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Structure-activity relationship of uridine-based nucleoside phosphoramidate prodrugs for inhibition of dengue virus RNA-dependent RNA polymerase

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ABSTRACT

To identify a potent and selective nucleoside inhibitor of dengue virus RNA-dependent RNA polymerase, a series of 2′- and/or 4′-ribose sugar modified uridine nucleoside phosphoramidate prodrugs and their corresponding triphosphates were synthesized and evaluated. Replacement of 2′-OH with 2′-F led to be a poor substrate for both dengue virus and human mitochondrial RNA polymerases. Instead of 2′-fluorination, the introduction of fluorine at the ribose 4′-position was found not to affect the inhibition of the dengue virus polymerase with a reduction in uptake by mitochondrial RNA polymerase. 2′-*C*-ethynyl-4′-F-uridine phosphoramidate prodrug displayed potent anti-dengue virus activity in the primary human peripheral blood mononuclear cell-based assay with no significant cytotoxicity in human hepatocellular liver carcinoma cell lines and no mitochondrial toxicity in the cell-based assay using human prostate cancer cell lines.

Dengue fever (DF) is an acute mosquito-borne viral disease that causes flu-like symptoms, and occasionally develops potential life-threatening dengue hemorrhagic fever (DFS) and dengue shock syndrome (DSS).[1] It is caused by the four serotypes of dengue viruses (DENV-1 to -4) and is widely spread throughout tropical and sub-tropical countries with an estimated 390 million people infected annually, including 20,000 deaths.[2] Secondary infection by a different DENV serotype can increase the risk of developing severe dengue diseases, therefore the ideal treatment for DF requires pan-serotype activity.[3] The first dengue vaccine, Dengvaxia® by Sanofi, was first registered in Mexico in 2015 and has been approved in 19 countries for use in endemic areas.[4] However recent analysis found that Dengvaxia® could cause more cases of severe disease for those who had never been infected by dengue virus.[5] On the other hand, no antivirals are currently available for the treatment of DENV infection, despite many medicinal chemistry efforts on drug discovery being pursued.[6]

DENV is a single stranded RNA virus of the flavivirus genus in the family *Flaviviridae* such as West Nile virus, Yellow fever virus, and Zika virus. The DENV genome encodes three structural and seven nonstructural proteins, of which the NS5 possesses methyltransferase and RNA-dependent RNA polymerase (RdRp) activities. The RdRp is a viral enzyme that catalyzes replication of RNA from an RNA template. It is essential for viral replication and is a proven antiviral target.[7] Nucleoside analogs are a clinically proven chemical class of viral polymerase inhibitors such as for HIV and HCV therapies.[8] It is anticipated that nucleoside DENV polymerase inhibitors can provide an increased barrier to developing resistance and have pan-serotype activity, given that they bind to the highly conserved active site of the RdRp.[9] As part of our research for dengue drug discovery,[10] we therefore investigated nucleoside class DENV RdRp inhibitors. We previously reported the adenosine-based nucleoside (NITD-008 (**1**), 7-deaza-2′-*C*-ethynyl-adenosine) which potently inhibited DENV both *in vitro* and *in vivo*. However, development of NITD-008 was terminated due to its insufficient safety profile.[11,12] 4′-Azido-cytidine (R-1479 (**2**)) and its ester prodrug, balapiravir (**3**), were originally developed by Roche for the

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treatment of HCV, but their development was terminated due to adverse hematologic events such as lymphocytopenia.[13] 4′-Azidocytidine (**2**) was found to be potent against DENV in both cellular and polymerase enzymatic assays.[14] Balapiravir (**3**) was repurposed for a phase II clinical trial for the treatment of dengue, but it failed to reduce viral load in patients, though reasons remain to be clarified (Fig. 1).[15]

Nucleoside viral RdRp inhibitors, which are chemical analogs of RNA ribonucleosides, require intracellular metabolism to their respective nucleoside triphosphates by cellular kinases. The triphosphate metabolites serve as competing substrates for a viral polymerase and are subsequently incorporated into the growing RNA chain, resulting in the termination of the RNA chain elongation. However, they can be also incorporated by the host polymerases, such as mitochondrial DNA-dependent RNA polymerase (POLRMT), potentially leading to mitochondrial dysfunction *in vivo*.[16] For the discovery of a new class of nucleoside DENV polymerase inhibitor, it is therefore critical to evaluate not only the inhibition of viral RNA replication by nucleotide triphosphate analogs but also the selectivity for their incorporation by POLRMT. R-1479 triphosphate (4′-azido-CTP) was reported to be a potent inhibitor for HCV and DENV RdRp.[14] However, 4′-Azido-CTP was incorporated by POLRMT at a rate similar to its corresponding natural counterpart, CTP (Fig. 1).[16] Likewise, NITD-008 triphosphate was observed to be incorporated by POLRMT at 14.2% of the single nucleotide incorporation rate (SNIR) measured at 500 μM (Fig. 1)

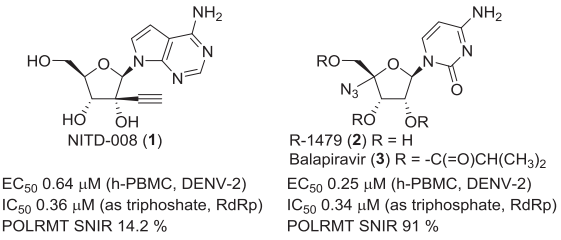


Fig. 1. Structures and *in vitro* biological profile of NITD-008 (**1**), R-1479 (**2**), and balapiravir (**3**). POLRMT = mitochondrial DNA-dependent RNA polymerase, SNIR = single nucleotide incorporation rate in comparison with the natural NTP of the nucleoside inhibitor (e.g. NITD-008 triphosphate versus ATP and R-1479 triphosphate versus CTP).

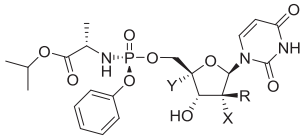
and the failure of NITD-008 in the pre-clinical safety studies was suspected to be associated with mitochondrial toxicity based on histopathological analysis of the tissues.[12] In contrast, the triphosphate metabolite **4** of sofosbuvir (2′-fluoro-2′-*C*-methyl uridine phosphoramidate prodrug),[17] which has gained approval for use as a HCV NS5B inhibitor, was incorporated by POLRMT at a rate of 1.8%, equivalent to background signal, however it was not efficacious against DENV RdRp (IC_{50} 18 μM) (Table 1).[18] In order to discover a potent and selective nucleoside DENV polymerase inhibitor, we synthesized 2′- and/or 4′-ribose sugar modified uridine-based nucleoside analogs and evaluated their anti-DENV activity in primary human peripheral blood mononuclear cells (PBMCs), which is one of the major target cells for dengue viral replication. In addition, the corresponding triphosphates were evaluated for their inhibition of RNA replication by DENV polymerase and incorporation by POLRMT. Herein we report the results of our studies on the structure-activity relationship (SAR) of uridine-based nucleotide analogs.

2′-*C*-Substituted uridine analogs were synthesized using a route described in the literature.[19] Dess-Martin oxidation of **5** gave 2-keto ribofuranose **6**, which was treated with the corresponding Grignard reagent to provide **7**. After conversion of the 2′-OH to its benzoate **8**, the sugar was coupled with uracil under Vorbrüggen glycosylation conditions, followed by deprotection of the benzoate to afford the 2′-*C*-substituted uridine analogs **10** (Scheme 1).

Synthesis of 2′-*C*-ethynyl/propynyl-2′-F-uridine analogs began with uridine **11** by applying the methods to introduce 2′-F reported in the literature.[20] Selective protection of 3′,5′-hydroxy of uridine **11** and oxidation of 2′-OH of **12** gave the 2′-keto intermediate **13**. This intermediate was reacted by the alkynyl Grignard reagents to provide 2′-*C*-alkynylarabino derivative **14**. After change of the protective group, nucleophilic fluorination of the *C*-2′ tertiary alcohol **16** with (diethylamino)sulfur trifluoride (DAST) gave the desired 2′-F intermediate **17**, which was treated with ammonia to afford the 2′-*C*-ethynyl/propynyl-2′-F-uridine analogs **18** (Scheme 2).

Synthesis of 2′-*C*-Substituted-4′-F-uridine analogs started with the 2′-*C*-ethynyl/propynyl-uridines **10**, which were converted to the 4′,5′-olefins **19**. Iodofluorination of **19** using *N*-iodosuccinimide (NIS) and 3HF·Et_3N yielded 4′-F-5′-iodo intermediates **20**. Benzoylation of **20** followed by oxidative hydrolysis or displacement of

Table 1
Anti-dengue activity of the uridine-based nucleoside phosphoramidate prodrugs and inhibition of DENV RdRp and selectivity over POLRMT by their triphosphates.



Phopsphoramidate	Y	R	X	h-PBMC DENV-2 EC_{50} (μM)[a]	HepG2 CC_{50} (μM)[b]	Triphosphate	RdRp IC_{50} (μM)	POLRMT SNIR (%)[d]
24[e]	H	Me	OH	0.19	>50	**26**	5.0	29.4
27	H	*	OH	0.45	>50	**28**	1.6	16.8
29	H	*	OH	1.9	>50	**30**	2.0	6.7
sofosbuvir	H	Me	F	1.2	>50	**4**	18	1.8
31	H	*	F	>25	>50	**32**	15.9	2.0
33	H	*	F	>100[c]	>50	**34**	>20	2.2
35[e]	F	Me	OH	1.1	>50	**36**	6.6	5.7
37	F	*	OH	0.57	>50	**38**	0.65	3.0
39	F	*	OH	9.0	>50	**40**	2.8	1.7

[a] DENV-2 plaque assay in h-PBMC.
[b] 4 days cytotoxicity.
[c] DENV-2 replicon assay in Huh-7.
[d] POLRMT SNIR was determined at 500 μM of the tested triphosphate.
[e] Tested as phosphorous diastereomixture (*Rp*/*Sp*).