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Synthesis and biological evaluation of novel flexible nucleoside analogues that inhibit flavivirus replication *in vitro*

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ABSTRACT

Flaviviruses, such as Dengue (DENV) and Zika (ZIKV) viruses, represent a severe health burden. There are currently no FDA-approved treatments, and vaccines against most flaviviruses are still lacking. We have developed several flexible analogues ("fleximers") of the FDA-approved nucleoside Acyclovir that exhibit activity against various RNA viruses, demonstrating their broad-spectrum potential. The current study reports activity against DENV and YFV, particularly for compound 1. Studies to elucidate the mechanism of action suggest the flex-analogue triphosphates, especially 1-TP, inhibit DENV and ZIKV methyltransferases. The results of these studies are reported herein.

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¹ https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?p=7&id=11050

1. h

Within the Flaviviradae family, the genus Flavivirus contains over 70 viruses with a growing number of unclassified members.¹ Many Flavivirus members are known to cause severe disease, such as Dengue hemorrhagic fever, sometimes associated to human mortality.¹⁻⁴ Members of this genus, including Dengue virus (DENV), West Nile Virus (WNV), Zika Virus (ZIKV), Yellow Fever Virus (YFV), and tick-borne encephalitis virus (TBEV), represent a tremendous health burden.¹⁻⁵ Of these lethal flaviviruses, DENV poses the most severe threat with over 50 million documented cases, and between 12,500 and 25,000 deaths reported annually.^{3, 6, 7} Recently it was discovered that ZIKV infections in pregnant women have led to numerous infant abnormalities including microcephaly and severe brain malformations, as well as the development of Guillain-Barré syndrome in adults.^{2, 5, 7-9} Furthermore, more recent studies have demonstrated that ZIKV infections are sexually transmittable, with a detectable viral load in semen up to 26 weeks post symptomatic onset.10-16 Unfortunately, due to increased globalization, it is inevitable that new, undiscovered flaviviruses will continue to spread, endangering populations worldwide. As a result, new and viable therapeutic options need to be developed in order to better combat these emerging infections.

Flaviviruses are single stranded, positive-sense RNA viruses, with capped genomes of approximately 11 kb in length.^{3, 17, 18} The genome contains an untranslated 5'-region followed by a single



Figure 1.General structure of the flavivirus genome including 5' and 3' untranslated regions and the polyprotein processing of both the structural and nonstructural protein regions.¹⁷⁻¹⁸

open reading frame and an untranslated 3⁻-end region.^{3, 17, 19} The open reading frame encodes for three structural proteins (capsid, pre-membrane, and envelope), as well as seven non-structural (NS) proteins forming the replication transcription complex. Among these NS proteins, the NS3 multifunctional protein has been shown to harbor serine protease, 5⁻-RNA triphosphatase (RTPase), nucleoside triphosphatase (NTPase), and helicase activities. The NS5 protein is another multifunctional protein essential for virus replication, which is responsible for the RNA-dependent-RNA-polymerase (RdRp) and methyl transferase (MTase) activities needed for RNA capping (Figure 1).^{1-3, 17-20}

Of the seven flavivirus MS proteins, one of the most important targets for drug design is the NS5 protein, which is the most conserved protein of the flaviviruses and plays an essential role in viral replication and capping. The C-terminal domain of the NS5 protein contains the RdRp domain and the N-terminal domain is responsible for the S-adenosyl-L-methionine (SAM) dependent N7 and 2'-O-MTase activity for the viral RNA.^{6, 21-23} The aforementioned MTase activities modify the cap structure of the flaviviral RNA through N7-methylation of the 5'-guanine of the



Figure 2. Conserved flavivirus 5'-cap structure.²⁵⁻²⁹

adenosine nucleotide (^{IN/ME}GpppA_{2'OMe}-KNA) (Figure 2). ^{0, 21}

The canonical RNA capping pathway of eukaryotic cells requires four main enzyme activities: (i) RTPase (NS3 in flaviviruses) that hydrolyzes the 5'-triphosphate end of the nascent transcript into a 5'-diphosphate;²⁵ RNA (ii) RNA guanylyltransferase (putatively NS5) which then transfers the GMP moiety of GTP to the 5'-nucleotide diphosphate end;²⁶ (iii) the RNA (guanine-N7)-MTase) that methylates the N7 position on the 5'-guanine; (iv) RNA (nucleoside-2'-O)-MTase methylates the 2'-OH (a conserved adenosine in flaviviruses) of the subsequent nucleotide, resulting in cap-1 structure for the viral RNA.^{6, 21, 22, 24,} ²⁷⁻²⁹ Both methylation reactions are catalyzed by a single MTase domain and SAM is used as a methyl donor, generating Sadenosyl-L-homocysteine (SAH) as a by-product.28,29

All flaviviral MTases share a conserved Rossmann-fold structure consisting of a SAH/SAM binding site, a cap/GTP binding site, and an RNA-binding pocket ²⁸⁻³⁰ Studies have shown that the presence of the methylated 5' cap is essential for the protection and stability of the viral RNA throughout the viral replication cycle; thus, disruption of the MTase activity would interfere with viral replication.^{6, 21, 22} Indeed, it has been demonstrated that the N7-methylation of flaviviral RNA cap structure is essential for viral mRNA translation into protein, whereas the 2'-O methylation is a "marker of self" limiting the detection of viral RNA by the host innate immune sensors of the RIG-like family such as RIG-1 and MDA5.^{28, 29, 31} As such, the essential roles played by viral MTases during the viral life cycle demonstrate the great potential of these enzymes as viable targets for drug design.

While the N-terminal domain of the NS5 protein is responsible for cap-MTase activities, the C-terminal domain of the protein is responsible for the RdRp activity.^{4, 32, 33} Unlike most polymerases flaviviral RdRp utilizes a *de novo* initiation mechanism, wherein a 5'-triphosphate AG RNA dinucleotide is first synthesized by the polymerase, even in the absence of RNA template. This AG dinucleotide is next used by the polymerase as a primer for RNA polymerization.^{4, 32-34} Proper function of the RdRp is critical for flaviviral replication, thus, impeding the ability of RdRp to synthesize viral RNA is also an attractive target for drug design. Furthermore, a therapeutic that could disrupt both the MTase activity as well as the RdRp activity of the NS5 protein could prove to be a highly effective broad-spectrum inhibitor for the treatment of numerous flaviviruses.

Unfortunately, there are currently no FDA approved therapeutics for treating flaviviruses infections.^{8, 19} Similarly, vaccine development for flaviviruses has been challenging, especially for DENV due to the necessity to provide a vaccine that would be effective against all four serotypes^{8, 35, 36} Furthermore, if a serotype of DENV is not fully protected against, a patient is more likely to develop severe Dengue hemorrhagic fever or Dengue shock syndrome.^{35, 36} As such, broad spectrum therapeutics are needed in order to better combat these viral infections.

Recent studies have focused on either developing novel therapeutics or repurposing previously approved drugs in order to expedite the development process.^{7, 8, 37-40} Of these therapeutics, nucleoside analogues initially garnered much attention due to their ability to disrupt the function of important viral replication enzymes.^{38, 41} One example of a potent nucleoside analogue is NITD008 (Figure 3), an adenosine mimic that has demonstrated the ability to inhibit the RdRp domain of all four serotypes of DENV with an average EC₅₀ value of 0.64 μ M.^{5, 38, 42} While these

is not a viable option for prophylaxis against DENV, as preclinical studies have demonstrated cytotoxicity associated with NITD008 treatment.^{5, 38}



Figure 3. Early examples of antiflaviviral nucleoside inhibitors.

Another example is Sinefungin (Figure 3), a natural SAM/SAH mimic that has demonstrated potent antiviral activity against numerous viral MTases, including those of flaviviruses with an IC₅₀ value of 0.03 μ M against N7 methylations and 0.041 μ M against 2'-O-methylations in DENV.^{42, 43} Unfortunately, Sinefungin has not been pursued further as a flavivirus therapeutic due to its low selectivity for viral MTases verses human MTases.^{37,43} While these analogues ultimately proved ineffective as potential therapeutics, they demonstrated the potential scope for utilizing nucleoside analogues in anti-flavivirus therapeutics.

nucleoside.^{40, 47, 49-51} Due to these interesting characteristics, the Seley-Radtke lab has recently applied the fleximer approach to FDA-approved nucleoside inhibitors in order to create more potent analogues for antiviral therapeutics. Acyclovir (ACV), for example, is an FDA-approved acyclic nucleoside analogue mainly used to treat herpes simplex virus and varicella zoster virus infections.⁵⁸⁻⁶⁰

Previously, fleximer analogues were synthesized utilizing the sugar moiety found in ACV, where broad spectrum screening of the Flex-ACV analogues revealed compound **1** to be active (10.1 μ M) against HCoV-NL63, an endogenous strain of human coronavirus (CoV) that displays similar symptoms to the common cold.⁵³ Further analysis of compound **1** and its acetylated prodrug **1-Ac** (Figure 5) demonstrated low micromolar *in vitro* antiviral activity against both Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) - two deadly human coronaviruses for which there is currently no cure. Compound **1-Ac** exhibited activity against MERS at 3.4 μ M (in Vero) and 11.9 μ M against SARS, while **1** inhibited HCoV-NL63 at 8.8 μ M. These findings were ground-breaking since these compounds were the first nucleoside analogues to exhibit low micromolar levels of anti-coronavirus activity.⁵³

These promising results prompted further investigation of these analogues against other viruses such as filoviruses, particularly given the dual anti-CoV and anti-Ebola activity recently noted by



Figure 4. Structure of proximal and distal guanosine fleximers compared to natural guanosine.43-46

Over the past two decades, the Seley-Radtke lab has focused on developing flexible purine base nucleoside analogues termed "fleximers".⁴⁴⁻⁵⁷ These compounds feature a purine ring that is "split" into the imidazole and pyrimidine moieties, with a single carbon-carbon bond between the C4 of the imidazole and the C5 of the pyrimidine (proximal fleximers), or the C5 of the imidazole and the C6 of the pyrimidine (distal fleximers) (Figure 4)⁴⁴⁻⁴⁷

These nucleoside analogues retain the hydrogen bonding and stacking elements necessary for nucleoside recognizing enzymes, while allowing for alternative interactions in the enzyme binding site.^{44-47, 49-51} This inherent flexibility allows for free rotation around the carbon-carbon bond between the imidazole and pyrimidine rings, thereby increasing the rotational degrees of freedom and allowing the fleximer to interact with other binding

the nucleoside analogue Remdesivir.^{61, 62} *In vitro* antiviral testing revealed that compound **1**, **1-Ac**, and the phosphoramidate prodrug **1-MG** were all active against Ebola (EBOV) virus, with compound **1** exhibiting the greatest activity ($EC_{50} = 2.2 \pm 0.3 \mu M$).⁵⁴ These results were quite interesting as they suggest the potential for dual activity for compound **1** and **1-Ac** against both CoVs⁵³ and EBOV.⁵⁴ Further studies also revealed promising anti-EBOV activity for compounds **2** and **2-Ac**, with the acetylated analogue **2-Ac** demonstrating an EC₅₀ value of 8.2 ± 1.8 μM (unpublished data).

Due to the remerging prevalence of DENV and ZIKV throughout the world, the ability of the Flex-ACV compounds to inhibit those viruses was pursued. Congruently, the compounds were also analyzed further in an effort to elucidate their





potent compounds. Herein, the synthesis, antiviral activity against both DENV and ZIKV, and biological studies designed to uncover their potential mechanism of action for several analogues are

Compounds 1 and 2 were then used to synthesize the acetate protected prodrugs 1-Ac and 2-Ac respectively (Scheme 2).⁶⁵

Synthesis of the phosphoramidate prodrugs 1-MG and 2-MG



Scheme 1. Reagents and conditions: (a) 4,5-diiodoimidazole, BSA, TMSOTF, ACN, rt for 4 h then 80°C for 18 h; (b) 30% EtOH, 5 eq Na₂SO₃, 120°C, overnight.



Scheme 2. Reagents and conditions: (a) DIPEA, 10% Pd/C, H₂, rt, 4 h; (b) NBS, CHCl₃, rt, dark, 5 h; (c) Br₂, NaHCO₃, 50% MeOH, rt, 3 h; (d) pin₂B₂, KOAc, Pd(PPh₃)₄, DME, 90°C, overnight; (e) 5, Pd(PPh₃)₄, NaHCO₃, 90°C, 4 h; (f) 1 or 2, Ac₂O, DMAP, DMF, rt, 3 h.

described.

2. Results

2.1 Chemistry

The compounds for this study were chosen based on the previous results for compounds **1**, **1-Ac**, and **1-MG** against MERS, SARS,⁵³ and EBOV,⁵⁴ as well as unpublished results for compound **2** and **2-Ac** against EBOV. The previously reported organometallic coupling procedures used by our group^{53, 54} to couple the two heterocyclic moieties involved tedious and multiple purification processes to remove the tin from the Stille coupling methodology, which led to very poor yields. As a result, attention turned to the Suzuki coupling methodology, which resulted in much cleaner reactions, facile purifications, as well as greatly improved yields. Starting with Scheme 1, coupling the imidazole to the commercially available 2-[(acetyloxy)methoxy]ethyl acetate (**3**) using BSA and TMS-triflate gave **4**, which, following selective deiodination, gave iodoimidazole **5**.⁵⁴

Compound **9** was synthesized starting with commercially available 2-amino-4-chloro-6-methoxypyrimidine for series 1 (Scheme 2).⁶³ Similarly, compound **10** was synthesized starting with commercially available 2,4-dimethoxypyrimidine for series 2.⁶⁴ Subsequent Suzuki-Miyaura cross-coupling of **9** and **5** gave **1** (30% over two steps), and coupling of **10** with **5** provided **2** (48% over two steps) (Scheme 2), each by way of **11** or **12** as the in-situ

began with commercially available L-alanine and utilized procedures previously described by our lab54 as well as those found literature⁶⁶ yield the 2ethylbutyl in the to ((perfluorophenoxy)(phenoxy)phosphoryl)-L-alaninate intermediate 13 (Scheme 3). Reaction of this intermediate with either 1 or 2 and tert-butyl magnesium chloride afforded the phosphoramidate prodrugs 1-MG and 2-MG as diastereomeric mixtures in moderate yields (74% and 86% respectively).



Scheme 4. Reagents and conditions: (a) i. 1 or 2, SalPCl, Pyr, 1,4-dioxane, rt, 45 min; ii. tributyl ammonium pyrophosphate, tributylamine, DMF, rt, 45 min; iii. I₂, H₂O, Pyr, rt, 30 min.

Finally, synthesis of the triphosphate analogues 1-TP and 2-TP were accomplished using a modified procedure by Hollenstein et. al. which utilized SalPCl and tributylammonium pyrophosphate.67 The methodology developed by Hollenstein et. al. noted important differences that ultimately greatly increased overall yields. For instance, prior to the reaction it is important that the fleximer nucleoside be coevaporated with anhydrous pyridine then dried in vacuo overnight, instead of storing the fleximer nucleoside in dried pyridine, 1,4-dioxane, and molecular sieves overnight. handling of 2-chloro-1,3,2-benzo-Furthermore, proper dioxaphosphorin-4-one (SalPCl) is important. SalPCl is a commercially available reagent that is typically a glassy green solid, however, once exposed to moisture, develops a powdery white coating on the outside of the crystals that should be scraped off prior to addition or the reaction goes poorly. Finally, in order to maximize the yield, HPLC purification should be done immediately to make the entire purification process more facile.

Synthesis of the triphosphate analogues 1-TP and 2-TP began with the addition of SalPCl to the fleximer scaffold to give a phosphite intermediate (Scheme 4).67 Then, addition of tributylammonium pyrophosphate and tributylamine induced cyclization of the phosphate moieties. After stirring at room temperature for 45 minutes, I₂ and water were added to the reaction mixture in order to promote the oxidation of the α phosphorous from a P(III) to a P(V) center.⁶⁶ Finally, the excess iodide was quenched with 10% sodium thiosulfate and the crude reaction was purified via HPLC to give either 1-TP or 2-TP. Following purification, triphosphates 1-TP and 2-TP were obtained as the triethylamine salts. As the triethylamine salts were not suitable for the enzymatic assays, these compounds were converted to their sodium salt forms using a Dowex 50Wx2 Na⁺ ion exchange column. This produced both triphosphates in good yields (50% for 1-TP and 62% for 2-TP). We have repeated this approach numerous times now and the yields have stayed consistent.

2.2 Antiviral Activity

The potent antiviral activity demonstrated by compounds 1, 1-Ac, and 1-MG against a wide array of viruses including SARS-CoV,⁵³ MERS-CoV,⁵³ as well as filoviruses such as EBOV⁵⁴ prompted further investigation with these analogues against additional viruses. These analogues, as well as the dimethoxy analogues 2 and 2-Ac, were then screened against various flaviviruses including DENV, ZIKV, and YFV. The analogues were analyzed utilizing a visual cytopathic effect assay on Vero76 cells infected with the live-virus isolates of DENV (New Guinea C), ZIKV (MR766), and YFV (17D).

The results showed that several flex-analogues demonstrated moderate to potent antiviral activity against all the flaviviruses tested, with compound **1** demonstrating the greatest antiviral activity against DENV (EC₅₀ = 0.057 μ M) (Table 1). Compound **1** also demonstrated potent antiviral activity against YFV (EC₅₀ = 0.37 μ M) with a selective index (SI) of 4.6. Although this is not ideal, preliminary minimum tolerated dose (MTD) studies have revealed no toxicity up to 250 mgs/kg, and we are currently pursuing those studies further to also explore the ProTide analogues.

A significant decrease in toxicity was observed with the acetate protected analogue **1-Ac** against DENV compared to the parent analogue **1** ($CC_{50} = 65 \ \mu M$ and $CC_{50} = 1.2 \ \mu M$ respectively), however, a decrease in activity was also observed as **1-Ac** demonstrated an EC₅₀ of 6.1 μM . While not as potent as compound **1**, compound **2-Ac** demonstrated moderate activity against DENV with an EC₅₀ of 19 μM , and little associated cytotoxicity. None of the analogues tested demonstrated any antiviral against ZIKV, and only analogue **1** demonstrated activity against YFV.

These results suggest that compound **1** could potentially act as a broad spectrum antiviral therapeutic across a wide range of viral

Commenced	DENV		ZIKV		YFV	
Compound -	^a EC ₅₀	^b CC ₅₀	EC ₅₀	CC ₅₀	EC ₅₀	CC ₅₀
1	0.057	1.20	>1.80	>1.80	0.37	1.7
1-Ac	6.1	65	>100	>100	>57	57
1-MG	>57	57	>31	31	>55	55
2	>50	50	>76	76	>58	58
2-Ac	19	53	79	>100	>58	58
2-MG	>53	53	>56	56	>53	53

 Table 1. Antiviral activity of flex-analogues against various flaviviruses including Dengue (DENV), Zika (ZIKV), and Yellow Fever Virus (YFV) in Vero76 cells.

Values are reported in µM. ^aEC₅₀: effective concentration showing 50% inhibition of virus-induced CPE. ^bCC₅₀: cytotoxic concentration showing 50% inhibition of cell survival.

2.3 Inhibition NS5 activities: RdRp and MTase Activity

As many nucleotide analogues act as chain terminators or mutagenic nucleotides incorporated into RNA, 1-TP and 2-TP were tested for their ability to be incorporated into RNA using DENV RdRp. No direct inhibition was observed at concentrations below 200 µM, however when studied for incorporation, as shown in Figure 6 on the next page, 1-TP did not serve as a competitive inhibitor in the presence of GTP. Compound 1-TP did, however, act as a delayed chain terminator. As there was no incorporation, but chain termination did occur, we then speculated that this was due to an allosteric inhibition, likely due to inhibition of a different but nearby enzyme.



Figure 6. Incorporation of 1-TP into the DENV genome, in the absence of GTP. An elongation complex was formed of DENV NS5 and a primer/template combination corresponding to the 5' end of DENV2 genome and the 3'end of the antigenome. Substrate (P10) and product bands were visualized by autoradiography, and quantification of primer [P10] illustrates inhibition of RNA synthesis.

Journal Pretlavivirus NS5 is an interesting and important target for the development of antiviral therapeutics. In the flaviviruses, the MTases and RdRp are in the same protein complex. However, unlike viral RdRps, which demonstrate a high mutation rate^{68, 69}, the viral MTase structure is highly conserved across most flavivirus species,^{21,70} making viral MTases an attractive target for drug design. As such, compounds 1, 1-Ac, 1-MG, and 1-TP were analyzed for activity against DENV, ZIKV, and human N7 MTases utilizing a radioactive filter-binding assay (Figure 7). The inhibition of the 2'-O-MTase activity of DENV and ZIKV, and that of the human N7 (RNMT) MTases was first analyzed against 50 µM of compound. Briefly, the MTases were incubated with synthetic RNA substrates (GpppAC5), radioactive ³H-SAM, and a Flex-analogue at 30°C for 30 minutes.²⁷ The reaction products were then filtered on DEAE membranes and the radioactivity

> While compound 1 did not exhibit a significant inhibitory effect on the different MTases activities, the triphosphate form 1- TP inhibited both DENV MTase and ZIKV MTase at 34% and 12% respectively (Figure 7). The triphosphate analogue 2-TP demonstrated the greatest inhibitory activity against ZIKV MTase at 9%. Furthermore, none of the analogues tested inhibited human N7 MTase activity, which also suggests these analogues selectively inhibit viral MTases.

> transferred on the RNA was quantified. Sinefungin was utilized as

inhibitory control due to its known inhibition of both viral and

human MTases.37, 42, 43

Analogues 1-TP and 2-TP were then further analyzed in order to determine IC₅₀ values against the MTases (Table 2). This data was congruent with the previous MTase data where compounds 1-TP and 2-TP demonstrated a greater inhibitory effect against ZIKV MTase compared to DENV MTase. The triphosphate 2-TP was most potent against ZIKV MTase (0.15 µM) whereas the triphosphate 1-TP (IC₅₀ = 1.7 μ M) was still active against ZIKV MTase but to a lesser degree than 2-TP. This data suggests that the antiviral activity seen with compound 1 is due to inhibition of the MTase activity rather than inhibition of the viral polymerases,



Figure 7. Percent inhibition of ZIKV and DENV MTase by series 1 and 2 (50 µM). None of the compounds inhibited human N7 MTase, suggesting that these analogues selectively inhibit the viral MTases.

Table 2. Inhibition of MTase activity of compounds 1-TP and 2-TP against DENV NS5-MTase, ZIKV NS5-MTase, and human N7 MTase.

Compound	ZIKV MTase IC50	DENV MTase IC50	hN7 MTase IC50
1-TP	1.7 μM	8.4 μM	49 µM
2-TP	0.15 µM	1.1 µM	13 µM

for



Figure 8. Predicted binding of A) 1-TP (carbon atoms in light blue) and B) 2-TP (carbon atoms in pink) to the GTP pocket of ZIKV NS5 MTase (PDB ID 5G0Z). Co-crystallized GTP is shown in light grey.



Figure 9. Predicted binding of A) 1-TP (carbon atoms in light blue) and B) 2-TP (carbon atoms in pink) to the GTP pocket of DENV NS5 MTase (PDB ID 4V0R). Co-crystallized GTP is shown in light grey.

since nucleosides must first be converted by kinases to the corresponding triphosphates in order to be active against and/or recognized.

2.4 Computational Molecular Modeling Studies

In order to gain further insights on the mechanism of action of the fleximers, their predicted binding to DENV (PDB ID 4V0R), ZIKV (PDB ID 5G0Z), YFV (PBD ID 3EVD), and human N7 (PDB ID 5E9W) MTase crystal structures were evaluated using a series of docking simulations. In particular, the capt/GTP binding site of these enzymes was explored since the triphosphates **1-TP** and **2-TP** were the most active against both ZIKV and DENV MTases. Furthermore, as this binding site is highly conserved among flaviviruses,⁷⁰ it was hypothesized that if the fleximers efficiently bind in this site, they could potentially serve as broad spectrum inhibitors.

As shown in Figure 8A, **1-TP** is predicted to maintain most of the key hydrogen bonding and stacking interactions shown by GTP in the ZIKV MTase structure demonstrating a very similar spatial occupation of the pocket overall. Notably, the hydrogen bonding between the free amine group of the fleximer with Met19 and Leu16 as well as between the oxygen in the sugar moiety of the fleximer and Lys13 of the enzyme binding site appears to be similar to the corresponding groups in GTP.

The triphosphate analogue **2-TP** was also analyzed (Figure 8B), in order to assess the potential effect of the replacement of the free amine at the 2-position with a methoxy group on binding to the ZIKV MTase. According to the docking results obtained, this modification is associated with the potential loss of hydrogen bonding with the backbone of Met19 and Leu16. However, the flex-nucleobase was still oriented such a way that it interacted with Phe24. In the case of both **1-TP** and **2-TP**, the triphosphate moiety was placed in the same region observed for GTP, and overall both compounds were predicted to occupy the pocket in a similar fashion to GTP.

While the ZIKV MTase GTP binding site shows the presence of an alanine at position 21, the residue in the corresponding position is replaced by an arginine (Arg22) within DENV-3 and DENV-4, and by a lysine (Lys22) in DENV-1 and DENV-2 (Figure 9A, DENV-3, PDB ID 4V0R). The arginine (or lysine) lateral chain allows for an additional hydrogen bond with the methoxy group of compound 1-TP, which could potentially explain its increased antiviral activity against DENV compared to ZIKV. When compared to the binding interactions found with 1-TP, 2-TP also displayed similar potential hydrogen bonding interaction between the 4-methoxy group and Arg22. As seen for ZIKV MTase, the replacement of the amine group in 1-TP with the methoxy group in 2-TP led to a loss of hydrogen bond formation with Leu17 and Leu20 (Figure 9B). Moreover, the overall binding of both 1-TP and 2-TP was consistent with the conformation observed for co-crystallized GTP. In summary, the molecular modeling results obtained for both ZIKV and DENV MTases are in accordance with the experimental data found in the enzymatic MTase assay. The lack of significant antiviral activity displayed by 2-TP could also be explained by poor phosphorylation of the parent fleximer analogue to its triphosphate form, likely due to the role of the nucleobase amine group in substrate recognition by the phosphorylating enzymes.

Similar to the DENV-1 and DENV-2 MTase binding sites, the YFV GTP MTase binding site (PDB ID 3EVD) showed the



Figure 10. Predicted binding of A) 1-TP (carbon atoms in light blue) and B) 2-TP (carbon atoms in pink) to the GTP pocket of YFV NS5 MTase (PDB ID 3EVD). Co-crystallized GTP is shown in light grey.



Figure 11. Predicted binding of A) 1-TP (carbon atoms in ight blue) and B) 2-TP (carbon atoms in pink) to the GTP pocket of human mRNA cap guanine-N7 MTase GTP binding site (PDB ID 5E9W). GTP is shown in light grey.

presence of a lysine residue, Lys21, in proximity to the nucleobase subsite of co-crystallized GTP. The results of the simulations revealed that the triphosphate **1-TP** is still predicted to maintain key hydrogen bonding interactions with Leu19 and Leu16. However, unlike the DENV-3 binding site, the Lys21 lateral chain does not appear to be at an optimum distance to interact with the 4-methoxy group (Figure 10A). This supports the decrease in activity seen with compound **1** against DENV and YFV (0.057 μ M compared to 0.37 μ M). By comparison, **2-TP** is unable to form any substantial hydrogen bonding interactions with Leu19, Leu16, or Lys21 (Figure 10B).

Finally, the potential interactions between the fleximer triphosphate analogues and the human mRNA cap guanine-N7 GTP binding site were analyzed (PDB ID 5E9W; GTP coordinates as defined in the E. cuniculi Ecm1 crystal structure 1RI1). The GTP binding pocket of human N7 MTase is significantly different from the one found in flaviviruses: it more closely resembles the SAM/SAH binding site and possesses a different amino acid residue composition. In line with the experimental data obtained, 1-TP was not predicted to have strong binding interactions in this site, even though the fleximer can adopt a similar general orientation in comparison with the natural ligand GTP (Figure 11A). The flex-nucleobase occupied a larger region of space than that defined by the GTP guanine moiety, and the residues surrounding this region of the pocket do not participate in an Hbond interactions. Moreover, there are no other notable interactions with the flex-nucleobase of the scaffold, thus supporting the experimental data observed for the reduced inhibition of this enzyme. In contrast, docking results in the GTP

binding pocket of human N7 MTase would suggest a better interaction of **2-TP** to this enzyme in comparison with **1-TP**, as the presence of the two methoxy groups appear to allow formation of a hydrogen bond with Asn176 (Figure 11B).

3. Conclusions

The design and synthesis of new and more effective antiviral drugs is of critical importance to the biomedical field in order to treat viruses such as flaviviruses. While ongoing studies have identified various therapeutics as potential treatments for diseases caused by flaviviruses, there are currently no FDA approved vaccines (except for YFV, however this vaccine has been associated with serious adverse effects⁷¹) or treatment, and as such, it is critical that an effective treatment option is developed. The flex-analogues reported in this study have demonstrated moderate activity against various flaviviruses, with analogue 1 being most active against DENV and YFV. While the mechanism of action has yet to be fully elucidated, these preliminary studies have shown that compound 1-TP inhibits the DENV and ZIKV MTases with IC₅₀ values of 8.4 µM and 1.7 µM respectively, potentially by binding in the GTP binding site of this enzyme. These results are promising due to the highly conserved nature of flavivirus MTases. Further research is currently underway in order to fully elucidate their mechanism(s) of action as well as to screen these analogues against other flaviviruses such as West Nile Virus and Tick-Borne Encephalitis, in order to see if these analogues could serve as broad-spectrum treatments against additional flaviviruses.

4.1 Chemical Synthesis

General Information: All reactions were performed using ovendried glassware under a nitrogen atmosphere with magnetic stirring. Reagents were purchased from Sigma-Aldrich, Alfa Aesar, and Combiblocks. Solvents were either purchased as anhydrous or were dried using the MBRAUN solvent purification system (MB-SPS). Reactions were monitored by thin layer chromatography (TLC) using EMD silica gel 60 F254 coated glass-backed TLC plates and visualized with a UV lamp and/or KMnO₄ stain. Column chromatography was performed on a Combiflash®Rf automated chromatography using REdiSep Rf silica. High Pressure Liquid Chromatography (HPLC) purification was performed on an LC-8A Shimadzu preparation liquid chromatograph using a Zorbax SB C18 column 9.4x250 mm, 5 μm. Samples were lyophilized utilizing a Labconco Freezone 1 L lyophilizer. ¹H NMR spectra was recorded on a JOEL Eclipse ECX (400 MHz) spectrometer. Chemical shifts are reported in parts per million (δ ppm) from tetramethylsilane with the solvent resonance as an internal standard. Data for the ¹H NMR are reported as follows; br = broad, s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet. ^{13}C NMR were recorded on a JOEL Eclipse ECX (126 MHz) or a Bruker Advance III HD (101 MHz) spectrometer with complete proton decoupling. Data for ¹³C NMR are reported in terms of chemical shifts (δ ppm) with the solvent resonance as the internal standard. ³¹P NMR was recorded on a JOEL Eclipse ECX (162 MHz) spectrometer with complete proton decoupling using 85% phosphoric acid as an external standard. Data for ³¹P NMR are reported in terms of chemical shift (δ ppm) (multiplicity, integration), with multiplicities reported the same as for ¹H NMR. Mass spectrometry was performed on a Bruker AmaZon X quadrupole ion trap mass spectrometer using electrospray ionization (ESI). The purity of all compounds tested in biological assays were verified by elemental analyses performed by Atlantic Microlabs.

4.1.1. Synthesis of 2-((4,5-diiodo-1H-imidazol-1-yl)methoxy)ethyl acetate (4).

4,5-diiodoimidazole (10.0 g, 31.25 mmol) was suspended in anh. Acetonitrile (200 mL) under nitrogen and stirred at room temperature for 10 mins. Then bis(trimethylsilyl)acetamide (46.0 mL, 187.68 mmol) and 3 (6.0 mL, 37.46 mmol) were added and the solution was allowed to stir at room temperature under direct nitrogen bubble 4 hours. for Trimethylsilyl trifluoromethanesulfonate (8.2 mL, 47.22 mmol) was then added slowly dropwise over 5 minutes. The reaction was then heated to 80°C under direct nitrogen bubble for 18 hours. After cooling to room temperature, the reaction was cooled further in a salt/ice bath to 0°C and quenched with NaHCO₃ (100 mL). After stirring for 10 minutes, the reaction was extracted 5x with CH₂Cl₂ (200 mL). The combined organic layers were dried with MgSO₄, gravity filtered, and the solvent was removed in vacuo to afford an amber oil with yellow solid. The crude mixture was then purified by flash column chromatography on silica gel (40-80% EtoAc in Hexanes) to give the pure product as a white solid with yellow oil (9.5 g, 70%); R_{f} = 0.39 (1:1 EtOAc/Hexanes); ¹H NMR (400 MHz, CDCl₃) δ 7.61 (s, 1H), 5.18 (s, 1H), 3.93-3.95 (t, J = 4.6 Hz, 2H), 3.45-3.47 (t, J = 4.6 Hz, 2H), 1.82 (s, 3H); ¹³C NMR (126 MHz, CDCl₃) δ 170.67, 142.06, 97.32, 82.71, 77.93, 66.69, 62.76, 21.07; MS (ESI+) m/z calc for C₈H₁₀I₂N₂O₃ [M+H⁺]: 436.99, found: 436.9.

4.1.2. Synthesis of 2-((4-iodo-1H-imidazole-1-yl)methoxy)ethan-1-ol (5). and H₂O (125 mL total volume) was added Na₂SO₃ (8.17 g, 64.82 mmol). The solution was then refluxed at 120°C for 18 hours. After cooling to room temperature, the reaction was concentrated *in vacuo* to remove the EtOH and was extracted 4x with CH₂Cl₂. The combined organic layers were dried with MgSO₄ and gravity filtered. The solvent was removed *in vacuo* to afford a white solid. The crude product was then purified by flash column chromatography on silica gel (0-20% CH₃OH in CH₂Cl₂) to give the pure product as a white solid (2.73 g, 79%); R_f = 0.79 (1:3 CH₃OH/CH₂Cl₂); ¹H NMR (400 MHz, DMSO-d₆) δ 7.74 (s, 1H), 7.46 (s, 1H), 5.31 (s, 2H), 4.65-4.68 (t, *J* = 10.5 Hz, 1H), 3.42-3.47 (m, 2H), 3.35-3.37 (m, 2H); ¹³C NMR (126 MHz, DMSO-d₆) δ 140.5, 125.5, 83.4, 76.2, 70.7, 60.3; MS (ESI+) *m/z* calcd for C₆H₉IN₂O₂ [M+H⁺]: 269.05, found 269.0.

4.1.3. Synthesis of 4-methoxypyrimidine-2-amine (7).

Commercially available 2-amino-4-chloro-6-methoxypyrimidine (8.0 g, 50.13 mmol) was suspended in 3:2 EtOAc: CH₃OH (175 mL total volume). After addition of diisopropylethylamine (18 mL, 100.25 mmol) and 10% Pd/C (1.6 g, 20% w/w), the reaction was mixed on a hydrogenator for 4 hours. Upon completion, the reaction was filtered over a celite pad, washed with EtOAc (100 mL), and the solvent was removed in vacuo to give a pale yellow/white solid. The crude product was then purified by flash column chromatography on silica gel (40-80% EtOAC in Hexanes) to give the pure product as a white solid $(5.3 \text{ g}, 85\%); R_f = 0.34 (1:1 \text{ EtOAc/Hexanes}); ^1\text{H NMR} (400 \text{ MHz},$ DMSO-d₆) δ 7.90-7.91 (d, *J* = 5.5 Hz, 1H), 6.52 (s, 2H)5.94-5.95 $(d, J = 5.5 \text{ Hz}, 1\text{H}), 3.74 (s, 3\text{H}); {}^{13}\text{C} \text{ NMR} (126 \text{ MHz}, \text{DMSO-d}_6)$ δ 170.16, 164.08, 158.99, 96.58, 53.25; MS (ESI+) m/z calcd for C₅H₇N₃O [M+H⁺]: 126.13, found 126.2.

4.1.4. Synthesis of 5-bromo-4-methoxypyrimidin-2-amine (9).

To a solution of compound 7 (1.5 g, 11.99 mmol) in chloroform, N-bromosuccinimide (2.14 g, 11.99 mmol) was added. After stirring in the dark for 5 hours, the solution was added to CH₂Cl₂ (200 mL) and 1M NaOH (100 mL). Upon mixing the layers were separated and the organic layer was washed with brine (100 mL). The organic layer was then dried with MgSO₄ and gravity filtered. The solvent was removed *in vacuo* to afford a yellow/white solid. The crude product was then purified by flash column chromatography on silica gel (0-5% CH₃OH in CH₂Cl₂) to give the pure product as a fluffy white solid (2.4 g, 95%); $R_f = 0.59$ (1:1 EtOAc/Hexanes); ¹H NMR (400 MHz, DMSO-d₆) δ 8.05 (s, 1H), 6.78 (s, 2H), 3.83 (s, 3H); ¹³C NMR (126 MHz, DMSO-d₆) δ 165.17, 162.91, 159.48, 91.02, 54.44; MS (ESI+) *m/z* calcd for C₅H₆BrN₃O [M⁺]: 204.03, found: 204.0.

4.1.5. Synthesis of 5-bromo-2,4-dimethoxypyrimidine (10).

NaHCO₃ (2g, 23.81 mmol) was slurried with compound **8** (1.74 mL, 14.27 mmol) in 50% CH₃OH in water (45mL total volume). Br₂ (1.32 mL, 25.69 mmol) was added dropwise over 1 hour with stirring. After 30 minutes of Br₂ addition, additional NaHCO₃ (3.5 g, 41.66 mmol) was added and the reaction mixture was stirred at room temperature for 2 hours. The resulting white precipitate was vacuum filtered and washed with CH₃OH (20 mL) and dried under vacuum to give the product as a shiny white solid (2.12 g, 68%); R_f = 0.97 (1:1 EtOAc/Hexanes); ¹H NMR (400 MHz, CDCl₃) δ 8.28 (s, 1H), 4.03 (s, 3H), 3.96 (s, 3H); ¹³C NMR (126 MHz, CDCl₃) δ 166.86, 164.36, 159.23, 98.26, 55.33, 55.00; MS (ESI+) *m/z* calcd for C₆H₇BrN₂O₂ [M⁺]: 219.04, found: 219.0.

4.1.6. Synthesis of 2-((4-(2-amino-4-methoxypyrimidin-5-yl)-1H-imidazol-1-yl) methoxy)ethan-1-ol (1).

T

MF

bis(pinacolato)diboron (0.75 g, 2.94 mmol), and potassium acetate (0.72 g, 7.35 mmol), were added and suspended in dimethoxy ethane (50 mL). Then the solution was subjected to freeze-pumpthaw cycles under N_2 (3x). Tetrakis(triphenylphosphine)palladium(0) (0.30 g, 0.25 mmol) was added to the reaction flask and subjected to one more freeze-pump-thaw cycle. The reaction was then heated to 90°C overnight with stirring. The reaction was then cooled to room temperature and 5 (0.65 g, 2.45 mmol), tetrakis(triphenylphosphine)palladium(0) (0.14 g, 0.12 mmol), and aqueous NaHCO3 (20 mL) were added. The flask was evacuated and pumped with N2 and heated to 90°C for 4 hours. After stirring, the reaction was cooled to room temperature and the solvent was removed in vacuo. The residue was suspended in CH-₂Cl₂/CH₃OH and NaHCO₃ was gravity filtered away from the solution. Solvent was removed in vacuo to give the crude product as an orange oil. The crude product purified via flash column chromatography (0-10% CH₃OH in CH₂Cl₂) to afford a yellow oil. The residue was then washed with minimal aqueous sodium thiosulfate and extracted with EtOAc 3-4x. The organic layer was dried with MgSO₄, gravity filtered, and the solvent was removed in vacuo to give a pink solid. The product was further purified via flash column chromatography on silica gel (5-10% CH₃OH in CH₂Cl₂) to afford the product as a light pink solid (0.19 g, 30%); $R_f = 0.26 (1:9 \text{ CH}_3\text{OH}/\text{CH}_2\text{Cl}_2); ^1\text{H NMR} (400 \text{ MHz}, \text{DMSO-d}_6) \delta$ 8.59 (s, 1H), 7.77 (s, 1H), 7.37 (s, 1H), 6.52 (s, 2H), 5.34 (s, 2H), 4.64-4.66 (t, J = 11.0 Hz, 1H), 3.91 (s, 3H), 3.41-3.44 (t, J = 10.0Hz, 2H), 3.36-3.38 (t, J = 9.6 Hz, 2H); ¹³C NMR (126 MHz, DMSO-d₆) § 165.78, 162.24, 155.48, 138.08, 134.72, 117.18, 104.65, 76.21, 70.39, 60.44, 53.67; MS (ESI+) m/z calcd for $C_{11}H_{15}N_5O_3$ [M+H⁺]: 266.27, found 266.2; Elemental analysis: Anal. Calcd for C₁₁H₁₅N₅O₃: C, 49.81; H, 5.70; N, 26.40. Found: C, 49.74; H, 5.76; N, 26.12.

4.1.7. Synthesis of 2-((4-(2,4-dimethoxypyrimidine-5-yl)-1Himidazol-1-yl) methoxy)ethan-1-ol (2).

To a dry Schlenk flask, compound 10 (0.50 g, 2.28 mmol), bis(pinacolato)diboron (0.69 g, 2.74 mmol), and potassium acetate (0.67 g, 6.84 mmol) were added and suspended in dimethoxy ethane (50 mL). Then the solution was subjected to freeze-pumpthaw cycles under N₂ (3x). Tetrakis(triphenylphosphine)palladium(0) (0.26 g, 0.22 mmol) was added to the reaction flask and subjected to one more freeze-pump-thaw cycle. The reaction was heated to 90°C overnight with stirring. The reaction was then cooled to room temperature and 5 (0.61 g, 2.28 mmol), tetrakis(triphenylphosphine)palladium(0) (0.13 g, 0.11 mmol), and aqueous NaHCO3 (20 mL) were added. The flask was evacuated and pumped with N₂ and heated to 90°C for 4 hours. After stirring, the reaction was cooled to room temperature and the solvent was removed in vacuo. The residue was suspended in CH₂Cl₂/CH₃OH and NaHCO₃ was gravity filtered away from the solution. Solvent was removed in vacuo to give the crude product as a yellow solid with a yellow oil. The crude product was purified via flash chromatography on silica gel (0-5% CH₃OH in CH₂Cl₂) to afford the product as a white solid (0.31 g, 48%); $R_f = 0.38$ (1:9 CH₃OH/CH₂Cl₂); ¹H NMR (400 MHz, CD₃OD) δ 8.77 (s, 1H), 7.87 (s, 1H), 7.63 (s, 1H), 5.44 (s, 2H), 4.10 (s, 3H), 3.98 (s, 3H), 3.62-3.64 (t, J = 9.1 Hz, 2H), 3.51-3.64 (t, J = 9.6 Hz, 2H); ¹³C NMR (126 MHz, CD₃OD) δ 167.13, 163.70, 154.60, 137.89, 133.13, 118.77, 108.89, 76.54, 70.07, 60.57, 54.08, 53.48; MS (ESI+) m/z calcd for C₁₂H₁₆N₄O₄ [M+H⁺]: 281.28, found: 281.2; Elemental analysis: Anal. calcd for C₁₂H₁₆N₄O₄: C, 51.42; H, 5.75; N, 19.99. Found: C, 50.99; H, 5.68; N, 19.72.

4.1.8. Synthesis of 2-((4-(2-amino-4-methoxypyrimidin-5-yl)-1Himidazol-1-yl)methoxy)ethyl acetate (1-Ac). (2 mL) and treated with acetic anhydride (0.053 mL, 0.56 mmol) and dimethylaminopyridine (0.0023 g, 0.019 mmol). The reaction was stirred at room temperature for 3 hours. After stirring, the solvent was removed *in vacuo* to give the crude product as a clear oil. The crude product was purified via flash column chromatography on silica gel (0-5% CH₃OH in CH₂Cl₂) to afford the product as a light pink solid (0.040 g, 70%); $R_f = 0.28$ (1:9 CH₃OH/CH₂Cl₂); ¹ H NMR (400 MHz, CD₃OD) δ 8.54 (s, 1H), 7.48 (s, 1H), 5.40 (s, 2H), 4.13-4.15 (t, *J* = 9.6 Hz, 2H), 4.00 (s, 3H), 3.64-3.66 (t, *J* = 9.6 Hz, 2H), 1.96 (s, 3H); ¹³C NMR (126 MHz, CD₃OD) δ 171.27, 166.39, 161.86, 153.87, 137.44, 134.30, 117.40, 104.76, 76.25, 66.53, 62.92, 52.78, 19.33; MS (ESI+) *m/z* calcd for C₁₃H₁₇N₅O₄ [M+H⁺]: 308.31, found: 308.2; Elemental analysis: Anal. calcd for C₁₃H₁₇N₅O₄ + 0.3% CH₃OH: C, 50.18; H, 5.78; N, 22.08. Found: C, 50.41; H, 5.79; N, 22.10.

4.1.9. Synthesis of 2-((4-2,4-dimethoxypyrimidin-5-yl)-1Himidazol-1-yl)methoxy) ethyl acetate (2-Ac).

Compound 2 (0.050 g, 0.18 mmol) was suspended in anh. DMF (2 mL) and treated with acetic anhydride (0.051 mL, 0.53 mmol) and dimethylaminopyridine (0.0022 g, 0.018 mmol). The reaction was stirred at room temperature for 3 hours. After stirring, the solvent was removed in vacuo to give the crude product as an orange oil. The crude product was purified via flash chromatography on silica gel (0-5% CH₃OH in CH₂Cl₂) and placed on a vacuum pump overnight to afford the product as a peak colored solid (0.076 g, 86%); $R_f = 0.41$ (1:9 CH₃OH/CH₂Cl₂); ¹H NMR (400 MHz, CD₃OD) δ 8.77 (s, 1H), 7.86 (s, 1H), 7.60 (s, 1H), 5.42 (s, 2H), 4.13-4.16 (t, J = 9.2 Hz, 2H), 4.09 (s, 3H), 3.97 (s, 3H), 3.66-3.68 (t, J = 9.6 Hz, 2H), 1.96 (s, 3H); ¹³C NMR (126 MHz, CD₃OD) δ 171.23, 167.09, 163.70, 154.62, 137.89, 133.19, 118.70, 108.81, 76.32, 66.62, 62.92, 54.11, 53.50, 19.35; MS (ESI+) m/z calcd for C₁₄H₁₈N₄O₅ [M+H⁺]: 323.32, found: 323.2; Elemental analysis: Anal. calcd for $C_{14}H_{18}N_4O_5 + 0.4\%$ H₂O: C, 51.18; H, 5.79; N, 16.93. Found: C, 51.03; H, 5.75; N, 17.00.

4.1.10. Synthesis of 2-ethylbutyl((2-((4-(2-amino-4methoxypyrimidin-5-yl)-1H-imidazol-1-yl)methoxy)-ethoxy)-(phenoxy)phosphoryl)alaninate (1-MG).

To a solution of 1 (0.04 g, 0.15 mmol) in anh. THF (3 mL) under a nitrogen atmosphere was added tertbutylmagnesium chloride (1M in THF, 0.24 mL, 0.24 mmol) dropwise, and the reaction was stirred at room temperature for 30 minutes. In a separated flask, 13 (0.08g, 0.17 mmol) was suspended in anh. THF (4 mL) and added to the reaction vessel. The reaction was allowed to stir at room temperature under a nitrogen atmosphere overnight. After stirring overnight, the reaction was quenched with CH₃OH (2 mL) and stirred for another 5 minutes before removal of solvent in vacuo. The crude mixture was then purified by flash column chromatography on silica gel (0-10% CH₃OH in CH₂Cl₂) to give the pure product as a pink oil (0.06 g, 74% mix of diastereomers); $R_f = 0.49 (1:9 \text{ CH}_3\text{OH}/\text{CH}_2\text{Cl}_2); ^1\text{H NMR} (400 \text{ MHz}, \text{CD}_3\text{OD}) \delta$ 8.64 (s, 1H), 7.79-7.80 (d, J = 5.4 Hz, 1H), 7.49 (s, 1H), 7.26-7.30 (m, 2H), 7.10-7.16 (m, 3H), 5.38-5.41 (app d, 2H), 4.13-4.22 (m, 2H), 3.86-4.04 (m, 6H), 3.65-3.70 (m, 2H), 1.40-1.48 (m, 1H), 1.27-1.35 (m, 7H), 0.82-0.86 (m, 6H); ¹³C NMR (126 MHz, CD₃OD) δ 173.85, 166.46, 161.66, 153.50, 150.83, 137.48, 134.14, 129.38, 124.72, 120.17, 120.10, 117.4, 104.86, 76.31, 67.44, 67.38, 66.70, 65.69, 52.88, 50.51, 50.22, 40.41, 22.92, 19.31, 19.25, 19.21, 19.14, 10.02; ³¹P NMR (162 MHz, CD₃OD) δ 4.39, 4.17; MS (ESI+) *m/z* calcd for C₂₆H₃₇N₆O₇P [M+H⁺]: 577.25, found: 577.3; Elemental analysis: Anal. calcd for C₂₆H₃₇N₆O₇P + 1.3% CH₃OH: C, 53.04; H, 6.88; N, 13.59. Found: C, 53.31; H, 6.66; N, 13.35.

4.1.

5-yl)-111-imiaazoi-1-yi)methoxyjethoxyj-(phenoxy)phosphoryl)alaninate (2-MG).

To a solution of 2 (0.042 g, 0.15 mmol) in anh. THF (3 mL) under a nitrogen atmosphere was added tertbutylmagnesium chloride (1M in THF, 0.30 mL, 0.30 mmol) dropwise, and the reaction was stirred at room temperature for 30 minutes. In a separate flask, 13 was suspended in anh. THF (4 mL) and added to the reaction vessel. The reaction was allowed to stir at room temperature under a nitrogen atmosphere overnight. After stirring overnight, the reaction was quenched with CH₃OH (2 mL) and stirred for another 5 minutes before removal of solvent in vacuo. The crude mixture was then purified twice by flash column chromatography on silica gel (0-10% CH₃OH in CH₂Cl₂) to give the product as a yellow oil (0.075 g, 86% mix of diastereomers); $R_f = 0.48$ (1:9 CH₃OH/CH₂Cl₂); ¹H NMR (400 MHz, CDCl₃) δ 8.96 (s, 1H), 7.58-7.60 (d, J = 5.9 Hz, 1H), 7.44-7.45 (d, J = 2.7Hz, 1H), 7.23-7.27 (m, 2H), 7.07-7.16 (m, 3H), 5.26-5.28 (app. d, 2H), 4.13-4.21 (m, 2H), 3.92-4.05 (m, 8H), 3.72-3.82 (m, 1H), 3.58-3.63 (m, 2H), 1.41-1.59 (m, 1H), 1.24-1.34 (m, 8H), 0.79-0.84 (m, 6H); ¹³C NMR (126 MHz, CDCl₃) δ 173.65, 166.94, 163.72, 155.64, 150.67, 137.30, 134.72, 129.70, 125.03, 120.20, 118.06, 109.32, 67.53, 65.66, 54.9, 54.16, 50.28, 40.25, 23.21, 21.16, 11.03; MS (ESI+) m/z calcd for $C_{27}H_{38}N_5O_8P$ [M+H+]: 592.60, found: 592.3; Elemental Analysis: Anal. calcd for C₂₇H₃₈N₅O₈P + 1% CH₃OH: C, 53.93; H, 6.79; N, 11.23. Found: C, 53.89; H, 6.55; N, 11.02.

4.1.12. General procedure for triphosphate synthesis (1-TP and 2-TP)⁶⁶.

Nucleoside 1 or 2 (0.3 g, 0.1 mmol) was coevaporated with anh. Pyridine and placed in a vacuum overnight. Simultaneously, tributylammonium pyrophosphate (0.13 mmol) was dried in a separate flask under vacuum overnight. The dry nucleoside was dissolved in anh. Pyridine (0.2 mL) and anh. 1,4-dioxane (0.4 mL) N₂ filled balloon. Then 2-chloro-1,3,2under а benzodioxaphosphorin-4-one (0.12 mmol) was added and the reaction was stirred at room temperature for 45 minutes (Ensure that the white powder impurity is scraped off of the 2-chloro-1,3,2benzodioxaphosphorin-4-one crystal, which should either be a glassy green or white solid, before use). Then anh. DMF (0.17 mL) and tributylamine (0.23 mmol) were added to the tributylammonium pyrophosphate flask and sonicated to mix. The solution was added to the reaction mixture and stirred at room temperature for 45 minutes. In a separate vial, iodine (0.041g, 0.16 mmol) was added to pyridine (0.98 mL) and H₂O (20 µL) and sonicated to mix. The contents were then added to the reaction flask and stirred at room temperature for 30 minutes. The reaction was quenched with 10% Na₂S₂O₃ (3 mL). The solvent was removed *in vacuo* while keeping the temperature of the water bath below 30°C. Then H₂O (4 mL) was added and the reaction sat for 30 minutes at room temperature. Solvent was removed under vacuum to afford crude triphosphate as a vellow oil. Products were purified via reverse phase HPLC (0-100% 0.1 M TEAB with 30% MeCN in 0.1 M TEAB, flow rate = 3.5 mL/min). Products were then purified further via ion-exchange chromatography utilizing Dowex 50WX2 Na⁺ charged resin. Column was washed with ultrapure H₂O, 1M HCl, ultra-pure H₂O, 1M NaOH, and finally ultrapure H₂O before the sample was run through the column and washed with more ultra-pure H₂O. All samples were then freeze dried by lyophilization for testing.

Characterization of 2-((4-(2-amino-4-methoxypyrimidin-5-yl)-1H-imidazol-1-yl)methoxy)ethyl tetrahydrogen triphosphate (1-**TP**). NMR (400 MHz, D₂O) δ 8.3 (s, 1H), 7.9, (s, 1H), 7.5 (s, 1H), 5.4 (s, 2H), 4.7 (s, 2H), 4.0 (m, 2H), 3.8 (s, 3H), 3.7 (m, 2H); ³¹P NMR (162 MHz, D₂O) δ -9.5 (d, 1P), -10.5 (m, 1P), -22.6 (t, 1P); MS (ESI+) *m/z* calcd for C₁₁H₁₈N₅O₁₂P₃ [M-H]: 504.02, found: 503.98.

Characterization of 2-((4-(2,4-dimethoxypyrimidin-5-yl)-1H-imidazol-1-yl)methoxy)ethyl tetrahydrogen triphosphate (2-TP).

Product is a white solid after lyophilization (0.035 g, 62%); ¹H NMR (400 MHz, D₂O) δ 8.4, (s, 1H), 7.9 (s, 1H), 7.6 (s, 1H), 5.4 (s, 2H), 4.0 (m, 2H), 3.9 (s, 3H), 3.8 (s, 3H), 3.6 (m, 2H); ¹³C NMR (126 MHz, D₂O) δ 167.3, 163.4, 153.9, 138.4, 133.6, 119.4, 109.4, 76.3, 68.2, 64.9, 55.0, 54.6; ³¹P NMR (162 MHz, D₂O) δ -6.2 (d, 1P), -10.4 (m, 1P), -22.1 (t, 1P); MS (ESI+) *m/z* calcd for C₁₂H₁₉N₄O₁₃P₃ [M-H]: 519.22, found: 519.01.

4.2. Enzyme Production and Purification

The coding sequence corresponding to the ZIKV and DENV-3 MTase domain were cloned in fusion with hist6-Tag in pQE30 expression vector as previously described^{72, 73} and the corresponding protein were produced Escherichia coli T7 Express Iq (New England BioLabs). Cells were grown in Terrific Brother at 97°C until the optical density of 0.6 at 600 nm (OD_{600}) and the Mtase expression was induced by adding 0.5 mM IPTG (isopropyl- β -d-thiogalactopyranoside) before overnight expression at 17°C. The bacteria were pellets and lysed in (50 mM Tris-HCl [pH 8], 300 mM NaCl, 5% glycerol, 0.1% Triton, 10 µg/ml DNase I, 2 tablets of EDTA-free antiprotease cocktail [Roche], 0.25 mg/ml lysozyme). After sonication the clarified proteins were purified by immobilized metal affinity chromatography (IMAC, GE Healthcare), and after, several washes of elution were performed in 50 mM Tris-HCl, 300 mM NaCl, and 250 mM imidazole (pH 8.0). The eluted proteins were then separated by gel filtration on a 16/60 Superdex 200 (GE Healthcare) equilibrated in a mixture of 10 mM HEPES, 500 mM NaCl, 5% glycerol, and 1mM dithiothreitol (DTT [pH 7.5]).

For the NS5 polymerase encoding both MTase and RdRp activities, the gene coding for a N-terminal His6-tagged DENV NS5 (serotype 2, strain New Guinea C) was cloned in a pOE30 expression plasmid as described previously in Selisko et. al.⁷⁴ Expression and purification was as described by Potisopon et. al.75 The synthetic ZIKV NS5 gene cloned in a pQE30 expression plasmid was obtained from Genescript. The sequence used was from strain H/PF/2013 from French Polynesia, Genbank acc# KJ776791. The ZIKV NS5, carrying His₆-tag at its N-terminus, was produced as described above for DENV NS5 with the exception that IMAC TALON beads were washed with 1.5 M NaCl. SEC was performed using a Superdex S75 HR 16/20 column (GE Healthcare) with SEC buffer 50mM HEPES pH 7.5, 750 mM NaCl, 10% glycerol, 10 mM DTT. After the second purification step, proteins were concentrated up to around 8 mg/ml (78 µM NS5) and stored at -20°C after adding glycerol to a final concentration of 40%. Protein purity was higher than 95% as judged by SDS-PAGE. Protein stock concentrations were determined by absorbance measurements at 280 nm using a Nanodrop 2000 (Thermo Scientific).

4.3. DENV and ZIKV 2'-O-MTase Assay

DENV-3 and ZIKV 2'-O-MTase activity was followed by incubating the MTase (0.5 μ M) with small capped RNA substrate GpppAC₅ in the presence of [³H]AdoMet.⁷⁶ The MTase activity assay was performed in 20 μ L samples containing 40 mM Tris-HCl pH 7.5, 5 mM DTT, 10 μ M AdoMet (0.2-2 μ Ci [³H]AdoMet), 0.5 μ M of MTase, 1 μ M GpppAC₅, and the inhibitors. The reaction was incubated at 30°C for 30 minutes and stopped by 20-fold

; 1H

then transferred onto a DEAE membrane (DEAE Filtermat; Wallac) by a Filtermat Harvester (Packard Instruments) washed with 0.01 M ammonium formate (pH 8.0), water, and ethanol, and the radioactivity transferred onto RNA was measured using a Wallac 1450 MicroBeta Trilux Liquid Scintillation Counter.⁷²

The inhibitor concentration at 50% activity (IC₅₀) was determined by performing DENV-3 and ZIKV MTase assays, in the presence of a serial dilution of the inhibitor. All data points were measure in triplicate. The IC₅₀ values were determined using Prism software and adjusted to a logistic dose-response function: % activity = $100/(1+[I]/IC_{50})^{b}$, where b corresponds to the slope factor and [I] to inhibitor concentration.⁷⁷

4.4. Molecular Modeling

All molecular modeling studies were performed on a Viglen Genie Intel®Core™ i7-3770 vPro CPU@ 3.40 GHz x 8 running Ubuntu 14.04. The MTase structures were downloaded from the PDB data bank (http://www.rcsb.org/; PDB codes 5GOZ,79 4V0R,⁸⁰ 3EVD,⁸¹ and 5E9W⁸²). GTP coordinates for the human N7 MTase crystal structure 5E9W were obtained from the E. cuniculi cap-MTase Ecm1 crystal structure 1RI1, which is in complex with GTP.70 This was done after structural superimposition of 5E9W with 1RI1, which reveals a highly conserved architecture and amino acid residue composition for the GTP binding pockets of the two proteins. Hydrogen atoms were added to the proteins, using the Protonate 3D routine of the Molecular Operating Environment (MOE).⁸² Ligand structures were built with MOE and minimized using the MMFF94x force field until a RMSD gradient of 0.05 kcal mol⁻¹ Å⁻¹ was reached. The docking simulations were performed using PLANTS applying the following parameters: search algorithm: aco ants 20, aco evap 0.15, aco sigma 2.0; binding site: 5GOZ: bindingsite center 45.608 -22.492], bindingsite radius [5.952 9; 4V0R: bindingsite center [-28.826 -19.856 -30.783], bindingsite radius 9; 3EVD: bindingsite center [27.897 24.789 -44.440], bindingsite radius 9; 5E9W: bindingsite center [15.299 37.929 95.164], binding radius 9; cluster algorithm: cluster rmsd 2.0, cluster structures 30; scoring function: chemplp.⁸³ Docking results were visually inspected using MOE.

Author Contributions

Synthesis, J.E.T, C.D.W, and A.F.; enzyme assays, C.V., B.M., W.A., B.S., B.C., E.D., and B.C.; computational modeling, M.B. and A.B.; writing- original draft preparation, J.E.T. and K.L.S.R.; writing- review and editing, K.L.S.R., B.C., E.D., M.C., M.B., and A.B.; K.L.S.R planned, designed, and organized the study.

Conflicts of Interest

The authors declare no conflicts of interest.

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Journal Pre-proofs compounds 1-TP and 2-TP against DENV NS5-MTase, ZIKV NS5-MTase, and human N7 MTase.

MI I ase.					
Compou nd	ZIKV	DENV	hN7		
	MTase	MTase	MTase		
	IC ₅₀	IC ₅₀	IC ₅₀		
1-TP	1.7 μM	8.4 µM	49 µM		
2-TP	0.15 µM	1.1 µM	13 µM		

 \boxtimes The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

□The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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