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BRIEF REPORT

Structural basis of Zika virus methyltransferase inhibition by sinefungin

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Abstract Zika virus is considered a major global threat to human kind. Here, we present a crystal structure of one of its essential enzymes, the methyltransferase, with the inhibitor sinefungin. This structure, together with previously solved structures with bound substrates, will provide the information needed for rational inhibitor design. Based on the structural data we suggest the modification of the adenine moiety of sinefungin to increase selectivity and to covalently link it to a GTP analogue, to increase the affinity of the synthesized compounds.

Zika virus has recently emerged as a significant threat to human health in our globalized society [1–3]. This member of the *Flaviviridae* family, genus *Flavivirus*, belongs a group of arthropod-borne viruses that are transmitted mainly by the bite of the *Aedes spp.* mosquitoes [4, 5]. However, sexual transmission [6, 7] and mother-to-fetus transmissions [8] have also been reported, which seem to be especially important because of the linkage between Zika virus infections and birth defects, namely microcephaly [9, 10]. Coupled with the fact that Zika virus RNA can be detected in semen for as long as six months [11], infection with Zika virus may present a serious threat to the safety of human reproduction. Apart from microcephaly and other common symptoms such as fever, rash and joint pain, Guillain-Barré syndrome was also reported to be associated with Zika virus infection [12]. Guillain-Barré syndrome is a serious condition characterized by rapidly progressing symmetrical muscle weakness. So far, neither a treatment nor vaccine against Zika virus is in clinical use, although significant effort has been invested into this challenging task within the last year [13–18].

As with other members of the *Flavivirus* genus, Zika virus is an enveloped single stranded positive sense RNA (+RNA) virus [19] whose genome contains a methylated cap at its 5′ end. This RNA molecule, after simple host cell-associated processing [20], serves as the mRNA for translation of a single large viral polyprotein, which is subsequently cleaved by both cellular and viral proteases into three structural (envelope, E; membrane precursor, PrM; and capsid C) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [21, 22]. The central protein of flavivirus replication is NS5, which is responsible for two distinct catalytic activities. Its first domain (approximately one third of the NS5 protein) is a methyltransferase (MTase) and the second domain is an RNA-dependent RNA polymerase (RdRp) [23].

The vast majority of cellular mRNAs possess a stabilizing cap structure at the 5′ end which usually consists of *N*-7-methylguanosine triphosphate combined with 2′-*O*-methylation [24] of the first one or two nucleotides (Fig. 1) [25]. Numerous viruses, including flaviviruses, have developed their own capping mechanisms in order to bypass host innate immunity, enhance the translation process and mimic host mRNA [26]. Apart from the installation of the guanosine triphosphate (Gppp) on the 5′ end of the RNA, the methylation of position *N*-7 of Gppp and 2′-*O*-hydroxyl of the following nucleotide(s) is essential for the whole capping process. In humans, the methyl groups are installed in the nucleus by two distinct enzymes (*N*-7

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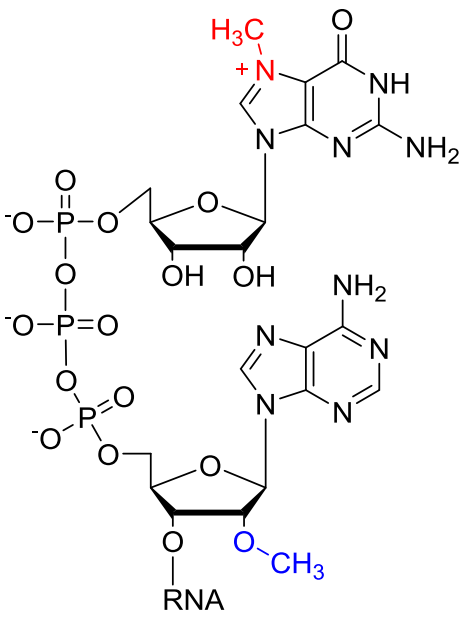

Fig. 1 The cap structure consisting of 7-N-methylated guanosine 5'-triphosphate and 2'-O-methyladenosine

MTase and 2′-*O*-MTase), however, Zika virus MTase catalyzes both methylation steps [27]. The methylation of the cap structure is essential for the whole replication process of flaviviruses, including Zika virus, and its inhibition leads to arrest of viral replication in cells [28]. Therefore, the MTase is one promising target for the discovery and development of novel therapeutic agents against Zika virus infection. We note some compounds targeting Zika virus MTase have already been reported based on an *in silico* screen [29].

Here we report the crystal structure of the Zika virus methyltransferase in complex with pan-methyltransferase inhibitor sinefungin, an adenosine derivative, originally isolated from *Streptomyces griseoleus* by Eli Lilly and Co. as a potential antifungal antibiotic [30]. From a mechanistic point of view, it competes with S-adenosyl-1-methionine (SAM), the natural substrate of numerous MTases [31], and, therefore, presents an interesting starting point for the development of novel competitive inhibitors of this essential enzyme involved in the Zika virus replication cycle.

The last few months have resulted in a frenetic struggle for the structural characterization of the Zika virus proteins, which resulted in the successful crystallization of proteins including Zika virus MTase [32–34]. However, this structure together with an inhibitor was not elucidated until now.

The sequence encoding the Zika virus methyltransferase from ZIKV virus (strain MR766) infected cells was amplified using primers: 5′-GAGGGATCCGGGGGTGGA ACGGGAGAGAC-3′ (forward) and 5′-GAGGCGGCCG CCTATTACCGCGTGCCAGAGCCGAGATT-3′ (reverse). The coding sequence was cloned into the pHis2 plasmid previously modified to encode an N-terminal 8xHis tag followed by the SUMO protein (a gift from Dr. Ren, Berkeley). The protein was expressed in *E. coli* Rosetta Gami B (DE3) cells in auto-induction medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol using standard protocols [35–37] and subsequently purified using metal affinity chromatography followed by TEV cleavage and size exclusion chromatography using standard methods [38, 39]. Briefly, the cells were lysed by French pressure cell press in lysis buffer (500 mM NaCl, 50 mM Tris pH 8.0, 3 mM 2-mercaptoethanol, 10% glycerol, 5 mM $MgCl_2$, 0.5 U/ml Salt Active Nuclease [ArcticZymes]) and centrifuged. The supernatant was incubated with Ni-NTA agarose (Machery-Nagel) and subsequently washed with the lysis buffer. The protein was eluted with lysis buffer supplemented with 300 mM imidazole. The 8xHis-SUMO tag was removed by Ulp1 protease cleavage at 4° C overnight and the protein was further purified using size exclusion chromatography on a Superdex 75 column (GE Healthcare). Finally, the protein was concentrated to 10 mg/ml and stored at -80° C until needed.

For crystallization trials sinefungin (Sigma-Aldrich), at a final concentration of 1 mM, was added to purified Zika virus MTase. Diffraction quality crystals grew within two weeks in hanging drops created by mixing 2 µl of the protein with 2 µl of the well solution (100 mM sodium acetate pH 4.6, 39% (v/v) PEG 400). The crystals were flash frozen in liquid nitrogen and data were collected using a home source. The crystals belonged to the monoclinic C2 spacegroup and diffracted to 1.95Å resolution. The structure was solved by molecular replacement using Zika methyltransferase bound to S-adenosylmethionine (PDB ID: 5KQR) as a search model using Phaser [40] and further refined using the Phenix package [41] to R_{work} = 22.67 % and R_{free} = 26.88 % and good stereochemistry as detailed in Table 1. The data and the model were deposited on the PDB database (http://www.rcsb.org) under deposition ID 5MRK.

Our crystals contained two Zika virus MTase molecules in their asymmetric unit. We traced the whole polypeptide except for the first four N-terminal residues in both molecules; however, we found the segment Arg37 – His53 disordered only in molecule B. This was probably caused by crystal packing; however, it still reveals that this segment is flexible, as reported by others [34, 42]. The overall fold from our structural analysis of Zika virus MTase is in good accordance with previously solved substrate-bound structures. The MTase domain is composed of eight α-helices and seven β-sheets (Fig. 2A). The β-sheet is mixed (parallel and antiparallel sheets are present) and forms a

Table 1 Statistics of crystallographic data collection and refinement

Data collection	
Crystal	MT + sinefungin
Space group	C2
Cell dimension	a = 144.8 Å, b = 52.0 Å, c = 84.2 Å, α = 90° β = 107.6° γ = 90°
X-ray source	Home source
Wavelength, Å	1.5419
Resolution, Å	29.67 - 1.9 (1.97 - 1.9)
No. of unique reflections	46804 (4328)
I/σ (I)	11.01 (0.7)
R_{merge}, %	12.4 (160.5)
$C_{1/2}$, %	99.5 (38.2)
Data completeness, %	98.67 (92.06)
Multiplicity	5.18
Refinement	
R_{work}, %	22.67
R_{free}, %	26.88
rms bond angle deviation, °	0.005
rms bond angle deviation, Å	0.72
Ramachandran (outliers/ favored)	0%/97.8%

central motif from β3 to β6. The *S*-adenosyl methionine (SAM) binding subdomain is defined by sheets β3, β2, β1, and β4 and helices α3, α4, and α5 while the remaining sheets (β5, β6, β7) and helices (α1, α2, α6, α7, α8) form the second subdomain. The density for sinefungin was clearly visible upon molecular replacement in both Zika virus MTase molecules and was located in the SAM binding pocket (Fig. 2B, Fig. 3A). It is held in place mainly by hydrogen bonds between sinefungin and residues Ser56, Gly86 and Trp87 (backbone), His110, Glu111, Asp146, Lys105 (backbone), and Asp131 as detailed in Fig. 3B. Comparison with SAM and SAH bound structures reveals that the binding of sinefungin and SAM differs mainly in the conformation of Arg84 and Glu111 (Fig. 3C) while the difference in the SAH bound structure is, again, changed in the conformation of Arg84 and also rather an insignificant change in the position of Ser56 (Fig. 3D).

The goal of this study was to obtain structural information that would be useful for drug design against Zika virus. Superposition of the currently obtained structure with the sinefungin inhibitor in the SAM pocket and previous structures with GTP and 7-methyl-guanosine-5'-diphosphate (m^7GDP) in the GTP/cap pocket (Fig. 3) reveals two distinct substrate binding sites. One site for SAM or its analogues and a second for GTP (Fig. 3A). Here, we speculate that a sub-nanomolar inhibitor of the Zika virus methyltransferase can be designed by a fragment-connecting approach; specifically, we propose a chimeric molecule that would bind to both the SAM and m^7GDP binding pockets. Both sinefungin and SAM can serve, in principle, as starting molecules. The chiral center in sinefungin is inherently a complication. However, this compound is commercially available and simple synthetic

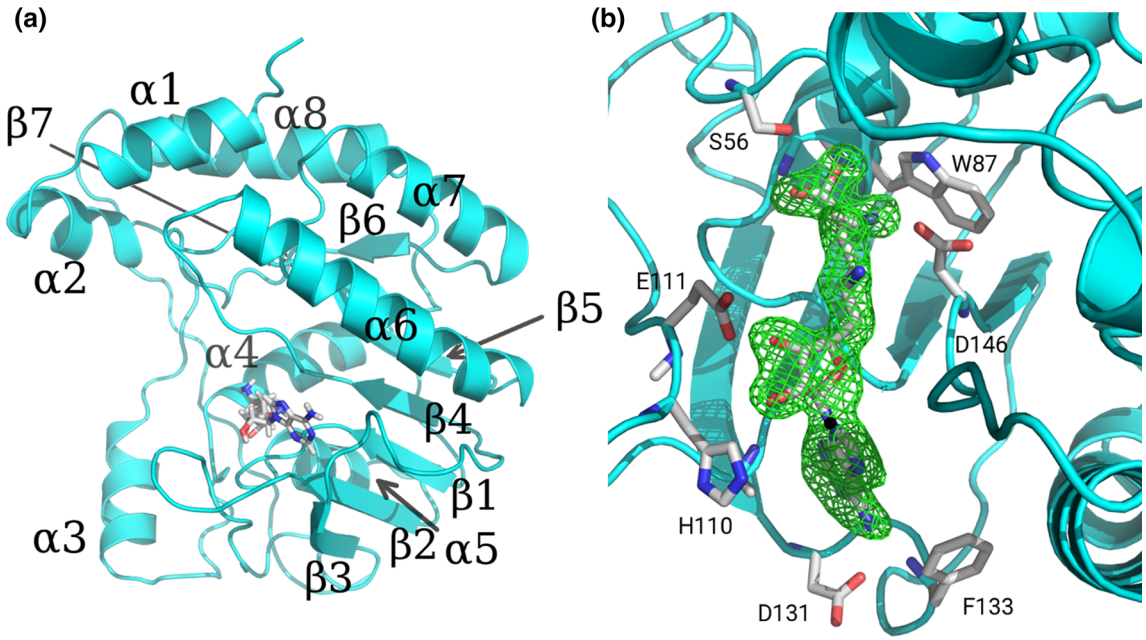

Fig. 2 Structure of the ZIKV methyltransferase in complex with sinefungin. A) The overall fold. B) The sinefungin molecule in the unbiased Fo-Fc omit map, contoured at 3 sigma. Selected residues are shown and colored according to atoms (color figure online)

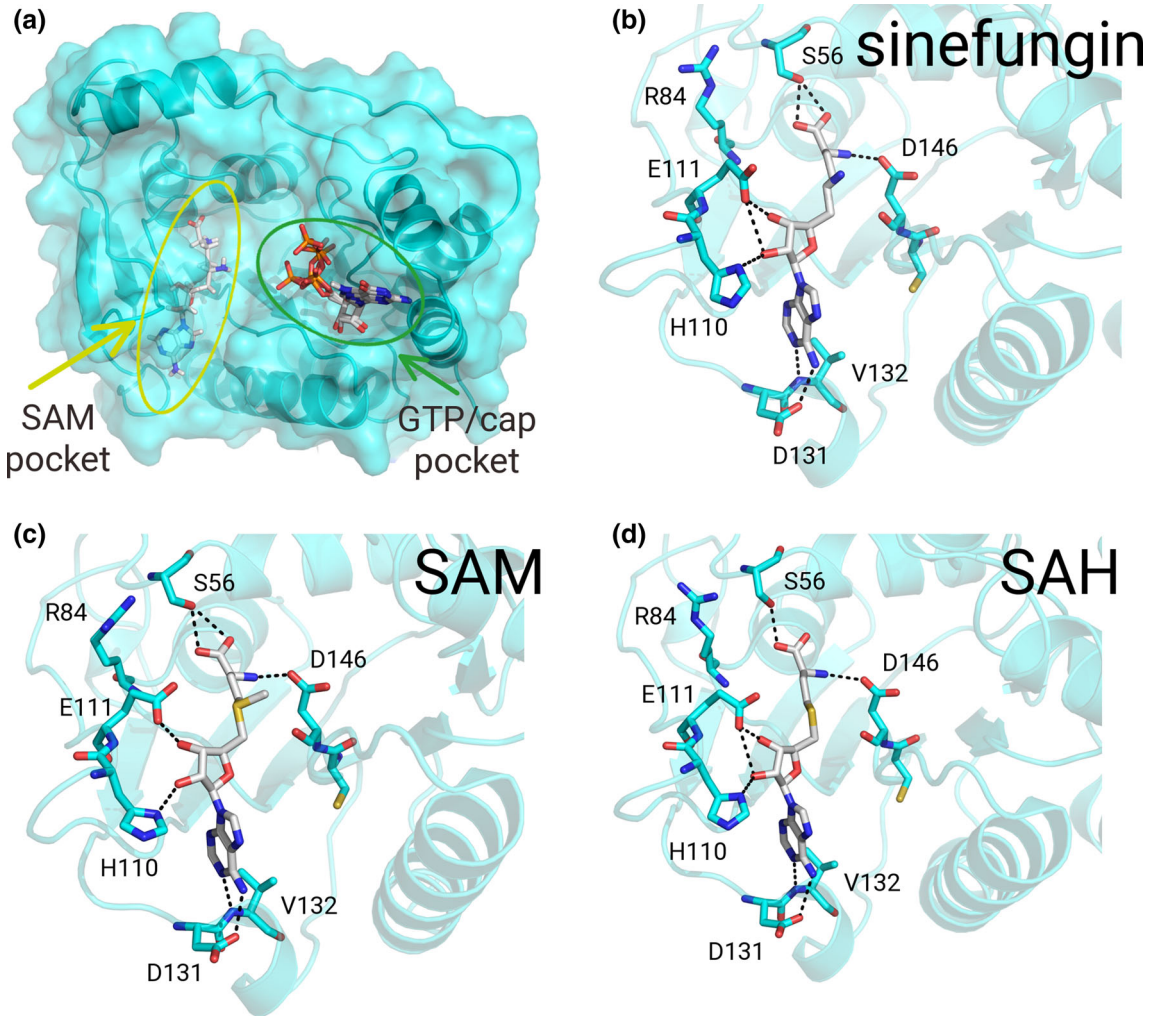

Fig. 3 A) Superposition of the sinefungin bound structure with previously solved GTP bound (pdb id: 5GOZ) and 7-methyl-guanosine-5'-diphosphate (pdb id: 5KQS) structures. B) Detail of sinefungin bound in the SAM binding pocket. C) Detail of SAM binding (prepared according to pdb id: 5KQR). D) Detail of SAH bound in the SAM binding pocket (prepared according to pdb id: 5GOZ). For figures B-D the important residues are shown in stick representation# and colored according to elements, hydrogen bonds are drawn as dashed black lines (color figure online)

approaches were reported [43]. To increase selectivity we propose to modify the adenine nucleobase because this approach was shown to be feasible in the development of dengue virus methyltransferase inhibitors [44]. The presented crystal structure shows that the Zika virus MTase possesses a similar lipophilic cavity which can be exploited in order to generate inhibitors specific for viral methyltransferases only.

Zika virus has been identified as a potential threat for human reproduction, especially due to its persistence in semen, putative sexual transmission and teratogenic defects associated with the viral infection during pregnancy [45]. Since there is no treatment for Zika virus infection, the design and development of novel strategies to fight this dangerous pathogen are of imminent importance. We report on a crystal structure of Zika virus methyltransferase in complex with sinefungin, a competitive methyltransferase inhibitor. The presented data can serve as a useful starting point for the further design of novel inhibitors of Zika virus replication. In particular, we conclude that covalently connecting sinefungin with a GTP or GDP analogue using an appropriate linker will result in outstanding affinity towards this protein, and, together with an increase in selectivity via proper substitution of the nucleobase, will result in highly potent and selective inhibitors of Zika virus replication.

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Compliance with ethical standards

Conflict of interests The authors declare no conflict of interests.

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