



## Adenosine triphosphate analogs can efficiently inhibit the Zika virus RNA-dependent RNA polymerase



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### ABSTRACT

We describe the expression and purification of an active recombinant Zika virus RNA-dependent RNA polymerase (RdRp). Next, we present the development and optimization of an *in vitro* assay to measure its activity. We then applied the assay to selected triphosphate analogs and discovered that 2'-C-methylated nucleosides exhibit strong inhibitory activity. Surprisingly, also carbocyclic derivatives with the carbohydrate locked in a North-like conformation as well as a ribonucleotide with a South conformation exhibited strong activity. Our results suggest that the traditional 2'-C-methylated nucleosides pursued in the race for anti-HCV treatment can be superseded by brand new scaffolds in the case of the Zika virus.

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Zika virus (ZIKV), a previously neglected mosquito-borne viral pathogen, has recently attracted global attention (Hajra et al., 2016; Lessler et al., 2016). ZIKV is a member of family *Flaviviridae*, genus *Flavivirus*, which includes several important mosquito- or tick-borne pathogens, such as dengue virus, yellow fever virus, West Nile virus, Japanese encephalitis virus, or tick-borne encephalitis virus (Gould and Solomon, 2008). Until 2007, ZIKV was thought to be present only in Africa and South-east Asia, but then the virus spread to Micronesia, French Polynesia, and more recently to the Americas, where it has displayed an explosive outbreak (Wikan and Smith, 2016). More than 80% of ZIKV-infections in humans are asymptomatic. Symptomatic cases usually manifest as a mild and self-limited rash, joint pain, and conjunctivitis (Hajra et al., 2016). However, during the recent outbreaks, severe cases of ZIKV-infection were reported, including myelitis (Mecharles et al., 2016), meningitis, encephalitis (Carteaux et al., 2016; Soares et al., 2016), and Guillain-Barré syndrome - a neurological disorder that may lead to paralysis and death (Brasil et al., 2016; Wise, 2016). Moreover, an association between ZIKV-infection and congenital

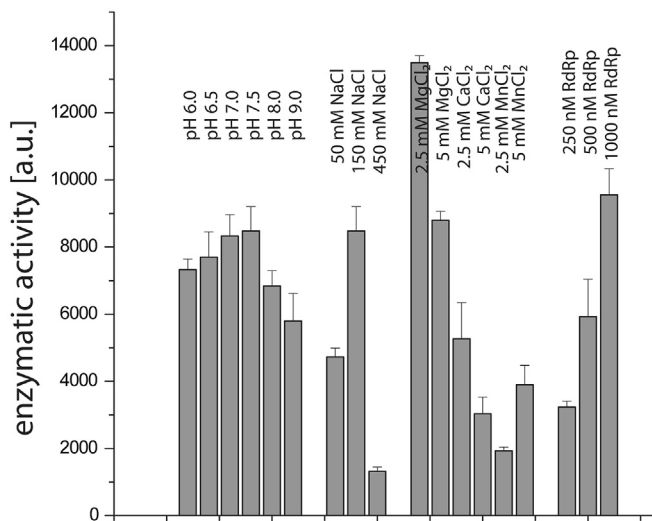
malformations, such as microcephaly in newborns, has been documented (Klase et al., 2016). An obvious strategy for prevention of these severe health complications would be the design of an anti-ZIKV vaccine, however, effective antivirals are urgently needed until such a technology has been developed.

The ZIKV single-stranded positive sense RNA (+RNA) genome is approximately 11 kb in length and contains one large open reading frame which is flanked by 5' and 3' non-coding regions. Translation yields an approximately 3400 amino acid polyprotein that is co- and posttranslationally processed by viral and cellular proteases into three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Barzon et al., 2016). NS5 protein together with NS3 are central to the viral replication-competent complex; these two non-structural proteins harbor most of the catalytic activity required to both cap and replicate the viral RNA (Bollati et al., 2010). NS5 consists of an N-terminal methyltransferase domain and the C-terminal RNA-dependent RNA polymerase (RdRp) domain. As such, it represents an attractive target for the development of antivirals that block the functions of this enzyme or cause premature termination of viral RNA synthesis (Bollati et al., 2010). We recently identified that 2'-C-methylated nucleoside analogs that likely target NS5 RdRp and terminate viral RNA replication after incorporation into the nascent viral RNA chain, represent effective ZIKV inhibitors (Eyer et al., 2016a).

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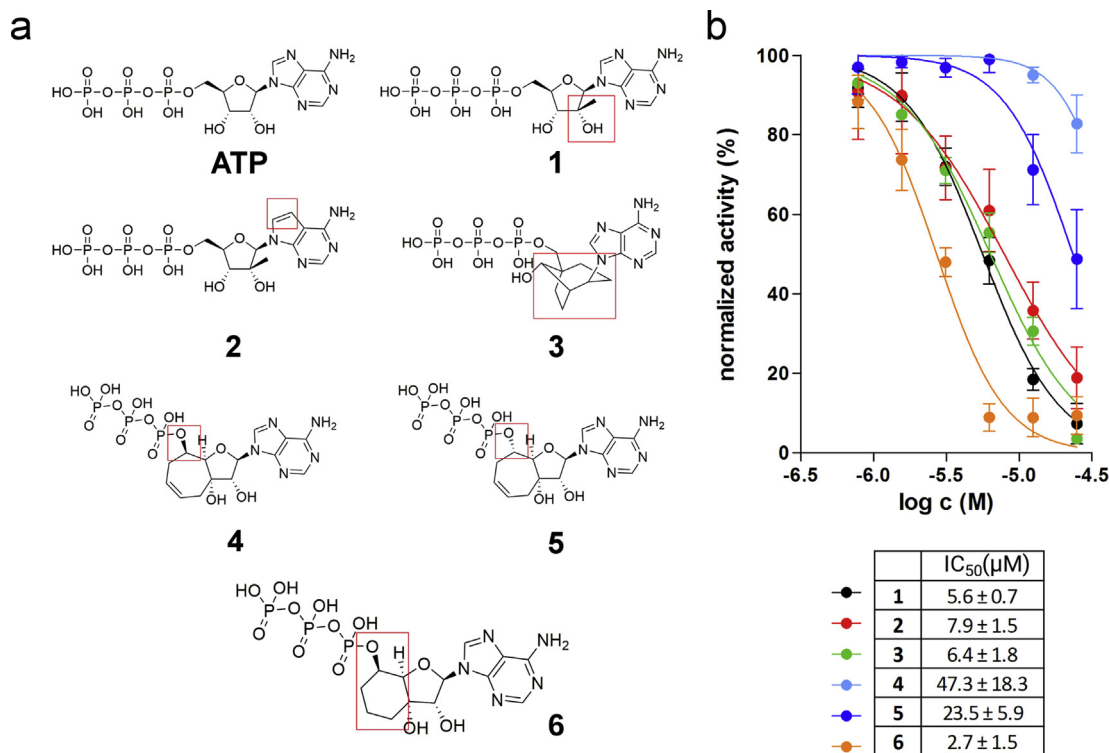
**Fig. 1. Optimization of the ZIKV RdRp activity assay.** Reaction buffer (150 mM NaCl, 5 mM MgCl<sub>2</sub>, pH 7.5) with 1000 nM ZIKV RdRp was changed one condition at a time as indicated in the graph.

While many ZIKV enzymes such as protease, helicase and methyltransferase have already been characterized (Coloma et al., 2016; Lei et al., 2016; Tian et al., 2016) the RdRp remains enigmatic. In this study, we have prepared active recombinant Zika virus (strain MR766) RdRp by expression in *E. coli* using our standard protocols (Baumlova et al., 2014; Rezakboka et al., 2010) as detailed in the Supplementary information. The recombinant ZIKV RdRp was more than 95% pure as judged by the SDS page (SI Fig. 1). Next, we developed and optimized a radioactivity based assay

(Fig. 1, detailed in SI). The ZIKV RdRp exhibited good enzymatic activity at 500 nM concentration with 0.2 ng/μl poly-U (Polyuridylic acid homopolymer, Sigma) as template and 10 nM 15-mer poly-A primer in the reaction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 0.1 mg/ml BSA and 0.2 U/μl RNase inhibitor, Promega). Higher or lower solution pH and lower or higher NaCl concentration as well as the use of Mn<sup>2+</sup> ions instead of Mg<sup>2+</sup> ions were detrimental to its enzymatic activity (Fig. 1).

Previously, it was shown by us and others that certain nucleoside analogs can effectively inhibit flaviviral replication (Eyer et al., 2016a, 2016b, 2015; Xie et al., 2016; Zmurko et al., 2016). Having a functional assay established we decided to resynthesize several triphosphate (TP) analogs of the compounds that exerted significant effect in our cell based assay against HCV as well as TPs of derivatives that were generally inactive (Hrebabecky et al., 2015) in order to assess their activity against ZIKV RdRp. Both the 2'-C-methylated nucleotides and the locked nucleoside triphosphate were obtained by the procedures described in the literature (Dejmek et al., 2015; Olsen et al., 2004; Sala et al., 2015) and the structures of the tested compounds are summarized in Fig. 2A. Next we tested the prepared TPs in our assay. Primer, template, and tested TPs were pre-incubated with ZIKV RdRp at 30 °C for 5 min. The reaction was initiated by adding a mixture containing 2.5 μM ATP and 0.12 μCi/μl of [ $\alpha$ -<sup>32</sup>P]ATP (3000 Ci/mmol). TPs **1**, **2**, **3** and **6** exhibited significant ability to inhibit ZIKV RdRp while compounds **4** and **5** were found to be virtually inactive (Fig. 2B).

2'-C-methylated nucleotides were described as efficient inhibitors of RdRp from several flaviviruses, e.g. HCV (Sofia et al., 2012), and the parent nucleosides of compound **1** and **2** exerted significant effect against ZIKV in cell-based assays (Eyer et al., 2016a), therefore, activity against ZIKV RdRp was anticipated. Both TPs **1** and **2** inhibited the enzymatic activity of ZIKV RdRp with an IC<sub>50</sub> in the single-digit micromolar level which nicely correlates



**Fig. 2. a: Triphosphates used in this study.** We used several analogs of ATP for evaluation of inhibitory potency against ZIKV RdRp. Derivatives **1** and **2** are the 2'-C-methylated nucleotides which are well known inhibitors of flaviviral replication. Compounds **3–6** are conformationally locked nucleotides (**3** in the North-like conformation, **4–6** in the South conformation of the sugar moiety). b: **In vitro** activity of selected TPs.

to our previous results in the anti-ZIKV cellular assay ( $5.26 \pm 0.12$  respectively  $8.92 \pm 3.32 \mu\text{M}$ ). To our surprise, locked TPs **3** and **6** inhibited the polymerase at a very similar level, with compound **6** being the most active ( $\text{IC}_{50} = 2.7 \pm 1.5 \mu\text{M}$ ). In contrast, derivatives **4** and **5** were only modestly recognized by the enzyme.

From the SAR point of view, the sugar moiety of TP **3** is locked in the North-like conformation, which is in direct contrast to TPs **4–6** that are locked in the South conformation. Therefore, both North- and South-locked nucleotides can act as potent inhibitors of ZIKV RdRp albeit we cannot be certain that the mode of action is the same. This North/South unselective behavior resembles HCV RdRp, which can also be inhibited by nucleotides locked in both conformations (Chapron et al., 2014; Clarkson et al., 2015; Sofia et al., 2010). However, the structural diversity of the potential inhibitors of ZIKV RdRp seems to be significantly wider than in the case of HCV. In particular, traditional nucleotide inhibitors of RdRp from HCV and related RNA viruses, compounds **1** and **2**, exert similar potency as the derivatives with significantly modified sugar moiety, compounds **3** and **6**. Interestingly, the most active derivative **6** is closely structurally related to rather inactive compounds **4** and **5**, which defines the boundaries of potential modification these bicyclic nucleotide derivatives.

In conclusion, we have shown that Zika virus RdRp can be effectively expressed in *E. coli* and that the purified protein is suitable for inhibitory assays. In addition, we proved that the triphosphate analogs of 2'-C-methylated nucleosides, i.e. compounds **1** and **2** exert significant inhibitory activity against ZIKV RdRp, and therefore can be used as a benchmark in subsequent searches for novel antiviral compounds. Furthermore, these results revealed that in fact the whole geometry and appropriate positioning of the nucleobase vs. triphosphate moiety is an essential structural feature of the anti-ZIKV nucleotide derivatives and it also revealed that both North- and South-locked TPs can be good inhibitors of ZIKV polymerase. Our results, therefore, provide not only a screening platform for inhibitory activity of novel nucleoside triphosphates but also bring promise for completely new therapeutics against flavivirus infections based on rational design using these scaffolds.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.antiviral.2016.11.020>.

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