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Discovery of β-D-2′-deoxy-2′-dichlorouridine nucleotide prodrugs as potent inhibitors of hepatitis C virus replication

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ABSTRACT

Discovery of sofosbuvir has radically changed hepatitis C treatment and nucleoside/tide NS5B inhibitors are now viewed as one of the key components in combination therapies with other direct-acting antiviral agents. As part of our program to identify new nucleoside inhibitors of HCV replication, we now wish to report on the discovery of β-D-2′-deoxy-2′-dichlorouridine nucleotide prodrugs as potent inhibitors of HCV replication. Although, cytidine analogues have long been recognized to be metabolized to both cytidine and uridine triphosphates through the action of cytidine deaminase, uridine analogues are generally believed to produce exclusively uridine triphosphate. Detailed investigation of the intracellular metabolism of our newly discovered uridine prodrugs, as well as of sofosbuvir, has now revealed the formation of both uridine and cytidine triphosphates. This occurs, not only in vitro in cell lines, but also in vivo upon oral dosing to dogs.

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Hepatitis C virus (HCV) is a global health burden estimated to have established chronic infections in nearly 3% of the world's population.[1,2] Individuals infected with the virus are at risk of developing life-threatening liver diseases e.g. cirrhosis and hepatocellular carcinoma. HCV belongs to the Flaviviridae family and its genome is a positive-sense single-stranded RNA molecule that encodes ten gene products whose functions are collectively required to replicate the viral genome and assemble progeny virons in the liver of infected humans.[3] Of the HCV-encoded gene products, three have achieved clinical validation as molecular targets for small molecule mediated inhibition; the NS3 protease, the NS5A phosphoprotein, and the NS5B RNA-dependent RNA polymerase (RdRp).[4] Compounds inhibiting the functions of these proteins are termed "direct-acting antivirals" (DAAs) and have transformed treatment of HCV-infected patients away from poorly tolerated, lengthy regimens containing injected pegylated-interferon towards all-oral therapies containing DAA combinations, which offer safer, shorter, and more efficacious alternatives. Nucleoside inhibitors targeting the HCV NS5B RdRp exhibit particularly appealing antiviral characteristics in vitro, which appear intrinsic and unique to this class of HCV DAA, namely; the capacity for broad and potent inhibition of all HCV genotypes together with a high barrier to the development of resistance. Known nucleoside inhibitors of HCV include those containing 2′-*C*-modified sugar moieties that, following phosphorylation by cellular kinases to corresponding triphosphates, specifically inhibit HCV NS5B-catalyzed RNA polymerization through competitive inhibition with natural nucleotide triphosphate substrates for the enzyme's active site.[5,6] The incorporation of 2′-*C*-modified monophosphates onto 3′ termini of growing HCV RNA strands promotes termination of elongation as a result of steric hindrance between the incoming natural nucleotide and the unnatural 2′-*C*-group of the inhibitor. Phosphoramidate prodrug approaches have been applied to the anti-HCV nucleosides containing 2′-*C*-modifications to improve cellular uptake and intracellular activation. This strategy likely bypasses the often rate-limiting first phosphorylation step by delivering the monophosphate form of the nucleoside analogue directly to cells, which can then be efficiently converted to the active triphosphate. Such an approach was successfully employed to discover a β-D-2′-α-fluoro-2′-β-*C*-methyluridine nucleotide monophosphate prodrug termed sofosbuvir **1**, that produces the triphosphate **2** (Fig. 1) and is so far the only nucleotide-based inhibitor approved for the treatment of HCV.

As a part of our continuous efforts to discover novel HCV nucleoside analogues we were interested in further explore the 2′-substitution pattern which we believed could potentially lead to compounds with improved potency and better genotype coverage. Several 2′-*C*-modifications have already been explored, and a few of these have reached clinical trials; TMC-649128,[7] INX-189,[8] and

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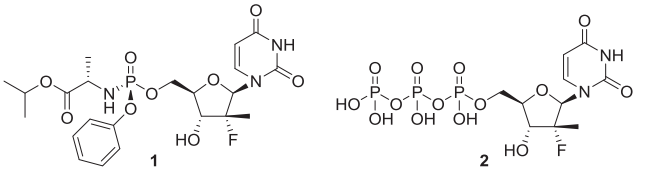


Fig. 1. Sofosbuvir (**1**) and its corresponding triphosphate (**2**).

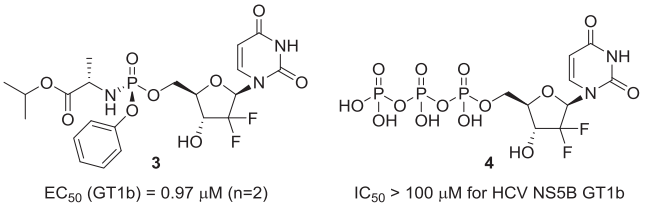
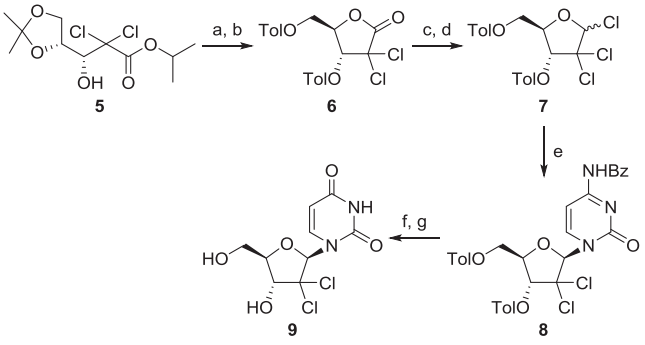


Fig. 2. 2′-Difluro-U prodrug (**3**) and its corresponding triphosphate (**4**).

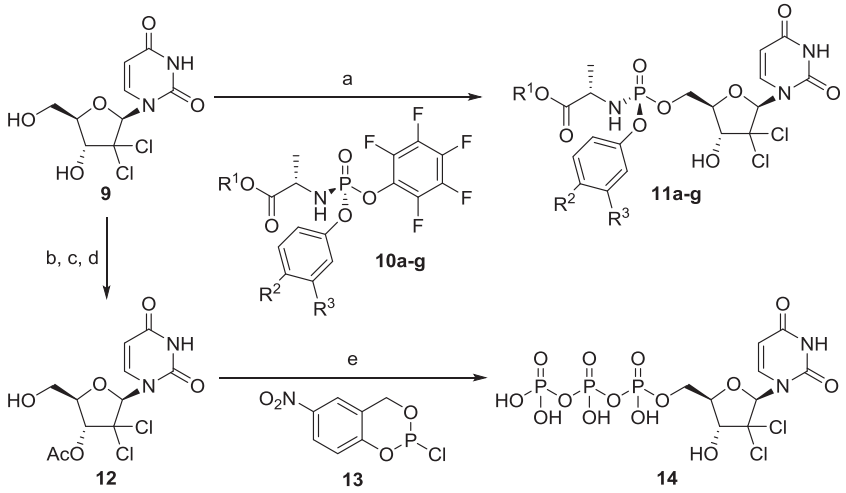


Scheme 1. Synthesis of β-D-2′-deoxy-2′-dichlorouridine. Reagents and conditions: (a) 0.12 eq. conc. HCl, 1.7 eq. water, ethanol, Δ. (b) 4-MeBzCl, Et_3N, EtOAc, Δ. (c) Li(*t*-BuO)$_3$AlH, EtOAc, 0 °C. (d) DMF, $SOCl_2$, EtOAc, Δ. (e) N4-Bz-C, HMDS, cat. $(NH_4)_2SO_4$, $SnCl_4$, PhCl, Δ. (f) AcOH/water: 7/3, Δ. (g) *n*-propylamine, MeOH.

IDX-184.[9,10] McGuigan et al. reported on the antiviral activity of 2′-deoxy-2′-difluoro-5-halouridines against a few different viruses.[11] We therefore prepared a phosphoramidate prodrug of the uridine analogue of gemcitabine (Gemzar®) **3** and its corresponding triphosphate **4** (Fig. 2). Despite observed activity against a HCV genotype 1b subgenomic replicon for compound **3**, the corresponding triphosphate **4** showed no measurable inhibition of purified HCV NS5B RdRp. Compound **3** showed consistent low μM toxicities across different cell-lines, values ranging from 2.9 μM to 7.5 μM, and compound **4** was found to strongly inhibit human DNA polymerase β. These findings are in agreement with those reported for gemcitabine activity against HCV replication.[12]

Interestingly, reports on the close analogue β-D-2′-deoxy-2′-dichloro **9** (Scheme 1.) appear to be absent. Reasoning that, the steric bulk of a chlorine is rather similar to that of a methyl group, we decided to investigate how these 2′-dichloro analogues would perform as inhibitors of HCV replication.

In order to prepare the desired nucleoside, Scheme 1 was envisaged. Compound **5** was prepared using an optimized protocol based on a literature report.[13] Condensation of readily available isopropyl trichloroacetate with a commercial solution of (*R*)-2,2-dimethyl-1,3-dioxolane-4-carbadehyde (50% in dichloromethane) using Turbo Grignard (1.3 M in THF) at −70 °C, without the addition of any other solvent, afforded **5** in 53% yield and 98% de. This crystalline intermediate was readily converted into **6** via a two-step sequence. Acid-catalyzed lactonization in ethanol and partial removal of the solvent followed by addition of toluene, afforded the free diol as a crystalline solid in 97.2% yield. This was acylated using p-toluoyl chloride in ethyl acetate with triethylamine as base. Crystallization from 2-propanol/dichloromethane: 9/1 afforded **6** in 89% yield. Reduction of the lactone in ethyl acetate yielded after extractive work-up, the intermediate lactol. The crude material was converted into chloride **7** using DMF/thionyl chloride in ethyl acetate. The reaction mixture was quenched with sodium bicarbonate solution, followed by extractive work-up and solvent evaporation to give a crude product that was taken into the following step without any further purification. Glycosylation with N^4-Bz-cytosine afforded a 1:1 mixture of the α and β anomers. The desired β-anomer **8** was isolated by crystallization from ethanol in 36% yield. The N^4-Bz-cytosine moiety was converted into uracil through treatment with hot acetic acid/water mixture and the product was precipitated upon cooling in 85% yield and 98.6% purity by HPLC. Finally, removal of the p-toluoyl protective groups, followed by precipitation from isopropyl acetate afforded compound **9** (86% yield) in a totally chromatography free sequence. Nucleoside **9** showed no inhibition (EC50 > 50 μM) of HCV replication (GT1b) in cell assay and no toxicity (CC50 > 100 μM) across different cell-lines. Phosphorylation of **9** using reagent **10a** (R1 = iPr; R2 = R3 = H),[14] provided direct access to compound **11a** as a single diastereomer (Scheme 2).



Scheme 2. Preparation of monophosphate prodrugs and corresponding triphosphate of β-D-2′-deoxy-2′-dichlorouridine. Reagents and conditions: (a) *t*-BuMgCl, DMPU, THF, −5 °C. (b) MMTrCl, py. (c) Ac_2O, py. (d) 80% AcOH, Δ. (e) Et_3N, Oxone, ACN/DCM: 2/1, water, followed by TBAPP, DMF and NH_4OH, water.

Table 1
HCV Huh7 GT1b replicon data.

Compd.	R^1	R^2	R^3	EC_{50} (μM)	CC_{50} (μM)
11a	*i*Pr	H	H	0.056	>100
11b	Cy	H	H	0.16	>100
11c	(*R*)-s-Bu	H	H	0.11	>100
11d	(*S*)-s-Bu	H	H	0.083	>100
11e	*i*Pr	H	Cyclobutyl	0.22	>100
11f	*i*Pr	H	1-Me-cyclopropyl	0.23	>100
11g	*i*Pr	1-Me-cyclopropyl	H	0.34	62

Table 2
Prodrug stability in human intestinal and liver extracts and human whole blood.

Compd.	Intestinal S9 $t_{1/2}$ (min)	Liver S9 $t_{1/2}$ (min)	Whole blood $t_{1/2}$ (h)
11a	1380	43	27
11b	70	3	20
11c	140	8	11
11d	140	7	11
11e	20	3	8
11f	20	4	12
11g	60	10	8

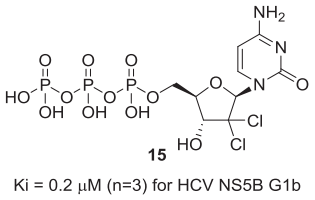


Fig. 3. The cytidine triphosphate (**15**) originating from **11a**.

Compound **9** was also converted into **12** through a three-step, one-pot sequence in 68% overall yield. Reaction of **12** with reagent **13**,[15] afforded an intermediate that was directly converted into the corresponding triphosphate **14** that was isolated as its lithium salt. Mean Ki of triphosphate **14** for purified HCV NS5B GT1b RdRp was 1.9 μM (n = 6), while no inhibition of the human DNA polymerases α, β and γ could be observed at 200 μM. The lack of cellular activity of nucleoside **9**, despite its triphosphate being an effective inhibitor of HCV RNA polymerase, clearly demonstrated the necessity of monophosphate prodrugs to bypass the first phosphorylation step as shown previously for sofosbuvir. Compound **11a** inhibited HCV replication with a mean EC50 of 0.056 μM (n = 33) on the cell-based replicon assay (GT1b). Similar analogues **11b-g** were also prepared[16] and these compounds showed consistent inhibition of HCV replication (Table 1). Of note, the analogue of **11a** prepared from unnatural (D)-alanine[17] was >100 fold less potent than **11a**. Compound **11a** also showed a lower susceptibility for the S282T mutation in a replicon assay setting when compared to sofosbuvir (**1**), 4.1 and 7.8-fold change in relation to parental GT1b respectively. Moreover, **11a** did show a consistently better genotype coverage than sofosbuvir; GT1a EC50 = 0.070 vs. 0.17 μM, GT2a EC50 = 0.018 vs. 0.050 μM, GT2a (virus) EC50 = 0.022 vs. 0.054 μM, GT3a EC50 = 0.068 vs. 0.13 μM, GT4a EC50 = 0.066 vs. 0.23 μM, GT5a EC50 = 0.053 vs. 0.12 μM and GT6a EC50 = 0.069 vs. 0.18 μM. Interestingly, despite **11a** being more potent than sofosbuvir (**1**) in the cell-based HCV replicon assay, its triphosphate (**14**) showed a higher Ki value for the HCV NS5B RdRp GT1b polymerase, when compared to **2** (Fig. 1).

Compounds **11a–g** were also screened for relevant in vitro stabilities, such as human intestinal and liver S9 extracts, as well as human whole blood (Table 2). All compounds proved stable in human whole blood and relatively unstable in human liver extracts. This is a desirable profile for prodrugs aiming for the treatment of HCV infection, since viral replication occurs in hepatocytes. Another important optimization factor was the stability of the compounds in the intestinal tract. Intestinal stability is desirable, to allow the compound to be effectively absorbed.

Analysis of the compound activities and stabilities, revealed **11a** as the compound with the most favorable profile. Incubation of **11a** in fresh human hepatocytes confirmed the formation of the active anti-HCV species, triphosphate **14**. Unexpectedly, the analysis of potentially active metabolites following incubation, also revealed high levels of the corresponding cytidine triphosphate **15** (Fig. 3) originating from **11a**, a phosphoramidate prodrug of an uridine analogue.

Cytidine triphosphate (**15**) is a more potent inhibitor of the HCV NS5B RdRp polymerase than the corresponding uridine triphosphate (**14**). This likely accounts for the discrepancy observed when comparing the activities of **2** and **14** in the HCV polymerase assay, with the potency of **1** and **11a** in the cell-based replicon assay. Fig. 4 illustrates the formation of **2** and the corresponding cytidine

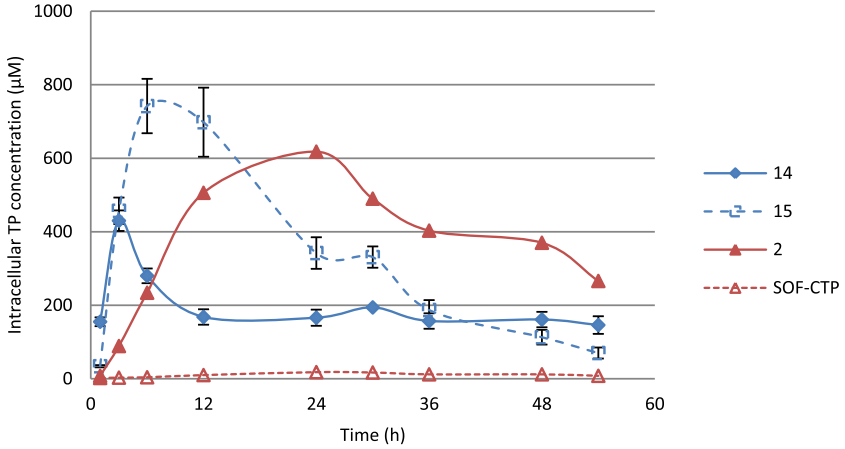


Fig. 4. Time course of uridine and cytidine triphosphate levels in primary human hepatocytes after extracellular incubation with 10 μM of **11a** and sofosbuvir (**1**).

triphosphate upon incubation of primary human hepatocytes with **1**, and formation of **14** and **15** upon incubation with **11a**.

As it can be seen from Fig. 4, both **1** and **11a** give rise to uridine and cytidine triphosphates, although formation of the cytidine metabolite is significantly higher in the case of **11a**. Moreover, we have seen that this transformation is not only observed in studies using cell lines, it was also observed after in vivo administration of **11a**. We have identified cytidine triphosphate (**15**) in dog livers after a four-day oral dosing of 50 mg/kg/day with **11a**. The dog liver exposures of triphosphate metabolites 4 h post-dosing at day 4 were of 3.5 μM and 0.93 μM for **14** and **15**, respectively. To our knowledge, this is the first report demonstrating intracellular generation of cytidine triphosphates following exposure of cultured cells, and dogs, to phosphoramidate uridine analogues. The exact mechanisms through which this phenomenon occurs are not known at present but it's possible that some uridine triphosphate analogues serve as substrates for cytidine synthetase; an enzyme involved in pyrimidine biosynthesis that interconverts uridine and cytidine triphosphates. To conclude, the findings described in the present study reveal that phosphoramidate uridine analogues can be converted into potent cytidine triphosphates, which may contribute to antiviral efficacy. Further, our data also clearly identify a need to include cytidine metabolites in the safety profiling of novel uridine analogues.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.05.075.

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