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Design, Synthesis and Discovery of *N,N'*-Carbazoyl-aryl-urea Inhibitors of Zika NS5 Methyltransferase and Virus Replication

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The recent outbreaks of Zika virus (ZIKV) infection worldwide make the discovery of novel antivirals against flaviviruses a research priority. This work describes the identification of novel inhibitors of ZIKV through a structure-based virtual screening approach using the ZIKV NS5-MTase. A novel series of molecules with a carbazoyl-aryl-urea structure has been discovered and a library of analogues has been synthesized. The new compounds inhibit ZIKV MTase with IC₅₀ between 23–48 μM. In addition, carbazoyl-aryl-ureas also proved to inhibit ZIKV replication activity at micromolar concentration.

Zika virus (ZIKV)^[1,2] is a single positive-strand RNA mosquito-borne pathogen belonging to the family of *Flaviviridae*, genus *Flavivirus* and it causes mild-severe diseases in humans and animals. ZIKV is closely related to Dengue virus (DENV), and just like DENV, is mainly transmitted to humans by bites of an

infected *Aedes* species mosquitoes (*Ae. aegypti* and *Ae. albopictus*).^[3] However, ZIKV can also be transmitted between humans through contact with blood or other body fluids, sexual transmission^[2,4] and by mother-to-fetus transmission during the pregnancy.^[6] While global epidemics of DENV have spread over the past few decades causing more than 20,000 deaths per year, ZIKV infections have emerged as a major public health concern only in the last few years. Before 2007, only sporadic human disease cases were reported in Africa and Asia. However, following the recent outbreaks in the Americas in 2015, ZIKV was declared by the World Health Organization (WHO) as a Public Health Emergency of International Concern (PHEIC) and the first case of sexually-transmitted ZIKV was reported in USA in 2008. While ZIKV infections generally cause a mild fever, headache, malaise, skin rashes and joint pain, they have been associated in several cases with severe neurological and fetal complications leading to microcephaly in new-borns^[4] and neurological diseases, such as the Guillain-Barré syndrome (GBS), in adults.^[5] The recent *Flavivirus* outbreaks, as well as the increasing number of cases of ZIKV infections worldwide, have raised the attention of pharmaceutical industries and healthcare providers toward the identification and development of efficient treatments against these diseases. Although a vaccine against DENV has recently been commercialized (DENVAXIA[®]),^[6] there is as yet no vaccine against ZIKV available. In addition, there are no drugs available to treat or prevent ZIKV infections, especially in the event of an outbreak. A limited number of early-phase discovery studies have identified few inhibitors of DENV and ZIKV replication,^[7–15] however there are no clinically approved drugs yet available to target flaviviruses directly nor any that may serve as vaccine adjuvants. The development of new antivirals thus represents a research priority.

Within this context, viral proteins represent appealing targets for the development of novel antiviral therapies. Examples of current flavivirus inhibitors, such as 1–5 (Figure 1), have been designed to target the DENV viral proteins NS3 (protease domain,^[16] helicase domain,^[17] and full-length NS3), NS5 (N-terminal Methyltransferase domain (MTase)^[18–19] and C-terminal RNA-dependent RNA polymerase (RdRp).^[20] The NS3 and NS5 proteins of DENV and ZIKV show a high degree of homology, and their crystal structures have recently been determined.^[21–23] While DENV NS5-polymerase has been investigated by a number of research groups as a potential target for

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Examples of DENV and ZIKV inhibitors

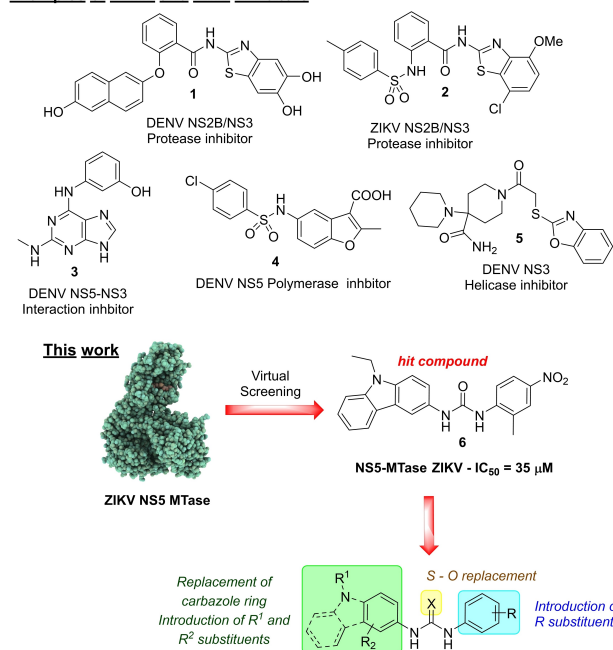


Figure 1. Examples of small molecules active against DENV and/or ZIKV, and overview of this work.

development of new antivirals, there has been very little work carried out on DENV and ZIKV NS5-MTase.^[23–24,18] The NS5-MTase is responsible for maturation of the viral RNA cap and catalyzes the methylation of the N7 position of a guanine and the 2'-OH of the first ribonucleotide to yield¹⁷MeGpppA₂OMe-RNA.^[25] These methylations of the RNA cap structure play a key role during virus replication and are critical to virus survival in infected animals.^[26] Indeed, biochemical studies and reverse genetic analysis have shown that N7-MTase activity is essential for mRNA translation into viral protein, and so viruses devoid of N7-MTase show a strongly reduced replication phenotype.^[18] By contrast, 2'-O-MTase defective viruses can replicate moderately well in infected cells, but are highly attenuated in mice or rhesus monkey and induce a strong antiviral response.^[27] Thus, the inhibition of both N7- and 2'-O-MTase activities should restrain viral replication, making the NS5-MTase a promising target for the development of new anti-ZIKV, and potentially anti-DENV, antivirals.^[24]

Herein, a structure-based virtual screening on a set of chemical libraries using the ZIKV NS5-MTase was performed, with the aim to identify structurally novel flaviviruses inhibitors.

The structure of the ZIKV NS5 MTase was built by homology modelling using SWISS-MODEL^[28–29] and the compounds in the NCI Diversity Set V database docked in the S-adenosyl methionine (SAM) binding site in order to identify molecules that might disrupt the activity of the enzyme. A set of 40 best ranking molecules were initially identified, among which the urea **6** emerged as the best candidate due to its favorable interactions with the SAM binding site. The urea **6** was thus evaluated in enzymatic inhibition assays against ZIKV NS5-MTase, showing an IC₅₀ of 35 μM (Table 1). A similar IC₅₀ of

Table 1. IC₅₀ values of compounds **6**, **9**, **11** and **21** against host and flavivirus MTases.

Compd	hRNMT IC ₅₀ [μM]	ZIKV NS5-MTase	DENV NS5-MTase
6	13.9 ± 0.7	35 ± 5	38 ± 6.7
9a	≈ 122	46 ± 1.1	≈ 133
9b	≈ 253	≈ 144	≈ 267
9c	27.4 ± 1.9	NA ^[b]	≈ 62
9d	5.3 ± 0.4	≈ 397	60 ± 32
9f	6.8 ± 0.5	NA ^[b]	NA ^[b]
11b	≈ 112	70 ± 1.3	≈ 236
21b	nd ^[a]	114 ± 1.3	NA ^[b]
21c	nd ^[a]	23 ± 1.2	NA ^[b]
21e	nd ^[a]	48 ± 1.3	NA ^[b]
21f	nd ^[a]	26 ± 1.2	NA ^[b]
Sinefungin	nd ^[a]	1.18 ± 0.05	0.63 ± 0.04

[a] Activity not determined. [b] No activity observed above 50 μM.

38 μM was observed when **6** was assayed against DENV NS5-MTase. Due to the novelty of the structure when compared to known flavivirus inhibitors, the urea **6** was thus selected as a hit compound for further studies. Figure 2b shows the most favorable binding pose of the hit compound **6** in the ZIKV NS5-MTase binding pocket. The urea spacer connects the carbazole with a 2-methyl-4-nitro-phenyl group, which occupies the region that interacts with the methionine backbone of SAM (Figure 2a). The nitro group of **6** interacts with the side chain of S62 in a similar way to the SAM carboxyl group, possibly stabilizing the interaction of the compound with the protein. The high sequence and structural identity between ZIKV and DENV NS5-MTases led to the identical binding of compound **6** in both proteins (Figure 2c).

Next, a library of analogues of compound **6** was designed and synthesized to explore the chemical space around the urea scaffold (Scheme 1). The carbazole derivatives **9a–f** were first synthesized through reaction of carbazole **7** with the appropriate isocyanate **8** in toluene at 60 °C with the aim to investigate the effect of different substituents (electron-donating and electron-withdrawing) on the phenyl ring bound to the urea moiety (Scheme 1a). Similarly, thioureas **11a–b** were synthesized from **10a–b** to investigate the role of the isosteric sulfur in place of the urea oxygen atom and to evaluate the

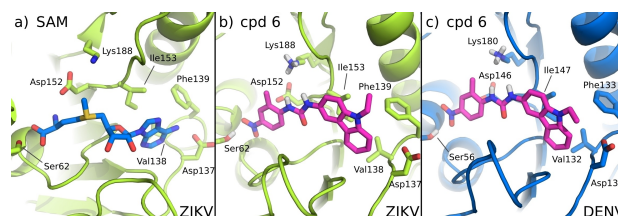
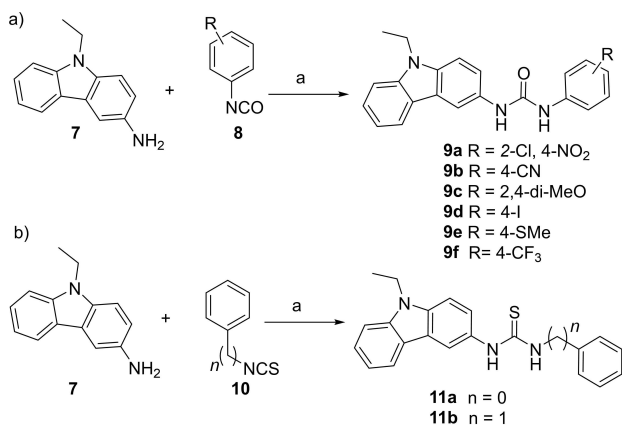


Figure 2. a) SAM bound ZIKV NS5 MTase in PDB 5M5B.^[23] b) Docking of compound **6** in ZIKV NS5 MTase. The carbazole pocket is accommodated in the hydrophobic environment that binds the adenine portion of SAM. The *p*-nitro group interacts with Ser62, mimicking the carboxy moiety of SAM. The carbamide spacer allows the two domains to be spaced appropriately for favorably interacting with the key residues of the binding pocket. c) Docking of compound **6** in DENV NS5-MTase (PDB 5E9Q).^[24]



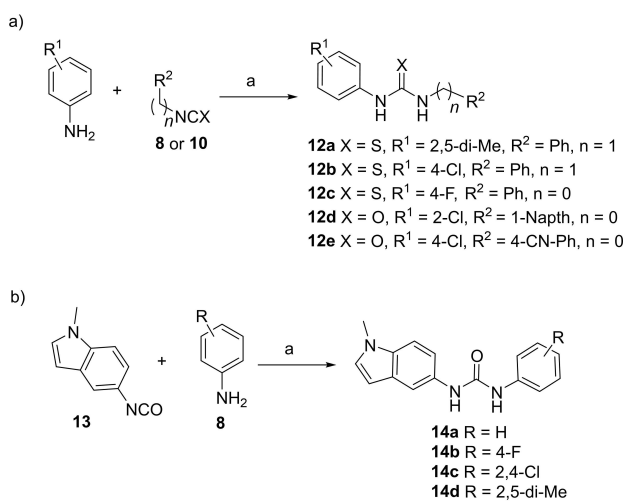
Reagents and conditions: (a) Toluene, 60 °C, 24h

Scheme 1. Synthesis of urea derivatives 9a–f and 11a,b.

influence of an extra methylene group in 11b on MTase inhibition (Scheme 1b).

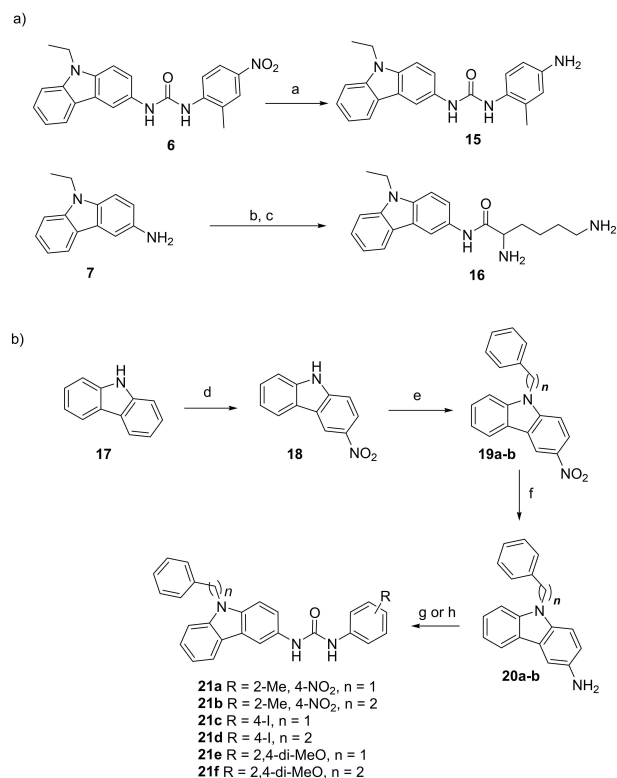
The role of the carbazole ring on the antiviral activity was also explored through its replacement with various phenyl moieties (derivatives 12a–e) and indole ring (derivatives 14a–d). Ureas 12a–e and 14a–d were obtained from appropriate isocyanates 8 and 10 in toluene according to Scheme 2.

In order to evaluate the role of the nitro group on antiviral activity, the amino derivatives 15 and 16 were also synthesized; compound 6 was treated with Fe⁽⁰⁾ in conc. HCl leading to 15 in high yields, while carbazole 7 was reacted under microwave irradiation with Cbz,Boc-lysine to give, after deprotection in HCl/AcOEt, the pure 16 (Scheme 3a). Finally, we also tried to modify the *N*-ethyl substituent of 6 in order to increase the selectivity of our inhibitors targeting the MTase SAM binding site. For this purpose, we compared the structure of the human



Reagents and conditions: (a) Toluene, 60 °C, 24h

Scheme 2. Synthesis of urea derivatives 12a–e and 14a–d.



Reagents and conditions: (a) Fe, HCl conc., MeOH, DCM, rt; (b) *N*-Cbz-*N*-Boc-Lysine, DIPEA, HOBT, EDC, DMF, MW, 60 °C, 10 min; (c) HCl/AcOEt, 48 h; (d) HNO₃ 60%, H₂O, 80 °C, 3h; (e) Benzylchloride or 2-phenylethylbromide, NaH, DMF, 90 °C, 2h; (f) SnCl₂, HCl conc., AcOH, 100 °C, 12h; (g) 2-Me,4-NO₂-isocyanate (prepared *in situ* from 2-Me,4-NO₂-aniline, triphosgene, Et₃N, 0 °C, THF, 4h), toluene, 60 °C, 12h; (h) isocyanate, toluene, 60 °C, 12h

Scheme 3. Synthesis of urea derivatives 15, 16 and 21a–f.

N7 MTase (hRNMT) involved in the capping of cellular mRNA, with that of both viral MTases. The X-ray structure revealed structural similarities between the viral and the human MTases (NS5 and hRNMT). However, both ZIKV and DENV NS5-MTase possess a hydrophobic pocket located near the exocyclic amide of SAM,^[18] which is absent in the hRNMT. In the ZIKV MTase, the region is defined by the amino acids F139, I153, G164, E155, R166, and V170, compared to residues F133, I147, G148, E149, R160, and V164 in the DENV MTase. We anticipated that the absence of this hydrophobic pocket in hRNMT could help to increase compound selectivity for the viral MTases and, hence, limit the interference of inhibitors with host MTases. In the docking snapshot, the ethyl group of compounds 6 is located in proximity of this pocket, clearly showing that its replacement with a benzyl or a phenylethyl moiety could lead to derivatives able to selectively recognize and inhibit only the viral enzymes over the host enzymes (Figure 3).

Thus, the synthesis of derivatives 21a–f bearing a phenyl-alkyl substituent on the carbazole nitrogen was planned. Carbazole 17 was treated with HNO₃ and converted into the nitro derivative 18, which was in turn alkylated on the aromatic nitrogen with appropriate benzyl or alkyl halide in the presence of NaH to give compounds 19a–b. Reduction of the nitro group of 19 with SnCl₂ led to amino derivatives 20a–b. The latter

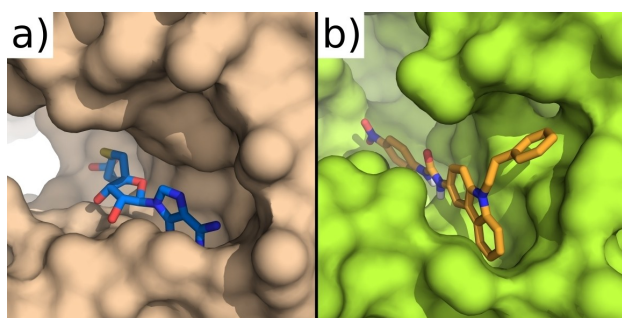


Figure 3. a) SAH bound to hRNTM (PDB 5E8J).^[30] b) Docking of compound **21b** in ZIKV NS5 MTase. The ethyl spacer on the carbazole nitrogen allows the phenyl ring to interact effectively with the hydrophobic pocket near the SAM binding site. The absence of the region in hRNTM is a potential determinant of target selectivity.

were then treated with 4-*l*-phenyl- or 2,4-(MeO)₂-phenyl-isocyanate in toluene at 60 °C affording ureas **21c–f** in high yields. A different strategy was adopted to synthesize the derivatives **21a–b**, due to the commercial lack of the appropriate isocyanate reagent. The 2-methyl-4-nitro-aniline was treated with triphosgene at 0 °C in the presence of Et₃N leading to the formation of the corresponding isocyanate, which was reacted *in situ* with carbazole **7** to give the desired ureas **21a–b** in good yields (Scheme 3).

The library of compounds **9**, **11**, **12**, **14**, **15**, **16** and **21** was then assessed on different purified recombinant MTases. All compounds were initially screened at 50 μM against ZIKV NS5-MTase and hRNTM as well as against DENV NS5-MTase due to its similarity with ZIKV protein. The SAM mimetic sinefungin was included as control.

The MTases were incubated with radiolabeled [³H]-SAM together with the GpppAC₄ RNA substrate and the different compounds at 50 μM. The reaction was stopped after 30 mins at 30 °C. The sample products were filtered on a DEAE membrane to remove non-incorporated [³H]-SAM, and radioactivity transferred on the RNA substrate was counted. Compounds **6**, **9a**, **9d**, **21b**, **21c**, **21e** and **21f** showed inhibition of ZIKV NS5-MTase higher than ~30%. The compound **21e** and **21f** showed the more potent reduction of ZIKV NS5-MTase activity of ~70%.

Conversely the compounds **15** and **16** and those of series **11** and **14** barely inhibited the ZIKV MTase. Interestingly compound **6** and those of the series **9** showed a similar inhibition profile on DENV NS5-MTase, but the compounds of series **21** barely inhibit the DENV MTase suggesting some specificity of this family. A dose-response assay was then performed for the most promising compounds and the IC₅₀ values deduced from titration curves after curve fitting are shown in Table 1. The results indicate that the replacement of the methyl group of **6** with a chlorine atom in **9a** did not affect the inhibitory activity of the compound against ZIKV MTase, whilst it proved to be detrimental for inhibiting the DENV MTase. Similarly, the replacement of the electron withdrawing nitro group of **6** with other substituents (i.e. the electron donating iodine or methoxy in **9c–d**, or electron withdrawing

–CN or –CF₃ in **9b** and **9f**) negatively affected the compound's inhibitory activity mainly against the ZIKV MTase. The different activity of compounds **6** and **9** can be explained by taking in consideration the crucial H-bond interaction between nitro groups of **6** with the hydroxyl group of S62, which is not possible for derivatives **9b–f**. The low activity of compounds **11b** can be attributed to the lack of appropriate substituents on the phenyl ring able to interact with S62. On the other hand, the carbazole ring proved to be crucial for the antiviral activity since its replacement with other rings in **12** and **14** led to inactive compounds. Derivatives **21c**, **21e** and **21f** showed good activity against ZIKV MTase at concentrations similar to **6** (IC₅₀ = 23–48 μM). It is likely that the *N*-benzyl or *N*-phenylethyl substituents in compounds **21** are helping these compounds to bind to the viral proteins even if no substantial improvement from **6** was detected. A dose-response curve for hit compound **6** is reported in Figure 4.

Finally, the ability of all the derivatives synthesized in this work to inhibit ZIKV replication was assessed. Results of the most active compounds, as well as their effect on cell viability, are reported in Table 2. Compound **6** and **9a–d**, which showed MTase inhibitory activity, were devoid of any antiviral activity against ZIKV.

Possible reasons for the lack of antiviral activity may be the inability of the molecules to enter the cell, or their conversion into inactive metabolites in infected cells. However, since compound **6** proved to be cytotoxic (CC₅₀ > 3.99 μM), it is likely

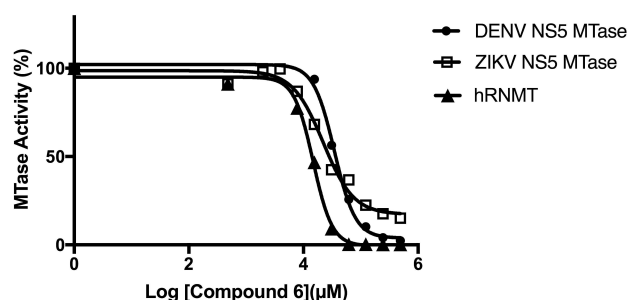


Figure 4. Dose-response curve for hit compound **6**.

Table 2. EC ₅₀ and CC ₅₀ values against ZIKV of the most active compounds of the library.		
Compd	ZIKV EC ₅₀ [μM]	CC ₅₀ [μM]
6	> 3.99	3.99
9a	> 50	30.7
9b	> 10	> 10
9c	> 10	> 10
9d	> 50	> 50
11b	4.78	19.57
15	1.67	> 12.5
21b	12.5	20
21d	25	100
21f	> 20	20
Compd	DENV EC ₅₀ [μM]	CC ₅₀ [μM]
15	9.75	20

that this compound and analogues thereof can enter the cells. Moreover, the inhibitory activity of **6** and **9a–d** against the host MTase (Table 1) hints toward an antimetabolic effect. Another possible explanation is that compounds **6** and **9a–d** mainly inhibit the 2'-O MTase activity without affecting the N7 MTase activity. This could explain the very poor inhibition of ZIKV replication in the cell-based assay, as the 2'-O methylation of the cap is not essential for viral replication in most cell lines. Derivative **11b**, which showed some MTase inhibitory activity, also exhibited antiviral activity against ZIKV in the cell-based assay ($EC_{50} = 4.78 \mu\text{M}$). Interestingly, derivative **15**,^[31] which did not show any activity against ZIKV NS5-MTase, was able to inhibit ZIKV replication with an EC_{50} of $1.67 \mu\text{M}$. It is plausible that **15** inhibits ZIKV replication via a different mode of action than via NS5-MTase inhibition, or that this compound is partially metabolized in the treated cells.

Interestingly, compound **15** also showed some activity against DENV ($EC_{50} = 9.75 \mu\text{M}$), even if the antiviral activity is clearly linked to an adverse toxic effect on the host cell ($CC_{50} = 20 \mu\text{M}$). Finally, compound **21b** which showed no enhanced activity against ZIKV MTases, proved to inhibit ZIKV replication at good concentration ($EC_{50} = 12.5 \mu\text{M}$). However, compound **21b** showed toxicity in cellular assay as well as **21f** which was found active on ZIKV MTase instead. This could suggest that these compounds might target another MTase (i.e. cellular MTase) involved in virus replication. Interestingly, compound **21d**, which showed poor inhibition of ZIKV MTase, inhibits ZIKV with good $EC_{50} = 25 \mu\text{M}$ and no toxicity ($CC_{50} = 100 \mu\text{M}$).

In summary, a structure-based virtual screening protocol on a set of chemical libraries using the ZIKV NS5-MTase was performed leading to the identification of a novel class of carbazoyl-urea inhibitors. Compounds **6** and **21c**, **21e**, **21f** showed inhibitory activity against ZIKV MTase as well as the related DENV MTase. Conversely, the urea derivative **15** showed an antiviral effect against ZIKV with EC_{50} of $1.67 \mu\text{M}$, despite its poor inhibitory activity toward the NS5-MTase *in vitro*. Studies to identify novel MTase inhibitors, to optimize the structure-activity relationships of carbazoyl-urea analogues of **6** and to fully unravel their mode of action are currently in progress in our laboratories.

Experimental Section

Experimental details are reported in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Zika · flavivirus · methyltransferase · antiviral agents · urea

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