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The application of phosphoramidate ProTide technology to the potent anti-HCV compound 4′-azidocytidine (R1479)

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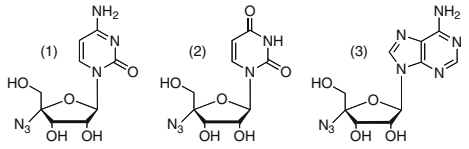
ABSTRACT

We report the design, synthesis and evaluation of a family of ca 50 phosphoramidate ProTides of the potent anti-HCV compound 4′-azidocytidine (R1479), with variation on the ester, amino acid and aryl moiety of the ProTide. Sub-μM inhibitors of HCV emerge. The compounds are all non-cytotoxic in the replicon assay. We herein report detailed SARs for each of the regions of the ProTide.

The discovery of new inhibitors of the hepatitis C virus (HCV) is a major current activity of both the pharmaceutical industry and academia. The Roche group have previously reported that various 4′-substituted ribonucleosides were endowed with potent and selective inhibitory activity against HCV[1] with 4′-azidocytidine (R1479) (**1**) emerging as a promising clinical candidate.[2]

As with the majority of nucleoside therapeutics (**1**) requires intracellular phosphorylation to its 5′-triphosphate form to interact at its viral (RNA polymerase) target.[2] As HCV lacks any suitable nucleoside kinase expression, the necessary phosphorylation steps must be host kinase mediated. Since such phosphorylations may be inefficient for modified nucleoside analogues a number of laboratories have developed phosphate pro-drug methods to allow the direct use of the masked monophosphate.[3] The Cardiff group first introduced phosphoramidate ProTides in 1996[4] and this motif has been reviewed by us[5] and now adopted for example by Gilead for phosphonate delivery[6] and Pharmasset for their 2′-modified anti-HCV family.[7] In a collaboration between the Cardiff and Roche laboratories we have previously reported the significant enhancement in the potency of the uridine analogue of **1**, compound **2** by the application of phosphoramidate ProTide methods.[8] Indeed, the triphosphate of **2** is a similarly potent inhibitor of HCV RNA polymerase as the triphosphate of **1**[2,8] even though the parent nucleoside (**2**) is inactive versus the virus; presumably due to poor phosphorylation. Thus, the ProTide method boosted **2** from inactive to sub-μM potency.[8] We subsequently noted a similar effect on the purine analogue 4′-azidoadenosine (**3**).[9]



Given the clinical progression of **1** as its ester pro-drug[10] we were interested to consider whether the antiviral activity of **1** might be further enhanced by our ProTide methodology and we herein report the early results of this study.

The synthesis of **1** was achieved entirely as has been recently reported,[11] involving the stereo- and regio-selective addition of TMS-azide to the appropriate 4′,5′-olefin related to **1**. We prepared a family of 5′-ProTides of **1** using phosphorochloridate chemistries we have extensively reported.[4,8,9]

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In the first instance, the synthesis of phosphoramidates was carried out in the presence of **1** with phosphorochloridate prepared from phenyl phosphorochloridates and amino acid ester hydrochlorides[4] in THF in the presence of *t*BuMgCl followed by repeated column chromatography gave the initial family of phenyl aminoacyl ProTides in low to moderate yield (Table 1, route 1).[12] Thirty phosphoramidates were synthesized in this way with problems of purification and solubility of the starting material (**1**) leading to low yields. In order to overcome this problem and considering our previous work, the phosphoramidate synthesis was performed with 2′/3′ protected nucleoside. In the first instance, the 2′,3-isopropylidine nucleoside was synthesized under standard conditions (Fig. 1, route 2). The synthesis of the phosphoramidates was then performed as above followed by deprotection in the presence of acetic acid (Fig. 1). In this way 10 phosphoramidates were synthesized with improvements in the purification and consequently in the overall yield. In order to further improve the yields of this synthetic pathway, the cyclopentylidine group was used as an alternative protecting group in 2′- and 3′-positions (Fig. 1, route 3).

The subsequent phosphoramidate synthesis was performed under standard condition followed by deprotection reaction in the presence of formic acid (80% v/v) at room temperature for 5 h. In this way 10 phosphoramidates were synthesized with a purification method that required only one column chromatography for each step and with the consequent improvements in the overall yield compared to routes 1 and 2.

Each of the phenyl ProTides **4a**–**at** was tested for inhibition of HCV in replicon as previously detailed with data being shown in Table 1.

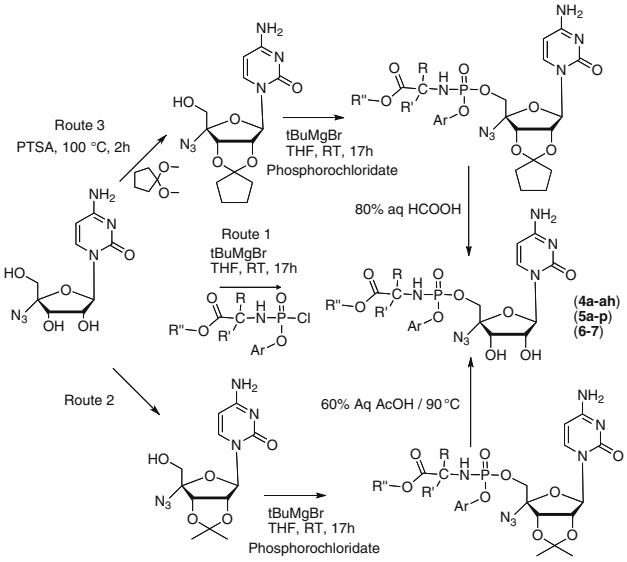

Figure 1.

Thus, the parent nucleoside (**1**) was active versus HCV replicon with a mean IC_{50} value of 1.28 μM. We have often noted the benzyl alanine phenyl ProTide to be a reasonable motif to embark with[4,8,9] and we did so on this occasion, and with some variation of the amino acid moiety.

Thus **4a**–**g** represent the benzyl esters of L-Ala, D-Ala, α,α-dimethylglycine, Gly, L-Val, L-Leu and L-Phe, respectively. Compound

Table 1
Anti-HCV activity and cytotoxicity of **4a**–**ah**

Route	Compd	Amino acid	R″	HCV replicon EC_{50}[a] (μM)	HCV replicon CC_{50} (μM)
1	**4a**	Ala	Bn	6.0	>100
1	**4b**	D-Ala	Bn	2.2	>100
1	**4c**	Me2Gly	Bn	9.2	>100
2	**4d**	Gly	Bn	2.1	>100
1	**4e**	Val	Bn	5.6	>100
1	**4f**	Leu	Bn	88% (4.2)[b]	>100
1	**4g**	Phe	Bn	89% (7.7)[b]	>100
2	**4h**	Ala	Et	0.38	>100
2	**4i**	D-Ala	Et	2.3	>100
1	**4k**	Leu	Et	11	>100
1	**4l**	Ile	Et	>100	>100
1	**4m**	Phe	Et	11	>100
1	**4n**	Met	Et	18	>100
1	**4o**	EtAsp	Et	88% (4.4)[b]	>100
1	**4p**	EtGlu	Et	74% (44)[b]	>100
1	**4q**	CycloPntGly	Et	69% (56)[b]	>100
2	**4r**	Pro	Et	>100	>100
1	**4s**	*N*-MeGly	Et	72% (48)[b]	>100
3	**4t**	BetaAla	Et	77% (47)[b]	>100
2	**4u**	Ala	*i*Pr	0.99	>100
1	**4v**	Me2Gly	*i*Pr	3.1	>100
1	**4w**	Leu	*i*Pr	>100	>100
1	**4x**	Phe	*i*Pr	2.9	>100
3	**4y**	Gly	*i*Pr	15	>100
3	**4y′**	Gly	*i*Pr	4.5	>100
2	**4z**	Ala	Me	3.1	>100
1	**4aa**	Ala	*n*Bu	11	>100
1	**4ab**	D-Ala	*n*Bu	0.94	>100
1	**4ac**	Ala	2-Bu	0.62	>100
1	**4ad**	Ala	*t*Bu	0.72	>100
1	**4ae**	D-Ala	*t*Bu	>100	>100
3	**4af**	Phe	*t*Bu	84% (6.7)[b]	>100
3	**4ag**	Ala	Ph	2.9	>100
1	**4ah**	D-Ala	C12H25	30	>100
R1479	**1**	—	—	1.28	—

[a] HCV Con 1 (GT1b) replicon inhibition. All data are means from ⩾2 experiments.
[b] Level of inhibition at top concentration (100 μM) in % (approximate IC_{50}).

4a is slightly less potent than **1** in this assay, implying a slightly lower eventual level of the triphosphate of (**1**) being delivered by (**4a)** as compared to (**1**) under the assay conditions. This is in contrast to the corresponding ProTide of (**2**), where we noted a >200-fold boost in potency.[8] This most likely relates to the relative efficiency of phosphorylation of (**1**) and (**2**) in the replicon system, with (**1**) being a good substrate for deoxycytidine kinase.[20] Although we have previously noted that D-amino acids, such as D-Ala, were poorly effective as ProTide motifs[13] we did observe that the benzyl D-Alanine derivative of (**2**) showed similar potency with only a twofold increased IC_{50} as compared to the L-analogue.[8] In the case of the cytidine analog (**1**), the D-Ala analogue (**4b**) was slightly (2.7-fold) more active as compared to the L-Ala analog (**4a**). Also, replacement of both glycine hydrogen atoms by methyl groups only led to a rather small loss of potency for the dimethylglycine compound (**4c**), with 4.2-fold as compared to the D-Ala analog (**4b**), similar to what had been seen with analogous prodrugs of (**2**).[8]

The glycine analogue (**4d**) showed similar potency as compared to the D-Ala analog (**4b**). The Valine analogue (**4e**) was similarly potent as compared to L-Ala (**4a**). Longer amino acids such as Leu (**4f**) and Phe (**4g**) could also be introduced without a large impact on apparent EC_{50}, although these compounds did not reach 90% inhibition levels under the assay conditions.

It was also of interest to explore other esters. Firstly, we prepared a series of ethyl esters of varying amino acids (**4h**–**t**). The ethyl alanine compound (**4h**) was the most potent of the series with a mean EC_{50} of 380 nM, thus 3.4-fold more potent than R1479 (**1**) and 16-fold more potent than the benzyl equivalent (**4a**). In the case of Et esters, the D-Ala analogue (**4i**) was sixfold less potent as compared to the L-Ala analogue. The introduction of bulky amino acid side chains significantly reduced potency in the Et-ester subseries; Leu (**4k**), and Phe (**4m**) showed 29-fold reduced potency as compared to the L-Ala analogue, whereas Ile (**4l**) was inactive. These results are in contrast to the case of nucleoside **2** where the Et-Phe analogue, was similarly potent as the Et-L-Ala analogue.[8] Several other amino acids were also explored as their ethyl esters. Thus, the Met (**4n**), ethyl aspartate (**4o**), and particularly ethyl glutamate (**4p**) analogues were relatively poorly active. The unusual spiro fused cyclopentyl glycine motif in **(4q**) has been found to be effective in other ProTide programmes in our laboratory, but was poorly active here, as was the Proline compound (**4r**). The poor activity of the latter could correspond to its unique cyclic structure amongst natural amino acids, or could arise from the need for a free NH. The poor activity of the *N*-methylglycine compound (**4s**) supports the latter point at least, although the Proline ring may also be detrimental. Lastly in this series we prepared a chain extended beta amino acid; beta alanine (**4t**), which was poorly active. Alpha amino acids have been considered essential by us for the putative activation mechanism of ProTides[14] and indeed early chain-extended examples were inactive when applied to the anti-HIV agent d4T.[15] Thus, it appears here too that an alpha amino acid with a free NH and a small amino acid motif is essential for potent activity.

Gilead have found success in applying our approach to acyclic nucleoside phosphonates[6] and have found particular advantage from alanines with branched aliphatic esters such as *i*Pr. For compound **2** we found that iPr esters were roughly equiactive with Et examples.[8] Thus, here we prepared iPr examples of Ala (**4u**), dimethylglycine (**4v**), Leu (**4w**), Phe (**4x**), and Gly (**4y**). In general activities fell within the range observed with the ethyl and benzyl series above. Notably, the Phe compound which showed 29-fold reduced potency as compared to L-Ala in the Et ester series had less of a detrimental effect in the benzyl and iPr series. In the case of the iPr glycine compound we observed a separation of the two diastereoisomers arising from mixed stereochemistry at the phosphate centre. Such an isomeric pair was present in every case, and roughly 1:1 as judged by ^{31}P NMR. In most cases the isomers were tested as mixtures. However, the fortuitous separation of the isomers in the case of **4y** lead to two fractions being evaluated separately. Although not fully resolved, **4y** was primarily the more polar isomer, based on reverse phase HPLC (9:1 'fast'/'slow') while **4y′** was primarily the less polar isomer (3.5:6.5 'fast'/'slow'). The 3.3-fold difference in potency noted between **4y** and **4y′** suggests a ca. sevenfold difference for the pure separate isomers, with the more lipophilic compound being more potent. In the case of two diastereomers of a ProTide of **2** we previously noted no significant difference in activity,[8] although we[16] and others[6] have seen more substantial isomers differences in some cases.

Shortening (**4z**) and linear extension (**4aa**–**ac**) of the ester chain was also explored. The activities and SARs noted were rather similar to those captured above, although the *n*-butyl case was anomalous, with the D-Ala example (**4ab**) being unusually more active than the L-compound (**4aa**).

The *t*-Butyl case was even more intriguing. Historically, we have generally observed a reduction, and in some cases a loss, of activity for *t*-Bu esters,[17] this being attributed to poor hydrolysis of these systems in vitro. In the case of ProTides of (**2**) we did see activity for a *t*-Bu ester,[8] although it was a log less active than the benzyl lead. In the case of (**1**), the *t*BuAla compound (**4ad**) was surprisingly similarly potent as compared to the Et-Ala lead, while the *t*Bu-D-Ala analogue (**4ae**) was inactive, as was the Phe compound (**4af**), thus differing from the SAR in the Et ester series. This is amongst the most striking and specific example of the apparent

Table 2
Anti-HCV activity and cytotoxicity of **5a**–**p**

Route	Compd	Ph subst	Amino acid	R″	HCV replicon EC_{50} (μM)	HCV replicon CC_{50} (μM)
3	**5a**	*o*-Cl	Ala	Bn	3.5	>100
2	**5b**	*m*-Cl	Ala	Bn	3.7	>100
1	**5c**	*p*-Cl	Ala	Bn	6.2	>100
3	**5d**	*o*-OMe	Ala	Bn	2.3	>100
3	**5e**	*m*-OMe	Ala	Bn	8.8	>100
1	**5f**	*p*-OMe	Ala	Bn	>100	>100
2	**5g**	*o*-Me	Ala	Bn	1.3	>100
3	**5h**	*p*-Me	Ala	Bn	0.51	>100
3	**5i**	*p*-Br	Ala	Bn	6.5	>100
2	**5j**	3,4-Cl_2	Ala	Bn	9.2	>100
2	**5k**	*p*-Me	Ala	Et	2.9	>100
3	**5l**	2,6-(OMe)	Ala	Et	4.1	>100
1	**5m**	*p*-Cl	Leu	Et	2.5	>100
1	**5n**	*p*-OMe	Leu	Et	9.3	>100
1	**5o**	*p*-Me	Leu	Et	4.3	>100
1	**5p**	3,4-Cl_2	Leu	Et	2.3	>100

Table 3
Anti-HCV activity and cytotoxicity of **6**–**7**

Route	Compd	Nap	Isomer	HCV replicon EC_{50} (μM)	HCV replicon CC_{50} (μM)
3	**6**	1-Nap	Polar	0.95	>100
3	**6′**	1-Nap	Non-polar	1.2	>100
1	**7**	2-Nap	Polar	4.3	>100
1	**7′**	2-Nap	Non-polar	2.9	>100

interplay between ester and amino acid motifs in ProTide SAR that we have observed.

In the case of ProTides of the anti-HIV agent d4T we have previously studied the effect of substitutions in the phenyl moiety,[18] and noted the beneficial effect of a *p*-Cl group for example. Thus we pursued Ph substitution in the present case. The same synthetic access was used as for the parent Ph systems **4a**–**ah** but it was now necessary to prepare the appropriate substituted phenyl phosphorodichloridates by reaction of the corresponding phenol and $POCl_3$ in inert solvent. The dichloridates were assayed by ^{31}P NMR and used without purification in most cases. Thus, as noted in Table 2, we explored a number of *o*-, *m*-, and *p*-mono- and di- substitutions on the Ph ring, with electron-withdrawing and donating groups, and examples in the BnAla, EtAla and EtLeu series.

Overall, phenyl substitution did not have a great affect on potency in this series, although **5h** did emerge as slightly more active than the parent nucleoside **1** (ca threefold) and its parent ProTide **4a** (ca 10-fold).

We previously noted a boost in efficacy for anti-cancer ProTides of BVDU[19] and also for the anti-HCV activity of ProTides of (**2**) on substitution of the phenyl moiety entirely by a 1-naphthyl unit. Thus, we prepared the 1-naphthyl analogue of (**4a**), using the phosphorochloridate prepared from naphthyl phosphorodichloridate and benzylalanine hydrochloride. The product was separated into its polar (**6**) and non-polar (**6′**) diastereomers by reverse phase HPLC. The compounds were essentially pure by ^{31}P NMR, with **6** resonating upfield of **6′** (δ_P 3.75, 3.87). We also prepared the corresponding 2-naphthyl analogue and also resolved it into its separate isomers **7** and **7′**. Data on the naphthyl family are presented in Table 3.

As we note in Table 2, the 1-naphthyl compound showed ~6-fold improved potency as compared to the parent Phenyl BnAla (**4a**) compound, with no significant difference in potency between the two diastereomers **6** and **6′**. By contrast, the 2-naphthyl compounds (**7** and **7′**) were slightly less potent (3–5-fold).

In conclusion, we herein report the application of the phosphoramidate ProTide method to the potent anti-HCV agent 4′-azidocytidine (**1**). Approximately 50 new compounds were prepared, with variations in the ester, amino acid, and aryl moiety. Structure activity relationships support the notion that an α-amino acid is essential for significant activity and a free NH on the amino acid also seems important. The ester moiety can be varied significantly. Interestingly, in this study *t*-butyl is an acceptable ester in the case of L-alanine, but not other amino acids. Small amino acids were best tolerated; L and (in most cases) D-alanine, and glycine. Larger and branched amino acids were generally less or poorly effective. Substitution in the phenyl ring was tolerated by a variety of substituents at various positions, though no substantial enhancements in potency were seen. 1-Naphthyl provided a small benefit over phenyl, whereas 2-naphthyl could substitute with no loss of potency. Lastly, in three cases the phosphate diastereoisomers were resolved and tested separately in replicon assay, and little difference in potency noted between the stereoisomers.

Many of the ProTides herein described retained or slightly (up to 3–4 fold) enhanced the potency of **1** in replicon, but the true potential of such ProTides is only likely to become apparent with further in vitro and in vivo study. For example, it is notable that the lead agent **5h** has a calculated lipophilicity (Clog *P*, ChemDraw Ultra 11.0) of 1.2 versus −1.9 for parent nucleoside **1**; the almost 3-log lipophilicity boost may well lead to more efficient passive diffusion into cells and independence from nucleoside transporters, which could convey significant in vivo advantage. Nucleoside deaminase resistance is also likely for these ProTides, based on prior examples.[21] Also, notably two cytidine analogues for HCV, NM 283 and R1626, have recently shown efficacy in HIV infected subjects, but have been withdrawn due to safety reasons.[22] Potential targeting of these ProTides to liver is quite likely based on recent data on some of our 2′-modified nucleoside ProTides,[23] and this could well lead to enhanced clinical safety profiles for such agents. As a sub-μM lead, the *p*-MePh analogue **5h** may represent a useful point of departure.

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12. *Synthesis of 4′-Azidocytidine-5′-O- [phenyl-(benzyloxy-L-alaninyl)]-phosphate* [**4a**] ^{t}BuMgCl (4.11 mL, 1 M solution in THF, 4.11 mmol) and 4′-azidocytidine (500 mg, 1.643 mmol) were dissolved in dry THF (15 mL) and stirred for 15 min. Then phenyl(benzyloxy-L-alaninyl)-phosphorochloridate (4.11 mmol, 4.11 mL, 1 M in THF) was added dropwise at RT and stirred at room temperature overnight. Then a solution of sat. NH_4Cl was added to quench the reaction. The solvent was removed under reduced pressure to yield a yellow solid which was purified by repeat column chromatography, using 10–20% MeOH/DCM eluent gradients each time, then a preparative TLC using a DCM/MeOH (9:1) solvent mixture. The obtained pure product was a white solid (49 mg, 5%). ^{31}P NMR (121.5 MHz, MeOH-d_4): δ_P 4.70, 4.49; ^{1}H NMR (300 MHz, MeOH-d_4): δ_H 7.65–7.58 (1H, m, H-6), 7.36–7.34 (7H, m, Ph-*CH*), 7.26–7.19 (3H, m, Ph-*CH*), 6.20–6.13 (1H, dd, J = 4.7 and 14.3 Hz, H-1′), 5.92–5.85 (1H, m, H-5), 5.20 (2H, s, Ph–CH_2), 4.37–4.29 (2H, m, H-2′ and H-3′), 4.23–4.11 (2H, m, H-5′), 4.01 (1H, m, Ala-CH), 1.41–1.25 (3H, m, Ala-CH_3). ^{13}C NMR (75.5 MHz, MeOH-d_4): δ_C 174.94, 174.88, 174.62, 174.56 (C=O), 167.62 (C-4), 158.34 (C-2), 152.05, 151.97 (Ph-C), 143.12, 142.93 (C-6), 137.41, 137.25 (Ar-C), 130.96, 130.17, 130.31, 130.171 (Ar-C), 129.66, 129.59, 129.41, 129.36, 129.29 (Ar-C), 126.44 (Ar-C), 124.34 (Ar-C), 123.92 (Ar-C) 121.66, 121.60, 121.47, 121.41 (Ar-C), 121.28, 121.22 (Ar-C), 98.83, 98.72, 98.60 (C-5), 97.04 (C-4′), 93.87, 93.42 (C-1′), 74.57, 74.37 (C-3′), 73.50 (C-2′), 68.82, 68.75 (Bn-CH_2), 68.12, 67.74 (Ala-CH), 20.52, 20.43, 20.30, 20.20 (Ala-CH_3).
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