 is a mixture of herbs used in traditional Tibetan medicine with anti-inflammatory activity. It was showed previously[1] that Padma 28 induced a concentration dependent inhibition of nitric oxide synthase (iNSO) protein expression and iNSO mRNA levels. NADPH-cytochrome P-450 reductase and iNOS have many structural similarities and consequently both can catalyze redox cycling and bioreductive anticancer agent[2]. The aim of this study was to investigate the activity of NADPH-cytochrome P-450 reductase in the presence of Padma 28. The NADPH-cytochrome P-450 reductase was obtained from human hearts, lungs and livers post mortem. The activity of NADPH-cytochrome P-450 reductase was evaluated by measuring the rate of cytochrome c reduction at 550 nm at 37 °C (pH=7.4). First, the activity of the enzymes alone was measured. Subsequently, activity of NADPH-cytochrome P-450 reductase was examined in the presence of different volumes of Padma 28 (1.0; 2.0; 5.0; 10.0 μl in total volume 1000 μl). Our study showed that Padma 28 increased NADPH-cytochrome P-450 reductase activity in the human samples with all tested doses. In all cases, the enzyme activity was enhanced with the rise of given concentrations. Padma 28 elevated a catalytic efficiency in increasing order of lung>liver>heart.

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TP04 ETHANOL INFLUENCE ON METHANOL OXIDATION VIA ADH AND MEOS

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The liver is the major organ responsible for methanol oxidation, and alcohol dehydrogenase (ADH) and microsomal alcohol oxidising system (MEOS) cytochrome P-450 - depended are the main enzymes involved. Methanol toxicity is directly caused by its toxic metabolites and the major task in clinical toxicology is to inhibit each of these enzymes to protect human life. Ethanol, well-known competitive inhibitor of ADH administered in methanol intoxication has raised many contradictory opinions. Generally it is not an ideal antidote. Our study is a part of the project involved in searching for new solutions in the methanol intoxication treatment based on postulate that MEOS is also involved in methanol oxidation, in addition to ADH. In the present study human liver samples were homogenised and used for enzymatic assays. ADH activity was assayed by methanol monitoring, GC method. MAOS activity was measured by formaldehyde monitoring, spectrophotometrical method. The percentage of enzymes activity changes was calculated for ethanol at concentrations 0,05, 0,022 , 0,01, 0,005 and 0,001 M. In order to calculate kinetic parameters the data were plotted according to Lineaweaver-Burk and Dixon. The activities of ADH and MEOS were measured by reaction with methanol ranging from 0,01 to 1,0 M or 0,03 to 0,5 M, respectively. Significant differences of enzymes activities were observed. The mean value of ADH activity was 1 μKat/g tissue and for MEOS activity 3 μKat/g tissue. The differences in K_m and V_m for both enzymes are significant. A higher ADH K_m value (53,6 mM) indicates that methanol has lower affinity to ADH as compared to MEOS (K_m 6,7 mM), *in vitro*. However, V_m is much higher for methanol oxidation catalysing by ADH. The results of the present study and data obtained from other authors allowed us to compare kinetic parameters for methanol and ethanol as ADH and MEOS substrates. The present results confirm that ethanol compared to methanol has higher affinity to ADH, by contrast to MEOS. As recent investigation, our study showed that ADH was competitively inhibited (K_i 24,8 mM) to different extents by all examined concentrations of ethanol in reaction with 0,05M and 0,10 M methanol. On the contrary, ethanol is responsible for increasing of MEOS activity with methanol at concentrations of 0,05 M and 0,20 M. There are a lot of doubt because the effectiveness of ethanol therapeutic administration in methanol intoxication. Specially, in alcoholics this problem could be observed. However, in light of our investigation we could try to explain these controversies, in theory. Ethanol can inhibit ADH activity but also it can activate MEOS activity and methanol oxidation could change the predominant pathway of biotransformation.

TP05 ACTIVATION OF NADPH-CYTOCHROME P-450 REDUCTASE IN THE HUMAN HEART, LIVER AND LUNGS BY UKRAIN – *IN VITRO* STUDY

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Ukrain is a semisynthetic drug with immunomodulatory prperties, derived from Chelidonium majus L. alkaloids and thiophosphoric acid. Previously studies showed that Ukrain can activate Cytochrome P-450 (CYP2E1). In the present study Ukrain was used to evaluate the effect on NADPH-cytochrome P-450 reductase activity. This enzyme was obtained from human hearts, lungs and livers post mortem. The causes of death were traffic accidents. The activity of NADPH-cytochrome P-450 reductase was evaluated by measuring the rate of cytochrome c reduction at 550 nm at 37 °C (pH=7.4). First, activity of the enzymes alone and subsequently, activity of NADPH-cytochrome P-450 was measured in the presence of different concentrations of Ukrain (1, 2, 5 μl in total volume of 1000 μl). Our study shows that Ukrain increased the NADPH-cytochrome P-450 reductase activity in the heart, lungs and liver at all investigated doses. In all cases the activity was enhanced with the rise of given concentrations. The rises strength of enzyme activity caused by Ukrain were in the following order: heart>liver>lung.

TP06 THE INTERACTION BETWEEN THE ANTICANCER DRUG ELLIPTICINE AND CYTOCHROMES P450 DICTATES ITS PHARMACOLOGICAL EFFICIENCIES

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Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) and several of its derivatives isolated from *Apocyanaceae* plants are alkaloids exhibiting significant antineoplastic and anti-HIV activities. We described that cytochrome P450 (CYP)-dependent metabolism of ellipticine leads to activation of this agent to more efficient metabolite(s) forming DNA adducts[1,2]. This shows the potential importance of several CYPs in producing these more active antitumor metabolite(s). The pattern of ellipticine metabolites formed by CYPs has not been characterized so far. Therefore, in the present work we investigated the metabolites of this anticancer drugs mediated by CYP catalyzed reactions. Moreover, ellipticine was also found to be an inhibitor of cytochromes P450, frequently used as a „selective" inhibitor of CYP1A1. Although ellipticine was reported to be a selective and strong inhibitor of CYP1A1, we found that its inhibitory potential is not specific.

Ellipticine binds with greater affinity to CYPs than most other compounds known to interact with CYPs. Ellipticine interacts with CYPs exhibiting a reverse type I binding spectrum (λmax 430 nm) in microsomes isolated from livers of uninduced rats and in those of rats pre-treated with β-naphthoflavone (β-NF), pregnenolon 16α-carbonitril (PCN), phenobarbital (PB) or ethanol with apparent dissociation constants (K_s) of 1.44, 2.38, 5.71, 16.63 and 2.26 μM, respectively. Five metabolites containing an oxygen atom in their molecules (M1-M5) are generated from ellipticine by CYPs; two of them were identified to be 9-hydroxy- and 7-hydroxyellipticines. Both these metabolites are predominantly generated by CYP1A1/2. The M5 metabolite was identified to be *N*(2)-oxide of ellipticine, while the structures of M2 and M3 metabolites have not yet been exactly characterized. All three ellipticine metabolites (M2, M3, M5) are mainly formed by CYP3A.

Control, β-NF-, PB-, ethanol- and PCN-induced microsomal CYP activities are inhibited by ellipticine. The degrees of CYP inhibition by ellipticine were quantified. 6β–hydroxylation of progesterone (K_i=0.021 μM) was most effectively inhibited by ellipticine, followed by CYP1A1/2-dependent EROD activity (K_i=0.038 μM) and CYP2B-mediated PROD activity (K_i=0.05 μM). Elliptine acts as a non-competitive or mixed-type inhibitor of these enzymes. Lower, but measurable, inhibition was detected for 1' hydroxylation of bufurarol, 21-hydroxylation of progesterone and 6-hydroxylation of chlorzoxazone catalyzed by CYP2D, CYP2C and CYP2E1, respectively.

The results indicate that the extent of inhibition by ellipticine might be explained by its differential potency to bind to individual CYP or its inhibition of another enzyme of the microsomal system, NADPH:CYP reductase. The mechanism responsible for CYP inhibition by ellipticine is discussed.

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TP07 NON-AROMATIC HYDROXYLATION OF BUPIVACAINE IN MAN

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Urine from patients receiving continuous epidural infusions of bupivacaine were investigated for evidence of non-aromatic hydroxylation. Urine extracts were analysed by chiral LC-MS (liquid chromatography with mass spectrometric detection) on a a1-glycoprotein column at pH 7 using a volatile buffer of hydroxylamine acetate in aqueous 2-propanol (3% v/v). (S)-Bupivacaine was more extensively metabolised than (R)-bupivacaine and dealkylation was the predominant pathway. However, at least 18 oxygenated metabolites of bupivacaine were detected by collision induced dissociation. Equal numbers of mono- and dihydroxybupivacaines were excreted of which there were equal numbers of aromatic and non-aromatic hydroxylated

metabolites. There was no evidence to suggest that any one of these was (S)-4'-hydroxybupivacaine, 2'-hydroxymethyl-bupivacaine, 3'-hydroxy-2',6'-pipecoloxylidide or a d-lactam. The metabolite previously identified as (S)-4'-hydroxybupivacaine is not hydroxylated on the aromatic ring. There was a wide inter-patient variation. The mean rate of excretion of (S)-3'-hydroxylbupivacaine relative to the (R)-desbutylbupivacaine excreted from three patients (1.76 0.48) was greater than for seven patients (0.19 0.09). Conversely, the rate of excretion of (S)-desbutylbupivacaine relative to (R)-desbutylbupivacaine from the group of three patients was 0.32 0.05 while for the group of seven it was 1.28 0.09. Studies are proceeding to identify the CYP isoforms responsible for the hydroxylations.

TP08 INHIBITION OF P450 ENZYMES PARTICIPATING IN P-NITROPHENOL HYDROXYLATION BY DRUGS KNOWN AS CYP2E1 INHIBITORS

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p-Nitrophenol hydroxylation is widely used as a probe for microsomal CYP2E1.[1] Several drugs are known as CYP2E1 inhibitors because of their capability to inhibit p-nitrophenol hydroxylation.[2] However, some of them did not reduce successfully other activities involving CYP2E1.

Our results suggest further participation of CYP2A6 and CYP2C19 enzymes in p-nitrophenol hydroxylation. Further aim of our study was to evaluate the selectivity of p-nitrophenol hydroxylase inhibitors towards P450 enzymes.[2] The effects of antifungals: bifonazole, econazole, clotrimazole, ketoconazole, miconazole; CNS-active drugs: chlorpromazine, desipramine, fluphenazine, thioridazine; and the non-steroidal anti-inflammatory drug: diclofenac were investigated on the enzyme activities selective for CYP2A6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4. None of the drugs could be considered as a potent inhibitor of CYP2E1 (Table). Strong inhibition was observed for CYP3A4 by antifungals with IC_{50} values in submicromolar range. However, ketoconazole was the only imidazole derivative that could be considered as a selective inhibitor of CYP3A4. The investigated CNS-active drugs were not found to be potent inhibitors of CYP2A6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4. Diclofenac efficiently inhibited CYP2C9 and to a less extent CYP3A4 enzyme.

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Table 1.: *IC_{50} values of the drugs towards selective enzyme activities.*
The incubations were carried out using human liver microsomes of three different donors. IC_{50} value is expressed as μM. Values represent mean + S.D.

	p-nitrophenol hydroxylation	Coumarin 7-hydroxylation **CYP2A6**	Tolbutamide 4-hydroxylation **CYP2C9**	(S)-mephenytoin 4'-hydroxylation **CYP2C19**	Chlorzoxazone 6-hydroxylation **CYP2E1**	Nifedipine oxidation **CYP3A4**
Bifonazole	**49.2**±44.04	**0.91**±0.27	**2.75**±1.71	**2.17**±1.36	**73.8**±21.32	**0.67**±0.42
Clotrimazole	**>250**	**11.25**±5.86	**5.91**±1.65	**73.2**±46.41	**>250**	**0.07**±0.03
Econazole	**166.7**±75.75	**180.8**±75.02	**5.03**±2.98	**0.84**±0.14	**129.4**±35.98	**0.41**±0.35
Miconazole	**>250**	**132.8**±110.86	**5.41**±3.52	**1.78**±0.84	**>250**	**2.04**±0.60
Ketoconazole	**>250**	**>250**	**52.1**±30.21	**122.1**±77.42	**>250**	**0.40**±0.16
Chlorpromazine	**>1000**	**535.7**±137.66	**797.3**±247.12	**338.2**±238.76	**~1000**	**798.1**±119.50
Desipramine	**~1000**	**830.3**±154.06	**>1000**	**546.1**±417.78	**719.9**±191.55	**339.7**±71.4
Fluphenazine	**>1000**	**477.4**±158.12	**398.1**±258.25	**528.3**±409.27	**735.2**±48.43	**441.2**±241.00
Thioridazine	**>1000**	**405.2**±45.48	**734.0**±119.52	**252.1**±181.75	**>1000**	**392.7**±25.96
Diclofenac	**>1000**	**>1000**	**69.7**±33.08	**>1000**	**>1000**	**698.4**±214.5

TP09 CYP19 ACTIVITY MODULATION: SMALL CHANGE OF FLAVONOID STRUCTURE RESULTS IN INHIBITOR-STIMULATOR TRANSITION

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An inhibitory capacity of flavonoids with respect to CYP activities has been extensively studied because of their potential use as agents blocking the initiation of carcinogenesis. Synthetic and naturally occurring flavonoids are effective inhibitors of at least four CYPs metabolizing xenobiotics: CYP1A1, 1A2, 1B1 and 3A4. On contrary, certain metabolic activities of CYP3A4 and 1A2 were also stimulated by several compounds of flavonoid structure. For example, synthetic 7,8-benzoflavone (ANF) is an inhibitor of human CYP1A1 and 1A2, but an activator of CYP3A4. CYP19, aromatase, is another member of cytochrome P450 enzyme super-family that is effectively inhibited by flavonoids since these compounds resemble the skeleton of steroids. This enzyme catalyzes a unique reaction, aromatization of the A ring of male sex steroids resulting in estrogens. Since estrogens are known cell proliferators and their metabolites, catechols, are carcinogens, a local expression of aromatase is suggested to be closely connected with tumor initiation, promotion and progression. Hence, aromatase inhibitors are used as pharmaceuticals in the prevention and/or treatment of especially hormone-dependent breast and prostate cancers. The most effective aromatase inhibitor of flavonoid structure are ANF and its 9-hydroxy-derivative, showing IC50 values comparable to a strong steroid aromatase inhibitor, 4-hydroxyandrostendione. The presence of C4 oxo-group of the flavonoid skeleton, which is likely approaching the heme iron (based on models), seems to be crucial for inhibition. One can expect that the substitution of oxygen for sulphur in this position will result in a stronger binding of the compound to aromatase increasing its inhibition capability. Surprisingly, a thio-analogue of ANF (SANF) formation of estrone from androstendione (160% of control) in human placental microsomes. The same stimulatory effect of SANF on CYP19 was determined with its non-physiological substrates, 7-ethoxycoumarin and dibenzylfluoresceine, in microsomes from baculovirus-infected insect cells containing co-expressed human CYP19 and NADPH:cytochrome P450 reductase (150% of control). In addition, in human placental microsomes almost 200% stimulation of dibenzylfluoresceine metabolism was detected. To our knowledge it is the first evidence for the stimulation of the enzymatic activity of a steroidogenic CYP19. Thus, flavonoids tested in this study can exert a rather complex effect on CYPs, either inhibit or activate human cytochromes P450 depending upon their structures, concentrations, and experimental conditions.

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TP10 POTENT MECHANISM-BASED INHIBITION OF HUMAN CYP2B6 BY CLOPIDOGREL AND TICLOPIDINE

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Thrombolytic drugs are widely used in patients with coronary heart disease (CHD). These include the thienopyridine derivates ticlopidine and clopidogrel, which are inhibitors of ADP-induced platelet aggregation. Since clopidogrel shows a more rapid onset of action and a lower incidence of adverse effects compared to ticlopidine, it is more frequently used today[1]. Clopidogrel is metabolized to its antithrombotic agent by CYP3A4[2] and a recently published clinical study demonstrated an inhibitory effect of the CYP3A4 substrate atorvastatin on platelet aggregation[3]. The aim of this study was to investigate the interaction potential of clopidogrel towards cytochrome P450 enzymes. Using human liver microsomes and recombinantly expressed enzymes we found that both clopidogrel and ticlopidine strongly inhibit CYP2B6 in a time-and concentration-dependent manner with IC50 values of 0.8 μM and 1.1 μM, respectively. As shown by dialysis experiments, inhibition was irreversible and dependent on the presence of NADPH, strongly suggesting a mechanism-based mode of action for both clopidogrel and ticlopidine. A high specifity of both substances for CYP2B6 was demonstrated by analyzing enzyme activities selective for other human liver enzymes including CYP1A2, CYP2E1, CYP2A6, CYP3A4, CYP2C8, CYP2C9 and CYP2C19, although the latter isozyme was also inhibited to some extent, as has been reported in an earlier study[4]. These results suggest the possibility of drug interactions between these two drugs and other drug-substrates of CYP2B6. Clopidogrel in particular may prove to be a useful *in-vitro* inhibitor probe for CYP2B6.

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TP11 CYP17 CATALYTIC IRON-OXYGEN SPECIES DICTATED BY CYTOCHROME B5

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Microsomal cytochrome P450 17α-hydroxylase-17,20-lyase (CYP17) has the ability to catalyse 17α-hydroxylation and acyl-carbon bond cleavage of the C_{21} progestogens. We have proposed that the hydroxylation reaction is catalysed by a heam-iron-monooxygen radical species whereas the cleavage process by a heam-iron-peroxide anion[1]. However, for the human isoform of CYP17 to catalyse the cleavage reaction a third protein, cytochrome b_5 (b_5), is required[2,3]. It is proposed that b_5 promotes a conformational change in CYP17 which culminates in the optimal juxtaposition of the haem-iron-peroxide anion with respect to the C-20 carbonyl of the substrate[2]. The ensuing nucleophilic attack at the carbonyl group then produces a tetrahedral intermediate that follows a side chain cleavage path. Using the X-ray structural information for $P450_{BM-3}$[4] and linear amino acid sequence alignments, the most likely docking site on CYP17 for b_5 was identified. Surface-exposed cationic residues within this putative b_5 docking site, that are proposed to form electrostatic interactions with anionic charges on b_5, were mutated to remove their charge and the expressed mutant proteins purified. The mutant CYP17 enzymes were subjected to kinetic analysis, utilizing the novel properties of three different substrates to probe b_5-CYP17 interaction. The results are presented and the determined metabolic profile is consistent with our proposal. Part of the docking interface between CYP17 and b_5 is putatively mapped onto the surface of a computer generated 3D homology model of CYP17.

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TP12 FARADAIC BIOELECTROCHEMISTRY FOR THE DRUG METABOLISM ASSAY

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Cytochromes P450 (CYPs) catalyze the oxidative metabolims of a wide variety of exogenous and endogenous chemicals such as drugs and carcinogens. Potential applications range from the use of cytochromes as drug targets to the use of these enzymes as tool to predict drug-drug interactions in humans. The industrial application of cytochrome P450-containing systems is tightly bound with the usage of alternative electron sources for P450s' reduction. Cytochrome P450-based electrochemical devices allowed to construct sensor systems for screening a wide variety of drugs on the possibility of drug-drug interactions via CYP, namely high throughput screening. Thick film screen-printed graphite electrodes served as working electrodes. Immobilized flavocytochromes and cytochromes P450 layers were prepared by cross-linking of enzyme with glutaric aldehyde or with entrapment in the [3-(2,3-epoxypropoxy)-propyl]-trimethoxysilane matrix. Sol-gel matrices were effectively used for the entrapment of CYPs. Voltammetric techniques were used to investigate the kinetics of P450 redox reactions. Kinetic parameters of redox system were calculated with algebraic transformation of the Michelis-Menten equation known as the Hill equation and with the electrochemical Lineweaver-Burk plots. The catalytic activity of electrochemical systems based on CYP 2B4, CYP 1A2 and CYP11A1 (P450scc) were characterized with the help of kinetic parameters of enzyme electrodes, such as apparent Michaelis-Menten constant, catalytic constant, reaction rates (K_m^{app}, k_{cat}, V_{max}). The ratio of the catalytic current to the diffusion controlled current $I_{cat}/I_{d\ was}$ has been taken as a measure of catalytic efficiency of the electrochemical system. From the integration of peaks in the cyclic voltammograms the apparent surface coverage of the electroactive cytochrome P4502B4 and P450scc was calculated. With the help of electrochemical artificial P450-based devices it is possible to analyse potential substrates or inhibi-

tors of P450 investigated. The catalytic current I_{cat} of the enzyme electrode changes with the concentration of substrate. Thus I_{cat} and steady state current response of P450-electrodes upon substrates addition could be used to measure drug (substrate) concentration. The inhibitory effects of some chemicals on the catalytic current of cytochrome P450-based bioelectrodes can be estimated. Enzyme electrodes described provide approach for the fabrication of specific, portable and low cost disposable cytochrome P450-based amperometric or potentiometric biosensors.

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TP13 STRUCTURAL AND FUNCTIONAL CHARACTERIZATIONS OF PUTIDAREDOXIN-INDUCED STRUCTURAL CHANGES IN CYTOCHROME P450CAM

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Cytochrome P450cam (P450cam) catalyzes hydroxylation of *d*-camphor by using two electrons from its redox partner, putidaredoxin (Pdx). In addition to being an electron shuttle to P450cam, Pdx acts as a conformational effector of P450cam, which is believed to be crucial for the enzymatic activity of P450cam. However, the detailed mechanism of the Pdx-induced structural changes in P450cam is not clear owing to the lack of the crystal structure of the P450cam-Pdx complex. Here, to characterize the Pdx-induced structural changes in P450cam and their functional role in the catalytic cycle, we utilized the Leu358→Pro (L358P) mutant[1] as a model for Pdx bound P450cam.

Even in the *absence* of Pdx, the ^{1}H NMR signals from the β-proton of the axial Cys and the substrate, *d*-camphor, were up-field shifted for carbonmonoxy L358P, as observed for wild type P450cam in the *presence* of Pdx[2], showing that both *d*-camphor and the axial Cys move toward to the heme iron in L358P. The structure of L358P in the *absence* of Pdx, therefore, corresponds to that of wild type P450cam in the P450cam-Pdx complex, which enables us to gain some insights into the Pdx-induced structural changes in P450cam without the crystallization of the P450cam-Pdx complex. The X-ray crystal structure of carbonmonoxy L358P showed the tilting of the heme plane, leading to the positional change of Arg112 at the putative Pdx binding site, through the hydrogen bond between Arg112 and the heme propionate. We can conclude that the binding of Pdx to P450cam induces the tilting of the heme plane by perturbing the hydrogen bond between the heme propionate and Arg112, resulting in the approach of *d*-camphor toward the heme iron. In order to clarify the functional significance of the Pdx-induced structural changes in P450cam, we also investigated the reactivities of L358P with electron donors. Because the Pdx-induced structural changes in P450cam are crucial for the electron transfer reaction, we hypothesize that L358P would exhibit enhanced reactivities with the non-physiological electron donors that cannot donate the electron to the oxygen adduct of wild type P450cam but have the lower redox potential than Pdx. As we expected, the mixing of oxy-L358P with the non-physiological electron donor such as dithionite in the presence of *d*-camphor produced significant amounts of the hydroxylation product, 5-*exo*-hydroxycamphor. Considering the lowered redox potential of L358P[1], we, therefore, propose that the Pdx-induced structural changes in P450cam would facilitate the electron transfer to oxy-P450cam by regulating the electronic coupling and/or reorganization energy.

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TP14 CHIRAL ASPECTS OF REDUCTION METABOLISM OF FLOBUFEN IN GUINEA PIGS IN VIVO

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Metabolic transformation of flobufen [4-(2',4'-difluoro-biphenyl-4-yl)-2-methyl-4-oxobutanoic acid], a chiral non-steroidal antiinflammatory agent[1], includes its conversion to a reduced metabolite[2]. Reduced flobufen [4-(2',4-difluoro-biphenyl-4-yl)-4-hydroxy-2-methylbutanoic acid] is the major metabolite generated by the subcellular fractions. It was detected upon incubation with liver microsomes from several animal species. Maximum yield of reduced flobufen (dihydroflobufen) was found after anaerobic incubation with NADPH. This finding combined with the knowledge of subcellular distribution of enzymes suggested that metabolite formation depends on the activity of microsomal reductase, cytochrome P-450[2]. Flobufen possesses an α chiral carbon atom next to the carboxyl group and its enantioselective bioelimination has been proved in rats[3]. Reduction of flobufen resulted in another center of chirality in the molecule and four stereoisomeric dihydrometabolites can be formed from two parent flobufen enantiomers[4]. We studied the metabolism of flobufen to its reduced metabolite in male guinea pigs *in vivo*. Especially, chiral aspects of the reduction metabolic process were analyzed. Flobufen was administered as *rac*-flobufen (R/S-enantiomer = 50/50) p.o. in a dose of 10 mg/kg. The drug was metabolized in a significant rate in the guinea pig. All four stereoisomers of dihydrometabolite of flobufen were detected in guinea pig plasma. However, the found concentrations were very different. The highest levels in plasma showed (2*R*;4*R*)-dihydroflobufen, the lowest levels were found in case of (2*S*;4R)-dihydroflobufen. The concentration ratio of these two circulating diastereoisomers in guinea pig plasma reached up to more than one order of magnitude. The main product of biotransformation of flobufen found in guinea pig plasma was nonchiral metabolite 17203 [4-(2,4-difluorophenyl)-phenylacetic acid]. The metabolite reached plasma levels comparable to parent flobufen enantiomers. However, metabolite 17203 is formed from reduced flobufen[2]. It means that the stereospecific reduction of flobufen by microsomal reductases seems to be the key step in flobufen metabolism in guinea pigs.

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TP15 MULTIPLE P450 SUBSTRATES IN SINGLE RUN: RAPID, COMPREHENSIVE AND CLINICALLY RELEVANT IN VITRO METABOLISM TEST

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The aim of this study was to develop a global *in vitro* screening test utilizing N-in-1 approach, wihich could answer the following questions: what is metabolic stability of a new chemical entity (NCE) studied, which enzyme(s) metabolise NCE and which enzyme(s) is/are target(s) for further interaction studies? By using appropriate concentrations and incubations this test should reveal affinities of NCE for different enzymes, kinetic properties of NCE (Km, Vmax) for each metabolising enzyme and prediction of in vivo behaviour.

A cocktail consisting of ten CYP-specific probes: melatonin (CYP1A2), coumarin (2A6), bupropion (2B6), amodiaquine (2C8), tolbutamide (2C9), omeprazole (2C19), dextromethorphan (2D6), chlorzoxazone (2E1), midazolam and testosterone (3A4) was incubated in a pool of human liver microsomes in the presence of NADPH. Analysis of samples was performed using LC/TOF-MS (total analysis time 20 minutes, 0.1%formic acid-methanol gradient, flow rate 0.25 ml/min). No post-incubation extraction or concentration procedures were needed.

Validation of the method has been performed with diagnostic CYP-specific inhibitors: fluvoxamine (CYP1A2), tranylcypromine (2A6), ticlopidine (2B6), quercetin (2C8), sulphaphenazole (2C9), fluconazole (2C19), quinidine (2D6), pyridine (2E1) and ketoconazole (3A4). The results were in good accordance with literature and our previous studies.

In addition, with liver microsomes this test has been succesfully applied to cDNA-expressed CYP enzymes,

mouse hepatocytes and permanent cell lines. Validation of the method with NCEs with known metabolic profiles is under preparation.

TP16 IMMOBILISATION OF P450 BM-3 AND AN NADP+ COFACTOR RECYCLING SYSTEM: TOWARDS A TECHNICAL APPLICATION

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P450 BM-3 (CYP102), which was originally cloned from *Bacillus megaterium*, is a 119 kDa natural fusion protein composed of a heme-containing monooxygenase domain and an FAD- and FMN-containing reductase domain[1,2]. The turnover frequencies determined for P450 BM-3 (>1000 min^{-1}) are, to date, among the highest reported for a P450 monooxygenase. The natural substrates of P450 BM-3 are long chain fatty acids (C_{12} to C_{20}). Artificial mutants of P450 BM-3 also hydroxylate non-natural substrates like short-chain fatty acids (C_8 to $C1_0$)[3], indole, polycyclic aromatic hydrocarbons and alkanes[4]. These hydroxylation reactions can lead to regio- and/or stereochemically pure compounds. As regio- and stereospecific oxidation of aliphatic carbons is a reaction, which is difficult to perform by means of classical organic synthesis, the biocatalysis by this monooxygenase is a promising option, especially with regard to fine chemistry. Key problems preventing the use of P450s in biotechnology are relative low stability and the supply of electrons to the heme iron. *In vivo*, the electrons are supplied by NAD(P)H, which is however far too expensive to be used in technical applications. To render P450 BM-3 more suitable for industrial biocatalysis, the immobilisation of P450 BM-3 (CYP 102) from *Bacillus megaterium* in a sol-gel matrix was combined with a cofactor recycling system based on $NADP^+$-dependent formate dehydrogenase (EC 1.2.1.2) from *Pseudomonas sp.* 101 and tested for practical applicability[5].

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TP17 HETEROLOGOUS EXPRESSION OF FULL-LENGTH NADH-CYTOCHROME B5 REDUCTASE AND ITS FUSION PROTEIN WITH CYTOCHROME B5

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NADH-cytochrome b_5 reductase is essential human liver and intestine enzyme, which involved in cytochrome b_5 reduction and participates in metabolism of a large number of chemicals of different structure, functions and size. The enzyme exists in two forms: a membrane-bound form that is comprised of both a hydrophobic membrane-anchoring region and a hydrophilic, or catalytic domain, and a soluble form which contains only a hydrophilic domain. Recently, soluble NADH-cytochrome b_5 reductase has been expressed in *E. coli*, purified and intensively studied. However, the role of hydrophobic N-terminal sequence of full-length NADH-cytochrome b_5 reductase, that anchors flavoprotein in the membrane, remains unknown.

The aim of the present work is to develop efficient expression system for full-length NADH-cytochrome b_5 reductase. To solve this problem we constructed several plasmids containing cDNA for full-length NADH-cytochrome b_5 reductase under *Tac* and *T7* promotors. We also introduced the His-tag fragment to the C-terminal sequence of flavoprotein to improve purification procedure. The expression level of NADH-cytochrome b_5 reductase under *T7* promotor proved to be significantly higher than that of under *Tac* promotor. The effective purification procedure for recombinant NADH-cytochrome b_5 reductase based on using immobilized recombinant cytochrome b_5 has been developed.

We also constructed, expressed and purified fusion protein between full-length NADH-cytochrome b_5 reductase and cytochrome b_5 and showed that fusion protein contains both heme and FAD prosthetic groups and able to intra-molecular electron transfer.

TP18 OVEREXPRESSION OF FULL-LENGTH OUTER MITOCHONDRIAL CYTOCHROME B5 IN *E. COLI*

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Outer mitochondrial membrane-bound cytochrome b_5, the isoform of microsomal cytochrome b_5, is a typical tail-anchored hemeprotein of outer mitochondrial membrane. Both mitochondrial and microsomal cytochromes b_5 have similar organization. They have hydrophilic N-terminal domain, which preserves spectral properties of cytochrome b_5 and C-terminal hydrophobic domain, which is responsible for interaction with membrane and functions as reporter for insertion hemeprotein to membrane as a tailed-anchored protein. The hydrophilic N-terminal domain of cytochrome b_5 consists of about 100 amino acids, contains heme and participates in electron transfer reactions. The N-terminal sequence of outer mitochondrial cytochrome b_5 contains extra 10 amino acids the role of which is unknown. Hydrophilic sequences of cytochrome b_5 and outer mitochondrial isoform of hemeprotein show 70% of identity. The hydrophobic C-terminal domain of cytochrome b_5 contains three subdomains: a. hydrophilic linker; b. intrinsic hydrophobic sequence (about 20 amino acids), which is embedded to the lipid bilayer; c. hydrophilic tail which contains information on protein translocation. The role of hydrophobic domain of outer mitochondrial cytochrome b_5 remains unknown.

The aim of the present work is to develop an efficient expression system for full-length outer mitochondrial cytochrome b_5 and to study the functional role of the hydrophobic C-terminal sequence. Surprisingly, our efficient expression system developed previously for microsomal cytochrome b_5 based on pCWori$^+$ vector and *Tac* promotor, did not result in expression of outer mitochondrial cytochrome b_5. Removal of extra N-terminal amino acid residues did not improve the expression. However, cloning the cDNA coding outer mitochondrial cytochrome b_5 under *T7* promoter (vector pT7) resulted in over expression of hemeprotein in some cases up to 6000 nmoles per l of culture. Recombinant full-length outer mitochondrial cytochrome b_5 and its N-terminal truncated forms have been expressed in *E. coli*, purified to apparent homogeneity using metal-affinity chromatography giving about 1.5-3.0 (mole of homogeneous full-length outer mitochondrial cytochrome b_5 which was used to assess the effect on cytochrome P4503A4 and P45017*a* catalyzed reactions.

TP19 ENGINEERING OF PROTEOLYTICALLY STABLE NADPH-CYTOCHROME P450 REDUCTASE

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NADPH-cytochrome P450 reductase (NCPR) contains hydrophobic N-terminal membrane-binding and hydrophilic catalytic domains. Truncated (trypsin-treated reductase), which does not contain the hydrophobic N-terminal fragment, is still able to transfer electrons to cytochrome ***c*** and other artificial electron acceptor, but is no longer capable to interact with cytochrome P450 and transfer electrons to it. The preferential site of tryptic attack on the rat reductase was shown to be at the Lys^{56}-Ile^{57} while the second recognition site for trypsin is Lys^{46}-Lys^{47}-Lys^{4}8 sequence that yields a small polypeptide (Lys^{46} or Lys^{47} through Lys^{56}). Moreover, Lys^{56}-Ile^{57} is a site for spontaneous digestion of CPR by intercellular trypsin-like proteases, which makes flavoprotein very unstable during purification or expression in *E. coli*. The aim of this study is to evaluate the effect of a single amino acid substitution at position Lys^{56} in attempt to create trypsin-insensitive flavoprotein. For that we replaced Lys^{56} for Asn, expressed and purified recombinant flavoprotein. Studies of kinetic mechanism of NCPR mutant showed no significant differences between

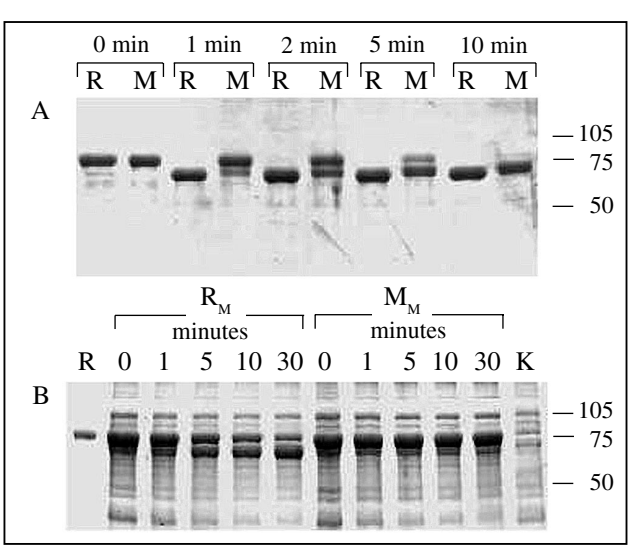

Fig. 1. *SDS-PAGE trypsin-treated purified (A) and membrane-bound reductases (B). Panel A: purified wild type (R) and mutant (M) reductase. Panel B: membranes prepared from E. coli expressing the wild type (R_M) and mutant form (M_M) reductases.*

wild type protein and Lys56Asn mutant. At the same time, replacement of Lys^{56} makes the full-length flavoprotein stable to spontaneous proteolysis. During prolong incubation or increased trypsin ratio, mutant form has the other limited proteolysis pattern, indicating the accessibility of the second site. The membrane-bound mutant form is absolutely stable to trypsinolysis. These results let us to conclude that the polypeptide fragments Lys^{46}-Lys^{48} is localized in membrane. Truncated form of mutant flavoprotein (AA's 46-676 of NCPR) unable transfer electrons to cytochrome P450c17.

TP20 C-TERMINAL RESIDUES OF CYTOCHROME P450SCC ARE REQUIRED FOR CORRECT FOLDING, HEME BINDING AND CATALYSIS

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To understand the functional role of C-terminal part of mitochondrial cytochrome P450scc (P450scc) in folding and heme binding, we used sequential amino acid deletion strategy. A set of mutants lacking 2, 4, 7, 9 and 10 amino acids from C-terminus of bovine P450scc were constructed, expressed and purified to compare with wild type protein. Removal of 10 amino acids from the C-terminus of P450scc results in complete disappearence of spectrally detected P450 in *E. coli* culture as well as in the cell solubilizate. However, immunoblotting analysis indicates the presence of apo-protein in cells. Alignment of P450's from different sources indicates that Arg^{472} might be the last residue in the protein sequence which is critical for P450scc expression in catalytically active holo-form. In contrast, protein shortened for 9 amino acids is fully competent to support electron transfer in reconstitution system with adrenodoxin and adrenodoxin reductase. Cytochrome P450scc lacking 9 amino acids is expressed in *E. coli* rather poorly, but could be affinity-purified as enzymatically active protein.

The expression level for mutants with deletion of 2 and 4 (nonconservative amino acids among mitochondrial P450's) is significantly higher as compared to the wild type protein and these mutants reveal activity towards cholesterol side chain cleavage reaction and suitable for functional and structural analysis. Deletion of seven amino acids yields a hemeprotein with properties similar to full-length P450scc. Kinetic and pattern of P450scc trypsinolysis as well as folding were not changed by C-terminal deletion. Thus, C-terminal truncation analysis showed that this region of P450scc (9aa) is not critical for enzyme activity, while removal 10 amino acids dramatically affects hemeprotein folding and heme binding. The role of C-terminal region in oligomerization/aggregation of P450scc is currently under investigation.

TP21 SPECTROSCOPIC STUDIES OF CYTOCHROME P4503A4 INTERACTION WITH FLAVONOIDS

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Flavonoids - low molecular weight phenols that are found ubiquitously in the vascular plants. They have pronounced antioxidant properties and ability to influence on

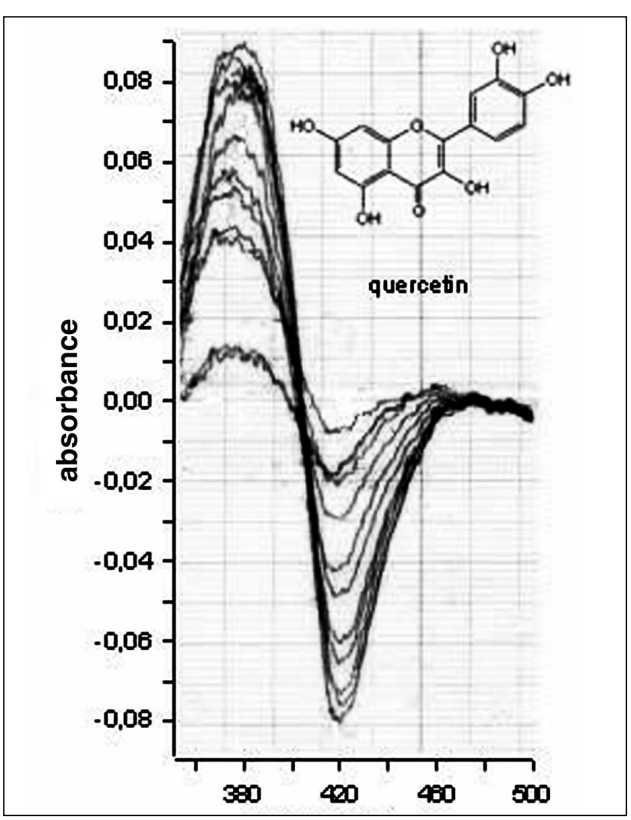

Fig. 1. *Titration curve of cytochrome P450 3A4 with quercetin.*

variety of enzymes and cell receptors. Among all flavonoids, quercetin is belived to be the most consumed flavonoid. It is known as one of the most potent natural antioxidant and effective metal chelator. Quercetin inhibits activities of calcium phospholipid-dependent protein kinase, phosphoinositol 3-kinase and DNA-topoisomerase, phospholipase A2. Currently there is growing evidence for the inhibitory effect of some flavonoids including quercetin on activity of cytochrome P4503A4. Cytochrome P4503A4 is the major P450 isoenzyme in human liver and intestine, which is involved in oxidations of a variety of xenobiotics and chemicals.

The aim of present study is to elucidate weather flavonoids like quercetine directly interact with the active site of cytochrome P450 and affect the reactions catalyzed by this hemeprotein. To answer this question we expressed in *E. coli* cytochrome P4503A4 as a His-tag derivative, purified recombinant hemeprotein using metal-affinity chromatography and used it in spectral binding studies.

The results of the spectral binding experiments indicate that quercetin is able to interact with cytochrome P4503A4 and induce the typical type I spectral changes with absorbance maximum at 390 nm and minimum at 420 nm, indicating the change of the spin state of cytochrome P4503A4. The A_{max} and K_s values calculated by nonlinear regression of the plot ΔA versus S using Origin 5.0 software were found to be 0,121 and 60 μM, respectively. The interaction of cytochrome P4503A4 with other types of flavonoids and their effects on cytochrome P4503A4 catalyzed reaction is underway.

TP22 HUMAN CYTOCHROME P450 2D6-CATALYZED REACTIONS: THE KINETIC INTERACTION BETWEEN TWO SUBSTRATES

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More than 80 drugs in clinical use are metabolized by polymorphic CYP2D6. It is important to evaluate whether new chemical entities are potent inhibitors of this CYP isoform. Bufuralol and dextromethorphan have been used as *in vitro* probes for inhibition studies. Recently, two fluorescent probes, AMMC and MAMC, were reported to be good CYP2D6 substrates which can be used in a high-throughput screening format. In order to investigate *in vitro* substrate-substrate interactions with CYP2D6, these four compounds were studied in pairs using recombinant CYP2D6 and human liver microsomes. For all studies, the IC_{50} values were determined using the substrate concentration equal to the apparent K_m value. In recombinant CYP2D6, AMMC was found to be a good inhibitor of bufuralol 1'-hydroxylation and dextromethorphan *O*-demethylation with IC_{50} values of 2.3 and 2.2 μM, respectively. Only moderate inhibition was observed by MAMC with IC50 values of 33 and 21 μM, respectively. The interaction between bufuralol and dextromethorphan was confirmed to be competitive with Ki = 1.2 μM for dextromethorphan on bufuralol 1'-hydroxylation and with Ki = 5 μM for bufuralol on dextromethorphan *O*-demethylation. However, in all cases, the inhibitory potencies were decreased when human liver microsomes were used in the study. Possible reasons explaining the apparent discrepancy between the two enzyme systems will be discussed.

AMMC: 3-[2-(N,N-diethyl-N-methylammonium)-ethyl]-7--methoxy-4-methylcoumarin
MAMC: 7-methoxy-4-(aminomethyl)-coumarin

TP23 REDUCTION OF OXYFERROUS CYTOCHROME P450 2B4 BY CYTOCHROME P450 REDUCTASE

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The rate of electron transfer from cytochrome P450 reductase to oxyferrous cytochrome P450 2B4 (cyt P450), i.e., donation of the second electron, has been measured experimentally by using stopped-flow spectrophotometry and a cyt P450 reductase containing a redox inactive FAD analogue. This FAD analogue-containing cyt P450 reductase can be 2 electron reduced to the FMN hydroquinone by dithionite. The $FMNH_2$ oxidizes monophasically in the presence of O_2. Cyt c oxidizes both the native reductase and the reductase reconstituted with the FAD analogue with the same second order rate constants indicating that the function of the FMN domain of the reductase is intact. Since the reductase reconstituted with a FAD analogue donates a single electron from $FMNH_2$, the spectral changes it undergoes when it oxidizes to the semiquinone are easily interpreted. This contrasts with what is observed in a 2e reduced FAD-containing cyt P450 reductase where the second electron is distributed between the FMN hydroquinone and FAD semiquinone according to their respective potentials. In this instance the spectral changes occurring on oxidation are complex and cannot be unambiguously interpreted. Ferrous cyt P450 2B4 and the 2 electron reduced

reconstituted cyt P450 reductase were premixed in one syringe of a stopped-flow spectrophometer and rapidly mixed with O_2 in a second syringe. Oxyferrous cyt P450 forms within 20 msec and is subsequently rapidly reduced by the FMNH2 of the reductase. Cyt P450 returns to the Fe^{+3} state with first order rate constants of 0.09 and $0.012s^{-1}$. Interestingly, the reductase oxidizes more quickly than cyt P450. In a parallel experiment with Fe^{+2} cyt b_5 the oxyferrous cyt P450 turned over and returned to the Fe^{+3} state with rate constants of 6.5 and $0.1s^{-1}$. When benzphetamine was the substrate, the reaction was 53% coupled in the presence of cyt b_5 and 33% coupled with the reductase.

TP24 HUMAN HEPATIC OXIDATION OF DOTHIEPIN MEDIATED BY CYP2D6

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Dothiepin, 3-(6H-dibenzo[*b,e*]thiepin-11-ylidene)propyldimethyl amine, is a tricyclic antidepressant, which is composed of predominantly the E-isomer (95%) and a minor amount of the Z-isomer (5%). Human *in vivo* metabolism of dothiepin was reported to involve the formation of nordothiepin, *S*-oxide, nordothiepin-*S*-oxide, and a *N*-glucuronide. The current study was undertaken to 1) further investigate the oxidative metabolism of dothiepin *in vitro*, and 2) to characterize the human hepatic CYP isoforms involved in the metabolism and the associated kinetic characteristics. Pooled human liver microsomes as well as individual cDNA-expressed human CYP isoforms were incubated with dothiepin at various concentration levels. The metabolites formed were quantitated by HPLC with UV detection, and structurally characterized by mass spectrometry and comparison to synthetic standards. The kinetic parameters were estimated using the Michaelis-Menten equation. Microsomal incubations produced an *S*-oxide and an *N*-desmethyl metabolite as the major products (*Fig. 1*). Minor products included an *N*-oxide, which is a newly identified metabolite, a monohydroxylation product, and a double *N*-demethylation product. Kinetic analysis indicated that the *S*-oxidation involved a high affinity component with a K_m of 1.7 μM and CL_{int} of 26 μL/mg/min, and a low affinity component with a K_m of 100 μM and CL_{int} of 1.9 μL/mg/min. Screening with cDNA-expressed CYP isoforms indicated the involvement of CYPs 2D6, 3A4, and 1A2 in dothiepin metabolism. Kinetic analysis indicated CYP2D6 as the high affinity component with a K_m of 2.0 μM and CL_{int} of 3.2 μL/pmol/min, and CYPs 3A4 and 1A2 likely the low affinity components with K_m of 192 and 68 μM, and CLint of 0.03 and 0.003 μL/pmol/min, respectively. The study suggests that CYP2D6 is likely the major isoform responsible for the hepatic *S*-oxidation of dothiepin in human.

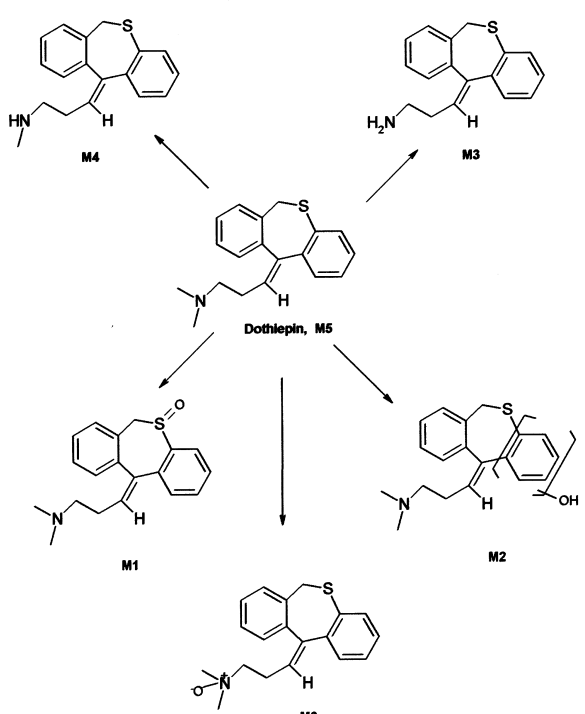

Fig. 1.

TP25 SELECTIVE DEHYDROGENATED INTERMEDIATES ARE MECHANISM-BASED INACTIVATORS OF CYP3A4, CYP2E1, AND CYP2F1

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P450-Mediated dehydrogenation of many substrates leads to the production of electrophilic intermediates, such as quinones, quinone methides, and imines. Some of these electrophiles are long-lived enough to be released from the catalytic site and initiate toxicity. Examples are acetaminophen, equine estrogens, troglitazone, 3-methylindole, valproic acid, and N-methylformamide. Conversely, several of the dehydrogenated intermediates are reactive enough to alkylate active-site nucleophilic residues of the P450 proteins, producing inactive enzymes (phencyclidine

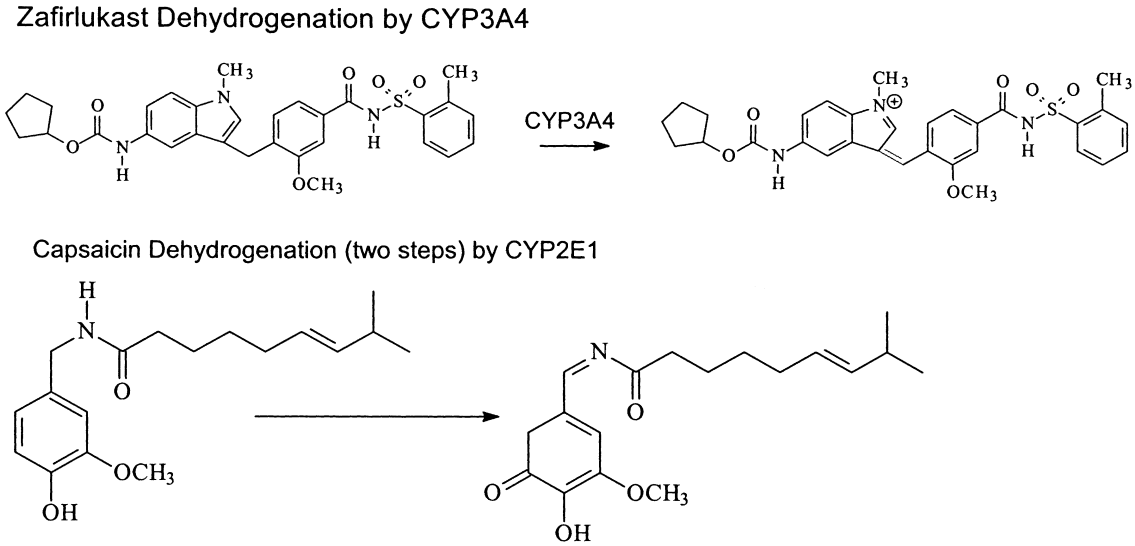

Fig. 1.

- CYP2B6, furafylline - CYP1A2, hydroxytamoxifen - CYP2B6). We have initiated an examination of the catalytic mechanisms that control enzyme selectivity in the production of dehydrogenated intermediates, and the propensity of these electrophiles to produce mechanism-based inactivation. Substrates and enzymes used for these studies were zafirlukast (CYP3A4), capsaicin (CYP2E1), and 3-methylindole (CYP2F1), see *Figure 1*. These substrates were oxidized by their respective enzymes to dehydrogenated imines which were characterized by trapping the electrophiles with N-acetylcysteine or glutathione, followed by mass spectral and NMR structural identification. In all three cases the dehydrogenated imines were major products, and were selectively produced by the enzyme, e.g. the zafirlukast dehydrogenated intermediate was not produced by CYP2E1 and the 3-methylindole dehydrogenated intermediate was not made by CYP3A4 or CYP2E1. In addition to catalyzing the selective formation of the dehydrogenated products, each enzyme was inactivated in a time- and concentration-dependent manner by its respective substrate. Again, selectivity was observed for the mechanism-based inactivation events, i.e. CYP3A4 was not inactivated by capsaicin or 3-methylindole, and CYP2E1 was not inactivated by zafirlukast or 3-methylindole. Thus, these studies demonstrated that the selective dehydrogenation of these diverse chemicals is catalyzed by certain enzymes, and the dehydrogenated electrophiles are potent mechanism-based inactivators of the unsuspecting enzymes. This research was supported by USPHS grants (HL60143 and HL13645) and by the National Institute of Standards and Technology (Contract# 60NANBOD0006).

TP26 HUMAN AND MINIPIG CYTOCHROME P450 2E1

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The search for a good model of drug metabolism in man has led to intensive studies on CYP systems in other species[1]. The pig (or minipig) liver microsomal fraction as well as the partially purified proteins has been shown to exhibit enzyme activities characteristic of human CYP enzymes[2].

The minipig cytochrome P450 form 2E1 has been recently purified to homogeneity and its primary structure has been obtained[3]. A detailed comparison of the properties of this enzyme with those of the human CYP2E1 is presented here. The minipig protein is well recognized by the antibodies against human CYP2E1. This contribution also shows the results of determination of both the prototypic CYP2E1 activities i.e. the chlorzoxazone 6-hydroxylating and the p-nitrophenol hydroxylating properties determined

in the microsomal fractions of minipig and human origin as well as in the respective reconstituted systems. Inhibition studies with diethyldithiocarbamate complement the study.

Both the chlorzoxazone 6-hydroxylating activity and the p-nitrophenol hydroxylating one has been determined using the methods described in literature[4,5]. The protein itself has been isolated according to procedure published earlier[2,3]. Enzyme kinetics has been analyzed using a LSW Data Analysis software (www.lsw.com). The results obtained show conclusively that the minipig cytochrome P450 2E1 is able to catalyze both reactions exhibiting the activity well comparable to this of the human enzyme. Diethyldithiocarbamate, a specific CYP2E1 inhibitor, has been also shown to be functional in minipig reconstituted system inhibiting its activity to 10% and less. Hence, the minipig enzyme shows the characteristics typical of a CYP2E1 enzyme.

Acknowledgment

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TP27 TWO NOVEL HUMAN P450 ENZYMES, CYP4Z1 AND CYP4X1

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The CYP4 family was originally discovered as the lauric acid omega-hydroxylase, which was induced in rat liver by peroxisome proliferators[1,2]. The cloning of members of the CYP4B and CYP4F families has revealed that there are additionally fatty acid substrates for the CYP4 family[2-5]. The CYP4 family has a potentially important role in metabolism of endogenous fatty compounds.

We prepared an extensive alignment of members of the CYP4 family using the program SAGA; this alignment included both vertebrate and invertebrate P450 proteins. The alignment showed unexpectedly high sequence similarity in the region of the C-helix for members of the CYP4 family. In order to test the significance of this finding, we used the sequence of CYP4A1 in the conserved region to search DNA databases. The conserved sequence detected exclusively members of the CYP4 family, and two additional partial EST sequences.

Subsequent cloning of the two novel gene sequences was achieved by RACE-PCR, and yielded full-length sequences for CYP4Z1 and CYP4X1. These data are consistent with subsequent human genomic sequences. The sequence of CYP4Z1 and CYP4X1 predict functional cytochromes P450. Antisera were raised against recombinant fragments of CYP4X1 and CYP4Z1. These antisera are characterised. The tissue-specific distribution of CYP4Z1 and CYP4X1 is discussed.

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TP28 POLYMORPHISM OF P450 IN *DROSOPHILA MELANOGASTER*

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Because of its impact on drug metabolism, cytochrome P450s polymorphism is under the scope in humans[1]. In insects, they are involved in numerous instances of insecticide resistance and adaptation to chemical stresses. Available large collections of strains, easyness of wild populations collections and genome knowledge make *Drosophila melanogaster* a suitable species for a global study of P450s polymorphism in insects. In this first study, we analyzed in 7 strains the polymorphism of Cyp6a2 and Cyp6g1 (xenobiotics metabolism and insecticide resistance[2,3]), Cyp302a1 and Cyp315a1 (steroid synthesis, dib and sad genes[4,5]) and of Cyp6w1 and Cyp6u1, two P450s physically close to Cyp6a2[6].

For each gene, primers were designed to amplify by PCR fragments no longer than 320 bp in the coding sequence. We first analysed these fragments by SSCP. Results showed that Cyp6a2, Cyp6g1 and Cyp302a1 (dib) were polymorphic with 6, 4 and 5 alleles respectively contrary to Cyp315a1 (sad) which had only 2 alleles. Work is in

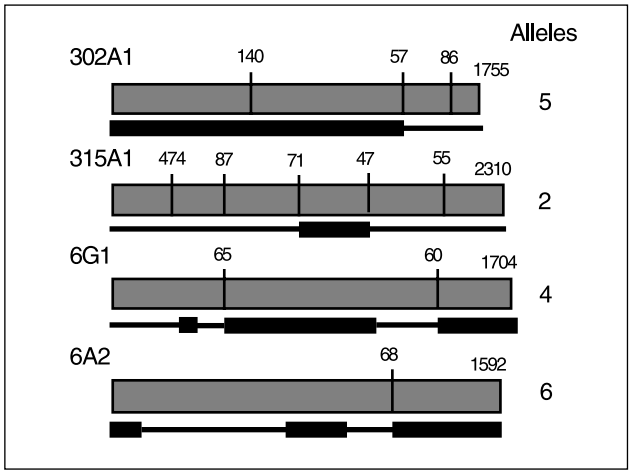

Fig. 1. *The 4 genes were represented wtih the position and the size of the introns. The polymorphic regions are indicated under the gene with black bars and narrow bars respectively.*

progress with Cyp6u1 and Cyp6w1. There was a region polymorphic in all the genes which code for the G, H and I helices but polymorphism was not distributed among the genes uniformously. Although polymorphism was expected for Cyp6a2 and to a lesser extend for Cyp6g1, we were surprised to classify Cyp302a1 in the polymorphic P450s category. Indeed, this gene is involved in steroid hormone synthesis and thus should be submitted to a high selection pressure. Nevertheless, we are sequencing these alleles to identify which polymorphism event is translated and thus putatively effective on protein function.

The amount of polymorphism of each gene, its repartition among the gene and its putative effects on the encoded protein activity will be discussed taking account of the function of the gene and P450 structure/activity relationships.

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TP29 BACTERIAL MONOOXYGENASES IN BIOTRANSFORMATION AND BIODEGRADATION OF ALIPHATIC AND AROMATIC COMPOUNDS

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Bacteria are able to degrade or transform many natural or xenobiotic compounds occurring in the environment using variety of oxidative reactions. An important group of enzymes are the monooxygenases including P-450 enzymes. The best known and characterized bacterial P-450 was the P-450cam isolated from Pseudomonas[1].

Study of dependent of the levels of cytochromes b_5 and P450 from structure of substrate was by purpose of this work. Two series of the compounds were used for these investigations. Aliphatic and alicyclic compounds represent the first, halogenated aromatic compounds represent the second series. *Pseudomonas fluorescens* B-22 was used for the transformation aliphatic and alicyclic compounds, Rhodococcus opacus B-2243 for the degradation of halogenated aromatic compounds. The levels of cytochromes b_5 and P450 was determined spectrophotometrically by the method Omura and Sato[2]. The concentrations of cyt P450 were calculated from reduced carbon monooxide difference spectrum, using an exinction coefficient 92,8 mM^{-1} cm^{-1} in according to the paper[3]. The results of these studies are represented in *Table 1* and *Table 2*.

The change of the levels of cytochromes b_5 and P450 in *Pseudomonas fluorescens* B-22 (*Table 1*) probably is caused by distinction of chemical reactions on cyt P450. Hexan is capable to hydroxylate with formation of alcohols; hexen-1 may be hydroxylated, but preferable it epoxydates; cyclohexen and nonen-4 epoxydate, what was confirmed in further study with GC.

Table 1. *Content of cytochromes b_5 and P450 in Pseudomonas fluorescens B-22*

Substrate	Contents of cytochrome b_5, nmol/mg of protein		Contents of cytochrome P450, nmol/mg of protein	
	Exponential growth phase	Stationary growth phase	Exponential growth phase	Stationary growth phase
Glucose	0,04	0,02	0,02	-
Hexan	0,12	0,05	0,10	0,08
Hexen-1	0,18	0,03	0,21	0,03
Cyclohexen	0,04	0,04	0,23	0,02
Nonen-4	0,05	0,01	0,18	0,02

Table 2. *Content of cytochromes b_5 and P450 in* Rhodococcus opacus *B-2243*

Substrate	Contents of cytochrome b_5, nmol/mg of protein		Contents of cytochrome P450, nmol/mg of protein	
	Exponential growth phase	Stationary growth phase	Exponential growth phase	Stationary growth phase
Benzen	0,010	0,003	0,033	0,012
Chlorbenzen	0,016	-	0,077	-
Bromobenzen	0,020	0,009	0,040	0,016

All compounds used in the experiments with *Rhodococcus opacus* B-2243 were the growth substrates. From our point of view, the change of the levels of cytochromes b_5 and P450 in *Rhodococcus opacus* B-2243 may be explained by next causes:

1) in the process of degradation of the growth substrates cyt P450 may be catalyzes the different stages of transformation of these compounds;

2) cyt P450 may be catalyzes the same stage of transformation of these compounds, but P450 has different substrate specificity to those compounds, that are substrate for it in this reaction.

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TP30 RABBIT AS AN ANIMAL MODEL TO STUDY CYP3A-MEDIATED DRUG-DRUG INTERACTIONS

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Cytochrome P450 3A (CYP3A) is an important class of enzymes highly involved in the metabolism of drugs. Any new drugs that can alter CYP3A levels through induction or inhibition can eventually cause drug-drug interactions in humans. There are several in vitro assays that have been developed to look at the inhibition or induction of CYP3A using human liver subcellular fractions or hepatocytes. The in vivo relevance of the results obtained from these assays is, however, difficult to address.

This presentation will focus on the use of rabbit as an animal model to predict CYP3A-drug drug interactions. First, in vitro studies were conducted to show that the metabolism of known CYP3A substrates (testosterone, DFP[1], DFB[2] (*Figure 1*)) and a variety of chemical inhibitors behaved similarly in human and rabbit. In vivo studies were then conducted and showed that DFB, being a fast clearance drug in rabbit, would be suitable to evaluate the effect of CYP3A inhibitors while DFP, a slower clearance drug, had the proper pharmacokinetic behaviors to test the effect of an inducer. When ketoconazole was co-administered with DFB to rabbit, the AUC of DFB increased by 4-fold. When rabbits were treated with rifamipicin, the

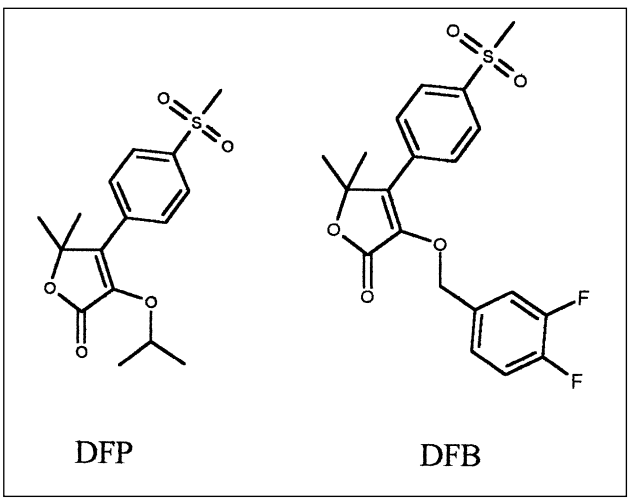

Fig. 1. *Structure of DFP and DFB*

AUC of DFP decreased by 50%, consistent with the effect of a CYP3A inducer. This CYP3A induction was also confirmed by Western blot analysis of rabbit livers. This model has also been used in our drug discovery program to test if new chemical entities can be victims of CYP3A interactions. In these studies, the effect of ketoconazole or rifampicin on the clearance of the NCE entities is monitored. This poster will provide the details of the in vitro and in vivo profiles of DFB and DFH in rabbits and will describe the rabbit protocol used to evaluate whether a NCE is likely to be victim of CYP3A interactions in humans.

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TP31 *CYP2D6* POLYMORPHISM AND EXTRAPYRAMIDAL SYMPTOMS IN PATIENTS ON LONG TERM ANTIPSYCHOTIC TREATMENT

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Cytochrome P450 enzyme CYP2D6 is one of the most important enzymes involved in metabolism of antipsychotic drugs. Due to genetic polymorphism of this enzyme coded by polymorphic *CYP2D6* gene individuals differ in drug metabolising capacity. *CYP2D6* genotyping for the most common defective alleles was reported to be a reliable prediction of decreased metabolic capacity for CYP2D6 substrates[1]. Our aim was to asses the predictive value of low and high resolution CYP2D6 genotyping for the development of extrapyramidal symptoms in patients in stable remission receiving long term maintenance antipsychotic therapy. 161 out-patients meeting the DSMIV criteria for schizophrenia receiving maintenance antipsychotic therapy with either zuclopenthixole, haloperidol or risperidone were included in the study. None of them was receiving any concomittant medication that would have potentially inhibitory effect on CYP2D6. All were in stable remission and on present medication for 44.7 ± 37.5 months. Extrapyramidal symptoms (EPS) were evaluated in 131 patients with Simpson-Angus rating scale (parkinsonism), Barnes akathisia rating scale, and Abnormal Involuntary Movement Scale (AIMS- tardive dystonia and tardive dyskinesia). Psychopathological symptoms were evaluated with Positive and Negative Syndrome Scale for Schizophrenia (PANSS). *CYP2D6* gene deletions (*CYP2D6*5*) and duplications were identified by long-PCRs[2,3]. Next, *CYP2D6* genotyping of *1, *3, and *4 (low resolution) and additionally of *2, *6, *8, *9, *10, *11, *12, *14 and *15 allele (high resolution) was performed by nested PCR-RFLP approach after amplification of the entire *CYP2D6* gene[4]. The frequency of *CYP2D6**1 allele coding for extensive metabolizer phenotype (EM) was 0.427. The frequencies of alleles coding for slightly (*2) or moderately (*9, *10) reduced activity (intermediate metabolizer phenotype- IM) were: 0.242, 0.016 and 0.041. The frequencies of defective alleles (poor metabolizer phenotype- PM) were: 0.178 (*4), 0.035 (*3), 0.022 (*5 and *6), while *8, *11, *12, *14 and *15 allele were not found. *CYP2D6* duplication alleles were found with frequencies of 0.007 (*1X2 and *2X2) and 0.003 (*4X2). The high resolution genotyping did not reveal any additional PMs, but helped to identify IMs. Regarding the EPS parkinsonism was observed in 56/131 (42.8%) and akathisia in 15/131 (11.5%) patients. The groups of patients with and withouth EPS did not differ regarding *CYP2D6* genotype frequencies, predicted phenotype or the number of functional *CYP2D6* alleles, or regarding the type and dosage of antipsychotics. PMs however scored significantly higher on the negative subscale for PANSS ($p = 0.008$). Our results indicate that *CYP2D6* polymorphism is not associated with the incidence of antipsychotic-induced EPS in patients with stabile remission on long term maintenance treatment.

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TP32 CYP2U1, A THYMUS AND CEREBELLUM EXPRESSED CYTOCHROME P450 CONVERTS ARACHIDONIC ACID INTO BIOACTIVE DERIVATIVES 19- AND 20-HETE

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The cytochrome P450 pathway for arachidonic acid (AA) bioactivation plays an important functional role in cell and organ physiology[1]. Arachidonic acid metabolites generated by cytochrome P450s (CYPs) have been described as powerful bioactive mediators in vascular and renal physiology and in the pathophysiology of experimental hypertension. However the full range of AA activities is not yet clear. Characterization of cytochrome P450s involved in AA metabolism is a critical step in this process.

We have recently cloned the cDNA encoding CYP2U1, a previously uncharacterized member of the CYP2 family. This protein shares 35, 34 and 31% amino acid identity with CYP2D6, CYP2R1 and CYP2J2 respectively. The tissue distribution of this CYP was investigated by northern-blot and by real-time PCR. Interestingly, we found that CYP2U1 is predominantly expressed in the cerebellum and thymus. CYP2U1 catalytic activity was tested in microsomal fractions from *sf9* insect cells expressing CYP2U1 re-

Tab. 1. *CYP2U1 substrates*

Exogenous	Endogenous
Linolenic acid (C18:3)	Palmitic acid (C16:0)
Arachidonic acid (C20:4)	Palmitoleic acid (C16:1)
Eicosapentaenoic acid (C20:5)	Stearic acid (C18:0)
Eicosatrienoic acid (C20:3)	Vaccenic acid (C18:1)
Docosahexaenoic acid (C22:6)	Eicosapentaenoic acid (C20:5)
Docosatetraenoic acid (C22:4)	Arachidonic acid (C20:4)
	Docosahexaenoic acid (C22:6)

combinant protein in the presence or absence of exogenous substrate. Medium and long chain fatty acids (C16 to C22) were metabolized by CYP2U1 (*Table 1*). In the case of AA, the two metabolites were identified as the 20- and 19-hydroxyeicosatetraenoic acid (20- and 19-HETE). Both ketoconazole and 17-ODYA inhibit CYP2U1 catalytic activity. Based on CYP2U1 amino acid sequence similarity and its ability to generate bioactive eicosanoid derivatives, we postulate that CYP2U1 plays an important physiological role both in the brain and thymus.

This is the first report of the cloning and functional expression of a new member of family 2, CYP2U1, which metabolize medium and long chain fatty acids.

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TP33 A NOVEL CYP26 FAMILY MEMBER METABOLIZES 9-CIS-RETINOIC ACID

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Retinoids (vitamin A analogs) are potent regulators of growth and differentiation of normal, pre-malignant and malignant cells. The synthesis and breakdown of retinoic acid are critical mechanisms governing the distribution and responsiveness of retinoic acid in target tissues both in the embryo and the adult. Retinoic acid catabolism is mediated by a highly specific cytochrome P450 enzyme family, called CYP26. Here we report the cloning and biochemical characterization of a new family member - CYP26C.

A novel cytochrome P450 was cloned using human adrenal poly(A) RNA. CYP26C amino acid sequence revealed 51 and 41% identity with human CYP26B and CYP26A, respectively. The genomic structure (intron-exon organization) of CYP26C is identical to that of CYP26B and differs from CYP26A by the number and the position of introns. The CYP26C gene is localized on chromosome 10 (chromosome 19 in mouse) in close proximity to the gene locus for CYP26A.

We have expressed recombinant CYP26C in insect cells and determined its catalytic activity in the presence of various retinoid substrates. All *trans*-retinoic acid (atRA) was metabolized by CYP26C in a fashion similar to that by CYP26A and CYP26B. However, CYP26C was also shown to metabolize *9-cis*-retinoic acid (9cRa) to the corresponding 4-oxo- and 4-OH-retinoic acid metabolites. This property seems to be unique to CYP26C since other retinoic acid metabolizing enzymes, CYP26A and CYP26B do not recognized 9cRA as a substrate. Similarly, although ketoconazole inhibits the activities of both CYP26A and CYP26B, CYP26C activity is unaffected, further evidence of differences in substrate binding properties amongst CYP26 family members.

We speculate that based upon the unique expression pattern during embryonic development and substrate binding that CYP26C plays a role distinct from those of CYP26A and CYP26B.

TP34 CDNA CLONING AND BIOCHEMICAL CHARACTERIZATION OF CYP4F11, A NOVEL HUMAN CYTOCHROME P450

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The CYP4F subfamily is known to be involved in the hydroxylation of arachidonic acid (AA), lipoxin, tocopherol (vitamin E), leukotriene B4 (LTB4), prostaglandins and ebastine (antihistamine). Members of the CYP4F subfamily are also known to play an important functional role in inflammatory responses by regulation of the level of inflammatory mediators and in the biotransformation of certain xenobiotics.

Here we report the biochemical properties of a new member of the CYP4F subfamily, CYP4F11. The CYP4F11 amino acid sequence has 82, 80, and 79% identity with other members of the CYP4F subfamily CYP4F3, CYP4F2 and CYP4F8 amino acid sequences, respectively [1].

CYP4F11 catalytic activity was tested in microsomal fractions from *sf9* insect cells expressing CYP4F11 recombinant protein in presence or absence of exogenous substrate. Our data show that arachidonic acid (AA), leukotriene B4 (LTB4) and other short to long chain (C12 to C22)

Tab. 1. *CYP4F11 substrates:*

Exogenous	*Endogenous*
Arachidonic acid (C20:4)	Lauric acid (C12:0)
Eicosapentaenoic acid (C20:5)	Myristic acid (C14:0)
LTB4	Palmitic acid (C16:0)
	Palmitoleic acid (C16:1)
	Stearic acid (C18:0)
	Vaccenic acid (C18:1)
	Eicosapentaenoic acid (C20:5)
	Arachidonic acid (C20:4)
	Docosahexaenoic acid (C22:6)

saturated or unsaturated fatty acids were metabolized by CYP4F11 (Table 1). In the case of AA, the unique metabolite formed was identified by mass-spectrometry as 20-hydroxyeicosatetraenoic acid (20-HETE). An apparent Km and Vmax of 41 μM and of 2 nmol/min/mg of protein were measured for AA. Based on CYP4F11 catalytic activity we can postulate that CYP4F11 might play an important functional role in inflammatory response. In specific tissues, it might also be involve in the formation of the physiologically active 20-HETE.

This is the first report of the functional characterization of a new member of the subfamily 4F, CYP4F11, which metabolize fatty acids and leukotriene B4.

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TP35 *CYP2C9* POLYMORPHISM AND WARFARIN DOSE REQUIREMENT

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Warfarin is the most widely prescribed oral anticoagulant drug in Slovenia. Its narrow therapeutic index and high interindividual variability in the dose required to achieve the desired therapeutic effect complicate the treatment. Cytochrome P450 2C9 (CYP2C9) is largely responsible for terminating the anticoagulant effect of racemic warfarin via hydroxylation of the pharmacologically more potent S-enantiomer to inactive metabolites. Genetic polymorphisms of the *CYP2C9* gene, which give rise to proteins with decreased catabolic activity, have been described. The variant alleles include *CYP2C9*2* with a point mutation in exon 3 causing an Arg144Cys exchange, and *CYP2C9*3* with a point mutation in exon 7 resulting in an Ile359Leu exchange[1].

The aim of this study was to asses the influence of *CYP2C9* polymorphism on the dose requirement of the anticoagulant drug warfarin.

In total 157 patients receiving warfarin from 6 months to 14 years (average 3,5 years) were included in the study. Indication for warfarin treatment were atrial fibrillation (n=145) and mechanical heart valves (n=12). The patients were divided into three groups according to the dose requirement: low (LD: < 2,15 mg/day, n=38), medium (MD: 2,5-7 mg/day, n=103) and high (HD: > 7 mg/day, n= 16). *CYP2C9* polymorphisms were analysed by PCR amplification of genomic DNA followed by restriction enzyme analysis as previously described by Yasar et al.[2]

In the LD group 13 patients (34.2%) were homozygous for wild-type allele (**1/*1*), 20 patients (52.6%) were heterozygous and 5 patients (13.2%) were homozygous for polymorphic alleles. In the MD group 74 patients (71.8%) had **1/*1* genotype, 27 patients (26.2%) were heterozygous and 2 patients (2.0%) were homozygous for polymorphic alleles. In the HD group all the patients were homozygous for wild-type allele. The frequencies of *CYP2C9*3* polymorphic alleles were significantly higher in LD as compared to MD group (26.3% as compared to 4.9%). The average dose of warfarin was the highest among patients with **1/*1* genotype (4.42 mg/day) and was decreasing with the number of polymorphic alleles: 2.96 mg/day in **1/*2*, 2.71 mg/day in **1/*3*, 2.14 mg/day in **2/*3* and 1.00 mg/day in **3/*3* genotype. The only exception was the patient who had **2/*2* genotype and the warfarin dose 4.29 mg/day.

CYP2C9 genetic polymorphisms significantly contribute to individual warfarin dose requirement. Since CYP2C9 polymorphisms are common in Slovenian population, occurring in 34% of individuals[3], it is of potential clinical importance to be able to identify patients carrying polymorphic alleles when aiming for rational and individualised anticoagulant therapy with warfarin.

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TP36 A STREPTOMYCES CYP105D1 EXPORTED IN *ESCHERICHIA COLI* INDUCES PERIPLASMIC ACCUMULATION OF PORPHYRIN

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Porphyrias are clinical conditions arising from genetic or sporadic defects of the enzymes in haem biosynthesis[1]. These diseases are associated with the accumulation of oxidised haem intermediates known as porphyrins. The commonest porphyric disorder, *porphyria cutanea tarda*, is characterised by hepatic accumulation and excretion of uroporphyrins (I and III). A number of etiological factors are thought to precipitate the sporadic form of the disease including iron overload, ascorbic acid deficiency, hepatitis c infection and induction of P450 isoenzymes. A structurally competent *Streptomyces griseus*, CYP105D1 was expressed in aerobically-grown *Escherichia coli* as an exportable form[2]. Expression of CYP105D1 was time-dependently coupled with accumulation of a stable, highly fluorescent compound in the periplasmic space, which was isolated and identified as uroporphyrin. The accumulation of uroporphyrin was over a hundred-fold higher than cells lacking *CYP105D1*. Expression of a cytoplasmic-resident CYP105D1 at a comparative level to the secreted form was far less effective in promoting porphyrin accumulation in the cytoplasm or periplasm. An *E. coli* strain that exported a mammalian holo-cytochrome b_5 at ten-fold molar excess over the exported CYP105D1 produced uroporphyrin levels similar to the control strain. This negated the possibility that the oxidative production of uroporphrin in the CYP105-secreted strain was a consequence of 5-aminolevulinic acid-promoted synthesis of heme precursor to provide the prosthetic group for the over-produced apo-cytochromes. Uroporphyrin production was abolished in the CYP105D1 secretory strain by azole-based P450 inhibitors. Furthermore, the isolated secretory CYP105D1, when reconstituted *in vitro*, was active in its ability to convert uroporphyrinogen I to uroporphyrin I. The present study demonstrates that the exported CYP105D1 catalyses periplasmic conversion of uropophyrinogen to uroporphrin in *E. coli*.

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TP37 NOVEL SNP'S IN THE CYP2B6 GENE AND THEIR FUNCTIONAL CONSEQUENCES

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The human cytochrome P450 2B6 is involved in the metabolism and activation of several therapeutically drugs and environmental toxins such as bupropion, cyclophosphamide, nicotine and aflatoxin B. The *CYP2B6* gene is highly polymorphic and at least 9 alleles with amino acid exchanges and associated with changes in liver protein expression are presently known[1,2]. By further sequencing of *CYP2B6* in samples from Caucasians we found five new point mutations (*A-E*) in coding regions. These novel SNPs are predicted to result in amino acid exchanges in exon1 (A: M46V), exon2 (B: G99E), exon3 (C: K139E; D: R140Q) and exon8 (E: I391N). Comparison of hepatic CYP2B6 protein levels and bupropion hydroxylase activity between polymorphic (n=10) and wildtype (n=31) samples demonstrated significantly reduced CYP2B6 content (p=0.0118) and enzyme activity (p=0.001). To investigate the functional concequences of the amino acid substitutions, enzyme variants A-E were recombinantly expressed in insect cells using baculovirus expression system and COS1 cells. Analysis of CO difference spectra of infected insect cells showed lower P450 content but higher P420 amount for the variant proteins 2B6.A, 2B6.B, 2B6.C, and 2B6.E, whereas 2B6.D was comparable to WT. Immunoblots of infected insect cells showed nearly the same amount of 2B6 protein for 2B6.A, 2B6.B and 2B6.D compared to wildtype. In the case of 2B6.C and 2B6.E, almost no immunostainable protein was detected. These results demonstrate that the novel 2B6 variants A, B, C and E are functionally severely compromized and may lead to a poor metabolizer phenotype if present in homozygous form. Further characterization of the new variants is currently in progress.

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TP38 COMPARATIVE ANALYSIS OF CYP3A EXPRESSION IN HUMAN LIVER SUGGESTS ONLY A MINOR ROLE FOR CYP3A5 IN DRUG METABOLISM

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In order to study mechanisms behind the interindividual variability in CYP3A expression and the relative contribution of the different CYP3A enzymes to the overall CYP3A activity we have analysed *CYP3A4, CYP3A5, CYP3A43* and PXR mRNA and CYP3A4 and CYP3A5 protein expression, catalytic activity and polymorphism in the *CYP3A5* gene in a panel of 46 Caucasian human livers. Protein quantification was performed by Western blotting using enzyme-specific antibodies directed to the C-termini of CYP3A4 or CYP3A5, and carrier protein-coupled peptides as standards. The mRNA levels were determined by quantitative real-time PCR. CYP3A activity was measured by analysis of the rate of testosterone 6(-hydroxylation. A correlation existed between all *CYP3A* and *PXR* mRNA transcripts measured. The interindividual variability in *CYP3A4* and *CYP3A5* mRNA expression was higher than of CYP3A protein and activity. The CYP3A5 protein was expressed at quantifiable levels in 5 (10.9%) of the livers. Four of those were heterozygous for the *CYP3A5*1* allele and had CYP3A5 protein at a mean level of 17% of that of total CYP3A, whereas one liver sample was from a *CYP3A5*3* homozygote individual having lower amounts of CYP3A5. In total, CYP3A5 only contributed 2% of the overall CYP3A protein among all samples. In conclusion, our data indicate that *CYP3A4, CYP3A5, CYP3A43* and *PXR* hepatic mRNA expression correlate, indicating common regulatory features, and that the contribution of CYP3A5 to hepatic drug metabolism in Caucasians is insignificant.

TP39 SEARCHING FOR THE GENE ENCODING STEROID 11β-HYDROXYLASE OF THE FILAMENTOUS FUNGUS *COCHLIOBOLUS LUNATUS*

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11β-hydroxylation of steroids is one of the key steps in the production of corticosteroids. The enzymes responsible for the hydroxylation of steroids by filamentous fungi belong to the cytochrome P450 superfamily. Despite the substantial biotechnological importance of these enzymes, the sequences of the genes encoding fungal steroid 11β-hydroxylase, or any other fungal steroid hydroxylase, have not yet been identified. The primary structures of these enzymes have also not yet been described. This is in part due to the low specific content of the protein, difficulties associated with their isolation (membrane bound enzymes), the presence of multiple cytochrome P450 isoenzymes, and the lack of homology with the analogue enzymes of higher eukaryotes. The filamentous fungus *Cochliobolus lunatus* is an important phytopathogen, capable of 11β-hydroxylation of steroids. Based on the conserved, cytochrome P450 specific regions, and the nucleotide sequence of the benzoate para-hydroxylase of the related filamentous fungus *Aspergillus niger*, a 273 bp long gene fragment has been obtained using the PCR method. The fragment was sequenced, and the BLAST database search clearly showed its cytochrome P450 nature. However, it could not be determined, whether the fragment belonged to the gene encoding steroid 11β-hydroxylase, or perhaps some other cytochrome P450 gene which could be expected in this fungus. Based on the sequence of the obtained 273bp fragment, new cytochrome P450 specific primers were designed. Using the rapid amplification of cDNA ends method, an entire 3'-end of the gene, and a part of the 5'-end were successfully amplified from the induced mycelium. After cloning, the sequence of the 3'-end was determined using an automatic sequencer. Using the BLAST database search, the obtained 3'-end was found to have a high homology with numerous cytochromes P450, including the benzoate para-hydroxylase of *A. niger*. The obtained 3'-end will be used as a probe for screening genomic and cDNA libraries. Thus the entire gene to which the 3'-end belongs will probably be identified, as well as, with a slightly lower hybridization intensity, other cytochrome P450 genes present in the fungus. It is likely that, among these, the steroid 11β-hydroxylase would be found as well.

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TP40 TRANSCRIPT ANALYSIS AND AZOLE INHIBITION OF MYCOBACTERIAL CYPS

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Mycobacteria contain novel CYPs of unknown function and revealing their roles and potential application is a challenge for the coming years. One potential application is in the treatment of mycobacterial infection. Known CYP inhibitors among the antifungal drugs have been observed to show effect against various fast-growing bacteria[1], but reduced effect against M. bovis BCG[2]. The presence of a CYP51 in mycobacteria is the most obvious potential target of such compounds as in fungi, but azole efficacy could easily be due to effect on other CYPs in these organisms.

We have looked at the CYP51 locus in mycobacteria and observed it is conserved except in *M. leprae*. CYP513 and the surrounding genes appear to be part of an operon. All the ORFs in the operon have been shown to be transcribed in growing *M. smegmatis* and *M. bovis* BCG. We are also examining the CYP51 azole affinity in *M. tuberculosis* and of *M. smegmatis* and have observed a correlation to the anti-mycobacterial activity of the compounds.

Other CYPs bind azole compounds including CYP162A2 the *M. smegmatis* homologue of the lone *M. leprae* CYP, but in some cases novel features are encountered as with CYP125. Further studies on ferredoxin (Rv0763c) and ferredoxin reductase (Rv3106) expression and function, including investigation of their interaction with CYP partners are presented.

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TP41 CYP 2D6 „INTERMEDIATE METABOLIZER" PHENOTYPE AS A CONSEQUENCE OF GENETICALLY DETERMINED ALTERNATIVE SPLICING

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CYP2D6 is responsible for the metabolism of many clinically used drugs including antidepressants, neuroleptics, beta-blockers and others. CYP2D6 expression is highly polymorphic with more than 70 known alleles which code for protein products with variant or lacking function[1]. In Caucasian populations, four phenotypes termed ultrarapid (UM), extensive (EM), intermediate (IM) and poor metabolizers (PM) are observed. Using liver samples of phenotyped patients, we have shown that these phenotypes are the result of different protein expression levels[2]. Our recent work concentrated on the IM phenotype, which affects about 10-15% of the population most of whom were shown to have the genotype **41 / *0*, with **41* designating a novel functionally impaired allele and **0* designating any nullallele[3]. The **41* allele is genetically distinct from **2* in a putative NFkappaB binding site at -1584 bp[3] but luciferase reporter-gene transfection experiments suggest that this promoter polymorphism may not explain the large difference in phenotypic expression between **2* and **41*. We therefore undertook a complete sequence analysis of the **2* and **41* alleles comprising the entire upstream region between CYP2D6 and CYP2D7 as well as all introns. Apart from several novel SNPs which are found in both alleles, one novel intronic mutation at 2988 (G>A) was found only in **41* alleles but not in **2*. To investigate whether this

intronic mutation may be responsible for the decreased expression of *41, we hypothesized that it may influence splicing. Using liver RNA samples of genotyped patients, a specific RT-PCR assay was developed to detect splicing events between exon 5 and 9, the region where the intron mutation is located. Individuals with one or two *41 alleles were subsequently shown to lead to an additional PCR product which was about 150bp shorter than the normal product. Sequencing of the splice variant revealed the deletion of the entire exon 6. All other genotypes showed only minute amounts of this splice variant. Because deletion of exon 6 interrupts the open reading frame,the alternative splicing should result in decreased expression of CYP2D6 protein, thus explaining the IM phenotype of individuals having the *41/ *0 genotype. To further investigate this hypothesis, quantitative analyses of splice variants and protein expression and further correlation studies between genotype and phenotype are in progress.

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TP42 IDENTIFICATION OF CYP2D6 GENOTYPE VIA A NEW SEQUENCING PROTOCOL

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The cytochrome P450 (CYP) 2D6 isoform mediates the oxidative metabolism of many different classes of drugs used commonly in clinical practice. The clinical consequences of the genetic polymorphism of CYP2D6 have been extensively reported; between 5-10% of Caucasians classified as poor metabolisers (PM). To date, approximately 75 variant alleles have been identified, the most frequent of which in the Caucasian population being **2* (28.5%), **4* (19.5%), and **5* (4.1%)[1]. Previous methods for identifying the various CYP2D6 genotypes have involved allele-specific polymerase chain reactions (PCR) followed by agarose gel electrophoresis[1-3], which when investigating the presence of multiple variant alleles are time consuming and expensive. This study aimed at establishing a sequencing method to determine the presence of multiple variant alleles in a Caucasian population. Following informed consent of the subjects (n=25) genomic DNA was isolated from either blood samples using a QIAamp DNA mini kit (QIAGEN Pty Ltd, Australia). Initial PCRs using primers previously published[2] were performed to produce two templates isolating regions in exons 3-4: position 1297 to 2034, and exons 5-6: position 2010 to 3112 (numbers based on translation start). PCR products were purified using a QIAquick PCR purification kit (QIAGEN) and sequenced using the following forward primers: position 1556 5'-GTG GGG CTA ATG CCT TC-3', and position 2379 5'-GAG ACT TGT CCA GGT GAA CG-3'. Sequencing reactions were performed with an ABI PRISM® BigDye™ Terminator kit, version 3 with analysis on an ABI PRISM® 3700 DNA analyzer (Applied Biosystems, Australia). Using this method we were able to identify CYP2D6 alleles **1* to **4* and **6* to **9* and the allele frequencies were comparable to those found previously in a Caucasian population1: **1*, 38%; **2*, 34%; **3*, 0%; **4*, 20%; **6*, 2%; **7*, 0%; **8*, 0%; and **9*, 4%. This is the first method to allow genotyping via sequencing methods, which is a time efficient and reliable alternative to conventional PCR methods enabling information regarding multiple variant alleles to be obtained rapidly. Further development of the method to allow identification of other variant alleles is ongoing.

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TP43 USE OF 2D - ELECTROPHORESIS AND MALDI - TOF IN THE IDENTIFICATION OF HEPATIC CYP1A ISOFORMES OF *PROCHILODUS SCROFA*, A BRAZILIAN FRESHWATER FISH

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Cytochromes P450 constitute a superfamily of the phase I enzymes whose primary task is the detoxification of both endogenous and xenobiotic compounds (Omura, 1999). Fish, among the non-mammalian species, received great interest because they are direct food source for human as well as conveyors of toxic chemicals to human beings. The aim of present study is the identification of hepatic isoformes of CYP in *Prochilodus scrofa*, with emphasis in CYP1A, in a Brazilian fish belonging the family Prochilodontidae and knowing as Curimbatá that has a high economic value in southeast region of Brazil. Purification of CYP1A was done by Reverse Phase HPLC on a C18 column. The fractions collected were applied on a SDS - PAGE 12,5% or in a 2D-PAGE. Spots were collected, and treated with Trypsin. CYP cleavage products were extract with acetonitrille. Peptide fragmentation were made using MALDI-TOF. Purified CYP1A was characterized with respect to electrophoretic, immunochemical and biocatalytic properties. CYP1A fractions produced a band on SDS-PAGE with an apparent molecular weigh of 54kDa. Purified CYP1A of *P. scrofa* also showed a strong cross-reactivity with antibody directed against CYP1A homologue purified from scup. Two major spots were identified in the 2D-PAGE. They have the same molecular weight, but pI were 5.5 and 6.0 respectively; which suggest the presence of 2 isoformes of the same protein, CYP1A1 and CYP1A2. Both spots showed that they

Tab. 1. *CYPIA tryptic peptides analyse*

Theor. AVG	Sequence position	Tryptic peptide	AV observed
34571.1+61,26	1-32	T1	3354,9+1,002
3131.2±82.13	44-73	T2	3103.03±0.648
2849.64+6,49	36-62	T3	2813.50+0.95
2372.5±13.53	409-427	T4	2340.76S±0.93
1804.90+0	194-210	T5	1792,03+0.38
1577,7±14.05	471-483	T6	1585.89±11.45
1375,11+44.80	270-280	T7	1385.64+0.32
1045,86±1869	428-436	T8	1041.42±0.53
851,45	17-177	T9	844.21±1.19

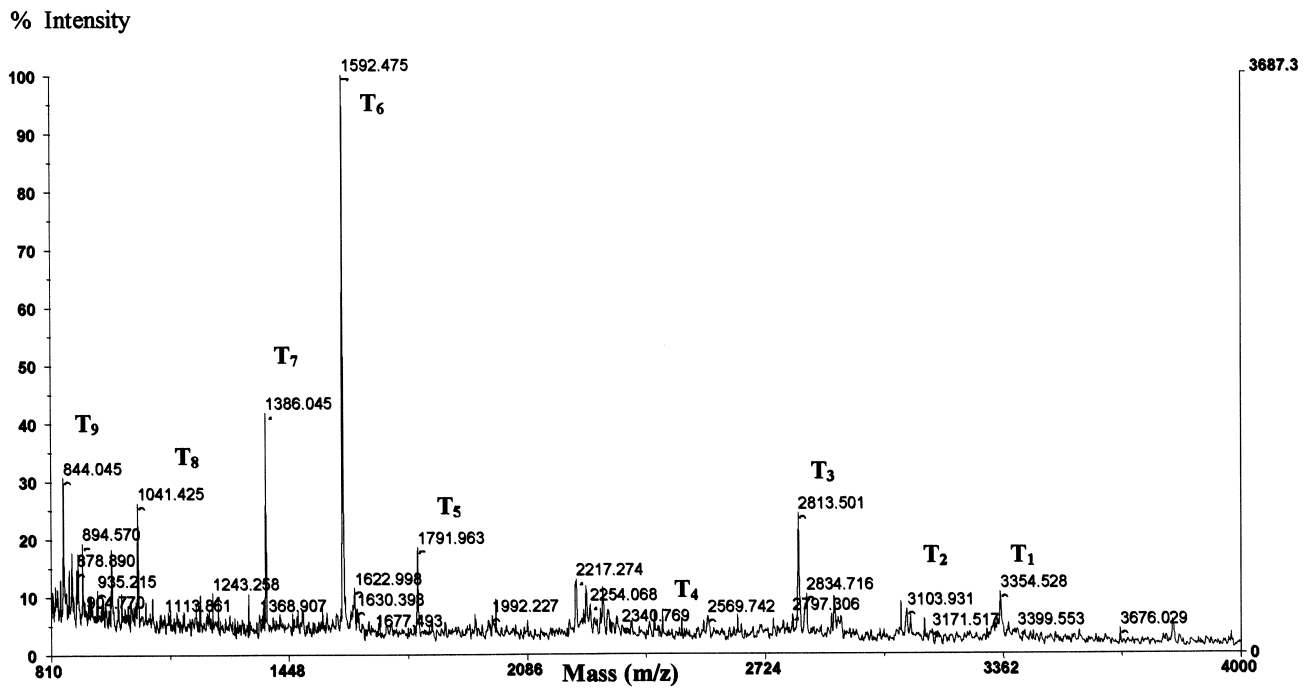

Fig. 1. *Tryptic peptide mass fingerprint of CYP1A*

have the same profile of fragmentation, which confirm that they are isoformes. Tryptic peptide mass fingerprint of CYP1A purified by HPLC showed the presence of 9 masses that matched the expected tryptic peptides obtained through theory digestion of database sequence (*Fig. 1*). These value corresponding to 30% of the translated amino acids of CYP1A family of other fishes (Buhler and Wang-Buhler,1998). Among those peptides, there are two of than that showed major importance T1, that could represent the NH2-terminal of CYP 1A1 and T2, which could represent a more conservative region (Graham and Peterson, 1999). *Table 1* shows CYP1A tryptic peptide analyze by MALDI-TOF. All those characteristics strongly suggest that this new procedure is efficient to purify simultaneously different isoformes of hepatic CYP1A from P. scrofa, identifying the conservative region of protein.

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TP44 NF-I REGULATES CYP2A5 TRANSCRIPTION IN MOUSE PRIMARY HEPATOCYTES

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Xenobiotic metabolising cytochrome P450 (CYP) enzymes are expressed in tissue specific manner and the highest levels are detected in the liver. The liver expression is believed to be controlled mainly by the liver enriched transcription factors[1] but few studies have focused on constitutive regulation of individual CYP genes. Cytochrome P4502a5 (Cyp2a5) gene is expressed principally in liver and olfactory mucosa[2]. In the current study, the transcriptional mechanisms of hepatocyte specific expression of Cyp2a5 were studied in mouse primary hepatocytes.

The Cyp2a5 5'-flanking regulatory region -3033 to +10 was cloned in front of a luciferase reporter gene and transfected into hepatocytes. Deletion analysis of the promoter revealed two activating sequence regions localized at proximal and distal parts of the promoter. The proximal activation region was further characterized by Dnase I footprinting. Incubation of the Cyp2a5 proximal promoter region with hepatocyte nuclear extracts protected a single clear footprint in the studied area centered over a sequence similar to nuclear factor I (NF-I) binding site. Protein binding to the detected footprint sequence was further analyzed with electrophoretic mobility shift assay. Three retarded protein bound DNA complexes were detected of which all were abolished by competition with consensus oligo for NF-I. Mutation of nucleotides critical for NF-I binding eliminated competition. Further, an antibody specific for NF-I supershifted one of the complexes. The functional contribution of NF-I site for transcriptional activation of Cyp2a5 gene was confirmed by mutation of the NF-I binding sequence in the Cyp2a5 promoter constructs.

In conclusion, these results indicate that the members of the NF-I protein family significantly contribute to Cyp2a5 expression in hepatocytes, and in addition to the previously suggested role in olfactory mucosa, are also involved in liver-enriched expression of Cyp2a5.

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TP45 CYP2C9 PHENOTYPING USING LOW-DOSE TOLBUTAMIDE

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Tolbutamide (TOL) has been established as a probe for the assessment of CYP2C9 activity *in vivo*. However, the validity and accuracy of the molar ratio of (carboxytolbutamide, CAR + 4-hydroxytolbutamide, 4-OH) / TOL in urine 6 - 12 h after TOL intake, proposed as a CYP2C9 metric, is unclear.

We therefore analysed plasma and urine samples of 25 healthy male volunteers using LC-MS/MS collected before and up to 24 h after intake of a single oral dose of 125 mg

TOL in the framework of two low-dose cocktail studies for phenotyping of important drug-metabolising enzymes. Subjects fasted before, and up to 6 h after drug intake. The limits of quantification were 15 and 12.5 ng/mL for TOL, 15 and 25 ng/mL for 4-OH, and 75 and 25 ng/mL for CAR in plasma and urine, respectively. Genotyping was carried out by direct sequencing of exon 3 and 7 for identification of the *2 to *5 alleles, respectively. Pharmacokinetic parameters were calculated with a compartment-free approach, and one way ANOVA was used for comparison between the genotypes.

CYP2C9 genotypes were *2/*2 in one, *1/*3 in three, *1/*2 in six, and *1/*1 in 15 volunteers, and reflected mean oral TOL plasma clearance (0.57, 0.60 (95% confidence interval, CI, 0.58 - 0.62), 0.79 (95% CI, 0.66 - 0.91), and 0.85 (95% CI, 0.80 - 0.89) L/h for the four genotypes, respectively). The urinary 6 - 12 h metabolic ratio did not correlate to TOL plasma clearance (r^2=0.136, p=0.07) or any of the pharmacokinetic parameters tested. TOL in plasma samples collected 6 to 24 hours after drug intake correlated significantly to TOL clearance. The best correlation was observed in samples drawn 24 h after TOL intake (r^2=0.83, p<0.0001). The mean 24 h TOL concentrations also reflected CYP2C9 genotypes: *2/*2, 3.27 μg/mL, *1/*3, 3.13 μg/mL (95% CI, 2.68 - 3.58 μg/mL), *1/*2, 2.03 μg/mL (95%CI, 1.51 - 2.56 μg/mL), *1/*1, 1.70 μg/mL (95% CI, 1.50 - 1.90 μg/mL). The TOL plasma AUC and the TOL elimination half-life were well correlated to TOL plasma concentrations 24 h after dosing (r^2=0.89 and 0.77, p<0.0001 each). No adverse event caused by TOL was noted.

In conclusion, phenotyping of CYP2C9 can be carried out with a TOL dose as low as 125 mg, which reduces the risk of undesired hypoglycaemia caused by TOL. For the simplification of CYP2C9 phenotyping, it seems more favourable to determine TOL in a single plasma sample taken 24 h after intake of a low 125 mg TOL dose than to determine urinary molar ratios. However, this procedure has to be validated in a larger population of volunteers and patients.

TP46 A NOVEL THYMUS-SPECIFIC CYTOCHROME P450, CYP2U1

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In the human genome about 55 active cytochrome P450 genes and 26 pseudogenes have been identified. Most of the corresponding enzymes have been rather well characterized, whereas some, mainly extrahepatic forms, remains to be characterized. Based on homology searches we identified a novel cytochrome P450 cDNA designated CYP2U1, and the corresponding gene was found to be located on chromosome 4. We found an open reading frame of 1635 nucleotides, which encodes a 544 amino acid long polypeptide. The predicted amino acid sequence of CYP2U1 displays up to 39% identity to other human family 2 members, with closest resemblance to CYP2R1. CYP2U1 is, like CYP2R1, conserved across species, showing a 58% homology to fugu fish and 78% homology to murine CYP2U1.

One of the structural differences of the CYP2U1 gene compared to other CYP2 family members is that CYP2U1 consists of only five exons. Furthermore it has a longer amino acid sequence than other CYP2s. When aligning the CYP2U1 amino acid sequence to CYP2R1, CYP2D6 and CYP2C5, it has 40-50 extra amino acids. Approximately 20 of these amino acids are found in the very NH2-terminal, and 25 are located after the NH2-terminal membrane spanning region.

The mRNA expression of CYP2U1 was examined using Northern blotting, and revealed high expression levels of CYP2U1 especially in thymus, but also in heart and brain. The high mRNA expression seen in both adult and fetal thymus was confirmed using dot blot analysis.

The observed high and specific expression of CYP2U1 in thymus as well as the high conservation across species might indicate an important endogenous function. The substrate specificity is currently evaluated after cDNA expression in heterologous systems.

TP47 RAPID TRANSLOCATION OF CYP11A1 INTO YEAST MITOCHONDRIA HAMPERS ITS NORMAL SORTING AND FOLDING

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Topogenesis of cytochrome P-450$_{scc}$, a resident protein of the inner membrane of adrenocortical mitochondria, is still obscure. In particular, little is known about the cause of its tissue specificity. In an attempt to clarify this point, we examined the process in *Saccharomyces cerevisiae* cells synthesising P-450$_{scc}$ as its native precursor (pCYP11A1) or versions in which its N-terminal addressing presequence had been replaced with those of yeast mitochondrial proteins: CoxIV$_{1-25}$ and Su9$_{1-112}$. We found the pCYP11A1 and CoxIV$_{1-25}$-mCYP11A1 versions to be effectively imported into yeast mitochondria and subjected to

proteolytic processing. However, only minor portions of the imported proteins were incorporated into mitochondrial membranes, whereas their bulk accumulated as aggregates insoluble in 1% Triton X-100. Along with previously published data[1], this suggests that a distinguishing feature of the import of the CYP11A1 precursors into yeast mitochondria is their easy translocation into the matrix where the foreign proteins mainly undergo proteolysis or aggregation. The fraction of CYP11A1 that happens to be inserted into the inner mitochondrial membrane is effectively converted into the catalytically active holoenzyme. Experiments with the $Su9_{1-112}$-mCYP11A1 construct bearing a re-export signal revealed that, after translocation of the fused protein into the matrix and its processing, the $Su9_{67-112}$ segment ensures association of the mCYP11A1 body with the inner membrane, but proper folding of the latter does not take place. Thus it can be said that the most specific stage of CYP11A1 topogenesis in adrenocortical mitochondria is its confinement and folding in the inner mitochondrial membrane. In yeast mitochondria, only an insignificant portion of the imported CYP11A1 follows this mechanism.

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TP48 SYNTHESIS AND PROPERTIES OF FUSED PROTEINS COMPOSED OF COMPONENTS OF THE CHOLESTEROL SIDE-CHAIN CLEAVAGE ENZYME SYSTEM

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We studied the properties of various fused combinations of the components of mitochondrial cholesterol hydroxylase/lyase, including cytochrome P450scc, adrenodoxin (Adx), and adrenodoxin reductase (AdR). To this end, recombinant DNAs encoding these constructs were expressed in *Escherichia coli* cells. We have found that both cholesterol side-chain cleavage activity and sensitivity to intracellular proteolysis of the three-component fusions depend on the origin and arrangement of their constituents. To shed some light on the assembly of catalytic domains in the fused molecules, we analysed catalytic properties of various two-component fusions (P450scc-Adx, Adx-P450scc, and AdR-Adx). Their ability to carry out side-chain cleavage reaction in the presence of corresponding missing component of the whole cholesterol hydroxylase/lyase system and the dependence of this reaction on the presence of added individual components of the double fusions themselves were in focus. This analysis has revealed that active centres in the double fusions are either separated from each another or somehow deformed; in some cases they were unreachable for exogenous partners. In total, our data suggest that upon folding of polypeptide chains including P450scc, Adx, and AdR, corresponding catalytic domains are not formed independently of each other, i.e., in a way that each domain assumes its normal structure with any arrangement of others.

TP49 P450 ALLELE AND HAPLOTYPE FREQUENCY STUDY IN DIFFERENT POPULATIONS USING THE CODELINK™ SNP ASSAY

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It is now evident that a significant part of variation in drug efficacy and safety is of a hereditary nature, and can be traced down to polymorphisms in genes involved in drug metabolism. Establishing the correlations between drug metabolizing phenotypes and P450 genotypes and haplotypes will make clinical trials safer and provide the next step towards a personalized medicine.

CodeLink™ SNP bioarrays from Amersham Biosciences are robust tools for broad-based single nucleotide polymorphism (SNP) genotyping, which can be used in linkage mapping or candidate gene profiling. The assay process is simple, straightforward, and highly efficient with automation allowing for high throughput genotyping. Data analysis using the CodeLink software provides rapid data processing yielding genotyping reports and other analysis capabilities. The combination of internal controls and software features allows for efficient data validation.

For SNP detection, CodeLink platform uses enzymatic allele specific extension (ASE) of oligonucleotide probes. In CodeLink SNP P450 assay, a template for ASE is generated by highly specific long-range multiplex PCR follo-

wed by amplicon purification and fragmentation. Unique proprietary PCR primer design ensures absolute specificity of amplification despite of high DNA homology within P450 gene subfamilies.

CodeLink SNP P450 v.2.0 allows for genotyping of 130 SNPs in nine genes: *CYP1A1, CYP1A2, CYP1B1, CYP2C19, CYP2C9, CYP2D6, CYP2E, CYP3A4,* and *CYP3A5*.

We sequenced 46 samples from three major populations at these loci, and sequence data was used as a „gold standard" to estimate assay accuracy.

From Coriell Human Diversity panels, 230 samples representing three major populations were genotyped and population-specific allele and inferred haplotype frequencies have been calculated.

TP50 ASSOCIATION OF GENETIC POLYMORPHISMS IN CYTOCHROME P450 2E1 AND OTHER BIOTRANSFORMATION ENZYMES WITH PROGRESSION OF LYMPHOMAS

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The genetically variable biotransformation enzymes: cytochromes P450 (CYP, EC 1.14.14.1), epoxide hydrolase (EPHX1, EC 3.3.2.3), NAD(P)H:quinone oxidoreductase (NQO1, EC 1.6.99.2), and glutathione S-transferases (GST, EC 2.5.1.18) metabolize and conjugate drugs, carcinogens, and natural products. In addition, high number of human cancer cases result from exposure to environmental carcinogens suggesting that individual effectiveness in the detoxification of these chemicals may influence susceptibility to malignant disease. The aim of our study was to determine frequencies of the above listed polymorphisms of biotransformation enzymes in healthy population of the Czech Republic and to compare these frequencies with data on group of lymphoma patients. Polymerase chain reaction-restriction fragment length polymorphism based genotyping assays were used to determine the frequency of polymorphisms in *CYP1A1* (3'-flanking region), *CYP2E1* (5'-flanking region and intron 6), *EPHX1* (exon 3 and exon 4), *NQO1* (exon 6), *GSTM1* (deletion), *GSTP1* (exon 5), and *GSTT1* (deletion) in a case-control study comprised of 219 patients with morbus Hodgkin (MH) and non-Hodgkin's lymphomas (NHL) and 455 age- and sex-matched healthy individuals. The distribution of genotypes of *CYP2E1*-intron 6 was significantly different between the control group and that of all lymphomas (P = 0.03), patients with NHL (P = 0.024), and especially that of aggressive diffuse NHL (P = 0.007). Grading of NHL seemed to be associated with this polymorphism as well (P = 0.041). The *EPHX1*-exon 3 genotype distribution was significantly different between control males and males with all lymphomas (P = 0.01) or with NHL (P = 0.019). The variant homozygous genotype of *GSTP1*-exon 5 was prevalent in all MH (OR = 2.08, 95% CI = 1.05 - 4.14, P = 0.035) and this difference was particularly evident in females (OR = 2.97, 95% CI = 1.16 - 7.61, P = 0.023). A significant difference in the distribution of *GSTP1*-exon 5 genotypes was found between NHL tumors larger vs. smaller than 5 cm (P = 0.03). In conclusion, the results suggest that genetic polymorphisms of biotransformation enzymes may play a significant role in the development and progression of lymphoid malignancies.

The work at this project was supported by grant IGA 6747-3.

TP51 GENOME-WIDE EXPRESSION PROFILING AND XENOBIOTIC INDUCIBILITY OF P450 MONOOXYGENASE GENES IN THE WHITE ROT FUNGUS *PHANEROCHAETE CHRYSOSPORUM*

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Phanerochaete chrysosporium belongs to white rot fungi which have the unique ability to biodegrade lignin (plant aromatic polymer), a rate-limiting step in the nature's carbon cycle, and a broad specificity to oxidize and mineralize (to CO_2) a wide range of environmental chemical pollutants. *P. chrysosporium* has been widely used as a model to study the physiology, biochemistry, and genetics of biodegradation of lignin and chemical pollutants by white rot fungi. Lignin degradation occurs under nutrient-limited conditions when the fungus enters secondary metabolism, whereas chemical pollutants are degraded under both nutrient-limited (ligninolytic) and nutrient-rich (non-ligninolytic) culture conditions. Recognized role of P450 monooxygenases in these environmentally and biotechnologically significant biodegradation processes has led to an increasing interest in the characterization of cytochrome P450s in this organism.

In an effort to characterize P450 monooxygenase systems of *P. chrysosporium*, we cloned the first two full-length P450 monooxygenase genes *pc*-1 and *pc*-2 belonging to the CYP63 family[1, 2] and the P450 reductase gene[3]. Subsequently, whole genome sequencing of this organism

by the Joint Genome Institute of the US Department of Energy led to the identification of the presence of over 150 P450 genes, of which 100 genes have been assembled full-length (http://drnelson.utmem.edu/nelsonhomepage.html). Except for the conserved fungal P450 genes CYP51 and CYP61 and the three CYP63 genes (CYP63 A1, A2, and A3) experimentally cloned in our laboratory, other P450 genes in *P. chrysosporium* genome have unknown identity as they do not bear significant homology to the known P450 genes indicating a unique CYPome of this organism. Based on our phylogenetic analysis, the 150 genes are groupable into 26 clusters, most of them with multiple members. The cloned CYP63 members (CYP63A1, A2, A3) belong to one of these clusters.

Functional characterization of the P450 genes revealed in the white rot fungal genome would require identification of their substrates and the physiological conditions regulating their expression. In this regard, we developed a custom P450 microarray and studied genome-wide expression of 87 P450 genes representing all 26 clusters under varying physiological states. A total of 66 genes showed expression under both nutrient-limited and nutrient-rich culture conditions, of which 19 and 13 genes were upregulated (2- to 12-fold) under the two physiological states, respectively. We investigated xenobiotic inducibility of the P450s in response to 35 individual chemicals belonging to aromatic/polyaromatic, alicyclic, and aliphatic groups. CYP63A1 (*pc*-1) and CYP63A3 (*pc*-3) showed several fold (2 to 11-fold) induction by alkyl-substituted aromatics and oxygenated aromatic and aliphatic xenobiotics. The results yielded information on potential substrates and nutritional regulation for the individual P450 genes tested. Additional information from our on-going studies on genome-wide expression and induction on other P450s in the white rot fungus will also be presented.

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TP52 HAPLOTYPE ANALYSIS OF CYTOCHROME P450 2B6 – LINKAGE BETWEEN PROMOTER- AND EXONIC MUTATIONS.

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The human cytochrome P450 2B6 (CYP2B6) isoenzyme is a highly polymorphic and inducible member of the CYP family. We have previously shown that some exonic mutations are associated with changes in expression in human liver but the underlying mechanism remained unclear. We therefore speculated that identification of haplotypes considering promoter and exonic mutations would be more informative than individual mutations. Using our liverbank, a total of 98 individuals were genotyped for 15 dimorphic sites. Ten of these SNPs (-1848C>A, -1778A>G, -1578C>A, -1489G>A, -1456T>C, -1186G>C, -801G>T, -750T>C, -591A>G and -82T>C) are positioned in the promoter region whereas the remaining 5 (64C>T, 516G>T, 777C>A, 785A>G and 1459C>T) lead to amino acid changes in the coding sequence. Three different kinds of phenotypic data are available for the examined liver samples: CYP2B6 mRNA content quantitated by TaqMan RT-PCR, protein content determined by Western Blotting, and CYP2B6 activity, measured by hydroxylation of the specific probe drug bupropion in liver microsomes. A haplotype analysis was conducted using the software package Arlequin[3]. Twentyone different haplotypes were calculated, and strong linkage disequilibrium between promoter SNPs (-1456T>C, -750T>C) and non-conservative exonic mutations (516G>T, 785A>G) was observed.

Our population sample was fragmented into more than 20 inferred genotypes which comprized between 1 and 12 individuals. Linkage between promoter- and exonic mutations could give a possible explanation for significantly decreased CYP2B6 protein content associated with some of the previously described alleles[2]. However, by analyzing expression data between different genotypes using ANOVA no statistically significant relationships were found, although there were marked differences between groups. This result is probably due to the small number of individuals in each group. It appears, therefore, that in the case of highly polymorphic genes like CYP2B6 complete haplotype analysis may lead into a statistical trap. For reasons of practicability it may be necessary to reduce the number of subgroups by merging functionally equivalent genotypes for genotype-phenotype correlation analysis.

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TP53 HIGH-LEVEL EXPRESSION OF PORCINE P450 REDUCTASE SOLUBILIZED DOMAIN IN *ESCHERICHIA COLI* BY MODULATING THE LOCAL SECONDARY STRUCTURE OF MRNA

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The high-level expression of heterologous protein in *E. coli* cells is necessary for *in vitro* study of the protein using site-directed mutagenesis. However, the expression of the protein depends on the individual protein, and various problems have to be often overcome to realize its high-level expression. In this study, direct expression plasmids for the solubilized domains of NADPH-cytochrome P450 reductase (sCPR) from porcine (PsCPR) and rat (RsCPR) were constructed using pCWori^{+}[1] in a similar manner previously described.[2,3] PsCPR was minimally expressed, whereas RsCPR was highly expressed. The substitution of the nucleotides encoding $Thr^{60}Ser^{61}Ser^{62}$ in PsCPR with those of $Ala^{60}Pro^{61}Pro^{62}$ in RsCPR markedly increased the expression level. The local secondary structures of mRNA predicted with a program GeneBee (http://www.genebee.msu.su) suggested that the intramolecular base-pairing at the ribosome binding site (RBS) in mRNA affected the protein expression. Silent mutations were systematically introduced into the codons of $Thr^{60}Ser^{61}$ in PsCPR to modulate the base-pairing. The expression levels of the silently mutated PsCPRs depended on the predicted local secondary structures of mRNA at RBS. The wild-type PsCPR, in which $Thr^{60}Ser^{61}$ was encoded by ACTTCT, was purified. The purification yield was 45.8 mg/liter of culture fluid. These results suggest that the introduction of silent mutation into the N-terminal region based on the local secondary structure prediction of mRNA at RBS is a useful approach to control the expression level of heterologous protein in *E. coli* cells.

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TP54 INTERACTION OF CONSTITUTIVE ANDROSTANE RECEPTOR-RETINOIC X RECEPTOR (CAR-RXR) HETERODIMERS WITH ELEMENTS OF THE CYP2B2 PHENOBARBITAL RESPONSE UNIT

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Hepatic cytochrome P450s (CYPs) play a critical role in the metabolism of hydrophobic xenobiotics and many are also selectively inducible by xenobiotics. Much progress has recently been made toward understanding the molecular mechanisms underlying phenobarbital (PB) inducibility of the homologous rat *CYP2B1* and *CYP2B2* genes, the mouse *Cyp2b10* gene and the chicken *CYP2H1* gene. A multicomponent 163-base pair enhancer in the 5' flank of the rat CYP2B2 gene confers PB inducibility on homologous and heterologous promoters and constitutes a PB response unit (PBRU)[1,2]. The PBRU contains three putative nuclear receptor (NR) binding sites, NR1[3], NR2[3] and NR3[4]. Within the PBRU there is a 51-base pair PB response enhancer module (PBREM) which contains the the NR1 an NR2 sites[3]. When the PBREM is placed directly adjacent to the heterologous *tk* promoter it confers maximal PB responsiveness[3], but in the natural sequence context the full PBRU is required to confer a maximal response[5]. The constitutive androstane receptor (CAR), in the form of a heterodimer with the retinoid X receptor (RXR), binds to the retinoic acid ß2 response element (ß-RARE)[6] and to the NR1, NR2 and NR3 sites of the PBRU[4,7] and activates NR1-driven transcription of reporter genes in cell lines[4]. CAR-RXR binding to the NR sites is thought to play

a key role in conferring PB responsiveness[8]. Individual base pairs of the NR sites were changed and the effects on CAR-RXR binding, as determined by gel shift analyses, were assessed. The CAR and RXR proteins were synthesized from expression vectors using coupled in vitro transcription and translation. The same mutant sequences were incorporated into the NR sites within the PBRU and their effects on conferring PB responsiveness in luciferase reporter gene assays after transfection into primary rat hepatocytes were evaluated. The results suggest that something other than, or in addition to, CAR-RXR binding to the NR sites is required for maximal PB responsiveness.

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TP55 THE ANTICANCER DRUG ELLIPTICINE ACTS AS AN INDUCER OF CYTOCHROMES P450 1A1/2 AND POTENTIATES ITS OWN PHARMACOLOGICAL EFFICIENCY

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Ellipticine is an alkaloid exhibiting significant antineoplastic and anti-HIV activities. Cytochromes P450 (CYPs) are believed to be the major enzymes catalyzing the metabolism of ellipticine. We described that CYP-dependent metabolism of ellipticine leads to activation of this agent to more efficient metabolite(s) forming DNA adducts[1,2]. This implies the importance of several CYPs in producing these more active antitumor metabolite(s). The aim of the present work is to study whether ellipticine could influence the expression of the major CYPs oxidizing this drug.

In order to investigate the induction of cytochromes P450 by ellipticine, Wistar male and female rats, suitable animal models mimicking the ellipticine activation in humans[3], were treated with 4, 40 and 80 mg ellipticine per kg. Because ellipticine was found to bind to the Ah receptor, which is known to be responsible for the induction of several enzymes including CYP1A1/2, we examined the expression levels of these CYPs in rat livers. In addition, the effect of ellipticine on expression of the CYP2B1/2, 3A1/2 and 2E1 enzymes was investigated. Selective antibodies against rat CYP1A1 and 3A1 and rabbit CYP2B4 and 2E1 were utilized for this study. The expression of CYP1A1/2 proteins in rat livers of both sexes was strongly induced ellipticine. The expression levels of these enzymes in treated rats are more than one order of magnitude higher than those in control animals. In analog, the increase of CYP1A1/2 expression correlates with an increased EROD activity, a marker for CYP1A1/2, and with that of MROD, a marker for CYP1A2, and with the oxidation of Sudan I, a marker for the CYP1A1 activity. The CYP1A1/2 induction is strongly dependent on the dose of ellipticine administered to the rats. The induction is transient, i.e. in the absence of ellipticine, the amount and activity of the induced CYP1A1/2 decreased to the basal level two weeks after exposure.

The results indicate that a long-term treatment of humans with ellipticine might stimulate its pharmacological efficiency against cancer diseases, if the CYP induction also occurs in the target organs of therapy, e.g. the breast.

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TP56 NEWLY-IDENTIFIED TRANSCRIPTION FACTOR BINDING SITES IN THE RAT CYP2B2 PHENOBARBITAL RESPONSE UNIT : HNF4 AND PBX-PREP1

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The *CYP2B1* and *CYP2B2* genes are dramatically induced in rat liver by phenobarbital (PB). A multicomponent enhancer confering PB responsiveness, the PB response unit (PBRU), is located between -2317 bp and -2155 bp in the *CYP2B2* 5' flanking region[1,2]. The PBRU contains three nuclear response elements (NR1, NR2 and NR3) that contribute to conferring PB responsiveness, plus other elements including an HNF4 binding site detected in this study. Mutational analysis of the HNF4 element was undertaken, using a reporter construct with the PBRU in the natural sequence context transfected into primary rat hepatocytes. The results suggest that HNF4 acts as a negative regulator by competing for binding to the NR1A half site thus leading to obstruction of the nuclear receptor or other activator that is presumed to activate transcripton via the NR1 element. Electrophoretic mobilty shift analysis (EMSA) and super shift analysis of the effects of mutant sequences on HNF4 binding revealed that HNF4 binds to the 5'-AGTACAGAGTCTGTG-3' sequence of the PBRU. At the NR2 site, mutation of the 4-bp spacer led to the loss of a prominent doublet in EMSA assays. Inspection of the NR2 spacer and NR2B half site revealed the presence of a putative binding site for Pbx-Prep heterodimers[3]. EMSA analysis using the wild type NR2 labeled oligo and antibody supershift experiments were thus undertaken to identify the proteins responsible for the characteristic doublet. The proteins were found to be Pbx1b-Prep1 and Pbx2-Prep1 heterodimers. This conclusion was confirmed by coupled in vitro transcription/translation analyses. The Pbx-Prep binding site was defined as 5'-TGACTGACAC-3'; it overlaps the NR2 spacer and the NR2B half site. The possible roles of HNF4 and PBx-Prep1 as negative regulators of PB responsiveness are presently under investigation.

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TP57 RABBIT CYP4B1 ENGINEERED FOR HIGH-LEVEL EXPRESSION IN *E. COLI*: LIGAND STABILIZATION AND PROCESSING OF THE N-TERMINUS AND HEME PROSTHETIC GROUP

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CYP4B1 is a largely extrahepatic form of cytochrome P450 that bioactivates numerous xenobiotics including 4-ipomeanol, 3-methylindole and several polyaromatic amines. CYP4B1 is also interesting from a structural perspective because this isoform, along with several others in the CYP4 family, possesses a covalently-bound heme prosthetic group. The unique environment of the CYP4B1 active site coupled to its proclivity for xenobiotic bioactivation make CYP4B1 an especially interesting target for detailed structural studies that will inevitably require significant re-engineering of the enzyme. Therefore, the full-length, wild-type, rabbit *CYP4B1* and three N-terminally modified genes were cloned into the pCWori+ vector and expression studies were initiated in *E. coli*. No spectrally detectable enzyme was observed from expression of the native sequence. However, incorporation of MALLLAVF as the first 8 residues (pCW4B1#1) or truncation of 17 residues from the N-terminus (pCW4B1#7) provided > 450 nmoles of membrane-bound, holo-CYP4B1 per liter culture. A third construct, in which the MALLLAVF sequence was aligned at amino acid residues 14-21 (pCW4B1#3), severely inhibited bacterial cell growth, resulting in very low yields of the expressed holo-enzyme. CYP4B1#1 and CYP4B1#7 were isolated in high purity following nickel-affinity and hydroxyapatite chromatography. Solubilization from bacterial membranes led to substantial converision to P420 which could be prevented by the addition of α-naphthoflavone as a stabilizing ligand. Mass spectrometry analysis and Edman sequencing revealed evidence of differential N-terminal post-translational processing of CYP4B1#1 and CYP4B1#7. Furthermore, both enzymes were found to covalently bind greater than 99.5% of their heme prosthetic group. The fully covalently-linked hemoproteins exhibited similar rates and regioselectivities of lauric acid hydroxylation to that observed previously for the partially heme processed enzyme expressed in insect cells. These studies establish conditions for the facile, high level expression of CYP4B1 in E. coli and demonstrate that the fully, heme-covalently linked enzyme is catalytically active.

TP58 TRANSCRIPTIONAL REGULATION OF THE CHOLESTEROL BIOSYNTHETIC PATHWAY IN THE MALE GONAD

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Lanosterol 14α-demethylase (CYP51) is the only cytochrome P450 involved in the housekeeping pathway of cholesterol biosynthesis. It is regulated at the level of transcription by transcription factors of the sterol regulatory element-binding protein (SREBP) family, in a similar manner as other cholesterogenic genes. Expression of cholesterogenic genes in somatic cells generally depends on cholesterol levels. In contrast to somatic cells, where the goal is to produce cholesterol, it was proposed that SREBPs do not play a significant role in transcriptional regulation in male germ cells, where cholesterol biosynthesis intermediates with signalling properties (sterols MAS) accumulate and the cAMP-dependent signalling cascade predominates. Male germ cells do not grow in a primary culture, which is a big limitation in experimental approaches. We have thus developed an *ex vivo* system, with the aim to simulate the cholesterol biosynthesis conditions in the testis. The level of transcription factors SREBP in human choriocarcinoma cell line JEG-3 was reduced by growing cells in cholesterol/fatty acid rich media. The trans-activation by the cAMP-dependent pathway was achieved by overexpressing transcription factor CREB. The simulation was only partially successful. cAMP-stimulation in cholesterol-repressed conditions lead to a decrease in cholesterol quantity, which is in accordance with metabolism of male germ cells that are not efficient in producing cholesterol *de novo*. In contrary to expectations, activation of the cAMP-dependent pathway did not result in a change of sterol composition and quantity. Recently, a soluble, 55 kDa cholesterol-insensitive form of SREBP2 (SREBP2gc) was discovered in male germ cells, which opened a new venue in understanding biochemical processes in the male gonad. To explore this issue, we have measured the level of SREBP proteins has in male germ cells of fasted and normally fed mice. Interestingly, three SREBP2-immunoreactive proteins (72, 63 and 55 kDa), that are not present in mouse liver nuclei, have been observed. The 55 kDa protein is likely the previously identified SREBP2gc, while the other two SREBP2 isoforms are novel and seem also to be insensitive to the level of cholesterol. The role of cholesterol-independent forms of SREBP2 on transcriptional regulation of CYP51 is currently investigated. It seems possible that cholesterol-independent forms of SREBPs together with cAMP-dependent stimuli, contribute to accumulation of cholesterogenic intermediates, the signalling sterols MAS.

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TP59 CONSTITUTIVE ANDROSTANE RECEPTOR-RETINOIC X RECEPTOR (CAR-RXR)-DEPENDENT TRANSCRIPTIONAL ACTIVATION BY THE CYP2B2 PHENOBARBITAL RESPONSE UNIT VARIES DRAMATICALLY WITH DIFFERENT REPORTER CONSTRUCTS

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Hepatic cytochrome P450s play a key role in xenobiotic metabolism. Some forms are expressed constitutively and others are inducible. In rat liver, phenobarbital (PB), well known for inducing CYP2B forms, produces a strong and rapid transcriptional activation of the *CYP2B1* et *CYP2B2* genes. A PB response unit (PBRU) is localized about 2.2 kb upstream of the *CYP2B2* transcriptional start site[1,2]. The PBRU confers PB responsiveness on the *CYP2B2* gene as well as on reporter constructs and possesses the characteristics of a multicomponent transcriptional enhancer[2]. The PBRU contains three putative nuclear receptor (NR) binding sites, NR1[3], NR2[3] and NR3[4]. The constitutive androstane receptor (CAR), is required for PB responsiveness *in vivo*[5] and, in the form of a heterodimer with the retinoid X receptor (RXR), binds to the the NR1, NR2 and NR3 sites of the PBRU and activates NR1-driven transcription of reporter genes in cell lines. The glucocorticoid receptor interacting protein 1 (GRIP1) mediates PB-dependent nuclear translocation and activation of CAR *in vivo* but has been reported to have only modest effects *in vitro*[6]. Furthermore, normal PB responsiveness of *CYP2B* genes is not maintained in any known cell line. In an effort to understand the molecular mechanisms whereby PB induces *CYP2B* genes *in vivo* but not in cultured cells of hepatic origin, expression vectors for CAR, RXR and GRIP1 were cotransfected into HepG2 cells along with luciferase reporter constructs carrying the PBRU in different sequence contexts. CAR activated reporter gene transcription when the PBRU was juxtaposed to a basal promoter, but was essentially inactive when the PBRU was tested in the natural sequence context. GRIP1 dramatically inc-

reased CAR activation when tested with reporter constructs in which the PBRU was adjacent to the basal promoter and also, but to a much lesser degree, when the PBRU was in its natural sequence context. It may be that HepG2 cells lack as yet unidentified transcription factors that are necessary, in the natural sequence context, for the PBRU to confer PB responsiveness on the luciferase reporter.

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TP60 EXPRESSION OF MAMMALIAN MICROSOMAL P450S IN PLANTS USING PLANT VIRUS VECTORS

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The P450 enzymes play important roles in plant physiology. The functional genomic and proteomic analysis of both known and newly discovered plant P450s should rely on a system for rapid and high-level production of these proteins in plants. To evaluate the potential of plant viral vectors for these purpose human CYP1A2 and rabbit CYP2B4 (both HIS-tagged) were expressed using potatovirus X (pVX201) and tobacco-mosaic (TMV70) vectors. The transfected *N.bentamiana* and *N.clevelandii* plants showed accumulation of CYP1A2 and CYP2B4 in microsomal fractions and displayed increased 7-MROD and 7-PROD activities respectively. The TMV-based vector had additional advantages of increased expression level, insert stability and decreased chlorosis as compared to pVX201 vector. This data justifies the production of functional-active recombinant P450s in plants using plant viral vectors.

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TP61 EXPRESSION OF LEUKOTRIENE B4 ω-HYDROXYLASE IN HUMAN LEUKOCYTE

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Leukotriene B_4 (LTB_4), a product of the 5-lipoxygenase-dependent arachidonic acid metabolism, plays a role as the most powerful mediator of inflammation. In human neutrophils, LTB_4 is rapidly converted into biologically less active products by the ω-oxidation pathway. The LTB_4 ω-hydroxylase, which catalyzes the first step of this pathway, is classified as the CYP4F subfamily. Five CYP4F isoenzymes have been found in human tissues. CYP4F3, an enzyme in human neutrophils, catalyzes ω-hydroxylations of various eicosanoids and related compounds as well as LTB_4. However, the *K*m value for LTB_4 (0.64 μM) is much lower than those for other substrates. Although LTB4 ω-hydroxylase was found in human neutrophils first, the expression of CYP4F isoenzymes in other leukocytes was not investigated well. In this study, we have analyzed the expression of CYP4F enzyme in human peripheral leukocytes and human myeloid leukemia cell line HL60 by immunocytochemistry.

Homogenates of the neutrophil lich fraction of human leukocytes catalyzed LTB_4 ω-hydroxylation activity (23.4 pmol/min/mg protein), and those of the monocyte lich fraction showed 11 times lower activity (2.1 pmol/min/mg protein). No activity was detected in the lymphocyte lich fraction. However, neutrophils contaminated into monocyte fraction can catalyze this activity. Then the expression of the CYP4F enzymes in human leukocytes was examined by immunocytochemistry. Western blot analysis with the antibody against CYP4F3 peptide showed the presence of a protein with the same apparent molecular weight as CYP4F3 in human neutrophil microsomes. When this antibody was used for the immunocytochemistry, fluorescence was observed in the cytoplasm of human leukocytes. Most of these positive cells had segmented nuclei. The double staining with anti-CD14 and anti-CYP4F3 antibodies showed that one third of CD14 positive cells, monocytes, were also stained by anti-CYP4F3 antibody. HL60 cells showed no LTB_4 ω-hydroxylase activity, and the cells stimulated by

all-*trans*-retinoic acid (RA) for 6 days catalyzed the LTB_4 ω-hydroxylation. HL60 cells stimulated by TPA for 6 days also showed the slight activity. By immunocytochemistry, fluorescence was observed in the cytoplasm of the HL60 cells stimulated by RA or TPA for 4 and 6 days.

TP62 NR3 AND ER-7 NUCLEAR RECEPTOR SITES OF THE CYP2B2 PHENOBARBITAL RESPONSE UNIT: MUTATIONAL ANALYSIS OF THEIR IMPORTANCE FOR PB-DEPENDENT TRANSCRIPTIONAL ACTIVATION

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Hepatic CYPs play a critical role in the metabolism of hydrophobic xenobiotics and certain forms are selectively inducible. CYP2B1 and CYP2B2 are dramatically induced by phenobarbital (PB) in rat liver. The *CYP2B2* enhancer, the PB response unit (PBRU), is situated between 2317 and 2155 base pairs upstream of the transcription start site[1,2]. The PBRU contains at least two elements essential for conferring PB responsiveness, NR1 and NR23. They are DR-4 elements located in the PB responsive enhancer module (PBREM), a 51-base pair sequence within the PBRU[3]. When the PBRU or the PBREM is placed directly adjacent to the basal *tk* promoter in reporter gene constructs there is no difference in their ability to confer PB reponsiveness in cultured primary hepatocytes[3,4]. However, when both are tested in the natural sequence context, the PB response conferred by the PBRU is at least four-fold higher than that of the PBREM[4]. These results and others[4] suggest the existence of a third sequence element in the PBRU required for maximal PB responsiveness. Two candidates for this third element are known, ER-7[2] and NR3[5], which are putative nuclear receptor sites that share a half site. Mutation of their shared half site reduces but does not abolish PB responsiveness, as does mutation of any half site of NR1 or NR2[4]. To determine which of NR3 or ER-7 are involved in conferring PB responsiveness, their unique half sites were mutated within the PBRU. The mutated plasmid reporter constructs were then tested in the natural sequence context for their ability to confer PB responsiveness in cultured primary rat hepatocytes. When the NR3 half site not shared with ER-7 was mutated, PB responsiveness was reduced but not abolished, and when either NR3 half site mutation was combined with a half site mutation of NR1 or NR2, PB responsiveness was essentially abolished. On the other hand, mutation of the ER-7 half site not shared with NR3 did not reduce PB responsiveness. Taken together, these results indicate that NR3 is the third element within the PBRU that is required to confer maximal PB responsiveness. The constitutive androstane receptor (CAR), in the form of a heterodimer with the retinoid X receptor (RXR), binds to the retinoic acid ß2 response element (ß-RARE) and to the NR1, NR2 and NR3 sites of the PBRU and activates NR1-driven transcription of reporter genes in cell lines. CAR-RXR binding to the NR sites is thought to play a key role in conferring PB responsiveness via binding to NR1[6,7]. Given that NR3 half sites are identical to those of NR1 except for a single base pair difference[5], it might be supposed that if CAR-RXR binds to NR1 to activate transcription, it would also bind to NR3. To determine if a high affinity CAR-RXR site could substitute for NR3, the NR3 site was converted into ß-RARE. The PB responsiveness conferred by this construct was similar to that conferred by mutational inactivation of either NR3 half site. Thus, as for NR1[4], ß-RARE cannot functionally replace NR3. In conclusion, NR3 is the third element required for conferring maximal PB responsiveness but the receptor which activates it remains to be discovered.

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TP63 CHARACTERIZATION OF CYTOCHROME P-450 26 (CYP26) ENZYMATIC ACTIVITY AND COMPARISON WITH OTHER CYPS INVOLVED IN ALL-TRANS-RETINOIC ACID (ATRA) METABOLISM

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Tumors are characterized by an abnormal cell multiplication accompanied by a loss of certain capacities of cellular differentiation. The anti-proliferative and pro-differentiating activities of retinoids, and in particular those of all-*trans*-retinoic acid (ATRA), have stimulated research towards their use as chemo-preventive and antitumor drugs. Human cytochrome P-450s (CYPs) 3A7, 1A1, 2C8, 4A11, and 2C9 have been identified as the most active CYPs involved in the metabolism of ATRA[1] and its isomers[2]. In addition to these CYPs, the novel CYP26 has been shown to be inducible by ATRA and to selectively oxidize this molecule to several metabolites. Since no activity comparison has been carried out so far between the principal CYPs involved in ATRA metabolism, the purpose of this work was to compare CYP26 activity to the other CYPs. We first stably transfected the *CYP26* gene into HEK293 cells. Of the various clones that showed ATRA metabolism, clones 293-26-15 and 293-26-2 were selected because they showed the best activity. Microsomes were prepared with cells from clones 293-26-15, and ATRA (10 μM) metabolism was carried out using 600 μg of protein/ml in a total volume of 500 μl, for a 1 h incubation period at 37 °C (NADPH, 1 mM). In these conditions, 6 metabolites were formed, 4 of which were identified as the 4-oxo-RA, the 4-OH-RA, the 18-OH-RA and the 5,6-epoxy-RA. Their relative rate of formation were as follows: 5.7 ± 4.4 nmol /24 h/mg protein for the 4-oxo-RA; 46.7 ± 6.2 nmol /24 h/mg protein for the 4-OH-RA; 2.2 ± 0.1 nmol /24 h/mg protein for the 18-OH-RA; and, 1.5 ± 0.4 nmol /24 h/mg protein for the 5,6-epoxy-RA. Compared to CYP3A7 which was found the most active until now in the oxidation of ATRA, CYP26 activity was at least 10-fold more active (CYP3A7: 0.6 nmol /24 h/mg protein for the 4-oxo-RA; 1.7 nmol /24 h/mg protein for the 4-OH-RA; 0.03 nmol /24 h/mg protein for the 18-OH-RA). In addition to the difference in activity, a qualitative difference was also noted in the formation of 2 novel metabolites formed exclusively by CYP26, and not by CYPs 3A7, 2C8, or 1A1.

Given the importance of the nature of the ligand to elicit specific biological responses to retinoids, it now appears possible to consider that CYPs probably play a role in the fine regulation of the nuclear retinoic acid receptors activation by modulating the biological properties and the quantity of ligands available.

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TP64 DISTRIBUTION OF GLUTATHIONE S-TRANSFERASES IN MULLET (*LIZA SALIENS*)

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Glutathione S-transferases (GST) are phase II biotransformation enzymes influenced by exposure to foreign compounds and are proposed as biomarkers of environmental pollution[1,2]. They catalyze the conjugation of glutathione to a great variety of xenobiotic compounds[3]. The distribution of GSTs in any tissue or cell type may be one of the determinants of its susceptibility to carcinogenesis[4]. In this study, the distribution of GSTs in various leaping mullet (*Liza saliens*) tissues has been investigated with the use of 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid (EA) as substrates. *Figure 1* shows the distribution of GST activity assessed by CDNB and EA in various leaping mul-

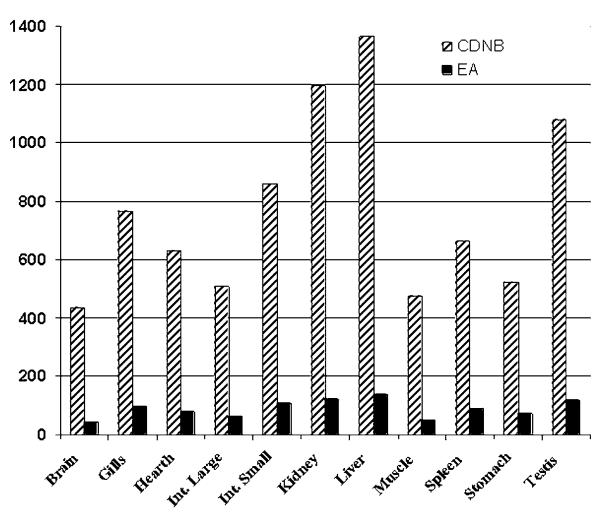

Fig. 1.: *Distribution of GST activities in leaping mullet tissues assessed by CDNB and EA*

let tissues. The maximum activity was found to be present in liver followed by kidney, testis and intestine with lower activities. These results suggested that both total GST and GST-π isozyme showed similar distribution pattern in mullet tissues. In addition, since GSTs play important role in protection against various toxic chemicals and environmental pollutants, we have studied the effects of various metals such as Hg, Zn, Cd, Sb, Cu, Co, Ni, Mn, Fe, K, Li, Ba, Na, Al and some detergents such as Brij 35, CHAPS, Cholate, Deoxycholate, Emulgen 913, Lubrol, SDS, Triton X-100, Tween 20 on mullet liver GSTs activities. *In vitro* incubations of fish liver cytosols with various concentrations of these metals and detergents showed that GST metabolism of both CDNB and EA in leaping mullet are sensitive to divalent cations and ionic detergents.

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TP65 BIOCHEMICAL, MOLECULAR GENETIC AND ENVIRONMENTAL ASPECTS OF MULLET (*LIZA SALIENS*) CYP1A1

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The CYP1A1 gene encodes microsomal cytochrome P4501A1 that plays important roles in the metabolism, detoxification and bioactivation of carcinogens and other xenobiotics, including polycyclic aromatic hydrocarbons (PAH)[1]. It is one of the most widely studied CYP because it metabolizes a large number of cytotoxic and/or mutagenic xenobiotics. Induction of CYP1A1 enhances the metabolism of those chemicals, and therefore, represents an adaptive response of cells to changes in their chemical environments[2]. We have obtained P4501A1 in a highly purified form with a specific content of 17 nmol/mg protein from liver microsomes of feral fish, leaping mullet (*Liza saliens*) captured from Izmir Bay on the Aegean coast of Turkey[3]. Purified mullet CYP1A1 showed very high substrate specificities for 7-ethoxyresorufin and 7-methoxyresorufin in a reconstituted system containing purified fish P450 reductase and lipid. In addition, EROD activity was strongly inhibited by a-naphthoflavone. Mullet CYP1A1 did not catalyze monooxygenations of other substrates such as aniline, ethylmorphine, N-nitrosodimethylamine and p-nitrophenol. Antibodies produced against CYP1A1 orthologues in fish such as trout and scup showed strong cross-reactivity with the purified mullet CYP1A1. Anti-mullet CYP1A1 antibodies showed very weak cross-reactivity with two proteins (presumably CYP1A1 and CYP1A2) in 3MC-treated rat liver microsomes. Moreover, 3MC-treated rat liver microsomal EROD activity was weakly inhibited by the anti-L. saliens liver CYP1A1. In addition to biochemical studies with purified mullet CYP1A1, a 2,037 bp CYP1A1 cDNA (GenBank AF072899) was cloned through screening of a cDNA library constructed from leaping mullet using rainbow trout CYP1A1 cDNA as a probe. This clone has a 1,563 bp open reading frame (ORF) encoding a 521-amino acid protein (58,972 Da)[4]. Alignment of the deduced amino acids of CYP1A1 cDNAs showed 58% and 69-96% identities with human and 12 other fish species, respectively. Southern blot analysis suggested that this CYP1A1 cDNA was from a single copy gene. The leaping mullet CYP1A1 gene is probably split into 7 exons. Alignment of the CYP1A1 cDNA encoded amino acids from 13 fish and 7 mammalian species disclosed differences in highly conserved amino acids between aquatic and land vertebrates. These results strongly suggested that the purified mullet CYP1A1 is structurally, functionally and immunochemically similar to the CYP1A1 homologues of other teleost species but functionally and immunochemically distinct from mammalian CYP1A1. Furthermore, induction of P4501A1-associated EROD activity of cytochrome P4501A in leaping mullet were used as biomarker for the assessment of organic pollutants around the Izmir on the Aegean Sea coast, Turkey. Fish were captured in February and May 2002 from four different sites, namely Pasaport, Foca, Cesme and Mordogan. The results indicated that these sites are highly contaminated with PAH and/or PCB type organic pollutants.

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T66 ALBENDAZOLE INDUCED CYP1A ACTIVITIES IN MOUFLONS (*OVIS MUSIMON*)

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Albendazole (ABZ) belongs to anthelmintics long-term and frequently used in veterinary therapy. In mammals ABZ undergoes a two-step sulphoxidation resulting firstly in formation of biologically active ABZ sulphoxide followed by formation of inactive ABZ sulphone. CYP3A (together with FMO) takes part in first oxidation while second oxidation is mediated by CYP1A[e.g.1]. In several species ABZ and its metabolites cause induction of CYPs, mainly CYP1A[e.g.2]. As the mouflons, food-producing animals used as farm and game species, are treated with ABZ often, the effect of ABZ on mouflon biotransformation enzymes was tested *in vivo*.

Adult mouflon ewes from game enclosure Vlkov (Czech republic) were divided in two groups: first group (5 animals) was treated repeatedly by therapeutic doses of ABZ (orally individually, in suspension, 5 x 7.5 mg/kg of body weight), second group (3 animals) represented controls. 24 hours after the termination of experimental treatment all animals were culled. Whole liver and proximal small intestine (1.5 m from stomach) were removed immediately and stored in liquid nitrogen during transport to laboratory. Microsomes were prepared from homogenates of both tissues of individual animals.

Activities of 7-ethoxyresorufin-O-deethylase (EROD), 7-methoxyresorufin-O-demethylase (MROD) (specific for CYP1A in rat, human), 6(-testosterone hydroxylase (specific for human CYP3A), 7-methoxy-4-trifluoromethyl-coumarin demethylase (specific for human CYP2C9) and p-nitrophenol-UDP-glucuronosyl transferase were measured in microsomes.

Significant increase (4-7 times) of CYP1A corresponding activities (EROD and MROD) was found in liver microsomes from ABZ treated animals comparing to control animals. ABZ treatment also caused significant increase of EROD activities (6-10 times) in intestinal microsomes (see Figure). All other activities tested did not significantly differ in ABZ treated and control animals. Increase of CYP1A proteins by ABZ treatment of mouflon was confirmed by Western blotting. With respect to our results, ABZ (in dosage scheme used) induces CYP1A in mouflon. Induction of CYP1A could result in increase of ABZ deactivation and could contribute to rise of parasites resistance.

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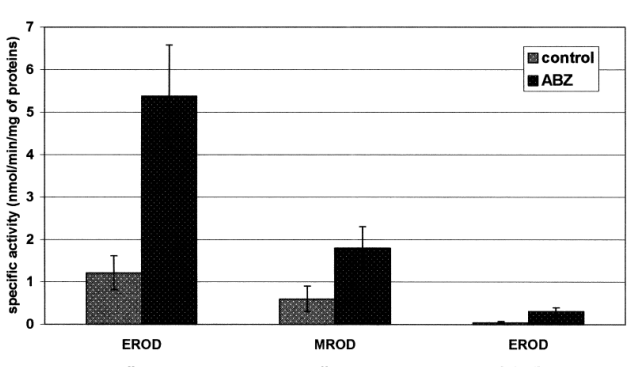

Fig. 1.

TP67 CYTOCHROME P450 3A4 EXPRESSION IS REDUCED IN THE PRESENCE OF CANCER-ASSOCIATED INFLAMMATION IN A HUMANISED CYP3A4 TRANSGENIC MOUSE MODEL

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Cytochrome P450 (CYP) enzyme activity, including that of CYP3A4, is reduced in the presence of various inflammatory states in man[1]. Pro-inflammatory cytokines, such as interleukin 6 (IL-6), have been shown to transcriptionally down-regulate CYP3A4[2]. CYP3A4 activity is also reduced in cancer patients as compared to healthy volunteers[3] and activity declines as a function of the acute-phase response as measured by serum C-reactive protein in patients with cancer[4]. It is important to understand, the mechanism of this interaction as reduced CYP3A4 activity may compromise the use of anti-cancer drugs due to their narrow therapeutic index. Therefore, we sought a suitable animal model for the investigation of the effect of cancer on the expression of human CYP3A4. Traditional rodent models are not ideal because of significant species differences in the activity and regulation of *CYP3A* genes. Instead, a tumour-bearing transgenic mouse model was developed. Briefly, suspensions of Engelbreth-Holm-Swarm sarcoma cells were injected i.m. into male mice harbouring a transgene construct comprising the 13kb upstream regulatory region of the *CYP3A4* gene linked to the *lacZ* reporter gene. This transgenic model has been validated using known inducers of the *CYP3A4* gene. Expression of the *CYP3A4/lacZ* transgene was measured by both X-gal staining of liver slices and assay of β-galactosidase activity in liver extracts. The expression of a mouse CYP3A4 homolog, *Cyp3a11*, was measured by real-time PCR following reverse transcription of hepatic mRNA. Serum IL-6 and TNFα levels were measured by commercially available ELISA kits. Comparison between control and tumour animals was performed using Mann-Whitney U test and p = 0.05 set for significance. Preliminary results show that tumour-bearing mice (tumour weight approximately 10% whole body weight) have reduced *CYP3A4/lacZ* transgene expression as determined by X-gal staining of liver wedges. β-galactosidase activity was reduced by 50% in tumour-bearing animals compared to saline-treated controls (p = 0.025). Furthermore, there was a corresponding 60% repression of mouse *Cyp3a11* gene expression in the presence of the tumour as compared to controls (p = 0.014). In addition, serum IL-6 concentrations were elevated from undetectable levels in controls to 25.45 +/- 3.76 pg/ml in tumour-bearing mice compared to controls (0 +/- 0 pg/mL vs 25.45 +/- 3.76 pg/mL [median +/- SEM], p = 0.002). In contrast, all animals had serum TNF_ concentrations below the limit of assay detection (23 pg/mL). Further experiments investigating the time course of the reduction in CYP3A4/11 activity are currently underway. The implications of these results suggest that the pharmacokinetics, and hence the pharmacodynamics, of the majority of drugs used to treat malignancies could be altered in the presence of cancer and associated inflammation.

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TP68 ACTIVITY OF CYTOCHROMES P450 2C IN RAT CARDIOMYOCYTES

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The function of cytochromes P450 in the heart is speculated to be in biosynthesis of mediator molecules from endogenous precursors[1,2]. We focused on the presence and activity of enzymes of the CYP2C subfamily, which are known to be involved in formation of derivatives of arachidonic acid[2].

The cardiomyocytes of the newborn rat were prepared by trypsin digestion; the cells were either cultured or used in a suspension of freshly isolated cells. The presence of CYP2C enzymes was studied by checking their enzyme activity (hydroxylation of a prototypical substrate, diclofenac). The results show that the activity of the CYP2C is clearly present both in the freshly prepared cells as well as in the samples from cultured cardiomyocytes.

Acknowledgment

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TP69 METABOLISM OF α-NAPHTHOFLAVONE AND ITS THIO-ANALOGUE BY MICROSOMAL SYSTEM

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Synthetic and naturally occurring flavonoids are effective inhibitors of several CYPs, e.g. CYP1A1, 1A2, 1B1, 3A4 and CYP19. In addition, certain metabolic activities of CYP3A4 and 1A2 were also stimulated by some compounds of flavonoid structure. Synthetic 7,8-benzoflavone (ANF) is an inhibitor of human CYP19, CYP1A1 and 1A2, but an activator of CYP3A4. Moreover, a newly synthesized thio-analogue of ANF (containing sulphur in flavonoid skeleton instead of C4 oxo-group) (SANF) is also an activator of CYP19. Modulation of CYP activities by flavonoids has been extensively studied because of their potential use as agents blocking the initiation stage of carcinogenesis. However, much less is known about their metabolism. Microsomes of rifampicine-treated rabbit were used as a model system to study of ANF and SANF metabolism. They contain high levels of CYP3A6 which is expected to be the major CYP present in intestinals and is in contact with flavonoids ingested. Using RP-HPLC combined with MALDI-TOF, 5,6-dihydrodiol, 7,8-dihydrodiol and 5,6-epoxid of ANF were determined as the major ANF metabolites. On the other hand, SANF is not metabolized into any hydroxy-derivatives in the microsomal system used. This compound is converted to ANF first and then ANF formed in this reaction undergoes its regular metabolism. Role of other CYPs in the metabolic conversion of both flavonoids is underway in our laboratory.

The work was supported by the grants 523/01/0840 from The Grant Agency of The Czech Republic, and MSM-113100001 from The Czech Ministry of Education.

TP70 APPLICATION OF α-NAPHTHOFLAVONE DURING THE GROWTH AND DEVELOPMENT OF BROILERS

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The modulation of CYP19 (aromatase) activity might significantly change the estrone/testosterone ratio and consequently change the poultry sex. Inhibition of CYP19 activity or expression causes a serious hormonal status change. For example, complete ovariectomy of female chicken results in a phenotypic sex-reversal accompanied by the development of testoids[1]. Similarly, in ovo administration of Fadrazole (CYP19 inhibitor), elicited in the case of genotypic females, testes formation. In the late development of the treated animals, however, the sex is frequently reversed to the genetic one, including the development of related sex organs[2]. Hence, it is clear, that after a single treatment of embryos, inhibitors have to be applied repeatedly to the hatched chicken to maintain the established sex reversal. Since the CYP19 was also detected in males, namely in testes, administration of CYP19 inhibitors might be expected to change hormone status of cocks with the respect to enhanced testosterone production on the account of a blocked androstenedione conversion to estrone. α-naphthoflavone seems to be the best model inhibitor of CYP19 for naturally occurring flavonoids[3]. In our experiments we verified the influence of repeated postnatal oral administration of α-naphthoflavone to broilers on the growth rate and possible physiology changes of their body. Application of α-naphthoflavone within 42 days of the fattening period of broiler chickens resulted in statistically significant difference ($P<0.01$) in the weight of the testes (4.7g) in comparison to controls (1.2g). We did not find statistic difference between the size of the left and right testes. Moreover, spermatogenesis in the group where α-naphthoflavone was applied began very early - the 10th week and cocks in this group started to crew at 13 weeks of age, at least 4 weeks earlier than control group. Ovary in experimental group treated with α-naphthoflavone was heavier ($P<0.05$) in comparison to control groups. The beginning of laying period in the (-naphthoflavone treated hens started at least one week earlier comparing to the control. For male groups, there was no difference in livers weight between treated and untreated animals. In contrary, female groups, showed significant increase ($P<0.05$) in liver weight in experimental group treated with (-naphthoflavone. There was no statistically significant difference between

the growth rate of treated and untreated male or female chickens. By the carcass analysis of the live body weight, kidney, gizzard, heart, abdominal fat and breast muscle weight we also did not find any differences from control groups.

The work was supported by grants GACR - 523/01/0840 from Grant Agency of the Czech Republic and MSM-113100001 from The Czech Ministry of Education.

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7 POSTERS – WEDNESDAY – WP

WP01 MOLECULAR BREEDING OF CYTOCHROME P450 ENZYMES INVOLVED IN XENOBIOTIC METABOLISM

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DNA shuffling, a novel technique for molecular evolution *in vitro*, can be used in designing new enzymes with improved properties. Xenobiotic-metabolizing P450s are especially well suited as starting materials for creating better catalysts because of their wide substrate specificity. In the present study, a novel method for DNA shuffling was developed using appropriate restriction enzymes in the initial digestion step, followed by size-selective filtration for selection fragments under 300bp. P450s 2C9, 2C11 and 2C19 were subjected to a single round of shuffling and co-expressed with NADPH-cytochrome P450 reductase in *E. coli*. A sample (54 clones) of the resultant library was assessed for sequence diversity, hemo- and apoprotein expression, and activity towards the substrate indole. All mutants showed restriction fragment length polymorphism patterns that were different when compared to all parents, suggesting that the library was free from contamination by any parental forms. Preliminary sequencing of mutants confirmed that significant mixing of different P450s had been achieved. Significant hemoprotein expression was detectable in 27/54 (50%) of mutants and in a further 13/54 (24%) of sampled mutants, expression was marginal. Indigo production was less than or comparable to the activities of the parental P450s, but certain mutants showed a shift towards indirubin production. In conclusion, a method is presented for effective shuffling of P450 sequences to generate libraries of novel mutant P450s. The library generated using P450s 2C9, 2C11 and 2C19 showed significant diversity and was free from contamination by parental forms.

WP02 AFLATOXIN B1 BINDING TO MUTANT PHE209ALA CYP2A5 EXPRESSING SACCHAROMYCES CEREVISAE YEAST CELLS

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Introduction

Carcinogenic aflatoxin B_1 (AFB1) is metabolized to the AFB1 exo-8,9-epoxide which is a primary metabolite responsible for its toxic effects[1]. This metabolite is conjugated to inactive glutathione derivative, although it reacts also with DNA forming stable adducts or is hydrolyzed to AFB1 dihydrodiol. Dihydrodiol is in equilibrium with dialdehyde, which can react with protein or be reduced to monoaldehydes. CYP enzymes and CYP2A5 in mice catalyze the formation of AFB1 8,9-epoxide. When CYP2A5 or its mutants are expressed constitutively in S. Cerevisiae yeast cells and simultaneously exposed to AFB1, the viability of the cells is decreased corresponding with the ability of CYP mutant to produce AFB1 8,9-epoxide[2]. Especially the mutant Phe209Ala CYP2A5 efficiently bioactivates AFB1. This study aimed at investigating (1) the effect of AFB1 concentration and exposure time on the viability of Phe209Ala CYP2A5 mutant expressing yeast cells (mutant CYP2A5) and (2) the binding of AFB_1 to these cells. To study the effect of time and concentration, CYP2A5 mutant yeast cells were incubated for 5 - 80 min with liquid growing media containing 0.25, 1 or 4 μM AFB1. Binding of AFB1 was studied by incubating yeast cells with tritium labelled AFB_1 and the radioactivity in the yeast cells indicated its binding. In both studies, yeast cells expressing no foreign CYP enzyme (pAAH5) were used as a reference.

Results

The viability of mutant CYP2A5 yeast cells was dependent on the incubation time and concentration of AFB_1. At 0.25 μM AFB1, after 5 min the viability was 80 %, this was decreased to 40 % at 80 min. At 4 μM the viability was decreased to 20 % already at 5 min, however, no further reduction in the viability was observed by prolonged exposure time. Such findings were not observed with pAAH5 yeast cells. When mutant CYP2A5 yeast cells were incubated with tritium labelled AFB1, radioactivity detected in yeast cells was significantly high and was dependent on the amount of yeast cells, concentration of AFB1 and incubation time. Moreover, washing the yeast cells with methanol could not remove this radioactivity. This was not the case for the pAAH5 yeast cells, where the radioactivity was small and was not dependent on variables mentioned above.

Conclusions

Mutant CYP2A5 in yeast cells metabolize AFB1 to its reactive epoxide and due to this metabolism it is bound covalently to yeast cells resulting lowered viability of these yeast cells. Mutant CYP2A5 expressing yeast cells are a good model to study the toxicity of AFB1.

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WP03 ENGINEERING SUBSTRATE RECOGNITION IN CATALYSIS BY CYTOCHROME P450CAM

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Abstract

We have a continuing interest in applying the current knowledge of P450cam substrate recognition to engineer the enzyme for the biotransformation of unnatural substrates with the long-term aim of applications in the synthesis of fine chemicals and bioremediation of environmental contaminants. Comparison of the structure of target substrates with camphor, the natural substrate, lead to the design of active site mutations that had greatly enhanced activity for the oxidation of chlorinated benzenes and selectivity of (+)-α-pinene oxidation. The crystal structure of the F87W/Y96F/V247L mutant with 1,3,5-trichlorobenzene and (+)-α-pinene bound revealed the enzyme substrate contacts and provided insights into the activity and selectivity patterns. The structures also provided a novel basis for further engineering of P450cam for increased activity and selectivity for the oxidation of related compounds.

WP04 VALIDATION OF COMMERCIALLY SUPPLIED CRYOPRESERVED HEPATOCYTES FROM A RANGE OF SPECIES FOR USE IN METABOLIC PROFILING STUDIES

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Isolated hepatocytes are a well established cellular model providing metabolic information on new molecular entities (NME). Traditionally, metabolic studies have used microsomes, however the main advantage of isolated hepatocytes is that they contain the cofactors and drug-metabolising enzymes required to study integrated Phase I and Phase II metabolism reactions. The use of cryopreserved hepatocytes has increased in recent years due to a number of reasons namely, the lack of human tissue available, a desire to decrease animal usage and improvements in the cryopreservation process.

We have validated a model for studying the metabolic profiles of NMEs utilising commercially available cryopreserved hepatocytes from a range of species. As human hepatocytes are the „gold standard“ to which metabolic information from other species is compared, freshly isolated human hepatocytes were also investigated for comparative reasons. Cryopreserved rat, rabbit, dog, primate and human hepatocytes were obtained from Xenotech, (KS, USA) or In Vitro Technologies (MD, USA). Freshly isolated human hepatocytes were supplied by the UK Human Tissue Bank.

Following thawing of the cryopreserved hepatocytes, the viability and the metabolic activity of the cells were determined over a 3 hour incubation period. Cell viability was assessed using the Trypan exclusion test. Metabolic activity of the hepatocytes was measured using testosterone 6β-hydroxylation as an marker of Phase I metabolism. Phase II metabolism was assessed by measuring the glucuronidation and sulphation of the probe substrate 4-methylumbelliferone. In all species, following thawing, Phase I and II metabolic activities were present. Furthermore these activities were demonstrated to be maintained throughout the 3 hour incubation period. There was only a small decrease in cell viability observed over the 3 hour incubation period. Therefor, this data indicates that commercially obtained cryopreserved hepatocytes are suitable for in vitro metabolic profiling studies with NMEs.

WP05 P450 2E1/BMR: STUDIES OF A HUMAN/BACTERIAL P450 FUSION PROTEIN

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The cytochrome P450s form a large family of monooxygenase enzymes that carry out an essential role in xenobiotic metabolism[1]. In humans they are found on the membranes of the smooth endoplasmic reticulum where they are attached by a N-terminal anchor[2]. Human P450s can be expressed in non-native hosts such as *Escherichia coli* in a membrane bound form but for optimal activity need to be fused to or co-expressed with the cytochrome P450 reductase[3]. To overcome these limitations we have made a chimerical protein containing the N-terminal of modified human P450 2E1[4] fused to the soluble bacterial cytochrome P450 reductase from *Bacillus megaterium* (BMR). The construct, P450 2E1/BMR, consists of amino acids 1-472 from modified P450 2E1 and amino acids 473-1049 from BMR. The fusion protein has been expressed using the pCW plasmid vector in the DH5α strain of *Escherichia coli* at a level of 900 nmol of P450 per litre of culture. The protein has been purified in a holo form and demonstrates p-nitrophenol and chlorzoxazone hydroxylase activities, both marker activities for P450 2E1[5,6]. The protein also shows a P450 peak upon carbon monoxide binding, characteristic of an active protein and exhibits spin shifts on substrate binding. In summary these results indicate that the construct retains the marker activities of human P450 2E1, i.e. the ability to metabolise p-nitrophenol and chlorzoxazone, but with the added advantage of being catalytically self-sufficient and soluble for electrochemical applications[7]. The P450 2E1/BMR construct should prove useful in studies of the human P450 2E1 enzyme in a convenient format in both whole *Escherichia coli* cells and as a purified enzyme.

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WP06 O_2 METABOLISM BY SOLUBLE CYTOCHROME P450 REDUCTASE AND ITS CHIMERAS WITH C-TERMINI OF NITRIC OXIDE SYNTHASES ASSAYED BY DIACETYLDEUTEROHEME HRP

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Diacetyldeuteroheme-substituted horseradish peroxidase (dHRP) reacts with both superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) to form its compounds III and II, respectively. These compounds are rather stable and spectrally distinguishable from each other. Hence it has been used to detect and quantify the oxygen metabolites of some oxidases[1]. Recently, we constructed chimeras of a soluble CYPOR and C-terminal tail portions of neuronal, endothelial and inducible NOS's, and have elucidated their kinetic properties[2]. In the present study, we have applied the dHRP method to analyze oxygen metabolites of these chimeric enzymes of CYPOR in their reactions with NADPH under aerobic conditions. The most common method to detect and quantify O_2^- based on SOD-sensitive cytochrome *c* reduction is difficult to apply to these reactions, because CYPOR reduces cytochrome c directly, not via O_2^-. Results obtained in combination with the measurement of O_2 consumption using an oxygen monitor indicate that the soluble CYPOR reduces O_2 exclusively to O_2^- and not to H_2O_2. The findings confirm our previous results using the adrenochrome method on steapsin-solubilized CYPOR[3], indicating that CYPOR produces only O_2^-. Available evidence suggests that the CYPOR chimeras with NOS tails produce both O_2^- and H_2O_2. Experiments using the dHRP method to analyze oxygen metabolites from wild type and chimeric constructs of holoNOS are in progress.

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WP07 AN HOMOGENEOUS ASSAY FOR SCREENING CYP2D6 INHIBITORS USING SCINTILLATION PROXIMITY ASSAY TECHNOLOGY

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The P450 cytochromes (CYP) are a superfamily of enzymes found in the liver which catalyse the oxidative conversion of drugs and other lipophilic compounds to hydrophilic metabolites. This conversion allows for the elimination or further metabolism and elimination of the drug from the body. CYP2D6 and CYP3A4, izoenzymes of this family, catalyse the metabolism of the major proportion of drugs available on the market.

Inhibition of CYP-mediated metabolism accounts for a number of adverse drug-drug interactions[1], which often leads to withdrawal from the market[2,3]. Promising new chemical entities (NCEs) are routinely tested for CYP inhibition during drug discovery or development.

A high throughput Scintillation Proximity Assay (SPA) to identify compounds that inhibit P450 CYP2D6 has been developed.

Tab. 1. *Results obtained with a panel of test compounds.*

	k_i values μM		
Compound	**SPA**	**95% confidence intervals**	**Literature**
Diazepam	Determined not to inhibit CYP2D6 in the SPA assay and in literature.		
Verapamil			
Omeprazole	295	256-342	240.7
Tranylcypromine	40.2 35.1	28.1-43.9 33.2-48.6	30
Tropisetron	16.5 16.0	12.6-21.6 1.3-21.0	14
Desipramine	3.7 4.1	3.1 - 4.3 3.8 - 4.4	12.5
Loratadine	1.99	1.75 - 2.27	2.7
Sulconazole	0.35 0.29	0.25 - 0.49 0.24 - 0.36	0.4
Quinidine	*0.0054* *0.0077*	*0.0058-0.010* *0.0050-0.0061*	*0.018 - 0.043*
Lansoprazole	*7.0* *8.2*	*6.3-7.7* *7.5-9.1*	*44.7*
Metoclopromide	*23.1* *18.2*	*17.5 - 30.5* *13.4 - 24.9*	*4.7*

SPA is a homogeneous technology that has been used extensively within the pharmaceutical industry. It has been used in a broad range of applications including radioimmunoassays, receptor-ligand and enzyme assays.

The assay that will be described measures the conversion of the CYP2D6 substrate, [^{14}C]dextromethorphan to [^{14}C]formaldehyde, by selective capture of this reaction product using modified Yttrium silicate SPA beads[4].

Validation of the assay against a panel of known CYP2D6 inhibitors has been carried out and is summarised in Table1.

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WP08 EXPLORATION OF NATURAL AND ARTIFICIAL P450 SEQUENCE SPACES

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Two complementary methods are described that associate *in vitro* and *in vivo* steps to generate P450 sequence diversity by segment directed mutagenesis and family shuffling. High-throughput DNA chip based and HPLC technologies have been developed for the characterization of combinatorial libraries and for the characterization of activities shuffling achieved, respectively.

Using these approaches, two combinatorial libraries of variants derived from the human CYP1A subfamily were constructed and their sequence diversity characterized. The results of functional screenings using high-throughput tools for the characterization of membrane P450-catalyzed activities is presented with a preliminary discussion.In contrast to mutants CYP1A1 in the 204-214 segment, CYP1A1-CYP1A2 mosaic structures with, on average, 5 crossovers exhibit a wide range of substrate selectivities. These mosaic structures also discriminate between closely related alkoxyresorufin and polycyclic aromatic hydrocarbon substrates. These results open the way to global high-throughput analysis of structure-function relationships in eukaryotic P450s using combinatorial libraries of enzymes together with a library of structurally related substrates.

This poster illustrates how *in vitro* and yeast *in vivo* steps can be associated to improve the diversity of combinatorial libraries either resulting from segment-directed mutagenesis or from family shuffling. The CLERY family-shuffling procedure combines an *in vitro* DNA-suffling PCR step to an *in vivo* recombination step in yeast aims to build a library of shuffled sequences containing a low proportion of parental structures. The recombination step in yeast is both a self-cloning tool and an additional shuffling step when overlapping and partial-length PCR-shuffled DNAs are involved. The production of such segment-limited or family-shuffled combinatorial libraries in yeast specially engineered to enhance P450-catalyzed activities is of great interest for the functional analysis of membrane-bound shuffled P450 variants.

WP09 CYTOCHROME P4502E1 ACTIVITY AND FREE RADICAL OXIDATION IN THE LIVER OF HYPOTHYROID RATS WITH ACUTE ALCOHOL INTOXICATION

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Ethanol-inducible cytochrome P450 2E1 (CYP2E1) is involved in free radical generation during ethanol oxidation by liver microsomes. A complicated relationship exists between the ethanol effects on the thyroid function and the thyroid hormone influence on the metabolism and toxicity of ethanol. The aim of our research was to study liver CYP2E1 activity, lipid peroxidation rate and antioxidant defence in acute alcohol intoxication of rats with hypothyroidism. The experimental hypothyroidism in rats was provoked by treatment with mercazolil (5 and 20 mg/kg, for 14 d.), ethanol was administered singly (6 g/kg, i.g.).The developtment of hypothyroidism was followed by measuring plasma thyroid hormone (T_3 and T_4) levels. In liver microsomes, CYP2E1 activity was 35% lower in the hypothyroid rats as compared to the control animals, whereas any differences in cytochrome P450-reductase (CYP-reductase) activity were not found in these groups. The ethanol administration caused an activation of CYP2E1 (23%) and CYP-reductase (19%) in euthyroid rats and a significant increase in CYP2E1 activity in liver microsomes of mercazolil-treated rats (5mg/kg).The group with more pronounced hypothyroidism showed no alcohol effect on CYP2E1 activity. The CYP-reductase activity was decreased proportionally to the reduction of T_3 and T_4 levels in the blood after the acute ethanol administration. In the liver cytosolic fraction from hypothyroid ethanol-fed rats the activity of alcohol dehydrogenase was increased. A decrease in MDA levels (28 and 56%) and an increase in glutathione content (22 and 102%), as well as an activation of glutathione peroxidase and glutathione reductase were found. A decrease in CYP2E1 and CYP-reductase activities in hypothyroidism can lead to a reduction in microsomal oxygen consumption and free radical production, thereby limiting ethanol-induced oxidative stress.

WP10 ASSIGNMENT OF OMEPRAZOLE-SENSITIVITY GENE LOCUS, WHOSE PRODUCT MEDIATES CYP1A1 INDUCTION, ON HUMAN CHROMOSOME 10

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Previously, we reported that CYP1A1 was induced in human GepG2 cells, but not in mouse Hepa-1 cells, by such benzimidazole compounds as omeprazole (OP), lansoprazole, and thiabendazole[1,2]. We also showed that protein tyrosine kinase is involved in the induction of CYP1A1 by omeprazole treatment in human HepG2 cells, and that the signal transduction pathway for omeprazole is different from that existing TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin). Furthermore, we reported that in HepG2 cells, the Ah-receptor antagonist (3'-methoxy-4'-aminoflavone) blocked the induction of CYP1A1 by TCDD, but not produced by OP[3]. Consequently, we proposed the presence of a ligand-independent pathway for the activation of the Ah receptor, and the omeprazole-sensitivity gene product may be involved in this pathway.

We utilized the lack of CYP1A1 induction by OP in Hepa-1 cells to map a putative human gene for OP-responsiveness in cell hybrids produced by fusion of Hepa-1 and HepG2 cells. OP-induced CYP1A1 expression was detected in four out of 32 Hepa-1 x HepG2 cell hybrid analyzed. To help identify the gene locus, a radiation-hybrid cell (E11) was constructed. Use of reverse-fluorescence in situ hybridization revealed that these five cell lines commonly retained human chromosome 10p4.

To narrow down the gene locus of OP-sensitivity gene on chromosome 10, we used site specific PCR primers and identified DNA sequences that are commonly present in the four OP-responsive hybrid cells and radiation-hybrid

cell. Finally, we assigned the two regions (2.1 and 3.0 magabp) that are responsible for the induction of CYP1A1 by OP treatment on the short arm of human chromosome 10.

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WP11 STUDY ON ORGAN SPECIFICITY OF CYP1A1 INDUCTION BY 1-PHENYLAZO-2-HYDROXY-NAPHTHALENE (SUDAN I) IN RATS

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Sudan I [1-(phenylazo)-2-naphthol, C.I. Solvent Yellow 14] was used as a food coloring in several countries, but it has been recommended as unsafe, because it causes tumors in the liver or urinary bladder in rats, mice, and rabbits, and is considered possible carcinogenic and mutagenic for man[1,2]. Besides its carcinogenicity, Sudan I is a potent contact allergen and sensitizer, eliciting pigmented contact dermatitis in human.

We found that this carcinogen is metabolized (activated and/or detoxicated) almost exclusively by CYP1A1; other CYPs oxidize Sudan I with efficiencies more than one order of magnitude lower than CYP1A1[1,2]. Therefore, Sudan I oxidation seems to be utilized as a marker for the CYP1A1 activity. While CYP1A1 is not constitutively expressed in human livers, it seems to be induced by planar aromatic compounds binding to the aryl hydrocarbon receptor (AhR), e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and/or by polycyclic hydrocarbons present in cigarette smoke[3]. The CYP1A1 enzyme is even strongly induced by Sudan I itself in rat livers by this mechanism[4]. The AhR is ubiquitously expressed in most organs and cells in the body. Nevertheless, the *de novo* synthesis of CYP1A1 molecules as a result of an increased transcription of CYP1A1 gene after stimulation by Sudan I interaction with AhR in various organs has not yet been studied in details. Here, we study an organ specificity of Sudan I as an inducer of CYP1A1 in rats. Selective antibodies against rat CYP1A1 were utilized for this study. An expression of CYP1A1 protein was found to be strongly induced in rat liver, kidney, lung, spleen and heart by treatment of animals with Sudan I, while almost no CYP1A1 induction was detectable in brain. The expression of this enzyme was the highest in liver of treated rats (almost one order of magnitude higher than those in control animals). Analogously, the increase in CYP1A1 expression correlates with an increase of EROD activity, a marker for CYP1A1/2, or with that of Sudan I own oxidation. In addition to CYP1A1, Sudan I induces DT-diaphorase, the cytosolic enzyme, whose gene(s) is(are) also transcriptionally activated by foreign chemicals through the Ah-dependent mechanism. Its induction was detectable in most rat tissues tested in the study except of brain. No increase of DT-diaphorase activity by Sudan I was detectable in rat brain cytosolic samples. The significance of CYP1A1 induction by Sudan I for an increase in risk of Sudan I exposition for humans working in dye industry is discussed.

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WP12 THE USE OF BACTERIAL MEMBRANES IN THE STUDY OF INTERACTIONS OF HUMAN CYTOCHROMES P450 AND XENOBIOTICS

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The need to identify the participation of individual human biotransformational enzymes in the metabolism of xenobiotics has lead to the use of heterologously expressed enzymes for these purposes. In our previous work[1] we have performed a series of experiments in which the decrease of cytochrome P450 (CYP) content and the extent of oxidative stress was related to the presence of metabolites of benzene (benzoquinone, hydroquinone) and NADPH in samples containing rat and human microsomes. In order to investigate the role of individual CYPs we decided to carry out a similar study using human enzymes expressed in bacterial membranes. Our goal therefore was to create a system suitable for *in vitro* testing of interactions of CYPs and xenobiotics.

We expressed human CYPs and NADPH-CYP reductase (NPR) in *Escherichia coli* and isolated the membrane fraction by differential centrifugation. The catalytic activity of the enzymes was verified and the membranes were used directly for incubations without further purification. After 60 minutes incubation at 37 °C in the absence or presence of NADPH and substrates (benzoquinone, hydroquinone, catechol), we measured CYP content, NPR content, the peroxidation of lipids and the formation of hydroxyl radicals. We observed CYP destruction that was dependent on the concentration of NADPH but as opposed to liver microsomes we found CYP destruction in control samples incubated without NADPH. CYP2E1 proved to be less stable than CYP2A6 and 2C9 and therefore not suitable for testing the effect of substrates on CYP content. We did not detect lipid peroxidation but we were able to measure the formation of OH radicals in the membranes. The effect of substrates on CYP destruction, NPR destruction and oxidative stress was in agreement with the results obtained with liver microsomes. Our results suggest that benzoquinone and hydroquinone can initiate the generation of OH radicals and damage proteins. It is possible to conclude that human CYPs expressed in bacterial membranes are in this form catalytically active although each CYP has different properties and is suitable for different types of experiments. These include the measurement of oxidative stress and with some restrictions also the measurement of the destruction of proteins.

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WP13 AZOLE ANTIFUNGAL DRUGS ACT UPON CYTOCHROME P450 MONO-OXYGENASES TO EFFICIENTLY INHIBIT GROWTH IN MYCOBACTERIA AND STREPTOMYCETES

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The genome sequence of several actinomycetes has revealed a surprisingly large number of genes encoding cytochromes P450 mono-oxygenases (P450s) indicative, perhaps, of essential physiological roles. Among these actinomycete P450s are homologues of 14α-sterol demethylases

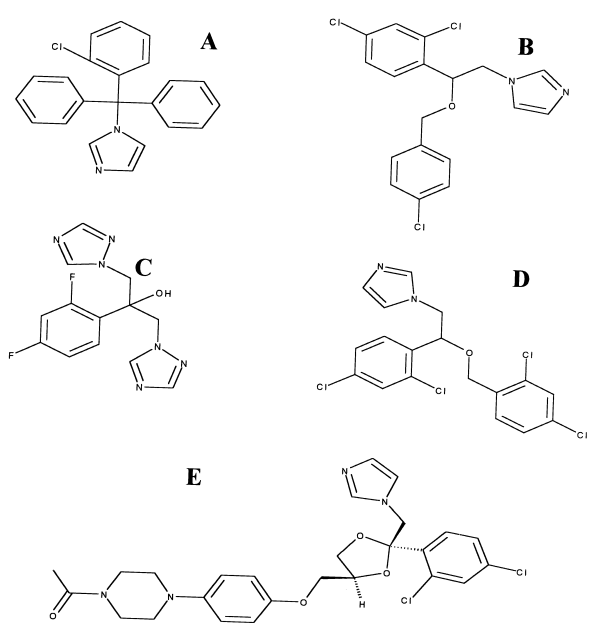

Fig. 1. *Chemical structures of azole antifungal drugs tested for binding to CYP121 and potency as anti-mycobacterials.*
A = clotrimazole, B = econazole, C = fluconazole, D = miconazole, E = ketoconazole.

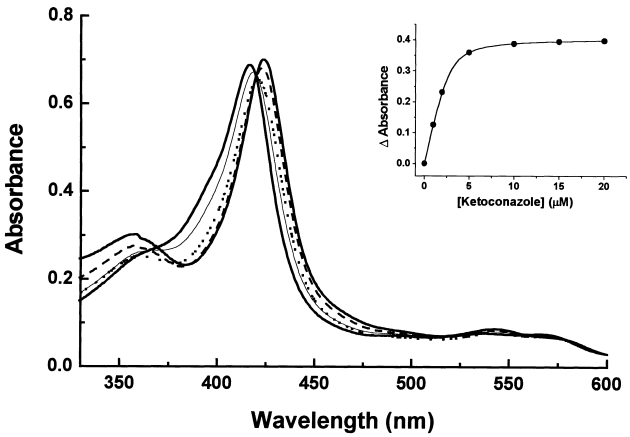

Fig. 2. *Binding of ketoconazole to Mtb CYP121 in the presence of 10 μM lanosterol. The main figure shows selected absolute spectra induced by addition of ketoconazole to CYP121 (6.5 μM), with progressive spectral shifts induced by addition of ketoconazole at 1.2, 2.2, 5.2 and 9.2 μM. The inset shows a plot of maximal shifts in absorption (A_{430} minus A_{408}) for binding of ketoconazole to CYP121,* versus *the relevant [ketoconazole]. Data were fitted to a hyperbolic function. K_d = 3.3 ± 0.3 μM.*

(CYP51 family) that are targets in yeasts and fungi for the azole class of drugs (*Fig. 1*). Others have shown that the *Mycobacterium tuberculosis* (Mtb) CYP51 homologue binds to azole drugs [1,2]. In this work we demonstrate that a second Mtb P450 (CYP121) binds azole drugs with even greater affinity than CYP51*,4* (*Fig. 2; Table 1*). Growth inhibition experiments using *Mycobacterium smegmatis* (a safe laboratory model for Mtb), in the presence of a number of azole drugs, gave MIC values of <0.2 and 0.3 μM for econazole and clotrimazole, respectively, compared to MIC values of 1.2 μM for rifampicin and 36.5 μM for isoniazid, the current front-line anti-tubercular drugs of choice[3] (*Table 1*). Of note was the fact that K_d values for binding of azole drugs to CYP121 mirrored MIC values in our growth inhibition experiments[3] (*Table 1*). Moreover, the fact that a *Streptomyces coelicolor (S. coelicolor)* CYP51 knock-out mutant remained as susceptible as wild-type to the inhibitory effects of azole drugs indicated CYP51 is unlikely to be the primary drug target[3]. Similarly, a CYP105D5 knock-out mutant of *S. coelicolor* we have constructed also remains equally sensitive to growth inhibition[3]. An attractive scenario is that at least two Mtb P450s are azole drug targets making the currently experienced problems with drug resistant mutants of Mtb less likely. In recent studies, we have solved the atomic structure of Mtb CYP121 to atomic resolution - providing the basis by which to rationalise the high affinity binding of azole drugs[5].

Tab. 1. *Comparison of K_d values for CYP121 binding with MIC values for* M. smegmatis *for selected azole drugs. ND = no spectral change observed. The K_d values for the binding of these drugs to* M. tuberculosis *CYP51 are all in the range 5-12 μM.*

Drug	MIC for *M. smegmatis*	K_d for Mtb CYP121
Econazole	< 0.2 μM (< 0.1 μg/ml)	< 0.2 μM (< **0.09** μg/ml)
Clotrimazole	0.3 μM (0.1 μg/ml)	< 0.2 μM (< **0.07** μg/ml)
Micronazole	2.6 μM (<1.25 μg/ml)	< 0.2 μM (< **0.10** μg/ml)
Ketoconazole	38 μM (20 μg/ml)	3.3±0.3 μM (1.75±**0.2** μg/ml)
Fluconazole	>325 μM (> 100 μg/ml)	9.7±0.2 μM (**3.0±0.1** μg/ml)
Isoniazid	36.5 μM (5 μg/ml)	ND
Rifampicin	1.2 μM (1 μg/ml)	ND

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WP14 SUDAN I METABOLISM BY HUMAN CYTOCHROME P450 1A1 IS STIMULATED BY MICROSOMAL CYTOCHROME B_5

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Many cytochrome P450 (CYP) - dependent reactions have been shown to be stimulated by another microsomal protein, cytochrome b_5. CYP3A4 - mediated reactions are the most sensitive from this point of view. Recent work of Guengerich and his co-workers[1] has also shown stimulation of catalytic activities of CYPs 2A6, 2B6, 2C8, 2C19, and 3A5 by cytochrome b_5. On the other hand, some CYPs

have not shown effects of cytochrome b_5, i.e. 1A1, 1A2, 1B1 and 2D6[1]. Here we report controversial results showing a strong sensitivity of CYP1A1 and 1A2 to cytochrome b_5.

1-Phenylazo-2-hydroxynaphthalene (Sudan I, C.I. Solvent Yellow 14) is a liver and urinary bladder carcinogen in mammals. We found that the most efficient CYP responsible for the metabolism of this carcinogen (activation and/or detoxication) in hepatic rat and human microsomes is CYP1A1. In human hepatic microsomes Sudan I is also oxidized by CYP3A4, but to a lesser extent[2,3]. Several other human CYPs as 1A2, 1B1, 2A6, 2C19 and 2D6 also oxidized Sudan I, but with efficiencies less than one order of magnitude lower. The capabilities of some CYP enzymes to oxidize Sudan I was significantly increased by cytochrome b_5. The CYP1A1, 1A2, 2A6 - dependent Sudan I oxidation was stimulated by cytochrome b_5, while reactions catalyzed by CYP1B1, 2C19, 2D6 were insensitive to this microsomal protein. The ability of CYP3A4 to oxidize Sudan I was almost undetectable without this protein. However, cytochrome b_5 caused increase of its efficiencies to oxidize Sudan I 3.9-fold. Stimulation of the CYP - mediated Sudan I oxidation reactions was dependent on concentration of cytochrome b_5. Mechanism of cytochrome b_5-dependent stimulation of the CYP catalyzed oxidation is discussed. The results presented in this study are the first report on the stimulation of CYP1A1-mediated oxidative reactions by cytochrome b_5. Caution is highly recommended in interpretation of the effect of this microsomal protein on catalysis mediated by some CYPs in studies utilizing only one of CYP substrates.

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WP15 P450 AROMATASE INHIBITION ASSAY BASED ON AN ELISA SYSTEM

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A convenient tool for P450 aromatase (P450arom) inhibition assay based on an Enzyme Linked Immunosorbent Assay (ELISA) system has been established. The assay consists of two parts. The first part is aromatase reaction, which converts a testosterone to a 17β-estradiol, using a recombinant human P450arom and a NADPH regenerating system. The second is an ELISA system using a highly specific and sensitive anti-estradiol monoclonal antibody immobilized on microplate and estradiol-3-CMO-horse radish peroxidase (E2-3-CMO-HRP). We could estimate pharmaceutical aromatase inhibitors (α-naphtoflavone, ketoconazole, aminoglutethimide) and organic tins (Tributyl tin: TBT and Triphenyltin: TPT) from their dose response curves by this ELISA based inhibition assay. The assay system is free from interference of fluorescence from tested chemicals themselves and does not have to use radio labeled ligands and does not have to care about radio active wastes. It has a potential for a high throughput screening of drug candidates and endocrine disrupting chemicals without place limitation and also without highly trained labors. Cross-reactivities of the antibody may cause some problem in some evaluations for some chemicals. It can be overcome by using different reagent parts (for example, E2-6-HRP and an antibody for E2-6-BSA or anti-Estriol antibody, Estriol-HRP and androstendione as an aromatase substrate).

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WP16 CYTOCHROME P450-MEDIATED ACTIVATION OF CARCINOGENIC 2-ANISIDINE LEADS TO FORMATION OF DNA ADDUCTS *IN VITRO* AND *IN VIVO*

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2-Anisidine is an important industrial chemical in the manufacture of azo dyes. This chemical exhibits strong carcinogenic activity, causing neoplastic transformation in the urinary bladder and, to a lesser extent in spleen, liver and kidney, of two animal species rats and mice. The mechanism of carcinogenicity of the chemical, unknown until recent time, was studied in this work.

We found that CYP-mediated oxidation of 2-anisidine is responsible for its metabolic activation to *N*-(2-methoxyphenyl)hydroxylamine as a proximate carcinogen. Using two independent methods, namely the ^{32}P-postlabeling technique and *14*C-labeled 2-anisidine, we show for the first time that 2-anisidine forms covalent adducts *in vitro* and in DNA of target tissues (urinary bladder, and to a lesser extent, kidney, spleen and liver) in rats. The nitrenium (or carbenium) ion is the reactive species generating covalent adducts with deoxyguanosine in DNA *in vitro* and *in vivo*. 2-Anisidine is also activated by peroxidases, besides CYPs.

Studies using hepatic microsomes of rats and rabbits pre-treated with several cytochrome P450 inducers showed that CYP2B2/4, CYP1A1/2 and CYP2E1 are mainly responsible for metabolism of 2-anisidine in these animal models. Human hepatic microsomal samples from 9 different human donors also oxidized 2-anisidine. In order to resolve which human CYPs are responsible for 2-anisidine oxidation several approaches were utilized: (i) correlation of CYP-catalytic activities (or CYP contents) in each microsomal sample with the levels of a proximate carcinogenic *N*-(2-methoxyphenyl)hydroxylamine metabolite formed by the same microsomes, (ii) selective CYPs inhibition in human microsomes and (iii) microsomes of baculovirus-transfected insect cells containing human recombinant CYPs (Supersomes®). On the basis of these studies we attributed most of the 2-anisidine oxidation in human hepatic microsomes to CYP2E1. This was confirmed with human recombinant CYPs expressed in Supersomes, but CYP 1A2 and 2B6 are other CYP enzymes effectively oxidizing 2-anisidine.

These results, the first report on the metabolism of 2-anisidine by human enzymes, strongly suggest a carcinogenic potency of this rodent carcinogen for humans.

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WP17 POSSIBLE ROLE OF CYP450S IN THE NABUMETONE METABOLISM

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The biotransformation of a non-steroidal anti-inflammatory drug (NSAID) nabumetone [4-(6-methoxy-2-naphthyl)butan-2-one] in the microsomal fraction of human hepatocytes was investigated. Nabumetone is a prodrug, which is metabolized in the organism to the principal pharmacodynamically active metabolite - 6-methoxy-2-naphthylacetic acid (6-MNA). This metabolic change includes the oxidative removal of a two-carbon fragment (probably in the form of acetate) from the side chain of the nabumetone molecule. Nabumetone and its principal metabolite 6-MNA may undergo another two metabolic changes during Phase I metabolism : a carbonyl reduction on the side aliphatic chain of nabumetone and *O*-desmethylation of nabumetone, of its reduced metabolite [4-(6-methoxy-2-naphthyl)butan-2-ol] as well as of the 6-MNA. There was no information found in the literature about the nature of the enzymes responsible for the above-mentioned metabolic changes.

The role of CYP450s in the metabolism of nabumetone was studied. Nabumetone and its principal metabolite, 6-MNA, were incubated with the microsomal fraction of human hepatocytes under various conditions (coenzymes, inhibitors, etc). Nabumetone and its metabolites in the extracts from the incubations were determined by a validated LLE-HPLC-DAD-MS method.

The results of our experiments predicate the possible involvement of CYP450(s) in O-desmethylation of nabumetone and its principal metabolite, 6-methoxy-2-naphthylacetic acid (6-MNA). Our results correspond with the findings that naproxen, a higher homologue of 6-MNA, un-

dergoes O-desmethylation catalysed by various isoform of CYP450[1,2].

Acknowledgment

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WP18 CYTOCHROME P450 1A2 AND N-ACETYLTRANSFERASE (NAT2) PHENOTYPES AS MARKERS OF CANCER SUSCEPTIBILITY BY WORKERS EXPOSED TO AROMATIC AMINES

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The aim of this work is a proposal of the new marker for individual susceptibility in humans exposed to aromatic amines. A group of workers (n = 9) with professional risk of exposition to aromatic amines and with high degree of chromosomal damage (3 - 5 % of abberant cells) was selected. Chromosomal aberrations were evaluated using standard method of cytogenetic analysis of peripheral lymphocytes. Simultaneously activities of CYP1A2 and N-acetyltransferase (NAT2) were determined using the caffeine test according to Butler et al. (1992). The CYP1A2 phenotype was expressed as the molar ratio of the sum of 1,7-dimethylxanthine [17X] and 1,7-dimethyluric acid [17U] to caffeine [137X]. The N-acetyltransferase (NAT2) phenotype was expressed as the molar ratio of 5-acetylamino-6-formylamino-3methyluracil [AFMU] to 1-methylxanthine [1X]. There was no correlation found between CYP1A2 phenotypes and determined percentage of abberant cells (y = -0,0434x + 4,1419 ; r = -0,255). However, the NAT2 activity marker correlated (y = -2,2632x + 4,1434; r = -0,646) with percentage of abberant cells better. The extent of percentage of abberant cells was hence shown to be indirectly proportional to the activity of NAT2 which is in accordance with literature. A new marker is proposed here - ratio of CYP1A2 and NAT2 activity marker, in other words {(17U+17X)/137X}/{AFMU/1X}. This new marker exhibits even closer correlation (y = 0,003x + 3,2009; r = 0,847) with found percentage of abberant cells. Although the tested group was very small, the results suggest, that index {(17U+17X)/137X}/{AFMU/1X} could be useful for determination of cancer susceptibility in human exposed to aromatic amines as it takes into account both the CYP1A2 and NAT2 activities.

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WP19 METHOD OF PRELIMINARY SELECTION OF SUBSTANCES SPECIFIC IN RELATION TO P450 ISOFORMS

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It is well known, that P450 isoforms responsible for the metabolism of xenobiotics plays the important role in drug interactions. Therefore, usually for drug development it can be required that some substance would be, for example, particular isoform substrate or, on the contrary, would not be such substrate.

The computer modeling of P450 isoforms substrate specificity is a difficult task because of the large variety of substrates structures. For this reason the development of computer model capable to predict with high quality all possible substrates of isoform can take a long time. On the other hand, the search of substance with specified metabolic properties can be performed using more simple predicting model. A new approach for construction of such model is offered in the present paper.

A 3D-QSAR algorithm BiS[1] has been used for the predicting model creating. The dataset of P450 isoforms substrates has been used as a training set. The same dataset has been used as a testing set. The quality of the prediction for

each isoform has been estimated using two parameters. The first parameter is quality of recognition has been defined as a relation of number of objects correctly referred by the prediction to total number of objects referred by the prediction to the considered class of substances. The second parameter is dataset coverage has been defined as a relation of number of objects correctly referred by the prediction to total number of objects actually belonging to this class.

The classification of P450 isoforms substrates of 100% quality of recognition has been achieved with dataset coverage about 5% for 1A2, 8% for 2A6, 40% for 2B6, 6% for 2C, 8% for 2C19, 44% for 2C8, 22% for 2C9, 18% for 2D6, 45% for 2E1, 26% for 3A, 18% for 3A4, 33% for 3A5. The recognition of substances which are not being P450 isoforms substrates of 100% quality of recognition has been achieved with dataset coverage about 11% for 1A2, 31% for 2A6, 57% for 2B6, 1% for 2C, 29% for 2C19, 34% for 2C8, 15% for 2C9, 37% for 2D6, 66% for 2E1, 19% for 3A, 11% for 3A4, 35% for 3A5.

Thus, it is shown, that the substances, which have specified metabolic properties in relation to P450 isoforms, can be revealed using the offered method. In spite of the fact, that the offered method is able to reveal only some part of such substances, the quality of recognition of the revealed substances in all cases achieves 100%. Hence the offered method can be used for preliminary selection of substances, which are specific in relation to P450 isoforms.

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WP20 PEROXIDASE AND CYP-MEDIATED ELLIPTICINE-DNA ADDUCT FORMATION EXPLAINS THE SELECTIVE EFFICIENCY OF THIS ANTICANCER DRUG AGAINST BREAST CANCER AND LEUKEMIA

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Ellipticine and its more soluble derivatives exhibit promising results in the treatment of breast cancer and acute myeloblastic leukemia. The inhibition of topoisomerase II after intercalation into DNA was considered an important property for its cytotoxicity. The CYP-dependent activation leading to DNA adduct formation, we found to be generated *in vitro*, is a novel mechanism for the ellipticine pharmacological action[1,2]. Target tumor cells express, beside CYP1A1, 1B1, 3A4, also peroxidases (myeloperoxidase or lactoperoxidase) in higher levels than cells of peritumoral tissues. Since the participation of peroxidases in metabolism of ellipticine has not been identified yet, the aim of the present work is to extend our knowledge on this issue. Lactoperoxidase, myeloperoxidase and a model plant peroxidase (from horseradish) were used in our study. Ellipticine is oxidized by all these peroxidases *via* a radical mechanism. Using mass spectrometry (MALDI-TOF, EI) we identify that ellipticine is primarily oxidized to a one electron reaction product (radical) producing a dimer as the major metabolite. Its formation is inhibited by radical trapping agents (glutathione, NADH) and by DNA or deoxyguanosine 3'-monophosphate. Another ellipticine metabolite formed by peroxidases is N(2)-oxide of ellipticine. The same metabolite is formed also by CYP-mediated reactions. During oxidation of ellipticine by peroxidases, two ellipticine-DNA adducts, which were generated by CYP-mediated reaction[1,2], are also formed. Identities of adducts formed by CYPs and peroxidases were confirmed by co-chromatography on HPLC. Deoxyguanosine was determined as a target deoxynucleoside for ellipticine covalent binding in DNA. The involvement of myeloperoxidase and lactoperoxidase in an increase of ellipticine pharmacological efficiency in the target tumor tissue is discussed.

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WP21 DISTINCT INHIBITION OF HUMAN CYP2A6 AND MOUSE CYP2A5 BY DERIVATIVES OF LACTONE, PHENYLETHYLAMINE AND BENZALDEHYDE

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Aim

The purpose of this study was to test the inhibition potency of lactone, benzaldehyde and phenylethylamine derivatives against human CYP2A6 and mouse CYP2A5 and analyse these results with Structure-Activity Relationship (SAR) methods.

Background

The orthologous human CYP2A6 and mouse CYP2A5 enzymes metabolise a number of drugs and toxic agents. Nicotine, the addictive component in tobacco, is mainly metabolized to cotinine by CYP2A6. Interindividual genetic differences in CYP2A6 activity affect on nicotine kinetics, smoking behaviour and lung cancer risk. Mimicking the CYP2A6 poor metaboliser phenotype by inhibiting CYP2A6 enzyme *in vivo* results in decreased smoking and renders nicotine orally available. Here we have determined IC50 values of coumarin 7-hydroxylation for lactone, benzaldehyde and phenylethylamine derivatives as CYP2A5 and CYP2A6 inhibitors and analysed the results using the Comparative Molecular Field Analysis (CoMFA) method.

Results

A rapid 96-well plate assay method was developed and validated to measure liver microsomal coumarin 7-hydroxylation *in vitro*, which was used to test inhibition properties of novel benzaldehyde and phenylethylamine derivatives. Almost all lactone derivatives inhibited more potently CYP2A5 than CYP2A6. 4-Methoxybenzaldehyde inhibited similarly mouse CYP2A5 and human CYP2A6. All of the other benzaldehydes and phenylethylamine derivatives were more potent inhibitors of human CYP2A6 than mouse CYP2A5. The IC_{50} values of 2-(p-tolyl)-ethylamine and 4-methoxybenzaldehyde were less than 10 μM for the CYP2A6 enzyme and the IC_{50} value of the weakest inhibitor, amphetamine, was 320 μM. Compounds with amine groups were more potent inhibitors of CYP2A6 than CYP2A5. 4-Alkyl substitution of benzaldehyde or 2-phenylethylamine increased the potency of these compounds for both enzymes. Benzaldehyde was an irreversible inhibitor of CYP2A6, since the inhibition was dependent on both its concentration and incubation time. Presence of a methyl or methoxy substitution at the 4-position in benzaldehyde changed the inhibition pattern, since 4-methylbenzaldehyde and 4-methoxybenzaldehyde were reversible inhibitors. CoMFA analysis demonstrated that the active site of CYP2A5 is larger than that of CYP2A6.

Conclusions

Inhibition potency of most tested compounds differ between the orthologous enzymes CYP2A5 and CYP2A6. The active site of CYP2A5 is larger than that of CYP2A6, but the size of inhibitor alone could not explain the different inhibition potencies of most lactone, benzaldehyde and phenylethylamine derivatives. Rather, their chemical character cause the different interactions at the active sites of these enzymes. CoMFA analysis gave initial steric and electrostatic field requirements for an inhibiting agent. The most potent of these compounds could be used as lead molecules for developing potent, selective and safe CYP2A6 inhibitors for clinical use.

WP22 CHARACTERIZATION OF HUMAN P450 INVOLVED IN OXIDATION OF MANIDIPINE, A 1,4 DIHYDROPYRIDINE CALCIUM CHANNEL BLOCKER

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Manidipine (MAN) is a 1,4 dihydropyridine calcium channel blocker used in the treatment of hypertension. MAN is metabolised by oxidative metabolism; the most important metabolic pathways are the dehydrogenation of the 1,4-dihydropyridine ring and the oxidative cleavage of the C-3 side chain, giving raise to MIX and MXIII metabolites. Similar metabolic pathways have been reported for several dihydropyridines and the oxidation of dihydropyridines is known to be catalysed by CYP3A4. However, the candidate human CYP isoform(s) responsible for MAN metabolism has not yet been identified. In vitro studies were then designed to clarify this point.

When MAN was incubated with 13 human liver microsomes (HLM), MAN disappearance as well as the forma-

tion of MIX and MXIII metabolites were highly correlated with CYP3A4-linked testosterone 6β-hydroxylase activity. Furthermore the presence of ketoconazole, a selective inhibitor of CYP3A4, effectively inhibited the disappearance of MAN and the formation of M-IX and M-XIII in HLM. Manidipine metabolism and M-IX and M-XIII formation were strongly inhibited by the addition of monoclonal anti-CYP3A4/5 antibodies. When MAN was incubated in the presence of the main human recombinant CYPs, only CYP3A4 was capable to metabolize MAN.

These results support the evidence that MAN Phase I metabolism is mainly mediated by CYP3A4.

As the combination of a calcium channel blocker and an angiotensin converting enzyme inhibitor is particularly advantageous in hypertension, a fixed combination of MAN and delapril (DEL) has been developed. Therefore, it was evaluated if DEL could determine significant drug-drug interactions with MAN.

The in vitro addition of DEL and its active de-esterified metabolite M-I to HLM incubations did not result in the inhibition of any of the main CYP450 linked activities. In addition, when M-I metabolism was studied, CYP3A4 did not result to be involved in its metabolism. Therefore these data indicate that DEL has no significant interaction with the major CYP-450 isoforms and no metabolic interaction is likely between manidipine and delapril.

WP23 HUMAN CYTOCHROMES P450 1A1/2 AND NADPH:CYTOCHROME P450 REDUCTASE ACTIVATE CARCINOGENIC ARISTOLOCHIC ACID TO FORM DNA ADDUCTS FOUND IN PATIENTS WITH CHINESE HERBS NEPHROPATHY

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Aristolochic acid (AA), a naturally occurring nephrotoxin and carcinogen, has been associated with the development of urothelial cancer in humans[1]. Understanding which human enzymes are involved in AA activation and/or detoxication is important in the assessment of an individual susceptibility to this natural carcinogen. We examined the ability of enzymes in microsome samples from eight different human livers and from one human kidney to activate AAI, the major component of a plant extract AA, to metabolites forming DNA adducts by the ^{32}P-postlabeling assay. Microsomes of both these human organs generated DNA adduct patterns reproducing those found in renal tissues from humans exposed to AA. 7-(Deoxyadenosin-N^6-yl)aristolactam I (dA-AAI), 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AAI) and 7-(deoxyadenosin-N^6-yl)aristolactam II were identified as AA-DNA adducts formed from AAI by all human hepatic microsomes. The same AA-DNA adduct pattern was observed upon incubation of AAI with microsomes isolated from kidney of one human donor. According to the structures of the DNA adducts identified, nitroreduction is the crucial pathway in the metabolic activation of AAI. To define the role of human microsomal reductases in the reductive activation of AAI, we investigated the modulation of AAI-DNA adduct formation by selective inhibitors of P450s and cofactors or inhibitors of NADPH:P450 reductase. The role of the enzymes in AAI activation was also investigated by correlating the P450- and NADPH:P450 reductase-dependent catalytic activities in different human hepatic microsomal samples with the levels of AAI-DNA adducts formed by the same microsomal samples. On the basis of these studies, we attribute most of the activation of AAI in human hepatic microsomes to P450 1A1/2, although a role of NADPH:P450 reductase cannot be ruled out. On the contrary, NADPH:P450 reductase is more effective in AAI activation in kidney. With human recombinant enzymes, the role of both these enzymes in AAI-DNA adduct formation was confirmed. Using difference spectroscopy and a molecular modeling and docking of AAI into the active sites of P450 1A1 and 1A2, the binding orientation of AAI to the Fe ion of the heme structure in the active sites of both human P450s was predicted. The results presented here demonstrate the potential of microsomal enzymes in human liver and kidney to reductively activate carcinogenic AAI by nitroreduction.

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WP24 INTERACTION OF OMEPRAZOLE AND CAFFEINE ON CYTOCHROME P450 1A2 EVALUATED USING 13C-CAFFEINE BREATH TEST IN RATS

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Omeprazole, a proton pump inhibitor, is an inducer of human cytochrome P450 1A (CYP1A) enzymes and it shows inhibitory effects on CYP2C19 and CYP3A4[1]. In this paper, a potential inhibitory effect of omeprazole on caffeine biotransformation, a validated CYP1A2 marker, was examined using ^{13}C-caffeine breath test in rats. Omeprazole was administered in one group of animals at a single oral dose of 4 mg/kg (therapeutic dose) and in the second group at a dose of 40 mg/kg. Caffeine breath test was performed 0.5 h after the administration of omeprazole. ^{13}C-caffeine was injected intravenously at a dose of 5 mg/kg. The expired air was constantly captured at selected intervals during four hours. $^{13}CO_2$ was assayed by mass spectrometry. The kinetics of expiration of $^{13}CO_2$ was evaluated in comparison with the control group (without omeprazole). Omeprazole causes slight inhibition of caffeine biotransformation in rats.

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WP25 MICROSOMAL TRANSFORMATION OF ORGANOPHOSPHORUS PESTICIDES BY WHITE ROT FUNGI

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Abstract

The enzymatic mechanism for the transformation of organophosphorus pesticides (OPPs) by different white-rot fungi strains was studied. With the exception of *Ganoderma applanatum* 8168, all strains from a collection of 17 different fungi cultures were able to deplete parathion. Three strains showing the highest activities were selected for further studies: *Bjerkandera adusta* 8258, *Pleurotus ostreatus* 7989 and *Phanerochaete chrysosporium* 3641. These strains depleted 50 to 96% of terbufos, azinphos-methyl, phosmet and tribufos after four-days exposure to the pesticides. In order to identify the cellular localization of the transformation activity, the extracellular and microsomal fractions of *Pleurotus ostreatus* 7989 were evaluated *in vitro*. While the activities of ligninolytic enzymes (lignin peroxidase, manganese peroxidase and laccase) were detected in the extracellular fraction, no enzymatic modification of any of the five pesticides tested could be found, suggesting the intracellular origin of the transformation activity. In accordance with this observation the microsomal fraction was found able to transform three OPPs with the following rates: 10 μmol mg prot^{-1} h^{-1} for phosmet, 5.7 μmol mg prot^{-1} h^{-1} for terbufos, and 2.2 μmol mg prot^{-1} h^{-1} for azinphos-methyl. The products from these reactions and from the transformation of trichlorfon and malathion, were identified by mass-spectrometry. These results, supported by specific inhibition experiments and the stringent requirement for NADPH during the in vitro assays suggest the involvement of a cytochrome P450.

WP26 DEVELOPMENT OF AN *IN VITRO* REPORTER SYSTEM TO ASSESS THE TRANSCRIPTIONAL REGULATION OF THE HUMAN CYP1A2 GENE

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The CYP1A2 gene is well conserved amongst mammalian species. It is expressed mainly in the liver and the olfactory mucosa. CYP1A2 metabolises several heterocyclic aromatic amines and amides and has been implicated in their carcinogenic effects. Additionally, CYP1A2 plays a significant role in drug metabolism. Changes in CYP1A2 expression in mice are associated with altered responses to the toxic and carcinogenic effects of a number of chemicals [1]. Studies have also linked inter-individual differences in CYP1A2 expression to elevated risks of colon cancer[2]. The human CYP1A1 and CYP1A2 genes are present on chromosome 15 and are separated by 23 kb with no open reading frame between the two genes[3]. The two genes are in opposite orientation to each other and share a common 5'-flanking region. Whilst the induction mechanism for

CYP1A1 has been relatively well established, much less is known about the induction of CYP1A2. Analysis of the intergenic region has shown the presence of several HNF sites that may influence expression of either gene[4]. In addition, several potential XRE sites, a 3-MC responsive element and two AP-1 sites, thought to be specific for CYP1A2 have been identified upstream of the transcription start site of CYP1A2 (5, 6,7, 8). The aim of the present study was to establish a reporter gene assay system with a view to determining the role of these putative regulatory elements in the expression of CYP1A2, and thus to develop a system that reflected, as closely as possible, the *in vivo* transcriptional regulation of CYP1A2 . For this purpose, we have developed reporter constructs composed of several fragments of the 5'UTR of the human CYP1A2 gene cloned upstream of a β-galactosidase reporter gene. One such fragment, located at -2973 and -1770 bp (relative to the transcriptional start site), contains two of the CYP1A2 specific XRE-sites that are responsible for 3-MC mediated enhancement of CYP1A2 promoter activity. Also localised to this fragment are two putative AP-1 sites, a HNF-1 site, a TATA box, C/EBP binding protein site, a CCGG site specific for DNA methylation, and a 259-bp positive control element with three major protein binding elements. Another fragment located at -2973 to +901 bp incorporates binding sites for transcriptional factors such as NF-1/CCAAT and two other HNFs upstream of the transcription start site. Also in this region is a 42bp sequence thought to be crucial for constitutive CYP1A2 promoter activity. It contains a TATA sequence for transcription factor IID binding, a SP-1 binding site (GC box) and a CCAAT box. We report the results of transfection of Hep G2 cells with these constructs and induction of the β-galactosidase activity following treatment with a panel of known inducers such as TCDD and 3-MC. Additionally, we shall discuss the implications for the CYP1A2 gene regulation, the potential for interaction with CYP1A1 and the application for a test assay for novel inducers.

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WP27 SAR-BY-NMR WITH CYP450ERYF: IMPLICATIONS FOR HOMOTROPIC/HETEROTROPIC COOPERATIVITY

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Allosteric phenomena have long been recognized in P450 isozymes, but the molecular mechanisms are not well understood. Recently, CYP450eryF, a prokaryotic P450 isolated from the soil bacterium *Saccharopolyspora erythraea*, has been shown to exhibit allosteric binding with the ligands androstenedione, 9-aminophenanthrene, and 1-pyrenebutanol. Although some ligand-bound crystal structures are available, they are not sufficient to describe all the observed allosteric phenomena. In an attempt to better understand P450 allosteric dynamics, we plan to use 2D ^{1}H-^{15}N-HSQC-NMR to monitor the ligand-dependent chemical shifts and peak intensities of both the bound and unbound enzyme. To this end, CYP450eryF was expressed in DL-39, a *tyrB- ilvE- aspC-* auxotrophic strain of *E. coli*, using minimal media supplemented with ^{15}N-Phe. The purified protein was confirmed have uniform isotopic labeling with ^{15}N-Phe using LC-ESI mass spectrometry. In addition, CO difference spectra showed a characteristic peak at 450 nm, indicating presence of the holoenzyme. ^{1}H-^{15}N-HSQC spectra were obtained from an 800 μM sample of ^{15}N-Phe CYP450eryF in the presence and absence of testosterone using a 500 MHz Varian Inova NMR spectrometer. Chemical shift perturbation mapping of the ^{15}N-edited HSQC spectra will allow for: (1) a determination of which of the three active site Phe residues (F86, F78, and F187) that are critical for substrate binding, and (2) an assessment of whether the pattern of perturbed Phe residues changes upon addition of a second ligand, thus providing a structural probe for the observed allosteric behavior of CYP450eryF.

WP28 IMPROVING THE AFFINITY OF ADRENODOXIN FOR ITS PARTNER PROTEINS BY DIRECTED EVOLUTION

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While over the last twenty years protein engineering based on site-directed mutagenesis has contributed to our understanding of enzyme catalysis, biological design capabilities have been greatly enhanced recently by the development of 'evolutionary' protein design methods which use random mutagenesis followed by screening or selection for a desired trait[1,2].

We applied this technique for adrenodoxin (Adx), a [2Fe - 2S] ferredoxin involved in steroid hormone biosynthesis in the adrenal gland of mammals. It is a small soluble protein that transfers electrons from adrenodoxin reductase (AR) to different cytochrome P450 isoforms where they are consumed in hydroxylation reactions[3].

The screening-system chosen for the directed evolution of Adx is the yeast two-hybrid system, a technique detecting interactions between two proteins[4]. It was developed to provide a genetic mean of identifying proteins that physically interact *in vivo*. Here we describe its application as screening-system in directed evolution, which we adapted to our purposes to screen new adrenodoxin forms with improved affinity to its redox partners. With this system we screened a library of 5000 Adx variants and selected new forms. The characteristics of these forms with regard to affinity and activity comparing to Adx wild type are presented in this poster.

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WP29 NOVEL REVERSIBLE ADDUCTION OF THE P450 HEME: INACTIVATION OF CYTOCHROME P450 2E1 T303A BY *TERT*-BUTYL ACETYLENE

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The constitutively expressed and ethanol-inducible cytochrome P450 2E1 catalyzes the oxidation of a large number of drugs and hepatotoxic xenobiotics, including halocarbon anesthetics and acetaminophen, and carcinogens, such as dialkylnitrosamines. The kinetics for the inactivation of cytochrome P450 2E1 by *tert*-butyl acetylene (tBA) and *tert*-butyl 1-methyl-2-propynyl ether (tBMP) were previously characterized[1]. The inactivation of P450 2E1 by tBA was shown to be a result of a combination of heme alkylation and protein adduction, while inactivation by the structurally similar tBMP was a result of only heme alkylation[1].

Mutation of a highly conserved threonine (T303) in the I helix of P450 2E1 has suggested roles for this residue in substrate interactions and orientation within the enzyme active site and as a possible proton donor in acid-base reactions. In order to investigate the role of T303 in the inactivation of P450 2E1 by acetylenic compounds, the kinetics for the inactivation of cytochrome P450 2E1 T303A by tBA and tBMP were characterized. The inactivation was NADPH-dependent and proceeded in a time- and concentration-dependent manner. The losses in P450 2E1 T303A enzyme activity occurred with concurrent losses in the P450 CO spectrum and P450 heme. These losses were accompanied by the appearance of two different tBA- or tBMP-modified heme products as detected by HPLC analysis. Electrospray ionization liquid chromatography - mass spectrometry (ESI-LC-MS) of the modified hemes showed masses of 661 Da or 705 Da, consistent with the mass of an iron-depleted heme plus the masses of either the tBA or tBMP reactive intermediate and one oxygen atom, respectively. Surprisingly, the activity and CO spectral losses observed with the tBA-inactivated T303A mutant were reversible with extensive dialysis. Similarly, HPLC analysis of the tBA-inactivated T303A mutant showed a decrease in the alkylated heme products and a significant recovery of the native heme after dialysis. The pH-dependence of the reversibility was investigated by dialyzing the inactivated samples against low and neutral pH buffers. Reduced levels in the alkylated products were again observed with concurrent increases in the native heme peak, suggesting that pH does not affect the reversibility.

The comparative studies on the inactivation of P450s 2E1 and 2E1 T303A by tBA and tBMP suggest the existence of three distinct mechanisms for inactivation: 1) covalent alkylation of the heme prosthetic moiety, 2) a combination of heme alkylation and protein adduction, and 3) a novel reversible alkylation of the P450 heme. These data support the notion that certain aspects of the structure of the inactivator and the identities of critical amino acid residues within the enzyme active site may significantly influence metabolism and therefore the inactivation of P450s 2E1 and 2E1 T303A by *tert*-butyl acetylenes.

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WP30 PROTEIN ENGINEERING OF HUMAN CYTOCHROME P450S FOR STRUCTURE DETERMINATION

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The 53 human P450s that have been identified play a critical by catalysing reactions involved in xenobiotic metabolism, biosynthesis of steroid hormones, oxidation of unsaturated fatty acids and metabolism of fat-soluble vitamins. However these enzymes are membrane bound and once detached from the membrane require the presence of detergent to keep them in an active state making studies with these enzymes problematic. These enzymes are not catalytically self-sufficient and as such necessitate accepting electrons from their membrane bound redox partner NADPH-cytochrome P450 reductase.

Here we report the rational design of human cytochrome P450s into soluble, catalytically self-sufficient chimeras using the reductase bacterial cytochrome P450 CYP102. Fusion of N-terminally modified human P450s to the large and negatively charge reductase domain of CYP102 ($P450_{BM-3}$) led to active enzymes (P450/BMR) that still retained a small degree of membrane association. Nevertheless upon removal from bacterial membranes, the enzymes remained stable in detergent free buffer and could be purified easily using a single anion-exchange column. This approach resulted in the soluble chimeras CYP2C9/BMR, CYP2C19/BMR, CYP2D6/BMR and CYP3A4/BMR. The CYP3A4/BMR was expressed to a yield of ≈ 830 nmol/ L of bacterial culture and the yield of purified enzyme was nearly 75% of the starting amount. The enzyme metabolised erythromycin forming 7.1 ± 0.9 nmol of formaldehyde/ min/ nmol of P450. These rates are comparable to those obtained with a human CYP3A4/ human NADPH-P450 reductase fusion protein constructed by Shet and co-workers[1]. Oxygen consumption rates were 13.5 nmol of oxygen/ min/ nmol P450 in the presence of substrate and 3.2 nmol of oxygen/ min/ nmol of P450 in the absence of substrate. Further characterisation of this and other human P450/ CYP102 reductase is currently underway.

These artificial fusion proteins provide a means to facilitate studies of these enzymes in solution as well as crystallisation screening leading to human P450 structures where the catalytic domain has not been altered by mutagenesis.

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WP31 STUDIES ON INTERACTION BETWEEN FIBRATES AND STATINS

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Recently, cerivastatin was withdrawn from the market due to disproportionate numbers of fatal rhabdomyolysis among patients who had received gemfibrozil concomitantly1). To gain a better understanding of the mechanism of drug-drug interaction between fibrates and statins, several experiments *in vitro* were performed. A remarkable metabolic inhibition of cerivastatin and atorvastatin was noted in the presence of gemfibrozil. However, the increase in unchanged form was fairly small for pitavastatin, compared with other statins. An extensive metabolism of gemfibrozil was noted in human hepatic microsomes. CYP1A2, CYP2C9 and CYP2C19 were shown to be principally responsible for the metabolism of gemfibrozil. However, no metabolism *via* CYP was observed on fenofibrate, bezafibrate, cipfofibrate and clofibrate. Gemfibrozil inhibited several CYP isoforms in contrast to other fibrates. In the Dixon plots, gemfibrozil was shown to be a potent competitive inhibitor of CYP2C9-mediated metabolism with an apparent Ki value of 18.6 μM. In contrast, gemfibrozil inhibited the CYP2C8- and CYP3A4-mediated metabolism non-competitively with apparent Ki values of 55 and

171 μM, respectively. Although, the mechanism of the drug-drug interaction was not completely clarified, we propose that the increase in creatine phosphokinase caused by co-administration of gemfibrozil and statins is at least partially due to a CYP-mediated inhibition.

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WP32 THE EFFECTS OF CHRONIC TREATMENT WITH ANTIDEPRESSANT DRUGS ON THE LEVEL AND ACTIVITY OF CYP2C6, CYP2C11 AND CYP3A1/2 IN THE RAT LIVER

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Antidepressants are given to patients for months or years, which creates possibility of profound alterations in hepatic enzyme system. Cytochrome P-450 subfamilies CYP2C and CYP3A play an important role in the biotransformation of many drugs and other xenobiotics. They also mediate the metabolism of endogenous substrates, such as steroids. Those subfamilies constitute the substantial pool of cytochrome P-450 in the human and rat livers.

The aim of present study was to investigate the influence of chronic treatment with tricyclic antidepressant drugs (TADs), selective serotonine reuptake inhibitors (SSRIs) and novel drugs (mirtazapine, nefazodone) on the hepatic level and activity of CYP2C6, CYP2C11 and CYP3A1/2 in the rat. The effect of antidepressants was studied *in vitro* in liver microsomes of control rats and of animals treated chronically (for two weeks, twice a day) with pharmacological doses of the drugs (imipramine, amitriptyline, clomipramine, nefazodone - 10 mg/kg ip.; desipramine, fluoxetine, sertraline - 5 mg/kg ip.; mirtazapine - 3 mg/kg ip.). Cytochrome P-450 activity was assessed by measuring the rate of testosterone hydroxylation in positions 2α and 16α (CYP2C11), 2β and 6β (CYP3A1/2), and the rate of warfarin 7-hydroxylation (CYP2C6). The amount of the metabolites formed in vitro was assayed using the HPLC method. The hepatic level of cytochrome P-450 proteins was determined by Western blotting using specific polyclonal antibodies.

The activity of CYP3A1/2 was significantly decreased by chronic treatment with TADs, while it was enhanced by SSRIs. The activities of CYP2C6 and CYP2C11 were elevated by imipramine, desipramine and SSRIs. Moreover, chronic mirtazapine increased the activity of CYP2C6, while nefazodone did not exert any effect on the three studied isoforms. At the same time, the investigated antidepressants (TADs, SSRIs, mirtazapine) increased the hepatic level of the cytochrome proteins. Thus, the elevated level of CYP3A1/2 protein by TADs corresponded negatively with a reduced CYP3A1/2 activity by these antidepressants, while in other cases positive correlations between the level and activity of CYP isoforms were observed, i.e. an increased level of cytochrome protein was accompanied by enhanced enzyme activity. In conclusion, the obtained results indicate induction of CYP2C6, CYP2C11 and CYP3A1/2 by many investigated antidepressants. However, an induction of CYP3A1/2 (an increase in protein level) by TADs seems to be masked by some other mechanisms (e.g. formation of CYP3A-TAD reactive metabolite complexes), which leads to decreased enzyme activity.

WP33 THE OPTICAL BIOSENSOR STUDY OF REDOX PARTNERS' INTERACTIONS IN THE P4502B4 SYSTEM IN HYDROXYLATION CONDITIONS

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Investigated were the interactions between cytochrome P4502B4 (d-2B4), NADPH cytochrome P450 reductase (d-Fp) and cytochrome b_5 (d-b5) within a monomeric reconstituted P4502B4-containing monooxygenase system in hydroxylation conditions and in the absence of phospholipid. It was shown that immobilization of the partner through their respective amino groups onto the carbodextran surface of the IAsys optical biosensor was accompanied by occurrence of the 7-pentoxyresorufin O-dealkylation reaction in the monomeric reconstituted (in 500 mM K-phosphate buffer) P4502B4 system. Under such conditions, no complex formation between the reductase and cytochrome b_5 was observable. At the same time the binary d-Fp/d-2B4 complexes were formed with the complex association/dissociation rate constants (k_{on} and k_{off}) = $(3\pm2)*10^4$ $M^{-1}s^{-1}$ and 0.05 ± 0.03 s^{-1} , respectively. The formation of ternary d-Fp/d-2B4/d-b5 complexes with the lifetime of about 40 s was also registered.

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WP34 ATOMIC FORCE MICROSCOPY(AFM) AND OPTICAL BIOSENSOR STUDIES OF CYTOCHROME P450'S COMPONENT INTERACTIONS

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It is shown that BIA-RM enables to reveal the binary as well as ternary complexes between redox proteins of P450cam, P402B4 and P450scc systems and measure kinetic parameters of protein-protein interaction. The AFM technique allows visualize these complexes. The AFM images of cytochrome P450cam (P450cam), putidaredoxin (Pd) and putidaredoxin reductase (PdR), binary (Pd/PdR, P450cam/Pd, P450cam/PdR) and ternary (PdR/Pd/P450cam) complexes were obtained and the heights on a mica support were measured. The AFM images of the redox proteins of P4502B4 system - cytochrome P4502B4 (d-2B4), NADPH-dependent cytochrome P450 reductase (d-Fp) and cytochrome b_5 (d-b_5) and images of the binary complexes d-Fp/d-2B4 and d-2B4/d-b_5 were obtained on HOPG. However, we failed to detect formation of d-Fp/d-b_5 complex. The ternary d-Fp/d-2B4/d-b_5 complexes were visualized and the typical height of the complexes were found to be 6.2 nm. The AFM images of the redox proteins of P450scc system were obtained and the heights of the binary complexes AdR/P450scc, AdR/Ad and Ad/P450scc were found to be 3.2-3.4, 3.1-3.4 and 2.8-3.6 nm, respectively.

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WP35 METABOLISM OF N-BENZYL-1-AMINOBENZOTRIAZOLE BY P450 2B1 RESULTS IN A MODIFIED ENZYME CAPABLE OF *N*-DEMETHYLATION BUT WITH ABOLISHED HYDROXYLATION ACTIVITY

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The 7-ethoxy-4-(trifluoromethyl)coumarin (EFC) activity of P450 2B1 is inactivated by *N*-benzyl-1-aminobenzotriazole (BBT) in a mechanism-based manner. Approximately 25% of the loss in enzymatic activity could be accounted for by a minor decrease in the P450 CO spectrum and the formation of a small amount of an MI complex. The majority of the inactivation was due to the formation of a covalent BBT adduct. Concurrently, the mass of the P450 apo-protein increased by 225 Da. Mechanistic studies that examined the rates of reduction, oxy-ferro intermediate formation, and product formation suggested that the reductive process was affected. However, the reduced rate of reduction alone could not account for the overall loss in EFC enzymatic activity. The BBT-inactivated enzyme also exhibited a comparable loss in testosterone hydroxylation activity. However, the ability of the BBT-inactivated enzyme to generate formaldehyde from benzphetamine metabolism was minimally affected. Substrate binding studies with benzphetamine and testosterone indicated that the BBT-modified P450 2B1 displayed similar spectral binding properties when compared to the native enzyme. Analysis of the benzphetamine metabolites showed a greater propensity of the BBT-modified enzyme towards the *N*-demethylation pathway and away from generating hydroxylated products. In addition, a change in the ratio of amphetamine to methamphetamine products was observed. With EFC or testosterone as a substrate the major defect appears to be a reduced rate of electron transfer and an inability of the enzyme to form hydroxylated products. In contrast to EFC and testosterone, benzphetamine can be metabolized by the BBT-modified enzyme via *N*-demethylation and *N*-debenzylation to levels comparable to the unmodified, control enzyme. (Supported by CA 16954 from the National Cancer Institute).

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WP36 A PROTON-SHUTTLE MECHANISM MEDIATED BY THE PORPHYRIN IN BENZENE HYDROXYLATION BY CYTOCHROME P450 ENZYMES

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One of the typical reactions catalyzed by Cytochrome P450 is arene hydroxylation. Although, the mechanisms have been studied thoroughly by experimental means,[1,2] it is still under debate. One of the issues of controversy is the observation of side-products such as arene oxide and cyclohexenone, of which the latter one is proposed to be obtained as the result of the so-called NIH-shift.[3] This has prompted us to carry out a quantum chemical study into the mechanism of benzene hydroxylation by a Cpd I model.[4] The calculations show a competition between radicalar and electrophilic mechanisms in which the π-system is attacked by the oxygen atom of the iron-oxo moiety to form two type of σ-complexes, one radicalar and one cationic. The dominant channel is the electrophilic substitution channel that produces a cationic σ-complex. Both type of σ-complexes can give arene oxide products via ring-closure. This product does not rearrange to form either phenol or cyclohexenone, so is an enzymatically dead-end product. We discovered a mechanism for an enzymatic mechanism to produce phenol or cyclohexenone via a proton-shuttle mechanism from the intermediate complexes.

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WP37 EFFECT OF MEMANTINE ON *IN VITRO* CYTOCHROME P450-MEDIATED METABOLIC ACTIVITIES IN HUMAN LIVER MICROSOMES

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Memantine is an uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist with therapeutic potential in dementia, spasticity and Parkinson's disease. This compound is predominantly excreted unchanged via the kidneys. Nevertheless, 10% to 25% of administered dose is excreted into bile, with 1% being metabolized to hydroxy metabolite. Therefore, potential for drug-drug interactions at the level of hepatic metabolism exists. This study was undertaken to examine the effects of memantine on several drug metabolizing CYP enzymes found in the liver. Pooled human liver microsomes were co-incubated with memantine and probe substrates for CYP1A2 (ethoxyresorufin), CYP2A6 (coumarin), CYP2C9 (tolbutamide), CYP2D6 (dextromethorphan), CYP2E1 (chlorzoxazone), and CYP3A4 (nifedipine). The formation of CYP-specific metabolites following co-incubation with various memantine concentrations was determined to establish IC_{50} and Ki values for these enzymes. While memantine did not inhibit CYP1A2, CYP2A6, CYP2C9, CYP2E1, and CYP3A4 activities at concentrations below 500 microM, this compound inhibited CYP2D6 with IC_{50} and Ki values of 368.7 μM and 250 μM, respectively. In conclusion, these results indicate that, although memantine can inhibit CYP2D6 catalytic activity, it would not be expected to cause any significant interactions with others CYP-metabolized drugs at clinically relevant concentrations achieved during long-term therapy.

This study was performed within the framework of the COST B15.30 project.

WP38 21-FLUOROSTEROIDS USED IN KINETIC AND 19F-NMR STUDIES TO PROBE CYP17 CATALYSIS

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Porcine microsomal cytochrome P450 17α-hydroxylase-17,20-lyase (CYP17) catalyses the conversion of pregnenolone (preg) to 17α-hydroxypregnenolone which is then a substrate for the enzyme's cleavage activity, resulting in the production of dehydroepiandrosterone with the side-chain being removed as acetic acid. In the presence of cytochrome b_5 (b_5) preg can be converted directly to 3β-hydroxyandrosta-5,16-diene (5,16-diene)[1]. We have shown that the substrate analogue, 3β-hydroxyandrost-5-ene-17β-carbaldehyde (aldehyde) is cleaved directly, in the absence of b5, to the 5,16-diene at a rate 8-fold higher than preg[2]. This was attributed to enhanced reactivity of the aldehyde carbonyl with a nucleophilic heam-iron-peroxy species. We now report that 21-fluoropreg is a substrate for CYP17 with the side-chain being removed as fluoroacetate. In contrast to the aldehyde, the 21-fluorocompound is cleaved at one third the rate of preg and the cleavage is not directed exclusively to the 5,16-diene. The reactivity of the aldehyde is, therefore, unlikely to be solely due to the enhanced electrophilicity of the C-20 carbonyl but may also be a result of an altered binding conformation. The lack of the 21-methyl group may facilitate the juxtaposition of the aldehyde carbonyl for attack by the active site nucleophilic iron-peroxy intermediate, which leads to the cleavage of the formyl side chain.

Also presented are preliminary Fluorine-NMR experiments that show that it is possible to measure the paramagnetic effect of the active site, high-spin iron on the T_2 relaxation rates of the fluoropreg enzyme-bound substrate. The data will be used to determine the distance from the active-site iron to the fluorine label in the substrate. The study can be extended with the use of substrates, labelled with fluorine at different positions, to determine the orientation of substrate binding.

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WP39 EFFECTS OF THALIDOMIDE ON THE CYP-MEDIATED METABOLISM OF CYCLO-PHOSPHAMIDE IN THE MOUSE

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Concurrent administration of racemic thalidomide (TH) (20 mg/kg, i.p.) potentiated the antitumour effect of cyclophosphamide (CP) (220 mg/kg, i.p.) with no increase in toxicity in the murine Colon 38 tumour model. This was accompanied by an increase in the area under the plasma concentration-time curve (AUC) for CP (3.5-fold), 4-hydroxycyclophosphamide (4-OH-CP) (2.7-fold), dechloroethylcyclophosphamide (dCE-CP) (1.8-fold) and 4-ketocyclophosphamide (4-keto-CP) (1.3-fold), but with no significant change in their C_{max}[1]. The aim of this study was to investigate whether the pharmacokinetic interaction arose from TH inhibition of the major metabolic pathways of CP associated with CYP-mediated 4-hydroxylation or dechloro-ethylation. In addition, the possibility that a TH metabolite or hydrolysis product was responsible for this interaction was investigated, as both metabolism and hydrolysis pathways are of major importance in the elimination of TH in the mouse[2].

Methods

CP, 4-OH-CP and dCE-CP were measured using solid phase extraction and LC-MS methods[1]. In the *in vivo* studies, C57B1/6 male mice (30 - 35 g) received i.p. injections of CP (220 mg/kg) followed by TH (20 mg/kg), with 3 mice used for each time point. Having established the concentration-time profile for 4-OH-CP with and without TH, a 60 min blood collection time point was selected to study the effects of 20 mg/kg of 5-hydroxy-thalidomide (5-OH-TH) (the major CYP-mediated metabolite), α-aminoglutaramide (AG), and phthaloylisoglutamine (PG) (the major TH hydrolysis product). At 60 min, plasma was collected for 4-OH-CP measurement. *In vitro* studies of CP oxidation were undertaken with mouse liver microsomes (0.5 mg/ml protein, 5 mM $MgCl_2$, 1 mM NADPH, CP [20 - 2000 μM] in pH 7.4 phosphate buffer) incubated for 30 min at 37°. This was followed by the addition of 500 μL methanol and 200 μL semi-carbazide, and the formation of 4-OH-CP measured. The effects of TH, 5-OH-TH, and PG (all at 50 and 500 μM) on the formation rate of 4-OH-CP were investigated. The formation of dCE-CP was also investigated in a separate set of incubations in a similar manner, except that the semi-carbazide was omitted.

A reduction of > 30% in the metabolite formation rate was considered significant.

Results

All compounds, except PG, caused a >2-fold increase in the plasma concentrations of 4-OH-CP at 60 min. Using 1 mM CP as substrate, the rate of formation of 4-OH-CP was not influenced by co-incubation with TH, 5-OH-TH or PG. In contrast, there was a significant reduction in the formation rate of dCE-CP in the presence of TH. Our results suggest that 5-OH-TH and hydrolysis products may play a part in the pharmacokinetic interaction with CP, but that the destruction of the glutarimide ring eliminates this activity. Direct inhibition of CYP-mediated CP activation pathway to 4-OH-CP does not appear to play a part in this interaction, but the deactivation pathway to dCE-CP may be involved.

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WP40 REDUCTION OF THE NITROAROMATIC ANTIANDROGEN NILUTAMIDE BY NO SYNTHASES. IMPLICATIONS FOR THE ADVERSE EFFECTS OF THIS ANTIANDROGEN DRUG

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Nitric oxide synthases (NOSs) are flavohemeproteins that catalyze the oxidation of L-arginine to L-citrulline with formation of the widespread signal molecule NO. Beside their intrinsic biological role in NO synthesis, these enzymes are also involved in the formation of reactive oxygen species and in the interaction with some xenobiotic compounds such as quinones[1-3].

Nilutamide **1** is a nonsteroidal antiandrogen derivative behaving as a competitive antagonist of the androgen receptors[4]. This nitroaromatic compound is proposed in the treatment of metastatic prostatic carcinoma. After oral administration in humans, Nilutamide is extensively metabolized in the liver, undergoing mainly reduction of its nitro group to primary amine, **2**[5]. Therapeutic effects of nilutamide are overshadowed by the occurrence of several adverse reactions. More serious complications include drug-induced hepatitis and pulmonary interstitial fibrosis. Absence of hypersensitivity manifestations suggests that Nilutamide hepatitis is mediated by a toxic mechanism which remains poorly known[6]. Previous studies have shown that NOSs could be involved in these toxic effects of Nilutamide[7].

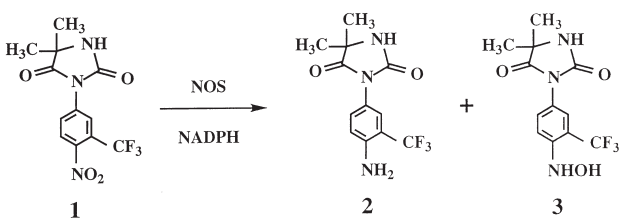

Fig. 1.

We studied the ability for recombinant NOSs to interact with Nilutamide and we showed that NOSs reduce this nitroaromatic compound with selective formation of the corresponding hydroxylamine **3**. This new reaction of neuronal NOS (nNOS) proceeds with intermediate formation of a nitro anion free-radical easily observed by EPR. The reduction of Nilutamide catalyzed by nNOS was insensitive to the addition of usual heme ligands and L-arginine analogues but strongly inhibited by O_2 and a flavin/NADPH binding inhibitor. Involvement of the reductase domain of the nNOS was further confirmed by the stimulating effect of Ca^{2+}/Calmodulin on the accumulation of hydroxylamine and nitro anion radical and by the ability of the isolated reductase domain of nNOS to catalyze this reaction. Inducible and endothelial NOS also displayed this nitroreductase activity, albeit with lower yields.

Selective reduction of Nilutamide to an hydroxylamine by NOSs could explain some of the toxic effects observed with this nonsteroidal antiandrogen and other nitrocompounds.

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WP41 DYNAMICS OF CYP2D6 ACTIVITY AND CLINICAL RESPONSE TO PAROXETINE, ALPRAZOLAM AND THEIR COMBINATION IN PATIENTS WITH DEPRESSIVE DISORDER

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Numerous antidepressants including tricyclic drugs and SSRI used in a long-term treatment of depressive disorder are substrates of polymorphic cytochrome P450 2D6 (CYP2D6). The therapeutic response and occurrence of adverse reactions to the substrate drugs may widely be dependent on the CYP2D6 activity. Paroxetine is known as one of the most potent CYP 2D6 inhibitors. Due to the strong inhibitory effect, particularly expressed during a long-term therapy, paroxetine is able to evoke phenotypic conversion to PM phenotype even in individuals with EM genotype (Hadašová E., Žourková A.: 18th Eur. Workshop on Drug Metabolism, Valencia, 16-20 September, 2002, Abstract Book, p. 52). Nevertheless, neither the extent of inhibition and its reversibility nor the factual clinical impact of decreased CYP2D6 capacity has definitely been established yet.

The aims of the study were 1) to characterize the dynamics of the paroxetine-induced CYP2D6 inhibition during the paroxetine therapy and the level of the enzyme restoration after discontinuation of the drug in patients with depressive disorder; 2) to follow the correlations between the actual CYP2D6 capacity, patient's genotype and clinical response.

The study was performed in three groups of patients (N=30) treated with alprazolam (group I), paroxetine (group II), and their combination (group III). All patients gave their written consent with participation in the study. Alprazolam was chosen as a comparator drug (positive control) because of its good antidepressant effect without a remarkable alteration of the CYP2D6 activity. Patients in all groups were repeatedly tested for CYP2D6 activity in the following periods of therapy: at the end of the washout period preceding the start of therapy, at the end of 6 weeks' therapy, and, finally, 4 weeks after discontinuation of paroxetine therapy in groups II and III and their change-over to alprazolam (week 10 of the study).

CYP2D6 genotype was determined by allele-specific PCR with detection of mutations in exons 3, 4, 5 and deletion in exon 6. Dextromethorphan (DEM, 25 mg p.o.) was used as a probe drug for CYP2D6 phenotype. Concentration of DEM and its metabolites dextrorphan, methoxymorphinan and hydroxymorphinan were measured in 8 h-urine by HPLC, the DEM/DOR metabolic ratio (MR) and proportional excretion of DEM and its metabolites were calculated and used as parameters of the actual CYP activity.

The therapeutic effect was assessed by HAMA, COVI and CGI 1 scales. Side effects were assessed according to UKU rating scale, sexual disorders using ASEX scale.

Homozygous PM genotype was not found within the group. Heterozygous mutation in CYP2D6 locus was found in 7 individuals (23.3 %), 4 patients (13.3 %) had deletion in exon 6 at one allele.

Paroxetine evoked significant inhibition of CYP2D6 or even „PM phenocopy" in 8 of 16 (50 %) patients treated with paroxetine alone or in combination with alprazolam. CYP2D6 inhibition was more pronounced in EM patients with heterozygous mutation in the CYP locus. Four weeks after discontinuation of paroxetine therapy, the enzyme activity was fully restored in 7 of 8 patients switched to PM phenocopy.

The therapeutic response was satisfactory in all tested groups. More rapid onset of paroxetine therapy was observed in PMs than in EMs. There was no significant difference between patients with EM and PM phenotype in the incidence and severity of adverse effects, but, PMs were more prone to sexual disorders.

The results of this small study suggest that even a profound inhibition of CYP2D6 in individuals with EM genotype may have low clinical relevance namely in drugs with higher therapeutic indexes like paroxetine and other SSRI antidepressants.

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WP42 INHIBITION OF SPONTANEOUSLY PRODUCED NITRIC OXIDE IN CONVENTIONAL AND PERFUSED HEPATOCYTE CULTURE IMPROVES CELL FUNCTIONALITY

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Introduction

Hepatocytes in culture synthesize increased levels of nitric oxide (NO) following isolation as a result of induction of nitric oxide synthase (NOS). The isoform of this enzyme (iNOS) is induced in response to stress that occurs during the two-step perfusion of the liver by EGTA/collagenase for hepatocyte isolation that results in sheer stress and structural changes of the hepatocyte architecture in the liver[1].

Goals

The present study was therefore directed to assess the spontaneous NO production under various culture conditions and compare its influence on functional status of hepatocytes in conventional cell culture and in recently described flat membrane bioreactor (FMB)[2], i.e. perfused culture.

Methods

Hepatocytes were isolated from rats using the standard two-phase collagenase perfusion method and seeded on petri dishes or into the bioreactor entire space formed by polycarbonate plate and gas-permeable 25-_m membrane. The hepatocytes were cultivated on the single collagen layer as a simple monolayer or between two layers of collagen as sandwich. For simple monolayer, supplemented William's E medium with 5% bovine fetal serum and 1 mM NH_4Cl in absence or presence of cyclosporin A (CsA, 2 μg/ml) was utilized. For sandwich culture, the above-mentioned culture medium and hormone enriched serum free Hybridoma Medium with prednisolone, glucagon, insulin and NH_4Cl were used throughout the study. The hepatocytes in FMB were perfused in a constant rate of 4.5 ml/h. Samples were collected in appropriate time intervals and nitrite levels were measured. Hepatocyte functions were tested by urea synthesis, ALT leakage and CsA biotransformation (HPLC-assessed) during 48 hours.

Results and Discussion

It was found here that perfused hepatocyte culture is metabolically more efficient when contrasted to the static conventional cell culture (*Table 1*). Sandwiching hepatocytes in double layer of collagen improved hepatocytes metabolic competence and reduced NO productivity with consequent preservation of cell integrity in culture. Moreover, the negative influence of NO on urea production was suppressed by the cultivation of hepatocytes in the culture medium containing prednisolone, which is known to inhibit iNOS expression. Similarly, the addition of the immunosuppressant CsA decreased the spontaneous NO production and enhanced urea biosynthesis. The effect of CsA on modulation of urea and spontaneous NO production demonstrated flexibility of the perfused culture in observing the time course of hepatocyte function status in a non-destructive way. The monitoring of NO levels during various

Tab. 1: *The rate of spontaneous NO production, urea synthesis (1 mM NH_4Cl), CsA disappearance and ALT leakage to the culture media (WE + FBS: William's E medium with serum; HM - FBS: hormone enriched serum free Hybridoma Medium with prednisolone) of the single-collagen-layer and sandwich conventional hepatocyte culture (SCL-/SW-HC) or flat membrane bioreactor (SCL-/SW-FMB). Data are expressed as mean ± SEM (n = 3-4).*

Metabolite	**Nitrite levels nmol/106cells /hr**	**Urea levels μg/106cells/hr**	**ALT levels mU/106cell/hr**	**CsA% initial value**	
Culture conditions	**0-48 hr**	**0-48 hr**	**0-48 hr**	**24 hr**	**48 hr**
SCL-HC: WE + FBS	5.10 ± 0.53	1.55 ± 0.03	2.47 ± 0.07		
SCL-HC: CsA 2 μg/ml	4.56 ± 0.57	1.74 ± 0.04	2.61 ± 0.07	24 ± 3	19 ±3
SCL-FMB: WE + FBS	1.07 ± 0.05	2.59 ± 0.44	2.37 ± 0.30		
SCL-FMB: CsA 2 μg/ml	0.60 ± 0.11	2.87 ± 0.59	3.23 ± 0.60	16 ± 2	9 ±1
SW-FMB: WE + FBS	0.54 ± 0.01	3.82 ± 0.46	1.33 ± 0.09		
SW-FMB: HM - FBS	0.34 ± 0.05	4.67 ± 0.97	2.18 ± 0.14		
SW-HC: HM - FBS	0.19 ± 0.01	2.27 ± 0.09	1.86 ± 0.05		

steps of isolation and cultivation suggests that spontaneously produced NO has a negative impact on hepatocyte metabolic and functional integrity.

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WP43 DIFFERENCES AND COMPARISONS OF THE ACTIVE SPECIES (CPD I) OF CYTOCHROME P450 AND HORSE RADISH PEROXIDASE AS REVEALED BY DENSITY FUNCTIONAL THEORETICAL STUDIES

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The enzymes Cytochrome P450 (P450) and Horse radish peroxidase (HRP) share many common features. The active species of both enzymes consists of an oxo-iron protoporphyrin system which is linked to the amino acid chain via the sixth ligand of the iron atom. In P450 this linkage is a thiolate of a cysteinate residue, whereas in HRP the linkage happens via an imidazole group of a histidine amino acid. Since, thiolate is an anionic ligand while histidine a neutral ligand this causes differences in reactivity and properties of the enzymes. Thus, we have initiated a density functional theoretical study using the unrestricted hybrid density functional method, UB3LYP, to model Cpd I of HRP[1] and compared the results to the ones obtained for a Cpd I model of P450.[2] As will be shown, the differences mainly originate from the „push" effect of the thiolate ligand, which raises the porphyrin a2u-orbital significantly due to interactions with the p_{σ}-orbital on sulfur.[3] Furthermore, the mixing is strongly dependent on environmental effects and results in chameleonic behavior of the active species. Because, the two active species shows differences in catalytic properties, we tested the reactivity differences of Cpd I of HRP[4] versus Cpd I of P450[5] by modelling the oxidation reaction of propene, which can result in either double bond epoxidation or C-H hydroxylation. Although, P450 has a clear preference for C-H hydroxylation in a protein environment,[5] by contrast HRP prefers epoxidation even under environmental conditions.

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WP44 INHIBITION OF INDUCIBLE NITRIC OXIDE SYNTHASE ACTIVITY BY OLTIPRAZ *IN VITRO* AND IN ACTIVATED MICROGLIA CELLS

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The clinically relevant drug oltipraz (OPZ) has previously been shown to be a mechanism-based inhibitor of cytochome P450 1A2 and an inducer of glutathione S-transferases. The current study shows that OPZ is also able to inhibit •NO formation by purified inducible nitric oxide synthase (iNOS) but not by purified neuronal nitric oxide synthase (nNOS) in hemoglobin assays. The inhibition of iNOS by OPZ is mixed-function with an IC_{50} of 8 μM. In murine BV-2 microglial cells, an immortalized cell line that produces •NO in response to lipopolysaccharide (LPS), OPZ is able to block the formation of nitrite in LPS-treated cells. The IC_{50} in activated BV-2 cells is ~5 μM. The inhibitory effect of OPZ on LPS-treated BV-2 cells is not due to cell toxicity. The concentrations of OPZ used in these studies are comparable to clinically relevant plasma concentrations in patients treated with OPZ. Finally, treatment of BV-2 cells with OPZ does not suppress production of iNOS protein. These results support an additional role of OPZ as an inhibitor of iNOS.

WP45 ENDOTHERIAL NITRIC OXIDE SYNTHASE REDUCTASE DOMAIN IS ACTIVATED BY CALMODULIN AND PHOSPHORYLATION

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The electron transfer reaction of NOS reductase domains is controlled by calmodulin (CaM)[1,2]. These domains are structurally similar to other dual flavin enzymes like cytochrome P450 reductase (CPR). Endothelial nitric oxide synthase (eNOS) function is also regulated by Ca/CaM, in part by post-traslational mechanisms including phosphorylation and protein modifications. In this study, the reductase domain was expressed in *E. coli* BL21 cells using expression vector pCWori$^+$. The purified enzyme contains the FAD, FMN, NADPH and CaM binding sites. Redox reactions of the reductase domain (FAD/FMN) were monitored at 457 nm (derived from oxidized flavins), 596nm (derived from semiquinone form of flavins) and 520 nm (derived from FAD semiquinone) by using stopped-flow and rapid-scan spectrometry methods. (1) Reduction of the oxidized enzyme (FAD-FMN) with NADPH occurred at least two phases. The both rates of reduction were accelerated in the presence of Ca/CaM. (2) Reduction of the air-stable semiquinone (FAD-FMNH.) with NADPH occurred at least two phases, and both phases were accelerated in the presence of Ca/CaM. and the increase at 520 nm was observed only in the presence of Ca/CaM. From the data of (1) and (2), we suggest that Ca/CaM accelerates both rates of reduction of FAD and intramolecular one-electron transfer from FAD and FMN. In addition to these experiments, we have analyzed the autoxidation spectra of reduced enzymes, which include the mutants of Ser 633 Asp and Ser1177Asp. The data suggest that the mimic mutation of phosphorylation sites of eNOS reductase domain activates one-electron transfer reaction between FAD and FMN.

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WP46 RECRUITMENT OF GOVERNING ELEMENTS FOR ELECTRON TRANSFER IN NITRIC OXIDE SYNTHASE FAMILY

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Nitric oxide synthases (NOS) exist as bidomain structures containing both N-terminal heme (oxygenase) and C-terminal flavoprotein (reductase) domains. The oxygenase domain contains the binding sites for iron protoporphyrin IX, pteridine cofactor (H_4B), and the substrate, L-arginine, and serves as the site for O_2 binding and substrate conversion to the oxygenated products. The flavoprotein domain shuttles electron equivalents from NADPH through FAD, then FMN, to the heme. Due to structural similarities among the NOS heme domains, other differences among the isoforms were sought to explain the unique catalytic turnovers in the production of NO. Each NOS isoform contains more C-terminal residues (iNOS: 21; nNOS: 33; and eNOS: 42) than NADPH-cytochrome P450 reductase (CYPOR), with which they share approximately 60% sequence similarity. CYPOR chimeras were constructed by incorporating the C-terminal tails of iNOS, nNOS, and eNOS, respectively, to the soluble CYPOR sequence to determine their effects on electron transport activities.

The ability of the CYPOR-soluble and CYPOR chimeras to catalyze the cyt c, DCIP, and ferricyanide reduction was examined. With the incorporation of residues from C-termini of the iNOS, nNOS, and eNOS, the reduction of all acceptors was lower, but NADPH oxidation increased by approximately 60% for all chimeras as compared to soluble CYPOR. The decrease in acceptor reduction was directly proportional to the length of the tail; i.e., cyt c reduction was decreased 20.2%, 25.8%, and 42.5% with the addition of the iNOS, nNOS, and eNOS tails, respectively. Stopped-flow spectrophotometry under turnover conditions showed that flavin reduction is five-fold faster with CYPOR-iNOS than the other proteins. These studies indicate that the C-termini of the NOS isoforms influence flavoprotein electron transfer to various electron acceptors.

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WP47 MODULATION OF ELECTRON TRANSFER IN NITRIC OXIDE SYNTHASE USING NON AMINO ACID GUANIDINES AND HYDROXYGUANIDINES

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Nitric oxide, NO, is a key inter- and intramolecular molecule involved in the regulation of vascular tone, neuronal signaling and host response to infection. The biosynthesis of NO in mammals is catalyzed by constitutive neuronal and endothelial NO synthases (nNOS and eNOS) and by inducible NOS (iNOS) expressed in macrophages following induction by inflammatory mediators. All three NOSs are heme-thiolate proteins related to cytochrome P450s that produce NO and citrulline from the two-step oxidation of L-arginine by NADPH and O_2, with intermediate formation of N-omega-hydroxy-L-arginine (NOHA). Many physiopathological situations are characterized either by an overproduction of NO, by a deficit in NO formation, or by the concomitant release of NO and superoxide, $O_2^{\cdot-}$, a situation that leads to the formation of the potent oxidizing agent peroxynitrite ($O{=}N{-}O{-}O^-$). Generation of peroxynitrite by NOSs is greatly enhanced in the absence of the cofactor tetrahydrobiopterin (BH_4) and/or substrate L-arginine, two compounds that are key-regulators of the electron transfer from the reductase to the oxygenase domain of NOSs.

We recently reported that some non amino acid N-alkylguanidines, N-aryl and N-alkyl-N'-hydroxyguanidines are efficiently oxidized by iNOS with formation of NO in reactions very similar to the oxidation of L-arginine or NOHA to L-citrulline and NO[1-3]. We studied the ability of some of these new non amino acid guanidines and hydroxyguanidines to modulate the electron transfer in the three recombinant NOSs. We measured their effects on NADPH consumption and formation of $O_2^{\cdot-}$ using a recently synthesized spin trap BMPO[4]. Some of these compounds strongly enhanced the NADPH consumption and $O_2^{\cdot-}$ generation by iNOS whereas others are potent inhibitors of the electron transfer.

These results show that modulation of the activities of NOSs (NO *versus* $O{=}N{-}O{-}O^-$ formation) can be achieved by the use of simple non amino acid guanidines and hydroxyguanidines. Their interactions with the active site will be more deeply investigated by UV/Vis, EPR spectroscopy, and molecular modelling. Some of these compounds could be of pharmacological interest.

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WP48 ON THE ROLE OF THE CYSTEINE LIGAND IN CYTOCHROMES P450

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What is the role of the cysteine ligand for the cytochromes P450 and what makes it different from other heme proteins such as the globins, peroxidases and catalases which have other axial ligands? To answer these questions we have made a systematic investigation of how the axial ligand in heme proteins influences geometry, electronic structure, spin states, and reaction energies. Using the density functional B3LYP method and medium-sized basis sets, we have compared models with histidine, histidine with a nearby aspartate, cysteine, and tyrosine (with and without an nearby arginine). We have studied twelve reactants and intermediates of the reaction cycles of these enzymes, including complexes with H_2O, OH^-, $O_2^{\cdot-}$, CH_3OH, O_2, H_2O_2, and HO_2^- in various formal oxidation states of the iron (II to V).

The results show that the most pronounced difference is found between the neutral histidine ligand an the other ligands. For example the histidine complexes have lower reduction potentials and different proton affinities than complexes with the other ligands. In particular, it is harder to reduce and protonate the $Fe^{II}O_2$ complex with histidine, in accordance with the O_2 carrier function of globins and the oxidative chemistry involving a $Fe^{III}O_2H$ intermediate of proteins with the other ligands. For most properties, the trend cysteine < tyrosine < histidine with aspartate < histidine is found, indicating the donor capacity of the various

ligands. However, the tyrosine complexes have a unusually low affinity for neutral ligands, giving them a slightly enhanced driving force in the oxidation of H_2O_2 by compound I. In vacuum, the formation of compound I is less favourable for histidine than for the other ligands, but this effect disappears in polar solvents. Likewise, we obtain the trend tyrosine < histidine < histidine with aspartate < cysteine for difference in reaction energies of the hetero- and homolytic brekage of the O-O bond.

WP49 IS NOS A NOX-SYNTHASE?

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NO-Synthases present a novel system of self-regulation. Due to a highly efficient geminate recombination, NO is trapped in NOS heme pocket instantaneously after biosynthesis. Therefore, the product of NOS catalysis is not NO but a heme-NO complex. Based on this approach, we recently unified the 3 NOS isoforms in a single kinetic model that explained their divergent catalytic behaviors[1]: Our model is based on the coexistence of two regenerative pathways linked to the nature of the heme-NO complex: i) Fe^{III}-NO gives rise to a rapid productive cycle that releases NO and ii) Fe^{II}-NO leads NOS into a slow and futile cycle that releases higher N-oxides[2]. Partitioning between these two pathways, modifies the catalytic rate, the nature of the catalytic product and the overall efficiency of all three NOSs. Two kinetic steps mainly control the whole process:

Fe^{II}-NO oxidation

We characterized the relationship between heme redox potential and Fe^{II}-NO reactivity by measuring the heme midpoint potential and the rate of Fe^{II}-NO oxidation for iNOS, nNOS, and mutants containing combinations of pteridines and substrate analogs to generate a wide range of potentials. Globally the rate of Fe^{II}-NO oxidation is controlled by heme redox status; heme potential lowering seems to favor a Fe^{III}-NO^- like behavior and to increase heme-NO reactivity with O_2[3]. Our results also highlight a complex correlation between this reactivity and electronic back-donation from the proximal thiolate bond.

Ferric heme reduction

In order to understand the reasons of the variability of heme reduction within the three major mammalian NOS isoforms, we determined the midpoint potential of flavin redox couples of their reductase domains[4]. iNOS $FMNH_2$/FMNH° potential is significantly higher than its heme midpoint potential. Consequently, for iNOS, the electron transfer to the oxygenase domain is controlled by the heme potential increase observed upon substrate binding. The 40-fold difference in heme reduction rate of nNOS and eNOS appears to be linked to modifications of their FAD/FMN redox couples and changes in hydride transfer rate and electron cycling within the reductase. Based on these results, we proposed a model of electron shuttling within NOS and an explanation for the specific oxygen control of heme reduction in eNOS.

Tuning of the two key kinetic parameters allows a given NOS to broadly modify its catalysis as a function of its physiologic environment. It particularly decides of the balance between NO-releasing complex (Fe^{III}-NO) and NOx-releasing species (Fe^{II}-NO). We are currently trying to determine the extrinsic and intrinsic parameters that determine the nature of the synthesized N-oxide.

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WP50 INDUCTION AND MODULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION FOLLOWING KERATOPLASTY IN MICE

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The inducible nitric oxide synthase (iNOS) is known to be expressed following exposure to cytokines or bacterial lipopolysaccharides. It has been suggested that nitric oxide (NO), in particular that which is generated via the induction of iNOS, is an important contributor to inflammatory processes and allograft rejection. Immunohistological studies showed a massive infiltration of rejected corneal allografts by macrophages and CD4+ lymphocytes.[1] CD8+

lymphocytes, activated by CD4+ T-cells, have been considered for a long time to be the main effector cells in the rejection process. Further studies brought evidence that activated macrophages are the key cytotoxic cell population in process of skin allograft rejection in mice.[2] The present study was directed to investigate iNOS expression following orthotopic allogeneic corneal transplantation in mice (C57BL/10; H-2^b to BALB/c; H-2^d) and to apply such a strategy of modulation of iNOS expression that will lead to the prolongation of corneal allograft survival. The expression of the gene for iNOS was assessed in non-rejected and rejected corneas using RT-PCR. The iNOS protein was detected by means of immunohistological techniques in crysections stained with anti-iNOS polyclonal antibody. 4,5-diaminofluorescein diacetate (DAF-2 DA) enables a direct detection of NO in tissues. Our results suggest that iNOS mRNA and iNOS protein are expressed during rejection of corneal allograft, whereas non-rejected transplants and non-transplanted corneas do not exert any expression of iNOS. Using DAF-2 DA, higher fluorescence was found in rejected grafts than in non-rejected grafts. The application of the selective immunosuppressant FK 506 (0.3 mg/kg/per day) or the specific inhibitor of iNOS aminoguanidine (100 mg/kg/per day) prevents corneal allograft rejection within 4 weeks in 71% and 57% of animals, respectively, (compared to 29% of clear grafts in controls) and suppresses iNOS at different levels. In conclusion, iNOS was strongly expressed in rejecting grafts of the control group and the suppression of iNOS prolonged the corneal allograft survival, under the present conditions.

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WP51 MECHANISTIC STUDIES ON THE INTRAMOLECULAR ONE-ELECTRON TRANSFER BETWEEN THE TWO FLAVINS IN INOS FLAVIN DOMAIN AND NADPH-CYTOCHROME P450 REDUCTASE

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Flavin electron transferases, including NOSs and NADPH-Cytochrome P450 reductase (CPR) catalyze one-electron reduction of quinones[1,2]. In this work, the inducible nitric-oxide synthase (iNOS) reductase domain was co-expressed with CaM. The purified reductase domain catayzed aerobic NADPH-oxidation in the presence of the model quinone compound menadione (MD). The MD-mediated NADPH oxidation was inhibited in the presence of NAD(P)H:quinone oxidoreductase(QR). The one-electron reduction potential of various Qs was related to their reduction rates by both the iNOS reductase domain and CPR. In both iNOS reductase domain and CPR, the semiquinone species (FAD-FMNH.) were major intermediates observed during the oxidation of the reduced enzyme by MD, but the fully reduced flavin species did not significantly accumulate under these conditions. Air-stable semiquinone form (FAD-FMNH.) and the FAD semiquinone (FADH.) do not react rapidly with MD, but the fully reduced species of both flavins, FAD and FMN, can donate rapidly one electron to MD. In the addition to air-stable semiquinone intermediate, the disemiquinone species (FADH.-FMNH.) as an intermediate was observed in the iNOS reductase domain. Finally, the iNOS reductase domain and CPR can shuttle between the 1e/3e level during one-electron reduction of MD. The CaM-bound state of the iNOS flavin domain showed similar rdox properties to those of CPR. Furthermore, the present data indicate that the low level of reactivity with electron acceptors of both FAD and FMN semiquinones could enable a successive one-electron transfer from the reduced FMN ($FMNH_2$) to Cytochrome P450.

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WP52 XANTHATES (DITHIOCARBONATES) METABOLISM BY SOME MONOOXYGENASES

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Xanthates (salts of alkyl or aryl derivatives of dithiocarbonic acid, $ROCS_2K$) are well known metal chelating agents. In different chemical and biological systems they behave also as very potent one electron acceptors. By pyrolytic reaction at 300 °C they decomposed to pure olefins.

Our studies have shown that such pyrolytic cleavage of xanthate molecule could be reproduced at 37 °C by any enzymatic or nonenzymatic system which generates active oxygen species as hydroxyl radicals ($Fe/EDTA/H_2O_2$, xanthine-xanthine oxidase, hemoglobin, cytochrome P450 (CYP)). The primary changes in xanthate molecule after CYP attack is one or two hydrogens abstraction from the first carbon atom of the alkyl chain. The resulting intermediate(s) irreversibly bound to enzyme protein. Such metabolic transformation was exert only by CYP 2B1/2B6 (with high affinity) and CYP 2E1 (with lower affinity). All others CYP are ineffective or only inhibited by xanthates. By this way xanthates behaved as potent and selective mechanism-based inactivators of some CYP enzymes. 3D QSAR studies showed that CYP xanthates inhibitory potency depends mainly by the derivative lipid solubility and molecular volume as the inactivation potency (rate of xanthate metabolism by CYP) depends mainly by the charge of the first carbon atom of the alkyl chain.

Differently from CYP, xanthates are oxidized by some FMO's at the sulfur part of the molecule to the corresponding S-peroxido dithiocarbonate derivatives (perxanthates). The same sulfur oxidation occurs in pure chemical system containing hydrogen peroxide.

The readiness of xanthate molecule to interact with different reactive oxygen species reflect in from one side their potent antioxidant and scavenger activity, and from the other side can explain the differences in their metabolic profile after attack from different monooxygenases. Knowing that the balance between the oxygen species in the cell and other active intermediates like NO, is playing crucial role in cell homeostasis, such xanthates properties could be on the background of their antiviral and anticancer activity triggered by the unusual xanthate metabolism in the cell.

WP53 THE METABOLIC RATIO CARBAMAZEPINE/CARBAMAZEPINE-EPOXIDE IN CHILDREN AND ADULT PATIENTS ON ANTIEPILEPTIC POLYTHERAPY

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Carbamazepine (CBZ) is considered a first-line drug in the treatment of most forms of epilepsy. Its pharmacokinetics shows both inter- and intraindividual variability with respect to age, dose, concomitant therapy and disease. The major metabolic pathway is the epoxide-diol enzymatic formation and carbamazepine-10,11- epoxide (CBZE) is an active metabolite with similar effects as the parent drug [1,2].

In this study we investigated metabolic ratios of CBZ /CBZE in children and adult patients on CBZ-antiepileptic polytherapy, as well as compared other pharmacokinetic parameters between the two groups.

Patients with epilepsy receiving CBZ in combination with other antiepileptic drugs (AEDs) were studied. AEDs taken in addition to CBZ included phenobarbital, valproic acid, lamotrigin or clonazepam. Thirty two patients: 21 children, 11 ± 2 years, mean ± SD and 11 adults, 31 ± 4 years, mean ± SD, were taken steady-state heparinized blood samples at 8 am, and at 1 and 6 pm. CBZ and CBZE were measured by UV-reversed-phase high-performance liquid chromatographic (RP-HPLC) method, after their extraction from plasma with ethyl acetate.

Pharmacokinetic parameters: metabolic ratio of parent drug and its metabolite (CBZ/CBZE), concentration-dose

Tab. 1. *Comparison of pharmacokinetic parameters of CBZ between children and adult patients with epilepsy*

Parameter	Group		Significance
	Children	Adults	
D_{CBZ} (mg/kg/day)	17,23 ± 5,36	17,32 ± 5,59	NS
C_{CBZ} (mg/L)	6,82 ± 3,24	8,60 ± 2,60	$p < 0,05$
C_{CBZE} (mg/L)	1,32 ± 0,80	1,37 ± 0,83	NS
$\frac{C_{CBZ}}{C_{CBZE}}$	5,99 ± 2,92	10,45 ± 10,35	$p < 0,05$
$\frac{C_{CBZ}\ (mg/L)}{D\ mg/kg/day}$	0,41 ± 0,20	0,66 ± 0,56	$p < 0,05$
CL_{CBZ} (L/h/kg)	0,347 ± 0,246	0,292 ± 0,131	NS
AUC_{CBZ} (mgh/L)	30,56 ± 30,26	75,40 ± 41,58	NS

ratio for CBZ (C/D), clearance (CL) as a direct measure of the activity of metabolizing enzymes, and area under the curve (AUC), were compared between the adults and children using Mann-Whitney test[3]. Summary results are presented in *Table 1*.

Diferences in CBZ/CBZE metabolic ratios between children and adults on equivalent antiepileptic polytherapy obtained in the study were dependent on differences in CBZ plasma concentrations between the two groups of patients. Metabolite, CBZE plasma levels, as well as plasma clearance values for CBZ, were comparable.

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WP54 NEW *IN VITRO* METABOLITES OF PACLITAXEL IN HUMANS, RATS, MINIPIGS AND REGULAR PIGS AND CYP INVOLVED IN THEIR FORMATION

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Paclitaxel is an important recently introduced antineoplastic drug. We investigated cytochrome P450 (CYP) - cytalyzed metabolism of paclitaxel in rat, minipig, regular pig and human liver microsomes. In the rat microsomes, paclitaxel was metabolized mainly to C3'-hydroxypaclitaxel (C3'-OHP), less to C2-hydroxypaclitaxel (C2-OHP), di-hydroxypaclitaxel (di-OHP) and another so far unknown monohydroxylated paclitaxel. In the minipig microsomes, this unknown hydroxypaclitaxel was the main metabolite, whereas C3'-OHP and C2-OHP were minor products. In the regular pig microsomes, the same metabolic pattern was demonstrated. In human liver microsomes 6α-hydroxypaclitaxel (6α-OHP) was the main metabolite, followed by C3'-OHP, C2-OHP and two other metabolites not yet fully characterized. However, the proportion of 6α-OHP and C3'-OHP in different human microsomes varied significantly. Kinetic parametres (K_m and V_{max}) of production of various metabolites in rat, minipig, pig and human liver microsomes revealed species and individual differences. The human microsomes oxidized paclitaxel at substantially higher rates than the rat, minipig and regular pig microsomes, with possible consequences for the effects of metabolically inactivated paclitaxel in these animal species. It became obvious that despite various similarities between human and pig metabolism, the profile of paclitaxel metabolites in the studied species is different and 6α-OHP remains uniquely human metabolite. In the study of paclitaxel metabolism by different CYP cDNA-expressed enzymes (CYP1A2, 1B1, 2A6, 2C9, 2E1 and 3A4), only CYP3A4 enzyme formed C3'-OHP, C2-OHP and one unknown metabolite.

The roles of CYP3A and CYP2C8 in the formation of paclitaxel metabolites were investigated using specific inhibitors: troleandomycin (inhibitor of CYP3A4) and fisetin, which we found to inhibit CYP2C8 activity[1]. CYP3A inhibitor troleandomycin inhibited the formation of C3'-OHP, C2-OHP, di-OHP as well as the unknown rat, minipig and pig OHP. Fisetin strongly inhibited the formation of 6α-OHP in human microsomes, which is catalyzed by human unique CYP2C8 enzyme.

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WP55 QUANTITATIVE EVALUATION OF RELATIVE CONTRIBUTION OF HUMAN CYTOCHROME P-450 ISOENZYMES (CYPS) TO THE METABOLISM OF PROMAZINE

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Our previous investigations indicated that the catalysis of promazine in humans do not exhibit a strict CYP isoform preference. The present detailed experiments performed at therapeutic concentrations of the drug were aimed to estimate quantitively relative contribution of CYPs to

promazine 5-sulfoxidation and N-demethylation. The amount of neuroleptic metabolites formed in liver microsomes, cDNA-expressed human CYPs or hepatocytes was assayed using HPLC with UV detection.

In human liver microsomes, the formation of promazine 5-sulphoxide and N-desmethylpromazine was significantly correlated with the level of CYP1A2 and ethoxyresorufin *O*-deethylase and acetanilide 4-hydroxylase activities, as well as with the level of CYP3A4 and cyclosporin A oxidase activity. Moreover, the formation of N-desmethylpromazine was correlated well with S-mephenytoin 4'-hydroxylation. Furafylline (a CYP1A2 inhibitor) and ketoconazole (a CYP3A4 inhibitor) significantly decreased the rate of promazine 5-sulphoxidation, while furafylline and ticlopidine (a CYP2C19 inhibitor) significantly decreased the rate of promazine N-demethylation in human liver microsomes. The cDNA-expressed human CYPs (1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4) generated different amounts of promazine metabolites. The kinetic parameters (Lineweaver-Burk analysis) obtained in cDNA-expressed human CYPs showed distinct inter-isoform differences (K_m = 37 - 350 μM, V_{max} = 0.15 - 2.47 pmo/pmol CYP isoform/min in the case of 5-sulphoxidation and K_m = 47 - 741 μM, V_{max} = 0.33 - 13.70 pmo/pmol CYP isoform/min in the case of N-demethylation), which was consistent with the multienzyme Eadie-Hofstee plots derived from liver microsomes. The highest intrinsic clearance (V_{max}/K_m) was found for CYP1A subfamily, CYP3A4 and CYP2B6 in the case of 5-sulphoxidation, and for CYP2C19, CYP1A subfamily and CYP2B6 in the case of N-demethylation. In a primary culture of human hepatocytes, TCDD (a CYP1A subfamily inducer), as well as rifampicin (mainly a CYP3A4 inducer) induced the formation of promazine 5-sulphoxide and N-desmethylpromazine.

Regarding the relative expression of various CYPs in human liver, the obtained results indicate that CYP1A2 (31%) and CYP3A4 (39%) are the main isoforms responsible for 5-sulphoxidation, while CYP1A2 (35%) and CYP2C19 (32%) are the basic isoforms that catalyze N-demethylation of promazine in human liver. Of the other isoforms studied, CYP2C9 (14%) and CYP3A4 (13%) contribute to a lesser degree to promazine 5-sulphoxidation and N-demethylation, respectively. The role of CYP2A6, CYP2B6, CYP2D6 and CYP2E1 in the investigated metabolic pathways of promazine seems negligible.

Since phenothiazine neuroleptics are combined with antidepressants or carbamazepine in the treatment of complex or „treatment-resistant" psychiatric disorders, the obtained results may have significant implications for the prediction of potential drug-drug interactions involving promazine.

WP56 STIMULATION OF HUMAN CYTOCHROME P450 3A4 ACTIVITY BY ANIONIC PHOSPHOLIPIDS AND THEIR MICRO-DOMAINS

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In humans, cytochrome P450 (CYP) 3A4 is the major enzyme expressed in liver and plays a major role in the metabolism of many drugs and procarcinogens.[1] CYP3A4 seems to have a large cytoplasmic domain anchored to the endoplasmic reticulum membrane by an amino-terminal transmembrane segment.[2] We has shown that the activities and conformations of rabbit CYP1A2[3,4] and CYP2B1[5] are modulated by phospholipids. It is also known that the composition of phospholipids in liver microsomes is dependent on physiological conditions.[6] Role of phospholipids in the catalytic activity and membrane binding of CYP3A4 was investigated with model membranes. When phosphatidylcholine (PC) matrix was replaced with anionic phospholipids [phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidylinositol] and phosphatidylethanolamine (PE), all anionic phospholipids increased the activity. PA was the most efficient and the enhancement reached 15-fold in the presence of 50% PA and 50% PC compared with that of 100% PC. Binding and insertion of CYP3A4 to the membranes was also increased by anionic phospholipids and PA exerted the most efficient effect. PE was found to act as an inert matrix phospholipid, similar to PC in PC/PE binary system without anionic phospholipids, as it showed very little effect on the catalytic activity of CYP3A4 and the binding of CYP3A4 to membranes. However, in ternary system of PC/PE/PA or PC/PE/PS with a fixed concentration of 10 or 20 mol% anionic phospholipid, the catalytic activity further increased as a function of PE concentration. From the observations obtained from the excimer fluorescence of pyrene-PA and NBD-PA and the resonance energy transfer between pyrene-PA and BODIPY-PA, it was deduced that PE caused PA-enriched domains in PC/PE/PA membranes like the case of PC/PE/PS previously suggested.[7] In ternary system of PC/PE/PA or PC/PE/PS, the activity further increased as a function of PE concentration. PE was found to cause PA- or PS-enriched domains in the ternary system. The phospholipid-dependent activity change coincides with conformational change including altered Trp fluorescence and CD spectra in visible region of CYP3A4. These results suggest that the formation of micro-domains enriched with anionic phospholipid is important for the initial binding to membranes and the cataly-

tic activity of CYP3A4. [This work was supported by Korea Research Foundation Grant (KRF-2000-015-FS0002].

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WP57 POTENT INHIBITION OF HUMAN CYTOCHROME P450-CATALYZED REACTIONS BY HEMIN

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An inhibition or destruction of CYP may cause prolongation of xenobiotic action, increase of decrease in xenobiotic toxicity, and even affect physiological functions, e. g. steroidal biosynthesis.[1] Selective destruction of various CYP types alters the activation/detoxication ratio of procarcinogens and thus may increase the cancer risk in conditions of combined exposure.[2,3] Heme taken up by hepatocytes is known to be oxidized to biliverdin by the microsomal heme oxygenase system which consists of heme oxygenase and NADPH-cytochrome P450 reductase (NPR).[4] Hemin, a stable form of heme, has an antimutagenic effect.[5] Inhibitory effects of hemin on the cytochrome P450 (CYP)-catalyzed reactions of human liver microsomes and reconstituted systems containing purified CYP and NPR were seen. Hemin non-specifically inhibited all of the microsomal CYP activities observed. Hemin also inhibited the reactions of 7-ethoxy-4-(trifluoromethyl)coumarin *O*-deethylation, 3-[2-(N,N-diethyl-Nmethylammonium)-ethyl]-7-methoxy-4-methylcoumarin *O*-deethylation, and benzo(α)pyrene 3-hydroxylation, catalyzed by purified CYPs 1A2, 2D6, and 3A4, with IC_{50} values of 25, 17, and 1.9 μM, respectively. Hemin also inhibited reduction of cytochrome *c* and ferricyanide by NPR up to 45%. Spectrally detectable CYP was destroyed in human liver microsomes and purified CYP in a reconstituted system in the presence of hemin and an NADPH-generating system. These results suggest the inhibitory effect of hemin on the CYP-catalyzed reactions may come from the inability of an efficient electron transfer from NPR to CYP. It also can be suggested that the antimutagenic effect of hemin might be due to inhibition of CYP and NPR enzyme, involved in the bioactivation of mutagens by hemin. [This work was supported by Korea Research Foundation Grant (KRF-2000-015-FS0002)].

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WP58 GENETIC POLYMORPHISMS IN HUMAN CYP2A6 GENE AND INTERINDIVIDUAL DIFFERENCES IN NICOTINE METABOLISM

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CYP2A6 catalyzes nicotine C-oxidation leading to cotinine formation, a major metabolic pathway of nicotine in humans. There are genetic polymorphisms in the human *CYP2A6* gene. We determined the phenotyping of in vivo nicotine metabolism and the genotyping of the *CYP2A6* gene in 92 Japanese and 209 Koreans[1,2]. Among them, 3 Japanese and 4 Korean subjects who were absolutely deficient in cotinine formation were genotyped as *CYP2A6*4* (whole deletion)/*CYP2A6*4*. The other poor metabolizers whose nicotine metabolism was impaired were genotyped as *CYP2A6*7*(I471T)/*CYP2A6*4* or *CYP2A6*10* (I471T, R485L)/*CYP2A6*4*, indicating that the CYP2A6 enzyma-

tic activity is lost in the subjects homozygous for either *CYP2A6*4, CYP2A6*7,* or *CYP2A6*10*, or heterozygous for these alleles in combination[3]. The *CYP2A6*1X2* (duplication) allele was found in only one Korean subject (0.5%) whose nicotine metabolic potency was not very high.

An allele possessing a point mutation in the TATA box termed *CYP2A6*9* (T-48G) has been reported to decrease the transcriptional activity *in vitro* as assessed by luciferase assay[4]. We investigated the effects of the *CYP2A6*9* allele on the *in vivo* enzymatic activity by evaluating nicotine metabolism. Furthermore, the effects on the expression level of CYP2A6 mRNA and coumarin 7-hydroxylase activities in human livers in vitro were also investigated. The mutation of T-48G was found only on the *CYP2A6*1A* allele but not on the *CYP2A6*1B* allele. In the *in vivo* study, the allele frequencies of *CYP2A6*9* in Japanese (n = 92) and Koreans (n = 209) were 21.3% and 22.3%, respectively. In both populations, the cotinine/nicotine ratio as an index of nicotine metabolism could be put in the order of subjects genotyped as *CYP2A6*1A/CYP2A6*1A* > *CYP2A6*1A/CYP2A6*9* > *CYP2A6*9/CYP2A6*9*. In the *in vitro* study, the expression levels of CYP2A6 mRNA in human livers and coumarin 7-hydroxylase activity in human liver microsomes that were genotyped as *CYP2A6*1/CYP2A6*9* and *CYP2A6*9/CYP2A6*9* were lower than those in human livers genotyped as *CYP2A6*1/CYP2A6*1*. Thus, the mutation in TATA box (*CYP2A6*9* allele) caused the decreased expression level of CYP2A6 mRNA and enzymatic activity in vivo and *in vitro*.

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WP59 GENETIC POLYMORPHISM OF CANINE CYTOCHROME P450 2D15 GENE

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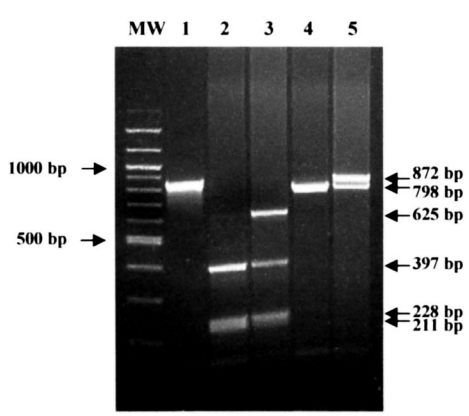
The human polymorphic isoenzyme CYP2D6 has a major role in the oxidative metabolism of many drugs[1]. The corresponding canine CYP2D15 was found to metabolize the COX-2 inhibitor, celecoxib, with different velocities suggesting that the gene may be polymorphic[2]. In this study, we have investigated the polymorphism of canine CYP2D15 that it is cloned using conventional PCR techniques from exon5 to the end of exon7 and sequenced. DNA was isolated from whole blood samples of 51 mixed breed dogs from the local animal hospital, amplified using PCR and digested with restriction enzymes.

PCR and RFLP conditions: a PCR reaction was performed for each DNA sample in a total volume of 50 μl using CYP2D15 primers (CYP2D15ex5-F: 5'-GCCCTGAACTCCATCCCCGTG-3', CYP2D15Aex7-B: 5'-CTTGGGGATGAGGAAGCCCTG-3') and 0,5 units Platinum *Taq* DNA polymerase. The PCR conditions consisted of an initial melting temperature 95 °C (2.5 min) followed by 35 cycles of melting (95 °C, 0.5 min), annealing (60 °C, 1 min), and extension (72 °C 1,5 min) with a final extension step (72 °C, 10 min). To determine the polymorphism of CYP2D15, the amplified DNA (872 bp) was digested with 1 unit of each restriction enzymes, separately, at 37 °C for 3 h:



a) *StuI* that cuts amplified DNA when it is mutated into 798 bp and 74 bp. When DNA is not mutated *StuI* do not cut, and b) *DrdI* that cuts amplified DNA sequences when it is mutated into three fragments of 625 bp, 211 bp and 36 bp and when it is not the 625 bp DNA fragment upon digestion gave two bands of 397 and 228 bp. Digestion products were then analyzed electrophoretically on an ethidium bromide-stained 2% agarose gel at 100 V for 90 min (*Fig. 1*).

Alignment of the cloned ex5-ex7 DNA sequence with the known canine CYP2D15 revealed that there are two polymorphic sites in the DNA coding region: 1) A834T (CYP2D15*1)

located in exon 5, and 2) A1005G (CYP2D15*2) located in exon 6 (numbered according to canine CYP2D15 cDNA with accession number D17397)[3]. These polymorphic sites have as result the amino acid change of Ile250➔Phe and Ile307➔Val. The Phe250 allele (T834) occurred at a frequency of 0.275 (homozygotes for the SNP) and none homozygote for A834 was found. Heterozygotes was occurred at a frequency of 0.725. For the other polymorphic site (A1005G), the Val307 allele (G1005) occurred at a frequency of 0.843 (homozygotes for the SNP) and none homozygote for A1005 was found. Heterozygotes was occurred at a frequency of 0.157. These preliminary results suggest that the canine CYP2D15 is polymorphic and phenotyping is needed in order to assess whether these polymorphisms may play a critical role in drug metabolism.

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WP60 THE INVOLVEMENT OF CYTOCHROME P450 IN THE METABOLISM OF CHLOROQUINE ON HUMAN LIVER MICROSOMES

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Chloroquine is the first line antimalarial drug for the treatment and prophylaxis of *Plasmodium falciparum* infection[1]. Although chloroquine is thought to be metabolized via the cytochrome P450 (CYP) enzymes[2], no data are currently available on the metabolism of chloroquine in human. Here we evaluated the involvement of CYPs in the metabolism of chloroquine using human liver microsomes and cDNA-expressed CYPs. Chloroquine was primarily metabolized to desethylchloroquine via N-dealkylation with the apparent *Km* and *Vmax* values of 1.03 mM and 0.425 nmol/min/mg protein. Quinidine (a CYP2D6 inhibitor), quercetin (a CYP2C8 inhibitor), and omeprazole (a CYP2C19 inhibitor) inhibited N-dealkylation of chloroquine in human liver microsomes. Among the cDNA-expressed CYPs examined, CYP1A2, 2C8, 2C19, and 2D6 exhibited desethylchloroquine formation with CYP2D6 being the most catalytically competent. The present study demonstrates that CYPs are involved in the metabolism of chloroquine in human and CYP2D6 is the principal enzyme responsible for chloroquine *N*-dealkylation in human liver microsomes.

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WP61 INHIBITION OF CYTOCHROME P450 ACTIVITIES BY OLEANOLIC ACID AND URSOLIC ACID IN HUMAN LIVER MICROSOMES

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Oleanolic acid (OA) and ursolic acid (UA) are triterpenoid compounds that exist widely in food, medicinal herbs including *Achyranthes bidentata*[1]. These substances have been well studied for the pharmacological activities including anti-inflammatory, anti-arthritic, and anticancer activities related to cytochrome P450 (CYP) (i.e. CYP1A2 and CYP2E1) as potential therapeutics[2]. In our previous report, extracts of *Achyranthes bidentata* showed inhibitory effects on CYPs[3]. So we evaluated whether active components, OA and UA, are related to the inhibitory effects of *Achyranthes bidentata* on CYPs using human liver microsomes. OA competitively inhibited CYP1A2-catalyzed phenacetin O-deethylation and CYP3A4-catalyzed midazolam 1-hydroxylation with the apparent IC_{50} (*Ki*) values of 143.5 (74.2) μM and 78.9 (41.0) μM), respectively. UA competitively inhibited CYP2C19-catalyzed S-mephenytoin 4'-hydroxylation with the apparent IC_{50} (*Ki*) values of 119.7 (80.3) μM. The present study demonstrates that both OA and UA have inhibitory activities on CYP isoforms in human liver microsomes and especially OA may modulate pharmacological and toxicological effects mediated by CYP1A2 in part. In addition, inhibitory effects of *Achyranthes bidentata* on CYPs may be related to that of OA.

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WP62 CLONING, SEQUENCING AND HETEROLOGOUS EXPRESSION OF THE GENES CODING FOR THE P450MOR SYSTEM FROM *MYCOBACTERIUM SP. STRAIN* HE5

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A cytochrome P450 and a ferredoxin, which are specifically induced during growth of *Mycobacterium* HE5 on morpholine, piperidine and pyrrolidine, have been purified (1,2). It was supposed that these proteins are components of a P450 system responsible for the hydroxylation of these heterocycles resulting in their ring-cleavage[2,3].

The corresponding genes of these proteins were cloned and sequenced. We identified a gene cluster exhibiting the genes for $P450_{mor}$ (*morC*), for the ferredoxin (*morF*), and for a putative ferredoxin reductase (*morFR*), which is thought to be the third component of this P450 system. These genes are nearly identical to the genes *morA, morB* and *morC* from *Mycobacterium* RP1, which were annotated in gene bank recently. This P450 system is also supposed to be involved in the breakdown of the above mentioned N-heterocycles[3].

In order to characterize the $P450_{mor}$ system biochemically and to reconstitute an enzymatically active system, we started to express all three components in *E. coli*. MorC was expressed as a fusion protein with an N-terminal or an C-terminal His-tag. The yields were 190 and 150 nmol P450/l culture, respectively, and induction was optimal on addition of 0.75 mM ALA. Unfortunately, we were not able to record any spectral changes with the purified proteins after addition of the supposed substrates. *MorFR* was also cloned in pET28b(+) to obtain an N-terminal His-tag fusion protein, but we could not detect any expression in BL21 codon plus. As it seems the protein might be toxic we will now change to another host strain. *MorF* was cloned into pET26b(+) without any tag and expression studies have just begun. Hopefully, we will be able to obtain all components of the P450mor system in an active enzymatic form.

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WP63 COUMARIN-7-HYDROXYLASE ACTIVITY IN LIVER AND BRAIN MICROSOMES OF MICE

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Coumarin is hydroxylated by the P450 isoform CYP2A5 in mice liver. CYP2A5 involves in the metabolism of several endogenous and exogenous compounds such as drugs, carcinogens and environmental pollutants. Although CYP2A5 is expressed in several tissues, no information is available with respect to brain. Therefore, we investigated coumarin 7-hydroxylase is present in brain and compared to that og liver in mice. Accordingly, optimum conditions have been established for both tissues. It was found that different reaction conditions are required to achieve maximum enzyme activity in brain and liver of mice.

WP64 CYP4F8 AND CYP4F12: TISSUE DISTRIBUTION AND CATALYTIC PROPERTIES

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Our aims were to compare the tissue distribution and catalytic properties of CYP4F8 (prostaglandin H 19-hydroxylase) and CYP4F12 in extra hepatic tissues. CYP4F8 was originally cloned from human seminal vesicles and catalyzes 19-hydroxylation of prostaglandin H1 and H2 *in vitro*. Antipeptide polyclonal antibodies were raised against the C-termini of the enzymes. Screening of 50 human

tissues for CYP4F8 immunoreactivity revealed protein expression, *inter alia*, in seminal vesicles, epidermis, corneal epithelium, sweat glands, the proximal renal tubules, and epithelial linings of the gut and urinary tract. The CYP4F8 transcripts were detected by Northern blot analysis and RT-PCR. A prominent induction of CYP4F8 immunoreactivity and mRNA was detected in psoriasis in comparison with unaffected epidermis. CYP4F8 and CYP4F12 occur along with cyclooxygenases in epithelia, and the transitional epithelium forms prostacyclin, thromboxane A2 and prostaglandins. Recombinant CYP4F8 metabolised prostacyclin and carbaprostacyclin to their 19-hydroxy metabolites, which were identified by LC- and GC-MS analysis.

Recombinant CYP4F12 did not metabolise prostacyclin. CYP4F12 immunoreactivity occurred in the epithelial cells of the gastrointestinal tract (stomach, small intestine, colon), endothelium of microvessels of placental villi, epidermis, and in the transitional epithelium. Western blot analysis also suggested that CYP4F12 was expressed in prostate and purified prostasomes, organelles of prostatic origin. CYP4F12 mRNA could be detected in placenta, epidermis, and prostate by RT-PCR.

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W65 BABOON CYP11B1: THE EXPRESSION AND STRUCTURAL ANALYSIS OF TWO CATALYTICALLY ACTIVE ISOFORMS

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In the adrenal gland the cytochromes P450 catalyse the synthesis of mineralocorticoids (aldosterone), glucocorticoids (corticosterone and cortisol) and, in some species, androgens. In human adrenals CYP11B2 catalyses the consecutive 11β-hydroxylation, 18-hydroxylation and 18-oxidation of 11-deoxycorticosterone to aldosterone in the zona glomerulosa. In the zona fasciculata/reticularis CYP11B1 catalyses the formation of cortisol from 11-deoxycortisol. CYP11B1 also catalyses the hydroxylation of 11-deoxycorticosterone yielding corticosterone. The subsequent hydroxylation to 18-hydroxycorticosterone occurs, but to a lesser degree[1]. In some species both CYP11B isoforms are present while in other species a single enzyme catalyses the formation of both glucocorticoids and mineralocorticoids[2].

Both CYP11B1 and CYP11B2 occur in the Cape baboon. We have previously reported the existence of three genes encoding baboon CYP11B1 which were cloned from adrenal tissue using RT-PCR. Expression of the recombinant cDNAs in COS1 cells, co-transfected with the gene encoding human adrenodoxin, showed that two of the cloned genes encoded functional enzymes, while the third was inactive. COS1 cells transfected with the functional cDNAs converted 11-deoxycorticosterone to corticosterone. Negligible amounts of 18-hydroxycorticosterone and aldosterone were detected. The conversion of corticosterone to aldosterone was not observed in the same expression system. We have also previously shown that deoxycorticosterone was converted to corticosterone in baboon adrenal cortex homogenates. The metabolism of corticosterone in those preparations was negligible and aldosterone was not detected[3,4].

The three baboon genes share 99% homology and showed 96% and 93% homology with human CYP11B1 and 11B2 respectively. Expression studies in COS1 cells were subsequently carried out to compare deoxycorticosterone and deoxycortisol as substrates for the two baboon CYP11B1 enzymes. In addition, the structures of both CYP11B1 isoforms were modeled using InsightII (Accelyrs, Inc). The P450 domain of CYYP102A1 (BM3) and CYP2C5 were used as the templates for threading the backbone followed by molecular simulation and docking of the substrate molecule to determine the residues involved in substrate binding and to correlate them with the two variants

Our results show that the expressed enzymes metabolised the conversion of deoxycorticosterone to corticosterone and deoxycortisol to cortisol respectively, at different rates when the recombinant baboon cDNAs (babc11a and babc11) were expressed in COS1 cells in the presence of recombinant human ADX. The expressed babc11 converted deoxycorticosterone to corticosterone at twice the rate of the expressed babc11a. Deoxycortisol, however, is converted to cortisol by the two expressed isozymes at a similar rate. The functional genes differ by 3 amino acid residues and structural analyses of the two isozymes may provide valuable information with regards to functional relevance of particular amino acids in the metabolism of specific substrates during the synthesis of mineralocorticoids and glucocorticoids in the baboon adrenal.

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WP66 AN INTERSPECIES COMPARISON OF THE METABOLISM OF THE ANTICANCER AGENT YONDELIS™ (TRABECTEDIN, ET-743)

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Yondelis™ (trabectedin, ET-743) is a potent anticancer agent isolated from the Caribbean tunicate *Ecteinascidia turbinata*[1], currently in development for the treatment of solid tumors[2,3]. The compound covalently binds in the minor groove of DNA through the 2-amino group of guanine, bending the DNA towards the major groove[4].

The in vitro metabolism of ^{14}C-labelled ET-743 was studied in liver subcellular fractions of man, male beagle dog, male Cynomolgus monkey, female New Zealand white rabbit, male and female Swiss albino mice, and male and female Sprague-Dawley rats, in the presence of an NADPH-generating system.

Various metabolites were detected in all the species. The metabolism of ET-743 in man resembled most that in monkey and was clearly different from that in dog and female rat. A major yet not fully identified metabolite, designated metabolite 16, was detected in 12,000 g supernatant fractions of human and monkey. Four metabolites could be identified by both LC-MSMS analysis and co-chromatography with reference substances. *N*-Demethylation, with formation of ET-729, was a major metabolic pathway in the male dog, the female rat and the female rabbit, and could also be detected in the other species as a minor pathway. Oxidation of the alcohol function to the carbonyl metabolite ET-759A was another metabolic pathway that was detected in each species. Sulphoxide formation (formation of ET-759B) was detected to a small extent in female mouse only, and hydrolysis of the acetate ester (formation of ET-701) was detected

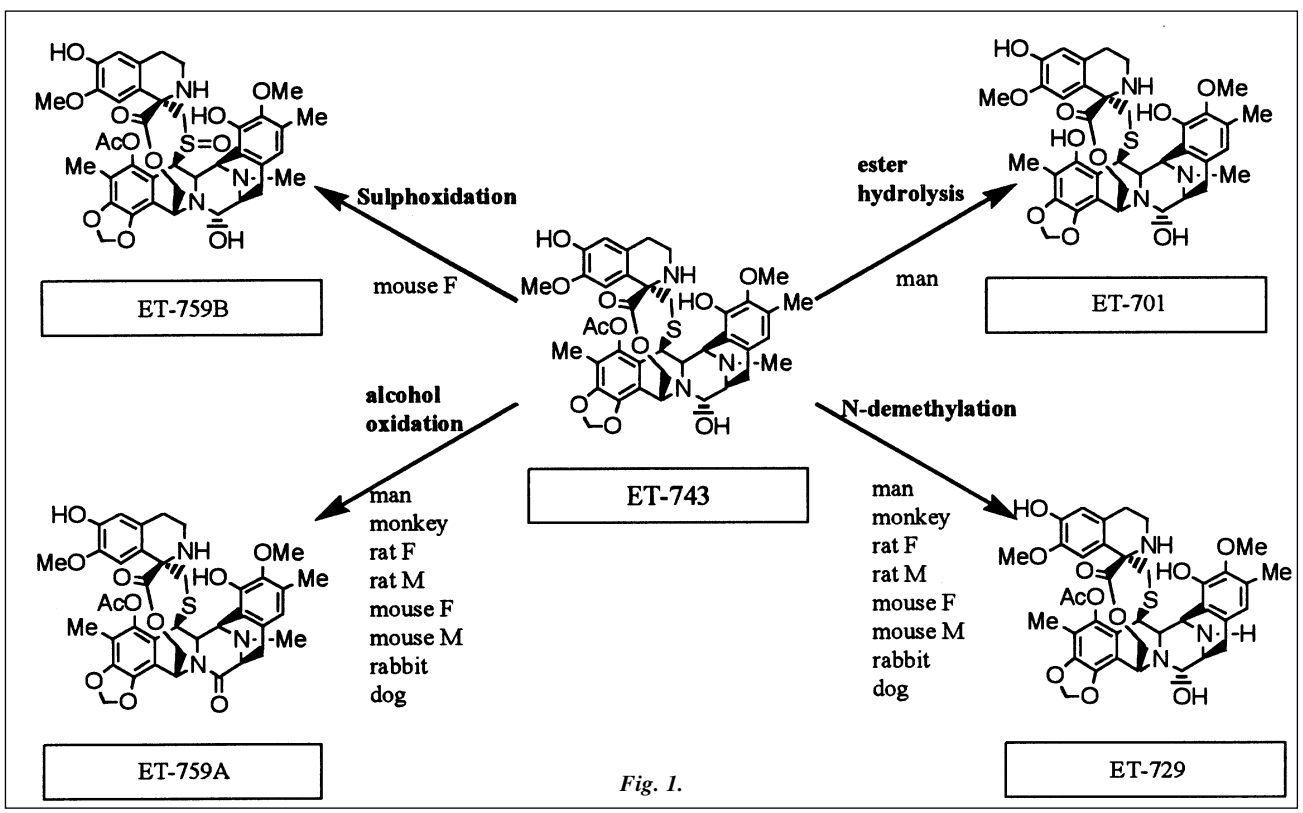

Fig. 1.

to a small extent in man. The unidentified metabolites in man were all detected in at least one animal species. These *in vitro* metabolism data suggest that the monkey is likely to represent the most relevant species to evaluate the toxicity of ET-743, at least from a metabolism point of view.

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WP67 DIFFERENCES IN BIOTRANSFORMATION OF TESTOSTERONE BY BRAIN MICROSOMES OF RAT AND RABBIT: A PRELIMINARY STUDY

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Introduction

Even if the liver is the main protagonist in metabolism, investigation of the extraepatic presence and function of cytocrome P450 isoenzymes has become a very important area of interest in recent years. Brain cytochrome P450 ezyzmes are involved in the metabolism of neurosteroids as well as other, biologically significant, endogenous compounds[1]. The aim of this study was the investigation on the main differences in P450 brain activities in two helpful laboratory species, rat and rabbit, using testosterone (TST) as marker substrate.

Materials and Methods

Brain microsomes were prepared from six males rats and four male rabbits as described by Voirol[2]. TST hydroxylated derivatives (OH-TST) were determined by HPLC. This study was carried out according to the Italian Legislation (D.L. 27/01/1992 n° 116) and to ISO 9001:2000.

Results and discussion

Five hydroxylated metabolites were identified from rat brain by comigration time and comparison of their absorption spectra with authentic standards as 6αOH-TST, 6βOH-TST, 11αOH-TST, 16βOH-TST, 2βOH. In rabbit we observed also the 16αOH-TST but not the 11αOH-TST (*Table 1*). These data confirm our previous observations[3] according with published results by Rosenbrock[4] in rat where 6αOH-TST was the main metabolite produced by brain microsomes. This hydroxylated compound was the most representative metabolite also in rabbit even if at lesser concentrations than in the rat. The biotransformation pattern of TST differs for brain in relation to liver that produces 6β-hydroxytestosterone as the main metabolite in rabbit[5] and in rat[6]. Although only few informations are available on P450 isoforms involved in its production in liver and nothing at all in brain, CYP2A1[6] and CYP3A18[7] seem to be involved in the production of 6(OH-TST. Further research could better explain which kind of izoenzimes are involved in this important testosterone biotransformation in the brain.

Tab. 1: *In vitro metabolites of testosterone by brain microsomes, in rat and in rabbit. Mean values ± standard deviation.*

Metabolites	Rat	Rabbit
6αOH-TST	0.80 ± 0.031	0.32 ± 0.023
6βOH-TST	0.24 ± 0.029	0.18 ± 0.021
16αOH-TST	n.d.	0.07 ± 0.002
16βOH-TST	0.13 ± 0.005	0.15 ± 0.003
11αOH-TST	0.24 ± 0.003	n.d.
2βOH-TST	0.10 ± 0.013	0.03 ± 0.001

n.d: non detectable.

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WP68 POLYMORPHISM IN CYTOCHROME P450 2D6-MEDIATED ACTIVATION OF MALATHION IN RATS

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Organophosphorus insecticides are widely used in agriculture although they are potentially toxic to humans for their ability to inhibit the enzyme acetylcholinesterase, which hydrolyzes the neurotransmitter acetylcholine at nerve synapse and neuro-muscular junction. They are used as phosphorothioate, to be converted in vivo to the active oxygenated form; this process of desulphuration is mediated by cytochrome P450 enzymes[1]. In the present study we evaluated the potential involvement of CYP2D6 in malathion biotransformation to the active form malaoxon, which even seems to induce free radical generation and lipid peroxidation[2]. CYP2D6 polymorphism may be important in understanding why certain individuals (extensive metabolizers) appear to be at greater risk from exposure to some organophosphorus compounds than others (poor metabolizers, 5-10 % of Caucasians)[3]. We used quinine (12.5 mg/kg, p.o. for 3 days) as a specific inhibitor of CYP2D6 in male Wistar rats[4]. The influence on malathion activation was verified *in vivo* evaluating the inhibition of serum acetylcholinesterase. We also investigated the effects of malathion exposure (1g/kg p.o.) on oxidative stress by assessing lipid peroxidation as malondialdehyde production (MDA), reduced glutathione (GSH) consumption, glutathione-S-transferase (GST) enzymatic activity, variation of catalase (CAT) and superoxide dismutase (SOD) in liver homogenate of rats treated with quinine and malathion. Acetylcholinesterase inhibition and lipid peroxidation determined by malathion were significantly reduced by quinine treatment, which also reduced GSH consumption and GST, CAT and SOD increment due to malathion. In conclusion, these data confirm that oxidative stress is involved in the toxicity of malathion and support the hypothesis that CYP2D6 has an important role in the bioactivation pathway of this pesticide. As a consequence, the polymorphism of CYP2D6 influences individual susceptibility to exposure to malathion.

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WP69 *IN VITRO* STUDIES ON THE ACTIVATION OF THE ORGANOPHOSPHATE PESTICIDE MALATHION BY CYTOCHROME P450 2D6 IN RAT LIVER MICROSOMES

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Malathion is one of the phosphorothioate pesticides mostly used in agriculture and public health programmes. It has been detected in the environment as contaminant of drinking water and pesticide residues foodstuff; thus, human exposure to malathion may occur through occupational settings, agricultural workers or pesticide applicators, as well as via a variety of environmental sources.

Malathion is a weak acetylcholinesterase inhibitor unlike its metabolite, malaoxon, which is a potent anticholinesterase agent. Moreover, recent studies raised concerns over the potential of malathion and its metabolite to cause genetic damage as strongly positive alkylating agents. As well known the activation of malathion to the oxygen analogue malaoxon occurs *in vivo* by the oxidative desulfuration of the parent compound and it is mediated by the cytochromes P450 (CYP), even if the specific isoform involved in the metabolizing process is not yet known.

In the present *in vitro* study we used quinine, a specific inhibitor of CYP2D6, to investigate if malathion activation in rat liver microsomes is catalysed by CYP2D6 subfamily. If this hypothesis is confirmed proving that CYP2D6 is involved in malathion activation, its inhibition by quinine may show a reduction of the oxon genotoxicity on human leucocytes in the single cell gel electrophoresis (comet assay), which is a sensitive genotoxicity test able to investigate DNA damage detecting single strand breakage. In the present study the results show that acetylcholinesterase inhibition and genotoxicity determined by malathion were significantly reduced by quinine treatment, due to a decre-

ment of malaoxon production, suggesting that CYP2D6 may have an important role in the bioactivation pathway of this pesticide.

According to the knowledge that CYP2D6 is characterized by a genetic polymorphism, it follows that if CYP2D6 is responsible of malathion activation the risk deriving from exposure to malathion may be dependent on the genotype of the population. As a consequence, the polymorphism of CYP2D6 influences individual susceptibility to exposure to malathion.

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WP70 PHOTOAFFINITY LABELING OF CYTOCHROME P450 2B4

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Photoaffinity labeling is a chemical modification technique used to study protein structure and interactions. Highly reactive intermediate which is able to modify amino acid residues of a protein is generated by UV-light irradiation at certain place and exact time. The goal of this technique is the identification of protein active centre or binding sites. Diamantane like compounds are highly specific substrates of phenobarbital-inducible cytochrome P450 2B4. Their affinity for the active centre is documented by spectral dissociation constants: adamantane, K_s=1.3x10^{-6} mol/l; diamantane, K_s=0.5x10^{-6} mol/l; triamantane, K_s=4.3x10^{-6} mol/l. Diamantane has the highest affinity for an active centre of cytochrome P450 2B4. Hence, diamantane skeleton was used for the construction of photoaffinity label. Its photolabile derivative, 3-azidiamantane [spiro-(diazirine-3,3'-diamantane)] was synthetized and evaluated. 3-Azidiamantane dissociation constant K_s=1.9x10^{-6} mol/l proves its affinity is comparable to diamantane. It guarantees effective binding to the active centre and labeling. Two UV-light sources for the label photoactivation were compared, first emitting light of 254nm and second 366nm. Photo activation of 90% of 3-azidiamantane by the 254nm source is accompanied by destroying more then 20% of cytochrome P450 2B4, while by 366nm one less then 10%. Upon photolysis (366nm, half live 1.5 minute) 3-azidiamantane gives highly reactive carbene intermediate. Photolysis of 3-azidiamantane in hexan results in formation of 3-hexyldiamantane (48.3%), demonstrating excellent incorporation of carbene intermediate to unactivated C-H bounds. When photoaffinity label was photolysed in water, the major products were 3-hydroxydiamantane (82.0%, K_s=49.2x10^{-6} mol/l) and 3-diamantanone (14.3%, K_s=4.6x10^{-6} mol/l); those side products of an active intermediate might interfere with active centre labeling. The experiment for photoaffinity labeling is conducted in three arrangements: photolysis of cytochrome P450 2B4, cytochrome P450 2B4 with 3-azidiamantane and cytochrome P450 2B4 in the presence both 3-azidiamantane and diamantane, used as a competitor to differentiate specifically bounded label in active center and label bound outside. Labeled cytochrome P450 2B4 is cleaved by trypsine and peptides are separated on HPLC RP C18. Separated peptides from all three arrangements are analyzed on MALDI TOF and MS-DECA and results are compared to distinguish peptides labeled by diamantane originating from the cytochrome P450 2B4 active centre, from those labeled nonspecifically.

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8 LATE SUBMISSION – POSTER

GENOTYPING OF DRUG METABOLIZING ENZYMES IN DNA OF ARCHIVED TISSUES FOR FUTURE ASESSMENT OF BREAST CANCER DRUG TREATMENT RESPONSE AND NON RESPONSE

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According to current scientific concepts polymorphisms of drug metabolizing enzymes and other potential drug targets may play a role in inter-individual differences in treatment responses. This is of major concern in breast cancer, a disease that kills more than 300,000 women each year worldwide. Constitutional variations in drug response, non response and toxicity may be studied on the genome level by comparing allele/genotype frequencies of potentially relevant drug metabolizing enzymes (DME) between breast cancer patient groups, i.e. treatment responders and non responders. In addition, these studies require long-term clinical follow-up under hormonal treatment, chemo- and immunotherapy, respectively. However, although clinical and survival data may be available, there is frequently no blood DNA available from eligible patients for genotyping. Therefore, these studies must rely on the genotyping of DNA isolated from formalin fixed and paraffin embedded regular breast tissues. At present it is not clear, if these tissues will be suitable for genotyping analyses since there has been frequently observed failure in PCR-based analyses of archival DNA due to poor quality. Here we tested the applicability of Taqman technology based allelic discrimination for the genotyping of DME, i.e. cytochrome P450: CYP2C19 (*2)[1,2], CYP2D6 (*3, *4,*6, *7, *8)[3,4,5,6,7], CYP2C9 (*2,*3)[8] and EPHX[9] involved in the metabolism of breast cancer drug treatments. We included paraffin embedded normal tissues of 44 non-cancer patients collected from 1992-2002, and 74 breast cancer patients collected from 1986-1988 (five-year survival: 18 patients without metastasis, 4 patients with metastasis, and 27 deceased). DNA was isolated from 6 to 30 10 µm tissue sections with the QIAamp(r) DNA Mini Kit and DNA amounts obtained ranged from 18 to 334 µg/ml. All allele/genotype frequencies tested so far could be called in all DNA samples and genotype frequencies of non-cancer patients were in Hardy-Weinberg equilibrium and comparable to published frequencies 1,2,4,5,6,9. Results of allele frequencies of CYP2D6 and CYP2C19 are listed in table 1. We observed a significantly higher frequency of the mutant CYP2D6*3 allele (delA) in breast cancer patients when compared to non-cancer patients (OR 8.28; CI 1.043-65.758; P=0.0287). This may indicate that breast cancer patients may be more frequently compromised in CYP2D6 enzymatic function. Our data suggest that DNA from formalin fixed and paraffin embedded tissues is suitable for Taqman technology based allelic discrimination analysis. Differences in allele/genotype frequencies may be observed when comparing patient groups. It will now be possible to apply this technology towards the genotyping of CYP2C19, CYP2D6, CYP2C9 and EPHX in large archived breast cancer tissue collections for future studies on inter-individual differences in breast cancer treatment response.

Tab. 1.: *Examples of Constitutional DME Allele Frequencies of Non Cancer Patients and Breast Cancer Patients*

DME	Non Cancer Patients	Breast Cancer Patients	OR (95%CI)	P
CYP2D6*3				
A	0.986	0.89		
del A	0.014	0.11	8.28 (1.04-65.7)	0.0287
CYP2D6*4				
G	0.75	0.703		
A	0.25	0.297	1.27 (0.62-2.6)	0.588
CYP2D6*6				
T	1	0.986		
del T	0	0.014	1.9 (0.076-47.6)	1.0
CYP2C19*2				
G	0.845	0.846		
A	0.155	0.154	1.0 (0.48-2.1)	1.0

Fisher`s exact test, two-sided P-value

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9 FORUM OF YOUNG SCIENTISTS – FYS

FYS01 HIJACKING *E. COLI*'S FERRICHELETASE: EFFICIENT, BIOSYNTHETIC AND NON-DENATURATING PREPARATION OF COBALT P-450 ENZYMES

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Denaturing methodologies have been used to exchange the porphyrin of a wide number heme proteins for decades, but success with P-450s has been limited. Rather than unfolding and re-constituting a wild-type protein with an iron heme, we report an efficient and biosynthetic method of preparing cobaltic CYP101 and CYP119. Heterologous expression of the P-450 genes on metal deficient M9 minimal media underlies the method. Substitution of iron with cobalt causes the porphyrin to be more labile and decreases enzyme thermal stability. High resolution crystal structures indicate this perturbation is likely due to an increased cobalt sulfur bond length. In contrast, nitrogen ligands such as imidazole and 4-phenyl imidazole bind with higher affinity to the cobalt CYP101 and CYP119. Exploiting the diamagnetic status of water bound CYP119, 2D NMR experiments were used to observe multiple conformations of CYP119 in solution. As revealed by earlier X-ray structures, the binding of imidazoles shifts the equilibrium of multiple conformers to one conformation which is open or closed. Iron CYP119 but not cobalt CYP119 hydroxylates lauric acid using the peroxide shunt. Hydroxylation activity by cobalt CYP119, however, was observed using NADPH and electron transfer proteins. In the presence of hydrogen and alkyl peroxides, cobalt CYP101 reacts quantitatively to yield porphyrin adducts which decompose into stable hydroxy porphyrins, a reactions likely driven by the unique radical chemistry of cobalt porphyrins. When expanded to a wider scope of P450 enzymes and transition metals, the biosynthetic preparation of metal substituted P450 enzymes promises to open new areas of both P450 chemistry and spectroscopy. Work supported by NIH Grants GM25515.

FYS02 LUMINESCENT MOLECULAR PROBES FOR CYTOCHROME P450

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Reduction of the ferrous-dioxygen intermediate of cytochrome P450cam by putidaredoxin is the rate-determining step in the catalytic cycle. In order to study the fleeting intermediates in P450 catalysis, we have developed a series of ruthenium-diimine based photoreductants that bind to the active site of P450cam and reduce the heme upon 470-nm excitation (*Fig. 1a*). The variation in electron transfer (ET) rates among the Ru-diimine:P450 conjugates strongly supports the through-bond electronic coupling model for the Ru:heme system. Light-triggered ET allows temporal resolution of events on the nanosecond timescale, 100 times faster than the timescales achieved by stopped-flow mixing.

Two dansyl-based, luminescent probes (D-4-Ad and D-8-Ad) were synthesized that target P450cam (*Fig. 1b*).[1] D-4-Ad luminescence is quenched by Förster energy transfer upon binding (K_d = 0.83 μM), but is restored when the probe is displaced from the active site by camphor. In contrast, D-8-Ad (K_d ~ 0.02 μM) is not displaced from the enzyme even in the presence of a large excess of camphor. Probes with properties similar to those of D-4-Ad potentially could be useful for screening P450 inhibitors. The re-

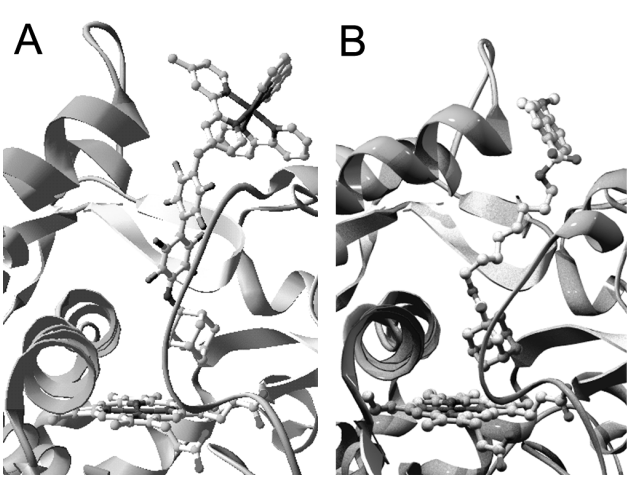

Fig. 1. *Crystal structures of a Ru-diimine photosensitizer (A) and D-8-Ad (B) bound to cytochrome P450cam.*

markable binding affinity of D-8-Ad suggests novel P450 inhibition strategies.

Crystal structures D-4-Ad, D-8-Ad, and Ru-diimine complexes2 bound to cytochrome P450cam show that the probes capture the enzyme in three different conformational states. Conformational flexibility in the B', F and G helices allow the enzyme to bind molecules much larger than the natural substrate camphor. Large changes in peripheral enzyme structure (F and G helices) couple to conformational changes in active center residues (I helix) implicated in proton pumping and dioxygen activation. Structural flexibility in the F/G helix region likely allows the 54 human P450s to oxidize thousands of substrates.

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FYS03 AUTOMATED DOCKING OF LIGANDS TO CYTOCHROME P450 ENZYMES

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In the absence of experimentally determined structures, homology models of human P450 isoenzymes CYP1A1, CYP1A2 and CYP2C9 are constructed using the only available mammalian P450 structure crystal structure (rabbit CYP 2C5)[1] as a template. These computer models are being validated and refined using automated docking, MD- simulations and essential dynamics (ED) analysis in combination with experimental data provided by mutation studies, SERRS and spin relaxation NMR. Automated docking methods can be used to predict energetically favorable conformations and orientations (poses) of (potential) ligands in the interior structure (binding cavities) of a protein. These methods consist of both an algorithm to generate different poses (docking), and a scoring function to subsequently consider the tightness of protein-ligand interactions (e.g., discrimination of favorable poses from unfavorable ones). Several docking algorithms and scoring functions have been described the past few years, so our first aim was to investigate which automated docking strategies are most appropriate for the refinement and validation of our human P450 homology models.

Using 39 X-ray crystal structures of P450-ligand complexes deposited at the Protein Databank as a test set, we evaluated several different docking algorithms (AutoDock[2] (simulated annealing (SA), 'Darwinian' genetic algorithm (GA) and 'Lamarckian' genetic algorithm (LGA)), FlexX[3] (incremental construction algorithm) and GOLD[4] ('Darwinian' genetic algorithm)) in combination with corresponding and additional (SCORE[5], X-CSCORE[6] and PMF[7]) scoring functions, and determined which docking approaches are most reliable in predicting the binding modes and binding affinities of these systems. In most of the cases studied, the usage of different charge models, incorporation of covalent binding information, and increasing the number of docking runs and enlarging the binding cavity compared to standard values did not have any significant effect on the performance of different docking approaches. In many cases, including crystal waters also had no or minor influence on docking accuracy. The GOLD-SCORE and Autodock (GA)-SCORE docking-scoring combinations seemed to be the most appropriate with respect to structure prediction, while no clear correlations were found between predicted and experimentally determined binding affinities of 16 P450-ligand complexes (neither by scoring of X-ray structures, nor docked poses).

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FYS04 CALMODULIN ACTIVATES INTERFLAVIN ONE-ELECTRON TRANSFER IN THE HUMAN NEURONAL NITRIC OXIDE SYNTHASE FLAVIN DOMAIN

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The flavin semiquinones, FADH. and the FMNH. participate in the regulation of one-electron transfer within the NOS flavin domain[1,2]. The FAD semiquinone form was unstable in the presence of O_2, and showed a characteristic absorption peak at 520 nm, which was not observed in the FMN semiquinone[1]. This makes possible to monitor the proportion of FAD semiquinone generated and directly determine the rate of one-electron transfer between the two flavins using stopped-flow spectrophotometry. CaM binding results in a greater than 10-fold increase in the rate of interflavin (i.e. FAD□ FMN) one-electron transfer.

The reduction of the air-stable semiquinone (FAD-$FMNH^{\cdot}$) of both CaM-bound human nNOS and iNOS flavin domains with NADPH showed that the extent of a conversion of $FADH_2$/$FMNH^{\cdot}$ to $FADH^{\cdot}$/ $FMNH_2$ in the iNOS flavin domain was greater than that of the nNOS flavin domain. The reduction of both oxidized domains (FAD-FMN) with NADPH resulted in the initial formation of small amount of disemiquinone, followed by its decay. The rate of interflavin electron transfer between the two flavins in the iNOS flavin domain was faster than that of the nNOS flavin domain. In addition, the formation of a mixture of the two- and four-electron reduced states in the presence of excess NADPH was different for the two NOS flavin domains. These data indicate a more favorable formation of the active intermediate, $FMNH_2$ in the iNOS flavin domain, as compared with the nNOS flavin domain. This may explain why the iNOS flavin domain has a high activity towards electron acceptors.

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FYS05 ELECTRON TRANSFER IN FLAVOCYTOCHROME P450 BM3 AND THE C999A MUTANT: KINETICS OF FLAVIN REDUCTION AND RELATIONSHIPS WITH MAMMALIAN CYTOCHROME P450 REDUCTASE.

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The cytochromes P450 are a superfamily of haem-containing mono-oxygenase enzymes found in all major domains of life. They catalyse the reductive scission of dioxygen, and generate an oxygenated product, water and $NADP^+$ [1]. The diflavin enzyme cytochrome P450 reductase (CPR) supplies electrons to eukaryotic P450 enzymes[2]. CPR has arisen by the fusion of ancestral domains related to flavodoxin and $NADP^+$-ferredoxin oxidoreductase[3]. Flavocytochrome P450 BM3 comprises a prokaryotic CPR and a soluble P450[4]. The diflavin reductase of flavocytochrome P450 BM3 is fused to a P450 fatty acid hydroxylase, and the enzyme has high levels of activity towards a range of long chain fatty acid substrates[5]. The crystallographic structure of the haem domain of P450 BM3 has been solved in the presence and absence of bound substrate[7,8], and solution studies have clarified the roles of a number of active site residues in substrate binding and catalysis[e.g. 6,9]. Structural and mechanistic studies of the diflavin reductase domains of flavocytochrome P450 BM3 are less advanced than for the P450 domain.

The primary structure of P450 BM3 reductase lacks the membrane anchor found in mammalian enzymes. Despite the inferred structural similarities, the thermodynamic properties of BM3 reductase are distinct from human and rabbit CPR, suggesting major differences in the catalytic cycles of the bacterial and mammalian enzymes[10-12]. BM3 reductase catalyses the reduction of artificial electron acceptors (e.g. cytochrome c and ferricyanide) more effectively than its eukaryotic counterparts. It also reduces its own heme domain at rates in excess of 200 s^{-1} [13], which far surpasses the reduction rates of mammalian P450s by their cognate CPRs [14]. The ability of BM3 reductase to deliver electrons rapidly to its P450 domain is due, in part, to the fast reduction of FAD by NADPH, which is considerably slower in mammalian CPR [15]. Notwithstanding, several residues known to facilitate FAD reduction by NADPH in mammalian CPRs are conserved in BM3 reductase. These include the „catalytic triad“ identified by Kasper and co-workers in rat CPR, comprising Ser 457, Cys 630 and Asp 675 [16]. These are equivalent to Ser 830, Cys 999

and Asp 1044 in BM3 reductase[17]. In the rat C630A mutant CPR, the cytochrome c reductase rate is decreased approximately 50-fold[16] and reduction of the flavin cofactors by NADPH is impaired. It has been suggested that Cys-630 either destablilizes the nicotinamide C4-H bond and/or donates a proton to the N5 atom of FAD during enzyme reduction[16]. Also, it has been suggested that flavin reduction is thermodynamically disfavoured in rat C630A CPR relative to wild-type[16].

Alignment of the available sequences for diflavin reductases indicates that Cys-630 of rat CPR is conserved throughout the family of proteins, except for human NR1. Modelling of the FAD domain of flavocytochrome P450 BM3 indicates that Cys-999 likely occupies a position analogous to that of Cys-630 in rat CPR. Given the predicted importance of Cys-999 (by analogy with Cys-630 of rat CPR), and that flavin reduction in flavocytochrome P450 BM3 is much faster than that seen with other diflavin reductases[e.g. 13, 15], we have examined the kinetic and thermodynamic properties of the C999A flavocytochrome P450 BM3. We show that the rate of FAD reduction is substantially impaired in C999A flavocytochrome P450 BM3. We provide evidence for a second, kinetically distinct nicotinamide coenzyme binding site, the occupation of which attenuates the rate of $NADP^+$ reduction by reduced flavocytochrome P450 BM3. We suggest that $NADP^+$ release from reduced flavocytochrome P450 BM3 is faster than with mammalian CPR. Unlike with mammalian CPR, this accounts for the lack of attenuation of the FAD reduction rate by NADPH through occupation of the second NADPH binding site, and this contributes to the superior catalytic rate of BM3 reductase. These data further our understanding of the complex phenomena underlying electron transfer and its control in the CPR family of enzymes.

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FYS06 DIRECTED EVOLUTION OF ALKANE HYDROXYLATION ACTIVITY IN CYTOCHROME P450 BM-3

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Cytochrome P450 BM-3 (CYP102) from Bacillus megaterium is a soluble, self-sufficient, highly active (C12-C18) fatty acid monooxygenase. We have recently converted this enzyme into a highly efficient catalyst for the conversion of alkanes to alcohols 1. The mutant enzyme exhibited initial rates faster than any other previously reported alkane hydroxylase. Using this mutant as a starting point we have applied directed evolution techniques and site-directed mutagenesis to further improve activity on short, gaseous alkanes such as propane, ethane and methane. Catalyzing this reaction requires efficient substrate binding and shifting the regioselectivity towards terminal hydroxylation. For this purpose we developed a colorimetric screening assay based on the conversion of alkyl methyl ethers that allows for detection of increased turnover number as well changes in regioselectivity. Propane-oxidizing mutant enzymes have been generated that show increased total turnover number without a shift in regioselectivity. However, these mutants do exhibit a shift in regioselectivity towards terminal oxidation in the hydroxylation of octane. The demonstrated ability of the P450 BM-3 framework to accommodate these enhanced activities towards small alkanes suggests that methane hydroxylation activity might be

accessible to this system upon the application of further rounds of directed evolution.

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FYS07 DIRECTED EVOLUTION OF P450 BM-3 FOR BIOTRANSFORMATIONS

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Cytochrome P450 BM-3 (EC 1.14.14.1) catalyses hydroxylation and epoxidation of a broad range of valuable compounds including alkanes, alcohols, fatty acids, amides, polyaromatic hydrocarbons and heterocycles, reactions which are difficult to be achieved by chemical means. However, the K_m values of P450 BM-3 towards most of these notoriously insoluble compounds fall in mM range, which greatly limits its practical application. An organic co-solvent is thus necessary to increase the substrate solubility and to achieve a higher catalytic efficiency. Applying P450 BM-3 in such non-natural working environment requires increase of its tolerance towards organic co-solvents. By directed evolution using pNCA assay[1,2], we improved the organic co-solvent resistance of P450 BM-3 towards DMSO and THF with increases in specific activity of: up to 10-fold in 2 % (v/v) THF and up to 6-fold in 10 % (v/v) DMSO[3]. The evolved mutants also showed a significantly higher tolerance towards other organic co-solvents including acetone, acetonitrile, DMF and ethanol3. In addition, colorimetric assays based on 4-aminoantipyrine, Fast Violet B salt and Fast Blue RR salt are being developed to detect hydroxylated aromatic biotransformation products and to access the industrial potential of organic co-solvent resistant mutants.

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FYS08 REGULATION OF EXPRESSION OF THE HUMAN LANOSTEROL 14α-DEMETHYLASE GENE (CYP51)

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Lanosterol 14α-demethylase (*CYP51*) is the most conserved cytochrome P450 with the major role in cholesterol biosynthesis in mammals. Having a housekeeping role in cholesterol biosynthesis, *CYP51* it is expressed ubiquitously, with highest levels observed in the testis, liver, ovary and the adrenal gland. These tissues differ in the sense whether they mainly produce cholesterol and excrete it in the form of bile acids (liver), or require cholesterol for steroid hormone biosynthesis (testis, ovary, adrenal gland). In addition to steroid hormones, testis and ovaries accumulate intermediates of the cholesterol biosynthetic pathway that are not observed in the liver. Meiosis activating sterols (MAS) are short-lived products of CYP51 demethylation reaction in somatic cells, but accumulate in gonadal tissues, suggesting their role in reproduction. The different roles of cholesterol and proposed roles of its intermediates suggest that regulation of enzyme activities of cholesterogenic genes differ between tissues. Consequently, it is expected that transcriptional regulation of genes encoding cholesterogenic enzymes is mediated in a tissue-specific manner. The complex promoter/regulatory region of mammalian *CYP51* genes permits trans-activation by a variety of signalling pathways, resulting is a tissue-specific mode of regulation. Sterol regulatory element (SRE) binds sterol regulatory element binding proteins (SREBPs) that are common trans-activatiors of cholesterogenic genes in the liver and mediate the negative cholesterol feedback signaling. SREBPs bind to SRE elements in low-cholesterol conditions, resulting in trans-activation of genes, including *CYP51*. When cholesterol is not limiting to the cell, SREBP-dependent genes are expressed only at basal level. To perform the trans-activation function, SREBPs require interactions with other transcription factors and with co-regulatory proteins, that different from gene to gene. In the case of the human *CYP51* gene we demonstrated SREBP interactions with Sp1 and with CRE-binding proteins. cAMP-responsive element (CRE2) binds cAMP-dependent transcription factors and is necessary for SREBP-dependent transcriptional activation. SREBP-1a is phosphorylated by PKA in ex vivo conditions and can interact with different co-activator proteins, such as CREB-binding protein (CBP) and activator of CREM in testis (ACT). Both mentioned co-activator proteins are not expressed in identical tissues, suggesting that SREBP transcriptional activity is modulated in different tissues according to choice/availabi-

lity of co-regulatory and coactivator proteins. The cAMP-dependent transcriptional activation of *CYP51* is from the physiological standpoint likely more important in germ cells and in adrenal gland compared to the liver. We have shown that a cAMP-dependent trans-activation of *CYP51* is acieved (by cAMP-dependent stimuli, such as forskolin, or by overexpression of transcription factors CREB and CREM) independently of the SREBP-dependent pathway. *CYP51* transcription is activated *ex vivo* in sterol-repressed conditions, when cells have enough sterols and unsaturated fatty acids and the mature forms of SREBP transcription factors are not available. This sterol independent/cAMP-dependent activation of *CYP51* transcription might also be important in somatic cells, such as liver, in some pathophysiological conditions. Understanding the *CYP51* gene transcription is important from the standpoint of its specific position in the cholesterol biosynthetic pathway. A better understanding of *CYP51* transcription will help to a better understanding of the tissue-specific modes of cholesterol homeostasis.

FYS09 STRUCTURAL GENOMICS OF CYP COMPLEMENT FROM STREPTOMYCES: APPLICATIONS FOR GENERATING NEW ANTIBIOTICS

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The genus *Streptomyces* produces approximately two-thirds of naturally occurring antibiotics as well as a wide array of other secondary metabolites. Polyketides form one of the largest and most diverse group of these natural products. They include numerous medicinal compounds e.g. antibacterial, antifungal, anticancer and immunosuppressant agents. Although diverse in their structures, polyketide molecules share common features in their biosynthetic pathways. Antibiotic diversity of polyketides is generated during their biosynthesis by several means, including post-polyketide modification performed mainly by group transferases and oxidoreductases, very broad group of enzymes, which among others includes cytochrome P450 monooxygenases (CYP). CYPs are common in polyketide synthase gene clusters. Thire activities include site-specific oxidation of macrolide antibiotics precursors introducing regiochemical diversity into the macrolide ring system, thereby significantly affecting antibiotic activity. Efficient manipulation of *Streptomyces* CYPs in generating new antibiotics will require identification and/or engineering of monooxygenases with activities toward a diverse array of chemical substrates. To better understand relation between structure and function of CYP monooxygenases from secondary metabolic pathways of industrially important *Streptomyces*, we have initiated the complete genetic, biochemical and structural evaluation of all 18 CYPs from *Streptomyces coelicolor* A3(2). X-ray structures for two *S. coelicolor* A3(2) CYPs assigned to the same CYP family, CYP154A1 and CYP154C1, have being determined. Both structures are analyzed in context with other available structures of related CYPs from polyketide synthase (EryF) and non-ribosomal peptide synthetase (OxyB) biosynthetic pathways. The generation of 3D-structures for evolutionarily related CYPs having the ability to hydroxylate different intermediates in macrolide antibiotic biosynthetic pathways leads to an opportunity to translate patterns of CYP amino acid sequences into structural patterns of folded proteins, a necessary step on the way to the rational design and modeling of CYPs of biotechnological as well as physiological importance.

FYS10 INACTIVATION OF P450 2B1 AND 2B2 BY *TERT*-BUTYL 1-METHYL-2-PROPYNYL ETHER

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Acetylenic compounds can inactivate P450 enzymes through binding of a reactive metabolite to either the apoprotein or the heme moiety. *tert*-Butyl 1-methyl-2-propynyl ether (tBMP) was tested for its ability to inactivate the rat P450 enzymes 2B1 and 2B2. P450 2B1 and 2B2 are 97% identical, differing by only 14 amino acids. P450 2B1 and 2B2 have similar substrate specificity and exhibit similar regioselectivity for the metabolism of most substrates; however, the rate of substrate metabolism by 2B2 is generally 5 to 10-fold lower than that of 2B1. tBMP inactivates the 7-ethoxy-4-(trifluoromethyl) coumarin (7-EFC) *O*-deethylation activity of P450 2B1 in a time-, concentration-, and NADPH dependent manner; however, no inactivation was observed with 2B2. The loss of 2B1 activity was accompanied by a loss in the reduced CO spectrum and the amount of native heme with the concomitant appe-

arance of two heme adducts. LC/MS analysis of the heme adducts resulted in an *m/z* of 705 for both adducts which is consistent with an iron-depleted heme and a tBMP molecule containing one oxygen atom. The loss in 7-EFC *O*-deethylation (75%) was always greater than the loss in the CO spectrum (31%) and native heme (39%). The ability of tBMP to inactivate P450 2B1 and 2B2 mediated benzphetamine N-demethylation was also determined. Inactivation of P450 2B1 by 10 μM tBMP resulted in a 22% decrease in benzphetamine *N*-demethylation which is significantly less than the 75% loss of 7-EFC *O*-deethylation that was observed. tBMP did not have an effect on P450 2B2 mediated benzphetamine *N*-demethylation and no loss in either the reduced CO spectrum or native heme was observed. tBMP is a mechanism based inactivator of P450 2B1. In contrast to P450 2B1, 2B2 is not inactivated by tBMP. These results indicate that the relatively minor differences in the primary sequence between these two isoforms have a dramatic effect on the active site architecture.

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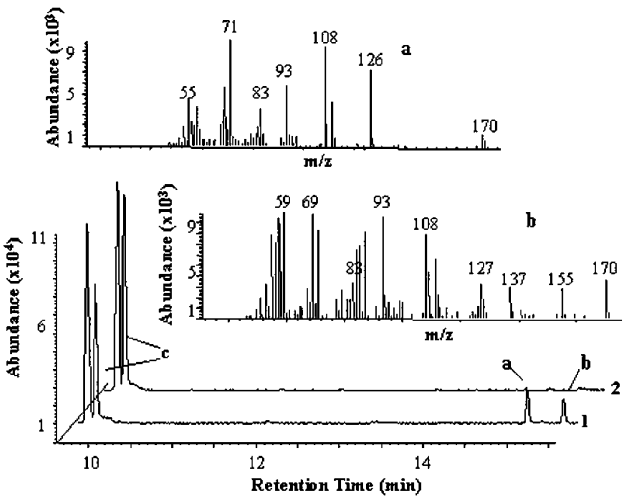

Fig. 1. *GC-MS-chromatograms with spektra of a sample after incubation without cytochrom P450 enzymes (2) and after incubatioon with human liver S9 (1). a) 2-hydroxy-1,8-cineole; b) 3-hydroxy-1,8cineole; c) 1,8-cineole*

FYS11 METABOLISM OF 1,8-CINEOLE IN THE HUMAN LIVER AND IDENTIFICATION OF THE COMPETENT CYPS

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Among numerous groups of naturally occurring compounds examined so far, monoterpenes are known as fragrances and flavouring agents for example. 1,8-cineole, a monoterpene cyclic ether which is named eucalyptol, is found in essential oils like *Eucalyptus polybractea*. It has a characteristic fresh fragrance and pungent taste and is extensively used for external application in pharmaceutical preparations, e.g. in nasal spray or as disinfectant[1]. The metabolism of 1,8-cineole by liver microsomes of rats, humans and by recombinant cytochrome P450 enzymes was investigated by Miyazawa et al.[2] The results suggested that 1,8-cineole is solely oxidized in high rates to 2-hydroxy-1,8-cineole and there was detected no other oxidation product.

In order to determine the toxic potential of monoterpenes we studied the human metabolism of monoterpenes in vitro and in vivo. We investigate the biotransformation of 1,8-cineole in vitro by pooled human liver S9 (*Figure 1*) and by recombinant cytochrom P450 3A4 and 3A5 enzymes coexpressed with human CYP-reductase in *Escherichia coli* cells. Beside the product mentioned above, 2-hydroxy-1,8-cineole, we found one more metabolite produced at high rates. It was identified by its mass spectrum as 3-hydroxy-1,8-cineole. There was a clear correlation between metabolite concentration and time of incubation and enzyme concentration, respectively. Additionally, we found different transformation rates of 2-hydroxy-1,8-cineole and 3-hxdroxy-1,8-cineole dependent on the cytochrome P450 3A enzyme used for incubation. In accordance to our results we detected after enzymatic hydrolysis and sample preparation[3] both metabolites in human urine after oral administration of cold medication.

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FYS12 CLEARANCE OF ANTIPYRINE IN SALIVA OF LUNG CANCER PATIENTS UNDER IRRADIATION THERAPY.

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Antipyrine clearance in body fluids is a recognized in vivo indicator for the function of liver monooxigenase system. It is a well-known fact, that the development of tumours is usually associated with the induction of certain CYP isoforms both in cancered tissie and in liver. We examined the clearance of antipyrine (Cl) in saliva of lung cancer patients (males 50-60 years old) before and after a therapy which was a course of irradiation in intensive mode. It is established, that Cl was apparently increased in the saliva of lung cancer patients in comparison with control group of volunteers of the same age and gender (6.04 versus 4.38 ml/kg/min). Consequently, half-time of antipyrine elimination (T1/2) in the saliva of patients was decreased (5.9 versus 8 hours in control) and the apparent volume of distribution (Vd) was increased (3.72 versus 2.88 l/kg in control group).

After the 2 week course of intensive radiation therapy the drop in Cl (down to 2.62 ml/kg/min), increase in T1/2 (up to 21.9 hours) as well as normalisation of Vd (2.8 l/kg) were observed in saliva of lung cancer patients.

It is concluded that enhanced antipyrine clearance in saliva of lung cancer patients reflects activation of its excretion as a result of induction of certain isoforms of cytochrome P-450 participating in antipyrine biotransformation process. The course of intensive therapy apparently inhibits the synthesis of CYP's de novo and slows down antipyrine elimination from organism.

FYS13 CYTOCHROME P450 REDOX SYSTEMS IN MYCOBACTERIUM TUBERCULOSIS

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The genome sequence of the pathogen Mycobacterium tuberculosis (Mtb) contains 20 distinct cytochrome P450 systems, an unprecedented number for a prokaryote. We have cloned and expressed several of the P450 systems from Mtb, along with redox partner enzymes - most notably the adrenodoxin reductase homologue FprA (encoded by gene Rv3106) and the ferredoxin located adjacent to Mtb's sterol demethylase P450 (CYP51) on the chromosome (encoded by Rv0763c).

Kinetic characterization of the flavoprotein FprA reveal intriguing features as regards interaction with pyridine nucleotide cofactors. While flavin (FAD) reduction rate shows „regular" hyperbolic dependence on concentration of NADH reductant (K_d = 42.9 ± 4.6 μM, k_{red} = 25.4 ± 0.7 s^{-1}), the situation is starkly different with NADPH (the tighter-binding coenzyme). Using stopped-flow methods, the FAD reduction rate was shown to be reciprocally related to [NADPH] at concentrations of the coenzyme (≤100 μM. The reduction rate is constant at 20 ± 2 s^{-1} at [NADPH] above 100 μM, but accelerates dramatically at lower concentrations, e.g. to 122.8 s^{-1} at 6.25 μM NADPH[1]. We consider that the phenomenon reflects dual binding sites for NADPH coenzyme, as observed previously in studies of human CPR enzyme[2]. The first site is catalytic, but the second (weaker) site is inhibitory, acting primarily to prevent release of $NADP^+$ from the catalytic site. FprA exhibits the most dramatic example yet seen of this complex regulatory behaviour.

Ongoing studies on the Rv0763c-encoded ferredoxin (Fer) reveal it to have a considerably more positive reduction potential than its primary redox partner (CYP51). Des-

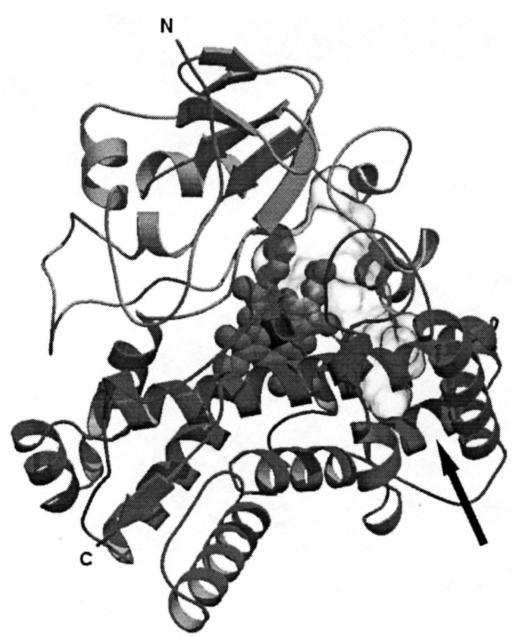

Fig. 1. *Atomic structure of CYP121 at 1.06 Å resolution. The heme (in* space fill*) is sandwiched between the two major domains of the structure: an α-helix-rich domain (*dark gray*) and the smaller _-sheet-rich domain (*light gray*). The large water-filled active site cavity of the enzyme is shown as a transparent surface. An* arrow *indicates the possible site of substrate entry between helices F and G.*

pite this, turnover of CYP51 occurs in the presence of Fer and an exogenous ferredoxin reductase[3]. This appears to be a rare example of uphill electron transfer in a redox system, perhaps reflecting a requirement to control carefully the turnover rate of the CYP51 (and possibly other Mtb P450 partners for Fer).

In recent work, we have determined the high resolution structure of a key Mtb P450 - CYP121 (encoded by the Rv2276 gene) (*Figure 1*). The structure (at 1.06 Å) reveals several novel features of cytochrome P450[4]. The heme cofactor is bound in two distinct conformations (related to each other by a 180^0 rotation about the CH_α-Fe-CH_δ axis of the macrocycle), and is „kinked" by an angle of approx. 30^0 at one of the pyrrole rings, due to interaction with the sidechain of Pro346. Several other important residues are immediately obvious, including the hydrogen-bonded Ser237 and Arg386, which dominate local structure above the heme iron. Two distinct pathways (involving amino acid residues and water molecules) by which protons can be relayed from the protein surface to the heme iron are evident. These converge at Ser279, which exists in two different conformations. In each of these positions it contacts one or other of the pathways to the surface, and in both positions it is able to pass protons to Arg386 (and on to the heme). Importantly, studies of drug-binding to CYP121 demonstrate extremely high affinity for several azole drugs, particularly econazole and clotrimazole[5]. The nanomolar K_d values for these drugs correlate well with MIC values for their growth inhibitory action against *Mycobacterium smegmatis*, providing strong evidence that the potency of these drugs against mycobacteria derives from their efficient inhibition of CYP121.

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FYS14 ROLE OF CYTOCHROME P450 2E1 IN ETHANOL OXIDATION IN THE BRAIN: DETERMINATION WITH KNOCK-OUT MODELS AND INHIBITOR ANALYSIS

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In contrast to the role of catalase, the role of CYP2E1 in ethanol metabolism in the brain is controversial[1,2,3,4]. But recent studies demonstrated constitutive expression of P4502E1 in brain, its differential induction in rat brain regions by chronic ethanol treatment, and its topographic distribution in rat and human brain[5]. The purpose of the study was to clarify the contribution of cytochrome P450 2E1 in oxidation of ethanol to acetaldehyde in the brain tissue. In the present study we tested the capacity of brain homogenates to oxidize ethanol through a catalase and cytochrome P450-dependent system using the genetic strains of acatalasemic mice (IACAT), mice with genetically specified deficiency of cytochrome P450 2E1 (ICYP2E1KO), double mutants with genetic deficiency of both catalase and cytochrome P450 2E1 (F3DKO) and control mice (C3H). The accumulation of ethanol-derived acetaldehyde in brain homogenates incubated for 30 min in the presence of 50 mM ethanol in IACAT mice was 47% of control value ($P<0.01$), in ICYP2E1KO 91% and in F3DKO mice 24% ($P<0.01$). The level of ethanol oxidation by brain tissue *in vitro* was significantly lower in F3DKO mice as compared to acatalasemic animals. Preincubation of homogenates with diallyl sulfide (CYP2E1 inhibitor) significantly reduced the acetaldehyde accumulation in control animals 23% ($P<0.05$). These experiments suggest that CYP2E1 as well as catalase can play an important role in ethanol metabolism in the brain.

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FYS15 POLYHALOGENATED COMPOUNDS OF APPROPRIATE CONFIGURATION INTERACT WITH MAMMALIAN OR BACTERIAL CYP ENZYMES TO INCREASE BILIRUBIN OXIDATION IN VITRO.

Role of an uncoupled catalytic cycle

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Polyhalogenated compounds, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin are associated with toxic *Uroporphyria*[1] and also causes alleviation of jaundice in the Gunn rat[2] (and references therein). These effects have been attributed to a microsomal oxidation of uroporphyrinogen and bilirubin for which supportive evidence has been obtained in vitro. CYP1A1 required planar PCBs for these oxidative reactions while CYP2B required non planar PCBs. Instead CYP1A2 was capable of oxidation in their absence[3].

We have now used genetically expressed rat CYP1A enzymes and confirmed with pure CYP1A1 that increased bilirubin oxidation was caused by the addition of 3,4,3',4'-tetrachlorobiphenyl (3,4-TCB). In contrast 2,4,2',4'-tetrachlorobiphenyl (2,4-TCB), the nonplanar isomer, was almost inactive. CYP1A2 was more active than CYP1A1 at oxidizing bilirubin in presence of NADPH alone and reacted to addition of 3,4-TCB with a depression rather than a stimulation of bilirubin oxidation (*Table 1*).

We have also tested a bacterial enzyme, CYP102 (a generous gift from Prof. Gordon C.K. Roberts of the University of Leicester). Dodecanoic acid and its polyhalogenated analogue (perfluorododecanoic acid) both stimulated NADPH oxidation by CYP102, but only the perfluoro analogue stimulated markedly bilirubin oxidation. The analogue exhibited much greater potency than the normal substrate in stimulating NADPH and bilirubin oxidation and also showed greater affinity for CYP102, as measured by the binding constant, Ks. The Ks values obtained for the substrate and halogenated analogue (230 μM and 2.2 μM) were in fairly close agreement with the EC50 values (approx. 300 and 1.5 μM) obtained for the oxidation of NADPH and bilirubin by the two compounds. This supports the view that occupancy of the activesite of the CYP enzyme by the halogenated substrate analogue is involved in the oxidative effects we have studied.

The molar stoichiometry ratio between NADPH and O_2 consumption was 1 in the case of the substrate, but approximated 2 with the perfluoro analogue, suggesting - in the case of the latter compound - an uncoupled catalytic cycle ultimately leading to full reduction of O_2 to water. In contrast, no significant difference was found between dodecanoic acid and perfluorododecanoic acid in the amount of H_2O_2 produced on incubation of CYP102 in presence of NADPH (Table 2). We conclude that halogenated substrate analogues interact with different CYPs to increase production of oxidative species, probably by an uncoupling mechanism. A role of the ferryl-oxygen intermediate is suggested in the oxidation of biologically important molecules, with possible implications for the therapy of jaundice and for toxic oxidative reactions, such as uroporphyria and cancer.

Table 1. *Effect of either 3,4,3',4'-tetrachlorobiphenyl (3,4-TCB) or 2,4,2',4'-tetrachlorobiphenyl (2,4-TCB) on the rate of bilirubin oxidation by „supersomes" preparations containing CYP1A1 or CYP1A2.*

Enzyme	Addition	Rate of bilirubin oxidation (pmol/min)
CYP1A1	DMSO	34.9 ± 8 (4)
	3,4-TCB	106.8 ± 11.5 (4)*
	2,4-TCB	52.8 ± 5.6 (3)
CYP1A2	DMSO	129.7 ± 45.2 (3)**
	3,4-TCB	59.2 ± 7.6 (3)
	2,4-TCB	113 ± 31.7 (5)

* P< 0.001, compared with corresponding values obtained after adding either DMSO (control) or 2,4-TCB
** P< 0.01, compared with DMSO values obtained with CYP1A1

Table 2. *Effect of dodecanoic acid or of perfluorododecanoic acid on the production of H_2O_2 by CYP102.*

Addition (final conc.)	H_2O_2 formed	
	nmol/m	% of NADPH oxidized
Dodecanoic acid (150 μM)	2.6 ± 0.97 (3)	-
Dodecanoic acid (150 μM) + NADPH (120 μM)	4.7 ± 1.1 (3)	1.8
Perfluorododecanoic acid (1.5 μM)	1.4 ± 0.2 (3)	-
Perfluorododecanoic acid (1.5 μM) + NADPH (120 μM)	3.7 ± 0.01 (3)	1.93

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AUTHOR INDEX

The following list is based on the Scientific Program of the Conference, i. e., the indicated code pertains chiefly to the code of a particular presentation (underlining indicating the presenting author). In most cases, but not always, this is the same as the code of the corresponding abstract, the differences being caused chiefly by late submissions and similar changes. The code FYSd was used to indicate the „short outline" contributions to the Forum of Young Scientists.

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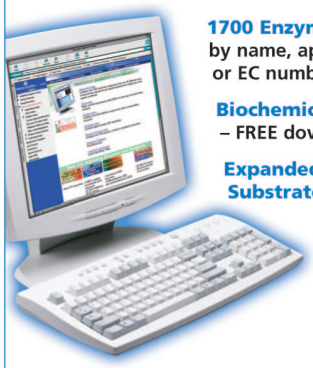

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