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STRUCTURAL BIOLOGY IN ANTIVIRAL RESEARCH – A STORY ILLUSTRATED BY THE TALES OF LIPID KINASES, STING AND VIRAL METHYLTRANSFERASES

The article is dedicated to the 70th anniversary of the founding of the Institute of Organic Chemistry and Biochemistry of the CAS in Prague.

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This article focuses on the role of structural biology in the development of small molecules that serve as protein ligands, which could potentially be used as drugs in the future. Experimental methods are utilized to obtain structural information about proteins, which then allow for virtual screening of extensive libraries of substances. The discovered ligands are further experimentally verified, with structural biology playing a crucial role in their refinement. The goal is to develop a ligand with high affinity and specificity, which could become an effective medicine. This is followed by the optimization of these substances from a chemical and pharmacological standpoint. The entire process is illustrated with examples from the recent past, such as lipid kinases, the stimulator of interferon genes (STING), and viral methyltransferases.

Keywords: antivirals, lipid kinase, STING, methyltransferase, virus

Content

1. Introduction

Structural biology is playing an ever-expanding role in drug design. Crystallography, cryo-electron microscopy (cryo-EM), and nuclear magnetic resonance (NMR) structures of biological macromolecules form the foundation for rational drug design. These experimental structures are also used in virtual screening campaigns, where very large libraries (often containing tens of millions of compounds) are screened against a protein, usually an enzyme, to discover hits for experimental validation and further chemical and pharmacological optimization. However, here the focus will be on the role of structural biology in improving ligands to obtain useful chemical tools or drug-like compounds characterized by high affinity and specificity. This process will be illustrated with medicinally important examples such as lipid kinases that are necessary for replication of certain viruses, the stimulator of interferon genes (STING) protein, which is a key player in innate immunity, and viral methyltransferases that viruses use to evade the innate immunity. Highly specific ligands for these targets were recently developed at IOCB with the help of structural information.

Mgr. et Mgr. Evžen Bouřa, Ph.D. graduated in physical chemistry and molecular biology and genetics at the Faculty of Science of Charles University, where he also received his Ph.D. in 2008 under the supervision of Professor Obšil. He worked for four years at the US National Institutes of Health (NIH) in Bethesda, where he conducted research on cellular transport and HIV using structural biology methods such as macromolecular crystallography. Since 2012, he has led a group focusing on RNA virus research and drug development at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic. He has published over 100 papers in peer-reviewed journals.

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2. Lipid Kinases

Lipid kinases are responsible for the generation of phosphorylated lipids from their non- or less-phosphorylated precursors, thereby changing the chemical composition and charge of the target and membrane. In addition, the phosphorylation state of certain lipids, such as phosphatidylinositol phosphates (PIPs), serves as a 'biological address' for a given membrane, which is crucial for orchestrating critical cellular events. By modulating the phosphorylation state of PIPs, lipid kinases govern a multitude of cellular events, including cell growth, differentiation, migration, and intracellular trafficking. Additionally, these kinases contribute to the regulation of membrane dynamics and vesicle trafficking. PIPs are key lipids produced by a class of lipid kinases known as phosphatidylinositol (PI) kinases. Some of these PIPs function as second messengers in signal transduction. Most importantly, as mentioned above, PIPs help define the identity of various cellular membranes. Few examples: the plasma membrane is rich in phosphatidylinositol 4,5 bisphosphate (PIP2), endosomes in phosphatidylinositol 3-phosphate (PI3P), and the Golgi in phosphatidylinositol 4-phosphate (PI4P) (for detailed review see ref.[1]). These PIPs are produced by the action of specific kinases. PI4P is produced by a phosphatidylinositol 4-kinases (PI4Ks) on the membrane surface (Figure 1). Humans have four of them: i) PI4KA that produces PI4P on the plasma membrane where it is further phosphorylated to PIP2 ii) PI4KB that is responsible for the synthesis of ~50% of the Golgi pool of PI4P iii) PI4K2A that synthesizes the other 50% of Golgi PI4P and also produces PI4P on endosomes as they mature iv) PI4K2B that is, most of the time, in an inactive form in cytosol and its physiological role is still unclear[2,3].

Many medically significant viruses, such as the Hepatitis C virus (HCV) and various picornaviruses including enteroviruses, poliovirus (the cause of polio), coxsackieviruses, and rhinoviruses, "hijack" PI4Ks, as they require PI4P-rich membranes for their replication. It turned out that almost all viruses hijack PI4KB, with the

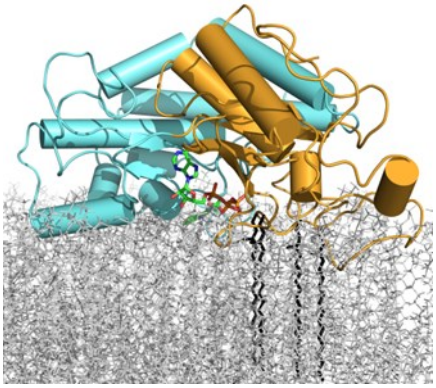

Fig. 1. **The structure of the PI4K2A on the membrane.** The kinase is docked on the lipid bilayer, with its anchoring palmitoyl groups being modeled. ATP is positioned near the membrane. The N- and C-lobes are colored orange and cyan, respectively

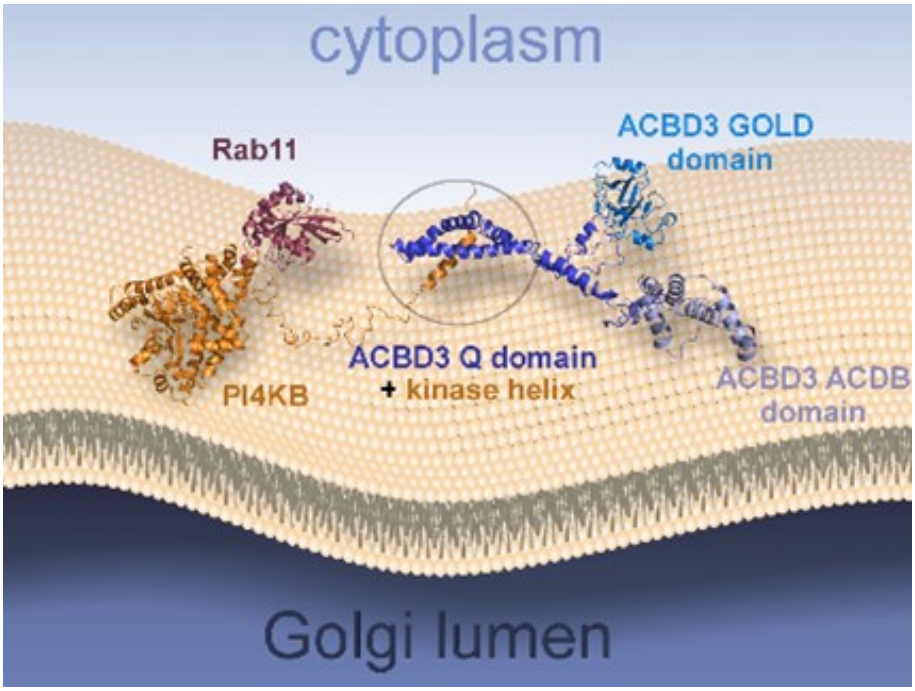

Fig. 2. **The multi-protein assembly of PI4KB**. A pseudo-atomic model of the heteromeric PI4KB/ACBD3/Rab11 complex, based on structural data (Klima et al. Sci Rep. 2016)[4]

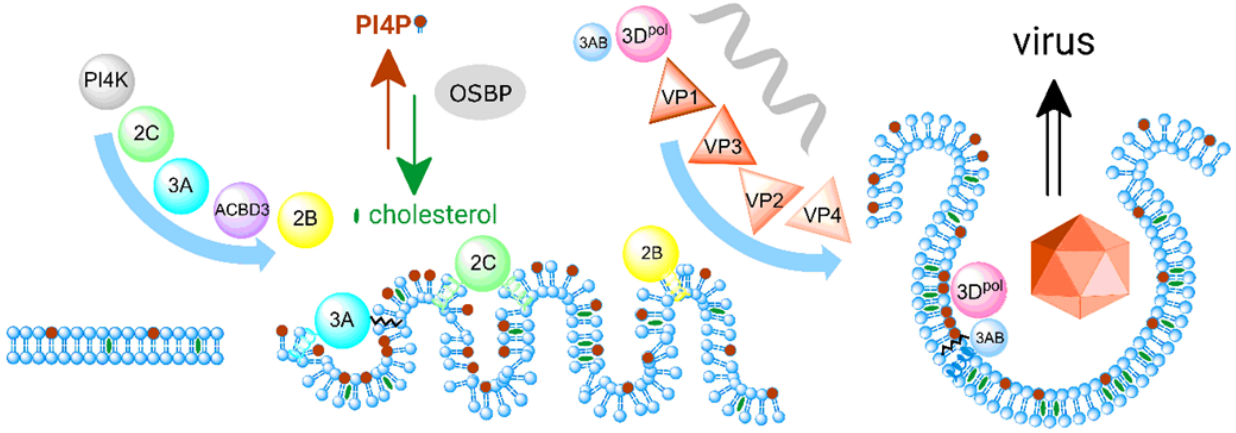

Fig. 3. **Scheme of a viral replication site**. Various viral proteins remodel the membrane and recruit PI4KB, leading to a membrane rich in PI4P and cholesterol. Subsequently, the viral polymerase, $3D^{pol}$, synthesizes new RNA molecules, which are then packaged into virions

exception of several HCV strains that hijack PI4KA[5,6]. This fact has placed PI4Ks at the center of scientific scrutiny, and the structures of these enzymes were readily become available[7–11]. Also the architecture of the PI4KB heterocomplexes was characterized. It forms a complex with the Golgi-resident protein ABCD3 (ref.[12]) which is important for its membrane localization – ACBD3 tethers PI4KB to the Golgi membrane. The Q (glutamin rich) domain of ACBD3 binds the N-terminal helix of PI4KB with nanomolar affinity[4]. Later it was shown that PI4KB forms highly flexible heterocomplex PI4KB:Rab11:ACBD3 (Figure 2) and when PI4KB is phosphorylated the complex also includes the 14-3-3 protein[13,14]. Interestingly, the interaction with 14-3-3 is conserved from yeast to man[15].

Picornavirus manipulate these PI4KB based complexes. All they need is a small 3A protein. This 3A protein interacts with ACBD3 and is able to bring the PI4KB kinase to the membrane (+RNA viruses always replicate on a membranous organelle)[12,16]. Structural analysis revealed that 3A proteins from different picornaviruses wrap around the GOLD domain of ACBD3 and, because, 3A has a transmembrane helix, this leads to anchoring of ACBD3 to target membrane and subsequent recruitment of PI4KB. It was believed that the enzymatic product of PI4KB, PI4P, specifically recruits viral polymerases[17]. However, later it was shown that not PI4P specifically but rather the negative charge in general is responsible for the recruitment of viral polymerases[18]. Another important feature of PI4P is that it can be exchanged for another lipid, such as phosphatidylserine (PS)[19–22], or cholesterol[23]. This is another reason why some viruses hijack PI4KB: they require membranes rich in PI4P and cholesterol[24].

When the PI4KB was identified as a target for the development of antivirals a search for its inhibitors has begun. The widely used PIK93 is a nanomolar inhibitor of PI4KB, however, it lacs selectivity and inhibits also other kinases such as PI3Ks. We have co-developed selective compounds against PI4KB based on a published high-throughput hit T-00127-HEV1 (ref.[25]). These compounds already exhibited activity in the sub-micromolar range in enzymatic assays *in vivo* and in the micromolar range against coxsackievirus B3 (CVB3), human rhinovirus (HRV), and hepatitis C virus genotype 1b (HCV 1b)[26]. Importantly, crystal structures of both pik93 and compound #**49** (ref.[26]) bound PI4KB were available[7,26]. This structural information was utilized and hybrid

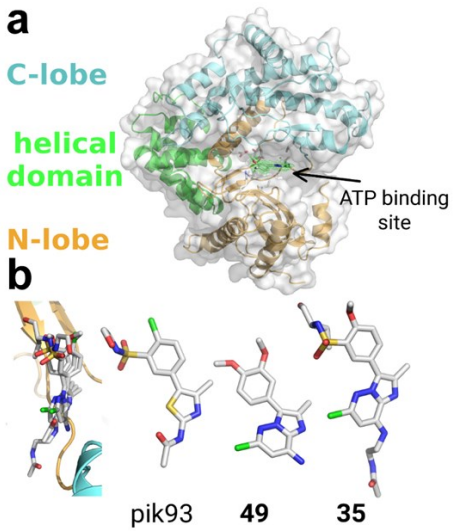

Fig. 4. **PI4KB and its inhibitors. a)** The kinase domain of PI4KB consists of the N-lobe (orange) and the C-lobe (cyan), which are in close contact with the helical domain (green). The ATP binding site is located between the N- and C-lobes. **b)** From left to right: inhibitors pik93, **49**, and **35** superimposed in the ATP binding site; structures of pik93, **49**, and **35**. Colored according to atoms, with carbon in white, nitrogen in blue, sulfur in yellow, and oxygen in red

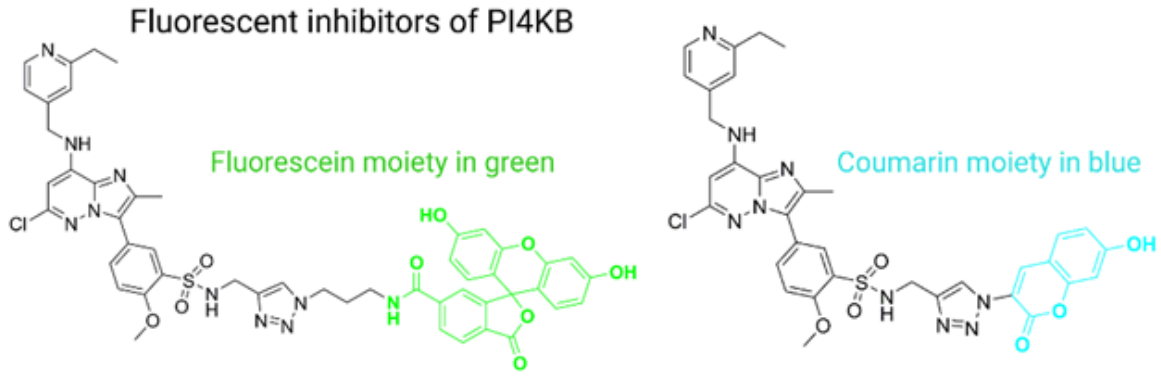

Fig. 5. **Fluorescent inhibitors of PI4KB.** Fluorescein moiety (depicted in green) or coumarin moiety (depicted in cyan) were added to the nanomolar scaffold of PI4K inhibitors

compounds bearing features of pik93 and #**49** compounds were designed (Figure 4). Functional and structural characterization revealed that the binding mode, as expected, resembles features of pik93 and and compounds **35** and **49** (Figure 4) and revealed activity in the single digit nanomolar range in enzymatic assays and strong (EC_{50} < 100 nM for all compounds) antiviral activity against CVB3, HRV1 and HCV 1b (ref.[27]). Importantly, these compounds exhibited exceptional specificity for PI4KB with no residual activity against the whole human kinome[27].

Another, important utilization that the structural information allows for is a specific modification. The crystal structures of PI4KB with several inhibitors revealed positions within the inhibitor, where a functional group can be attached without interfering with the binding of the ligand. In this case, a fluorophore was attached to the sulphonamide moiety (Figure 5) giving rise to a specific fluorescent label of the PI4K that could be used to determine K_d values *in vitro* or in live cell imaging[28].

3. Viral Methyltransferases Modifying RNA Caps

Human RNA is capped at its 5′ end. This cap is chemically an *N*7-methylated guanine base linked to the 5′ end of RNA via a triphosphate linker. This structure is termed cap-0. In higher eukaryotes, including humans, the ribose ring of the first and second RNA nucleotides can also be methylated at the 2′ position of the ribose. This process gives rise to cap-1 (where the first ribose ring is methylated) and cap-2 (where both the first and second ribose rings are methylated) (Figure 6). These RNA caps are important for RNA stability, nuclear export, and are also essential for efficient RNA translation[29]. Recently, other rare non-canonical caps, such as NAD or dinucleoside polyphosphates, have been described[30,31], but their biological relevance, especially in viruses, remains to be determined[32,33].

RNA-cap methylation was, at least partly, discovered thanks to viruses[34]. Soon it became clear that almost all viral families must protect the 5′ end of their RNAs. However, not all viruses use the cap; for instance, picornaviruses covalently link their 3B protein (also known as Vpg, viral protein genome-linked), which serves as a primer—more specifically, a protein primer—for the synthesis of both positive (+) and negative (–) RNA strands[35].

Similarly to humans, viral RNA (vRNA) capping is also important for the translation of viral RNA, at least in some viral families and at certain stages of viral infection. However, another prominent reason for vRNA capping is that successful viruses must be able to defend against

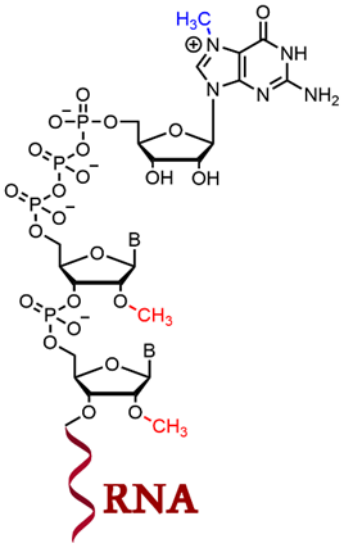

Fig. 6. **Capped RNA.** Methyl group at the guanine base is highlighted in blue, methyl groups at the first and second ribose rings are highlighted in red

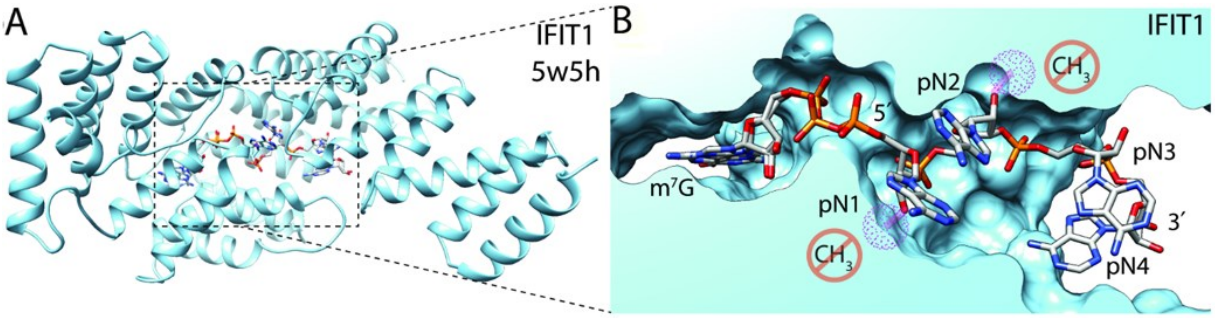

Fig. 7. **IFIT1 mediated recognition of non-fully methylated RNA. a)** The crystal structure of IFIT1 with cap-0 RNA based on PDB ID 5w5h. **b)** Details of the IFIT1 binding site, where methylated ribose rings would create steric clashes. Adopted and modified from Nencka et al.[36]

innate immunity[37]. In fact, we have many pattern recognition receptors (PRRs) that can detect vRNA, including uncapped or incompletely capped RNA. For instance, interferon-induced proteins with tetratricopeptide repeats (IFITs) recognize and bind to vRNA[38], inhibiting viral translation and replication (Figure 7). Another example is retinoic acid-inducible gene I (RIG-I), which is a critical PRR in the innate immune system responsible for detecting vRNA. RIG-I is activated by the presence of double-stranded RNA, triggering a signaling cascade that leads to the production of type I interferons and pro-inflammatory cytokines, which is essential for an effective antiviral immune response[39].

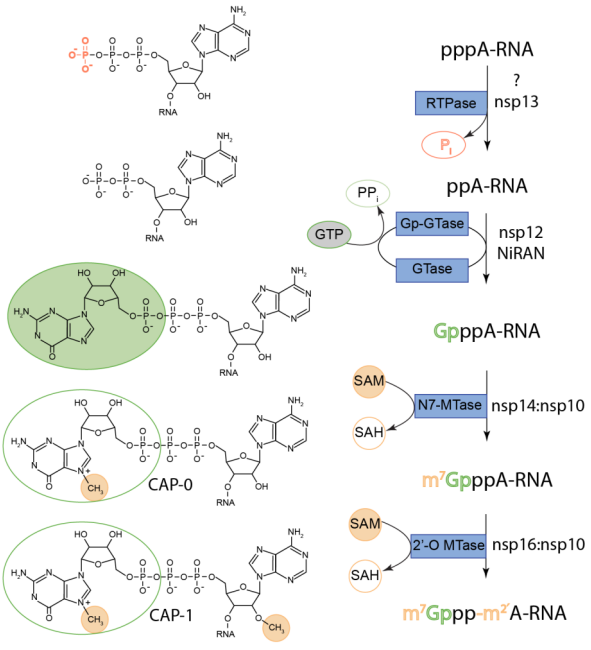

Fig. 8. **RNA cap synthesis in coronaviruses.** Adopted and modified from Nencka et al.[36]

Some viruses, most notably influenza virus, 'steal' the RNA cap from cellular RNAs and ligate them to vRNAs[40]. However, most viruses, including dangerous viral families with pandemic potential such as flaviviruses, poxviruses, and coronaviruses, possess their own enzymes for cap synthesis. These enzymes include the *N*7- and 2′-*O* methyltransferases (MTases) and can be covalently linked to the polymerase[41] or co-localize with it[42]. These facts naturally lead to the idea that inhibiting viral MTases would render the virus vulnerable to the innate immune response, potentially leading to viral clearance or, at the very least, an asymptomatic infection.

3.1. Inhibitors of Coronavirus Methyltransferases

Coronaviruses, including the infamous SARS-CoV-2, have two RNA-cap methyltransferases: nsp14, which methylates the guanine base at the *N*7 position to form cap-0, and nsp16, which, in complex with its activating protein nsp10, methylates the first ribose ring of the initial RNA nucleotide, leading to the formation of cap-1. Both of these enzymes are considered promising targets for antivirals, which led to significant progress in inhibitor design upon the beginning of the COVID-19 pandemic by us and others[33,43–58]. Structural biology played a significant role. We used a homology model of SARS-CoV-2 nsp14 based on its strong similarity to SARS-CoV nsp14 MTase (at that time crystal structure was not yet available) which led to discovery of new SAH (*S*-adenosyl-L-homocysteine) derivatives with modifications at the adenine nucleobase. These compounds were synthesized and tested *in vitro*, revealing their remarkable inhibitory potential in the single-digit nanomolar range against this enzyme. Our docking studies effectively explain how the aromatic component, linked to position 7 of the 7-deaza analogs of SAH, contributes to their activity[50] (Figure 9).

Similarly, a computational study suggested the existence of cryptic druggable pockets within the coronaviral nsp16 enzyme[58]. This information guided structure-based inhibitor design process. Initially, a high-throughput screen identified compound 5a, which was

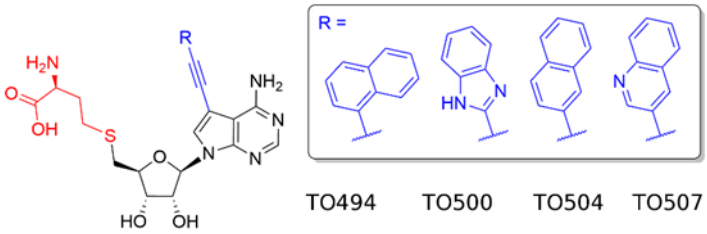

Fig. 9. **SAH derived inhibitors of SARS-CoV-2 nsp14 MTase.** The large aromatic substituents at the 7-deaza position are highlighted in blue, the aminoacid moiety is highlighted in red

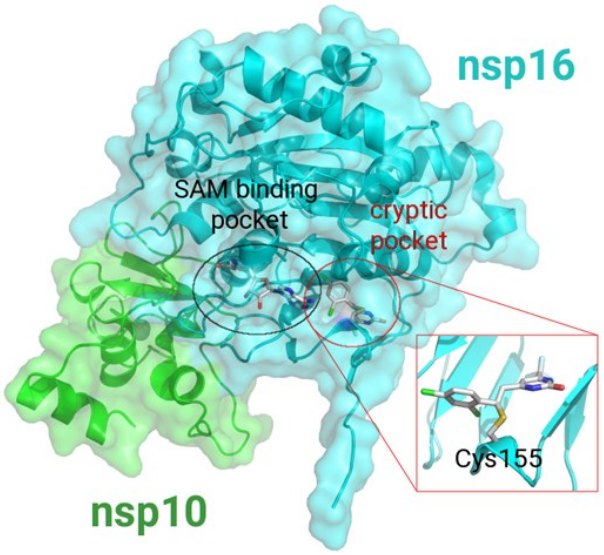

Fig. 10. **Cryptic pocket in the SARS-CoV-2 nsp16 protein.** The SAM binding site and the cryptic pocket are highlighted. A detailed view of the cryptic pocket shows the covalent bond between the small inhibitor compound 5a and residue Cys155. We used a homology model of SARS-CoV-2 nsp14, based on its strong similarity to the SARS-CoV nsp14 MTase. At that time, the crystal structure was not yet available. This approach led to the discovery of new *S*-adenosyl-L-methionine (SAM) derivatives with.

subsequently crystallized in complex with the nsp16/nsp10 complex. Notably, it was observed within a pocket in close proximity to the enzyme's active site (Figure 10). Utilizing this structural information, derivatives were developed that also acted as allosteric inhibitors[59].

4. STING, Innate Immunity and Poxviruses

Recently, a new virus, monkeypox or mpox virus (MPXV), has rapidly spread across all continents[60]. This outbreak is likely linked to the discontinuation of smallpox vaccination, which had been effective not only against smallpox but also against other poxviruses, preventing the global spread of MPXV. Nevertheless, the swift spread of MPXV highlights how easily a new pandemic could emerge, posing a threat to both our lives and the global economy.

Poxviruses are DNA viruses that replicate in the cytoplasm. Their large genome encodes the entire DNA replication and RNA processing machinery, including the capping machinery[61,62]. DNA should not be present in cytoplasm and its presence there indicates a DNA virus infection. Double-stranded (dsDNA) is detected by the cGAS-STING pathway. cGAS (cyclic GMP-AMP or cGAMP synthase) detects dsDNA and synthesizes cGAMP, which activates STING (stimulator of interferon genes)[63]. This, in turn, leads to the expression of interferon -induced genes, inducing an antiviral state in the cell. To counteract this pathway, poxviruses encode an unusual nuclease called poxin, which rapidly degrades cGAMP[64,65]. Recently, many STING agonists were developed at IOCB[66–74], some of which are resistant to poxin cleavage (Figure 11).

4.1. Poxvirus Methyltransferases and Their Inhibitors

Poxviral MTases are also considered *bona fide* drug targets. The crystal structure of the MPXV 2′-*O* MTase, VP39, revealed a cavity in close proximity to the SAM adenine base that could be exploited to prepare effective inhibitors[75]. Two approaches were used: *i*) a screening campaign against VP39 and *ii*) structure-based design. Both approaches yielded sub-micromolar inhibitors that exploited this cavity and, interestingly, some of these inhibitors had the same chemical structure as nanomolar inhibitors against the SARS-CoV-2 MTase nsp14 (ref.[75]). These results illustrate that the same compound can target different enzymes (*N*7- and 2-*O*-MTase) from unrelated viruses. All of these inhibitors occupied the SAM binding site and would not interfere with RNA binding[76]. However, experiments with the live virus in our biosafety level 3 laboratory revealed that these compounds efficiently block the replication of the MPXV virus[77].

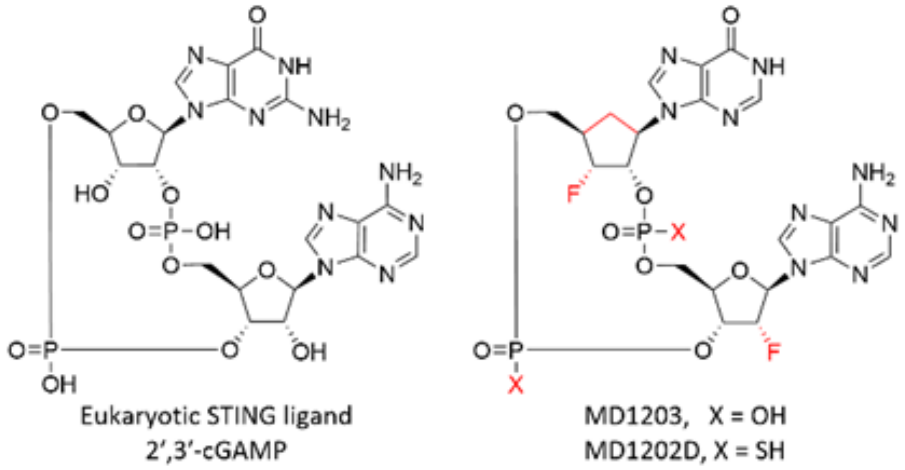

Fig. 11. **STING ligands.** Left: The natural cGAMP ligands. Right: Example of ligands prepared at IOCB that are resistant to poxin cleavage

5. Future directions

Structural biology will remain crucial in the design of inhibitors. However, a significant shift towards *in silico* is expected due to advancements in artificial intelligence (AI). AlphaFold 2 already excels in protein structure prediction, and the emergence of AIs that can accurately predict ligand binding in the near future is anticipated. Such AIs would be particularly beneficial in scenarios where generating experimental crystal structures is very challenging. For example, while identifying inhibitors of viral polymerases is relatively straightforward[78–81], procuring their crystal structure can be exceedingly challenging because also the RNA needs to be incorporated and its sequence optimized. In such situations, previously, we often relied on models derived from computer simulations[82].

Another instance is with intrinsically disordered proteins or those comprising well-folded domains linked by intrinsically disordered segments, like the coronaviral N protein. While structural biology can characterize these, often by combining methods like small-angle X-ray scattering (SAXS) with computer simulations[83] – as was recently done for the N protein[84] – the outcome is an ensemble of thousands of structures. These structures are then grouped into several clusters. Here, AI could play an invaluable role by identifying a druggable cluster and suggesting a compound capable of "locking" a protein in a physiologically inactive conformation.

As with many scientific disciplines, AIs are expected to transform and enhance the conventional pipeline of structure-based drug design. Nevertheless, experimental structures will always be essential to validate in silico results; no one would embark on an expensive drug development endeavor based solely on the predictions of an AI with 90 to 95% accuracy.

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