An investigation into the anti-HIV activity of 2′,3′-didehydro-2′,3′-dideoxyuridine (d4U) and 2′,3′-dideoxyuridine (ddU) phosphoramidate 'ProTide' derivatives†

Youcef Mehellou,[a] Jan Balzarini[b] and Christopher McGuigan*[a]

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As part of our studies on the anti-HIV activities of 2′,3′-didehydro-2′,3′-dideoxyuridine (d4U), 2′,3′-dideoxyuridine (ddU) and their 'ProTides', we have prepared and evaluated the anti-HIV activity of a range of d4U and ddU aryl triester phosphoramidates. Besides elucidating SAR characteristics, we performed molecular modelling studies on both d4U and ddU in order to probe the first phosphorylation step required for the activation of these two nucleoside analogues. Overall, the application of the phosphoramidate approach turned the inactive ddU to a moderately active anti-HIV agent, while this was not the case with d4U. Enzymatic assays investigating the metabolism of d4U phosphoramidates revealed an efficient cleavage of the phosphoramidate motif to release the d4U monophosphate. Thus, a poor second and/or third phosphorylation step may be the most likely reason for the virtual lack of anti-HIV activity in this case.

Introduction

Human immunodeficiency virus (HIV), the etiologic cause of AIDS,[1,2] is still one of the most fatal infections, and responsible for killing millions of people worldwide. Currently dideoxynucleoside analogues form a major class of drugs that are approved for combating this infection.[3] Such agents are structurally characterised by lacking the 3′-hydroxyl group, which gives them the ability to be used as a substrate by HIV reverse transcriptase (HIV-RT) resulting in DNA chain termination after being converted to their corresponding 5′-triphosphates by cellular enzymes.[4,5] However, the emergence of resistance and toxicity as well as dependence on host nucleoside kinase-mediated activation to generate the bioactive phosphates, have limited the value of these drugs. This highlights the need for new, potent anti-HIV drugs with acceptable toxicity profiles. Encouraged by the success of 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) as an anti-HIV agent, we initiated a study aimed at investigating the anti-HIV activity of 2′,3′-didehydro-2′,3′-dideoxyuridine (d4U), which is structurally similar to d4T, as well as its 2′,3′-dideoxy analogue, 2′,3′-dideoxyuridine (ddU, Fig. 1).

Both of these nucleoside analogues, d4U and ddU, have been found to possess poor anti-HIV activity.[6,7] In this study, we investigated whether or not the first phosphorylation step is behind the poor anti-HIV activity seen with these agents. We accomplished this *via* two different strategies. Firstly, we performed the docking of both d4U and ddU into the human thymidine kinase (TK-1), which has been shown to be responsible for the phosphorylation of several clinically approved anti-HIV drugs, and compared their docking to that of thymidine. We also prepared a series of d4U and ddU 'ProTides' in order to bypass the first phosphorylation step, which out of the three phosphorylations required for the activation of nucleoside analogues, is often considered to be the bottleneck for activation of such compounds. In this 'ProTide' approach, the phosphate group is masked to improve the poor membrane permeability seen with the free (charged) nucleotides and the inherent susceptibility to dephosphorylation. Upon entering the cell, the group masking the phosphate moiety may undergo enzymatic metabolism to release the corresponding nucleoside monophosphate, which may be subsequently phosphorylated by human kinases into the di- and triphosphates.[6]

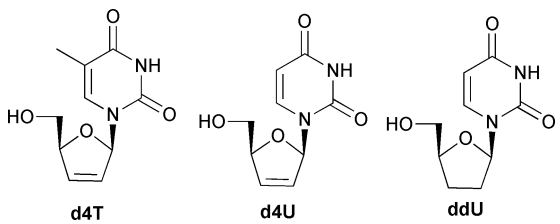

Fig. 1 Chemical structure of d4T (an FDA-approved anti-HIV drug), d4U and ddU.

In this work, we will report both the results of the molecular modelling studies as well as the effect of the 'ProTide' approach on the anti-HIV activity of d4U and ddU. In addition, we will report on the results of the enzymatic metabolic assays carried out to investigate the efficiency of the d4U monophosphate release from the ProTide.

Results and discussion

1. Docking

The fact that d4U and ddU exerted poor anti-HIV activities has led us to hypothesise that this could be due to the failure of the host cell

[a]*Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff, UK CF10 3NB. E-mail: mcguigan@cf.ac.uk; Fax: +44 2920874537; Tel: +44 2920874537*
[b]*Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000, Leuven, Belgium*

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thymidine kinase to efficiently phosphorylate these two nucleoside analogues to their 5′-mono phosphate derivatives. This hypothesis was more of a conceivable explanation after the observations by Hao *et al.*,[7] that 2′,3′-dideoxyuridine (ddU) was a poor inhibitor of HIV, while its triphosphate derivative was found to possess potent activity *versus* HIV reverse transcriptase (K_i, 0.05 μM). Working upon our hypothesis, we carried out some molecular modelling studies investigating the first phosphorylation step required for the activation of d4U and ddU. Thus, both 2′,3′-didehydro-2′,3′-dideoxyuridine (d4U) and 2′,3′-dideoxyuridine (ddU) were docked (see experimental) separately into the active site of thymidine kinase (human TK-1) and compared with the natural substrate thymidine. The resulting structures for the two nucleoside analogues were ranked according to the relative position of the uracil base with the corresponding position of the thymine base in the crystal structure of thymidine, and only the results with RMSD values below 1.0 Å were further analysed. The best results (Fig. 2) showed that both d4U and ddU had their 5′-hydroxyl group placed significantly far away from the optimal (ideal) site of phosphorylation where thymidine placed its 5′-hydroxyl group, 1 Å for ddU and 2 Å for d4U. Thus, this would most likely have a negative effect on the efficiency of the first phosphorylation step and as a result decreases the chances of d4U and ddU to be further phosphorylated to their di- and (potentially active) triphosphate derivatives. In addition, the conformations of the sugars of ddU and thymidine were different. Indeed, ddU adopted a *North* conformation while the natural substrate, thymidine, adopted a *South* conformation. The sugar conformation of nucleoside analogues is well documented to be crucial for the efficiency of phosphorylation,[8–10] as the non-preferred sugar conformation for kinases may result in reduced efficiency of the phosphorylation process or even prevent phosphorylation at all. Overall, the reasons presented discussed above led us to hypothesize that bypassing the first phosphorylation step, using the phosphoramidate ProTide approach, might improve the biological activity of d4U and ddU.

2. D4U and ddU phosphoramidates

Since the docking results (Fig. 2) suggested the first phosphorylation might be responsible for the poor anti-HIV activity of both d4U and ddU, we decided to synthesise d4U and ddU and then apply to them the 'ProTide' approach in order to bypass the first phosphorylation step. A series of d4U and ddU phosphoramidates were prepared bearing different ester and aryl moieties, while the amino acid was kept as L-alanine. Phosphoramidates bearing L-alanine were found to be effective in improving the biological activity of different nucleoside analogues, particularly d4T,[11] as were phosphoramidates with either phenyl or naphthyl as their aryl moieties.[12] Thus, we chose to make phosphoramidate derivatives of both d4U and ddU with different amino acids, mainly L-alanine, and two aryl moieties of phenyl and 1-naphthyl.

D4U can be synthesised *via* different routes.[13,14] In this study, it was prepared *via* the same procedure adopted by McGuigan *et al.*[15] (see ESI†), which was originally described by Horwitz *et al.*[13] Thus, 2′-deoxyuridine was firstly 3′,5′-dimesylated and then reacted with aqueous sodium hydroxide in 52% yield, which was followed by treating the product with sodium hydride in DMF to give the desired product (d4U) in a moderate yield, 43%. Hydrogenation of d4U in the presence of palladium on activated carbon gave ddU in a 69% yield.

D4U and ddU phosphoramidates were synthesised according to the previously reported synthetic routes developed by McGuigan *et al.*[15–18] (Scheme 1). Aryl phosphochloridates were prepared by the reaction of phenyl/naphthyl dichlorophosphates with the appropriate amino acid ester hydrochloride. The obtained phosphochloridates were allowed to react with d4U or ddU in THF and ^{t}BuMgCl to give target phosphoramidates in moderate yields. ^{31}P NMR investigations of the phosphoramidates displayed two closely spaced signals, corresponding to two diastereoisomers resulting from mixed phosphate stereochemistry. All the phosphoramidate samples presented in this work (Table 1) were further investigated as a 1 : 1 mixture of phosphate diastereoisomers, unless stated otherwise (see experimental and ESI†).

The parent nucleoside d4U and its 'ProTide' derivatives were evaluated for their activity against HIV-1 and HIV-2. The biological data revealed that neither the parent compound nor any of its phosphoramidates possessed significant anti-HIV activity (Table 2). Generally, all the tested compounds were shown to have low, if any, toxicity.

The failure of d4U phosphoramidates to exert significant anti-HIV activity could be attributed to three different reasons. Firstly, the inability of d4U 'ProTides' to deliver d4U monophosphate. Secondly, the failure of d4U-monophosphate to be further phosphorylated to its di- and triphosphate forms and/or thirdly, the triphosphate of d4U being inactive as an inhibitor of HIV-RT. Recently, researchers from Gilead sciences reported the inhibitory

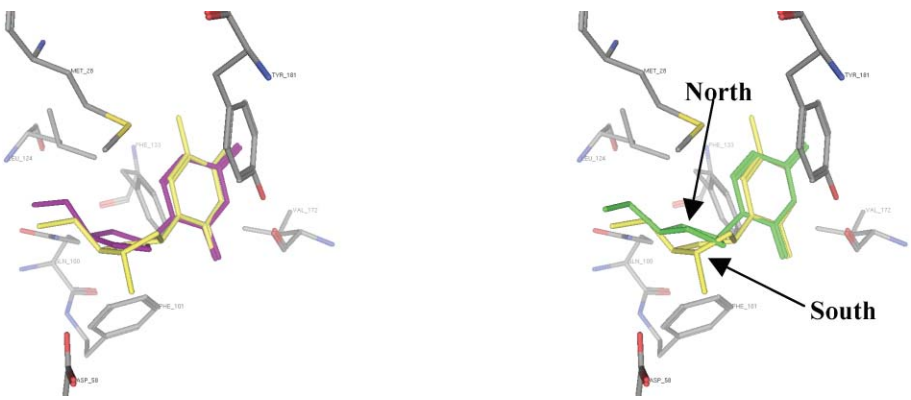
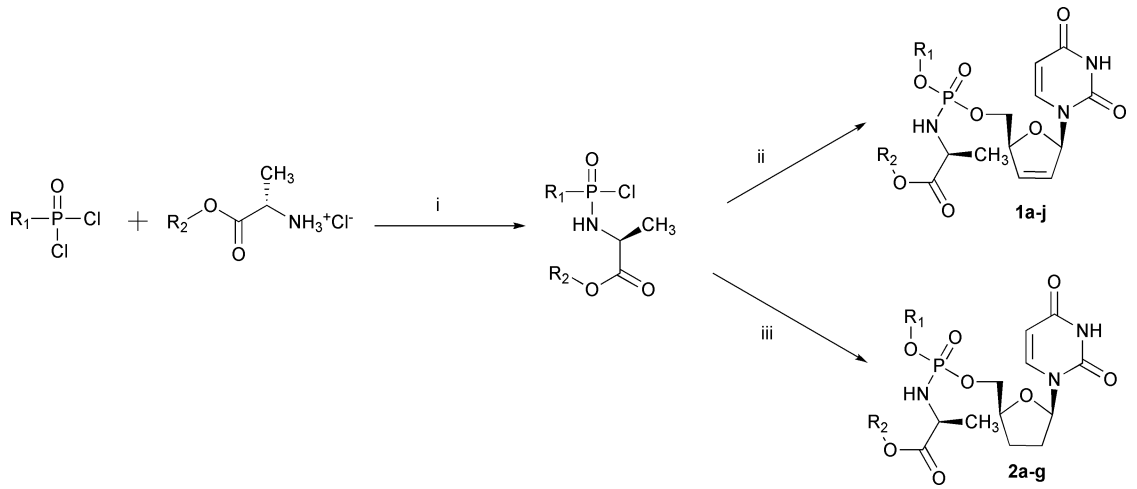


Fig. 2 Docking results of d4U (purple) and thymidine (left) as well as ddU (green) and thymidine (right).

Table 1 Structures of d4U and ddU phosphoramidates

Compound	R_1	R_2	Compound	R_1	R_2
1a	Phenyl	Methyl	**2a**	Phenyl	Methyl
1b	1-Naphthyl	Methyl	**2b**	1-Naphthyl	Methyl
1c	Phenyl	Ethyl	**2c**	Phenyl	Ethyl
1d	1-Naphthyl	Ethyl	**2d**	Phenyl	*t*Butyl
1e	Phenyl	^{i}Propyl	**2e**	Phenyl	Benzyl
1f	1-Naphthyl	^{i}Propyl	**2f**	1-Naphthyl	Benzyl
1g	Phenyl	*t*Butyl	**2g**	4-Methoxy-1-naphthyl	Benzyl
1h	1-Naphthyl	*t*Butyl	—	—	—
1i	Phenyl	Benzyl	—	—	—
1j	1-Naphthyl	Benzyl	—	—	—



Scheme 1 *Reagents and conditions*: (i) Et_3N, −78 °C (ii) THF, d4U, ^{t}BuMgC1, N_2, rt (iii) THF, d4U, ^{t}BuMgC1,N_2, rt.

Table 2 Anti-HIV activity and cytostatic properties of d4U and its phosphoramidates in CEM cell cultures

	EC_{50} / μM[a]		
Compound	HIV-1	HIV-2	CC_{50} / μM[b]
1a	>50	>10	97 ± 13
1b	>10	>10	17 ± 13
1c	>50	>50	212 ± 54
1d	>50	>50	214 ± 62
1e	>250	>250	>250
1f	200 ± 70	185 ± 92	>250
1g	>250	>250	>250
1h	>50	>50	102 ± 0
1i	>50	>50	106 ± 7.1
1j	ND[c]	ND[c]	ND[c]
d4U	>50	>50	207 ± 61
d4T	0.65	0.77	174

[a] EC_{50}: the effective concentration (μM) required to protect CEM cells against the cytopathogenicity of HIV by 50%. [b] CC_{50}: the cytostatic concentration (μM) required to inhibit CEM cell proliferation by 50%. [c] ND: no data.

effect of d4U triphosphate on HIV reverse transcriptase, and noted potent inhibiton of this enzyme, $IC_{50} = 0.55$ μM.[19] Thus, in this study we decided to investigate the first possibility, which is the failure of d4U phosphoramidates to be metabolised to d4U monophosphate, as will be discussed later.

By comparison, the 'ProTide' derivatives of ddU were found to exert modest anti-HIV activity despite the parent nucleoside, ddU, being inactive against HIV (Table 3). In particular the 1-naphthyl derivatives were more active than their phenyl counterparts. This indicates that a successful bypass of the first phosphorylation step can turn ddU from an inactive compound to a moderately active anti-HIV agent. Interestingly, only compound **2d**, which has a *t*butyl as an ester moiety was inert against HIV. This strongly supports phosphate delivery *via* ester cleavage as the mode of action of the other ddU ProTides.[20] Notably, ddU phosphoramidates retained their anti-HIV activity in cells deficient of thymidine kinase, thus confirming the ability of the phosphoramidates to deliver ddU monophosphate into cells. As for d4U, the tested compounds showed low, if any, cytostatic activity.

In terms of SAR, we have found the naphthyl derivatives to be more active than the phenyl counterparts. This could be attributed to the higher log*P* of the naphthyl 'ProTides' compared to those of the phenyl compounds. These higher log*P* values could translate *in vitro* to a better membrane permeability and eventually better delivery of the nucleoside analogue monophosphate. Generally, the *t*butyl ester phosphoramidates are poor substrates for ester hydrolysis as suggested by Perrone *et al.*[20] Therefore, phosphoramidates bearing *t*butyl esters are unlikely to undergo the first hydrolysis activation step required for the activation of phosphoramidates.

3. Metabolic enzymatic assay

The metabolism of aryl triester phosphoramidates is thought to proceed in four steps to eventually release the nucleoside analogue

Table 3 Anti-HIV activity and cytostatic properties of ddU and its phosphoramidates

	EC_{50} / μM[a]			CC_{50} / μM[b]
	CEM/0		CEM/TK$^-$	CEM/0
Compound	**HIV-1**	**HIV-2**	**HIV-2**	
2a	36.7 ± 11.5	≥50	40.0 ± 014.1	121 ± 1.4
2b	≥50	≥20	>250	101.9 ± 4.7
2c	43.3 ± 20.8	93.3 ± 32.1	175 ± 106	≥250
2d	>250	>250	>250	>250
2e	40.8 ± 31.7	33.2 ± 5.1	50.8 ± 41.6	96.4 ± 82
2f	15.0 ± 0.0	20.0 ± 7.1	22.5 ± 10.6	96.9 ± 0.85
2g	20.0 ± 7.1	30.0 ± 7.1	21.0 ± 8.5	90.9 ± 10.0
ddU	>250	>250	>250	>250
d4T	0.65	0.77	—	174

[a] EC_{50}: the effective concentration (μM) required to protect CEM cells against the cytopathogenicity of HIV by 50%. [b] CC_{50}: the cytostatic concentration (μM) required to inhibit CEM cell proliferation by 50%.

monophosphate (Scheme 2). The first step in this metabolic process is mediated by esterase-type activity, which cleaves the ester motif of the aryl triester phosphoramidate molecule. One enzyme responsible for this intracellular cleavage has been recently described as cathepsin A.[21]

Since we found d4U phosphoramidates to be devoid of any anti-HIV activity, we hypothesised that one of the reasons for the absence of activity was that d4U phosphoramidates did not undergo intracellular metabolism to release the nucleoside analogue monophosphate. To investigate this possibility, we conducted the enzymatic metabolism assays of d4U phosphoramidates using cathepsin A. In addition, we synthesised d4U-monophosphate to be used as a reference in this enzymatic assay.[22]

The naphthyl L-alanine benzyl ester phosphoramidate of d4U (**1j**) was incubated with cathepsin A for 12 h at room temperature, and the progress of the reaction was monitored by ^{31}P NMR. The results (Fig. 3) show that d4U phosphoramidates were metabolised primarily to the aminoacyl intermediate **iii** (δp *ca.* 6.7, Scheme 2)[23] and to some extent to the corresponding d4U-monophosphate.

To reveal whether CEM cells contain the enzymes required for hydrolysis (activation) of the prodrugs, the naphthyl L-alanine benzyl ester phosphoramidates of d4U and ddU were exposed to CEM cell extracts for 30, 60 and 120 minutes at 37 °C. The prodrugs were hydrolysed for more than 90% (d4U prodrug) or 95% (ddU prodrug) within 30 min. After 60 to 120 min, no intact parent compound remained. Since marked differences in cellular uptake of both prodrugs would be rather unlikely, and given the fact that d4U-TP has been reported to be a potent HIV-1 RT inhibitor,[7] our data suggest that the bottleneck for the eventual antiviral activity of the prodrug of d4U-MP may reside in the phosphorylation to the di- and/or triphosphate derivative.

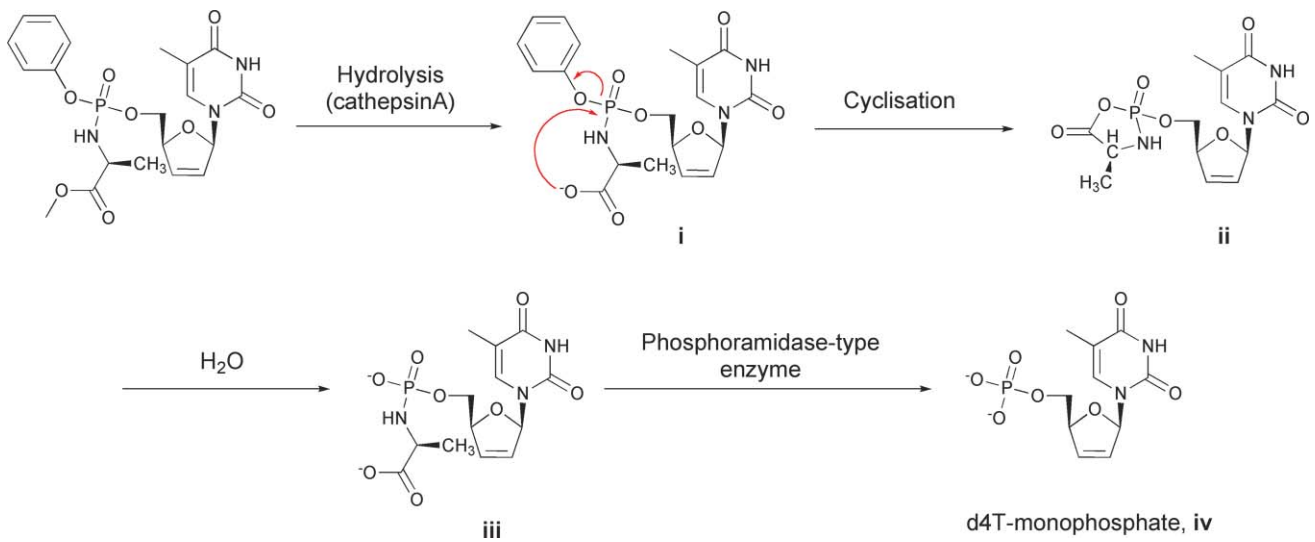
Conclusion

In conclusion, d4U and ddU are two nucleoside analogues with poor anti-HIV activity. We have shown in this study, using molecular modelling, that these two nucleoside analogues occupy a position at the active site of thymidine kinase, which is not favourable for phosphorylation. Applying the 'ProTide' approach to bypass the first phosphorylation step turned ddU from an inactive compound into a moderately active anti-HIV compound. However, this was not the case for d4U as none of its 'ProTides' showed any appreciable anti-HIV activity. Our enzymatic metabolic assays revealed that d4U phosphoramidates were metabolised to release d4U monophosphate, and since d4U triphosphate is a potent inhibitor of HIV reverse transcriptase, we concluded that d4U phosphoramidates lack potent anti-HIV activity because of poor, if any second and/or third phosphorylation steps.

Experimental

Standard procedure for phosphoramidate synthesis

*t*BuMgCl (1.3 equivalents) was added to a stirring solution of the nucleoside analogue, d4U or ddU (1 equivalent) in THF (10 mL) under nitrogen. After 15 minutes and at room temperature, the appropriate phosphochloridate (2 equivalents) was added and



Scheme 2 Postulated mechanism of action of aryl triester phosphoramidates.

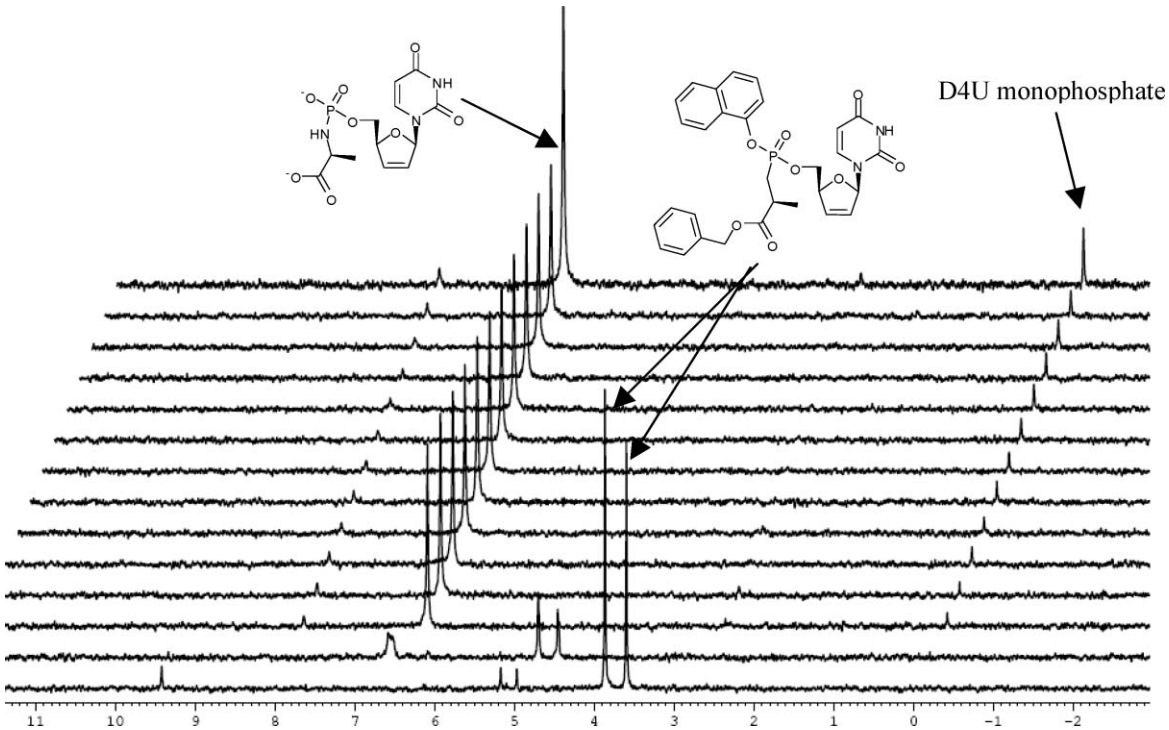


Fig. 3 The metabolism of naphthyl L-alanine benzyl ester d4U phosphoramidate in the presence of the esterase enzyme cathepsin A. (Spectra were recorded at 1 hour intervals except when stated otherwise.)

the progress of the reaction was monitored by TLC using MeOH–DCM (1 : 9) as an eluant. At the end of the reaction, the solvent was evaporated and the crude product was dissolved in DCM and purified by flash column chromatography with eluant MeOH–DCM (3 : 97). Pooling and evaporation of the appropriate fractions gave the desired phosphoramidate as a solid.

Molecular modelling

All the molecular modelling studies were performed on a RM Innovator with Pentium IV 2.8 GHz processor, running Linux Fedora Core 3 using Molecular Operating Environment (MOE) 2006.08 (Tripos Inc., 1699 South Hanley Rd, St Louis, MO 63144, USA. http://www.tripos.com) and FlexX module in SYBYL 7.2.[24] The thymidine kinase structure was downloaded from the PDB data bank (PDB code: 1kim).[25] All the minimisations were performed with MOE until an RMSD gradient of 0.05 kcal mol^{-1} $Å^{-1}$ was reached with the MMFF94x force field. Docking experiments were carried out using the FlexX docking program of SYBYL 7.2 using the default settings. The output of FlexX docking was visualised with MOE, and the scoring.svl[26] was used to identify interaction types between ligand and protein.

Enzymatic assays

5 mg of the appropriate phosphoramidate derivative was first dissolved in 200 μL of d_6-acetone and then 400 μL of TRIZMA (tris(hydroxymethyl)aminomethane) buffer solution (pH = 7.4) was added. A ^{31}P NMR was conducted at this stage to see the peaks of the phosphoramidate in d_6-acetone and TRIZMA, so that it acts as a reference ($t = 0$ h). To this mixture was added 0.3 mg of cathepsin A (carboxypeptidase Y, >50 units mg^{-1}, EC number 3.4.16.1) that had already been dissolved in 200 μL of TRIZMA buffer. ^{31}P NMR of the reaction mixture was then carried out every 30 minutes for 12 h at room temperature.

For the preparation of human T-lymphocyte extracts and stability measurements of test compounds, human T-lymphocyte CEM cells were grown in RPMI-1640 culture medium in a 1 litre culture bottle to a density of 2×10^6 cells per mL. Cells were centrifuged at 1200 rpm at 4 °C for 10 min, washed three times with cold PBS, and resuspended in 20 ml of PBS at 100×10^6 cells per mL. After 2 rounds of 6 × 20 sec sonication on ice, the cell extract was centrifuged at 13 000 rpm for 30 min at 4 °C and the supernatant divided in aliquots of 1 mL and frozen at –80 °C until use. Stability of the test compounds was performed as follows: 200 μL of cell extract were mixed with 200 μL of PBS and 200 μL of test compound (300 μM) to obtain a total volume of 600 μL (100 μM test compound). The reaction mixture was incubated for 0, 30, 60 and 120 minutes at 37 °C. At each time point, 100 μL were withdrawn, added to 200 μL cold methanol, centrifuged and the supernatant analysed for compound stability by HPLC (Reverse Phase RP-8 (Lichrocard 125–4), Merck, Darmstadt, Germany). The following gradient was used: 2 min buffer A (acetonitrile) 2% + buffer B (50 mM NaH_2PO_4 + 5 mM heptane sulfonic acid pH 3.2); 6 min linear gradient to 20% buffer A + 80% buffer B; 2 min linear gradient to 25% buffer A + 75% buffer B; 2 min linear gradient to 35% buffer A + 65% buffer B; 8 min linear gradient to 50% buffer A + 50% buffer B; 10 min same buffer mixture; 5 min linear gradient to 2% buffer A + 98% buffer B; 5 min equilibration at 2% buffer A + 98% buffer B. The naphthyl L-alanine benzyl ester phosphoramidate enantiomers of d4U and ddU had retention times of 29.2 and 29.6 min (d4U prodrug) and 29.9 and 30.2 min (ddU prodrug).

Antiretroviral evaluation

Human immunodeficiency virus type 1 (HIV-1) was originally obtained from a persistently HIV-infected H9 cell line, as described previously and was kindly provided by Dr R. C. Gallo (then at the National Institutes of Health, Bethesda, MD, USA). Virus stocks were prepared from the supernatants of HIV-1-infected MT-4 cells. HIV-2 (strain ROD) was kindly provided by Dr L. Montagnier (then at the Pasteur Institute, Paris, France), and virus stocks were prepared from the supernatants of HIV-2-infected MT-4 cells. CEM cells were obtained from the American Tissue Culture Collection (Rockville, MD, USA). CEM cells were infected with HIV as previously described. Briefly, 4×10^5 CEM cells per mL were infected with HIV-1 or HIV-2 at ~100 $CCID_{50}$ (50% cell culture infective dose) per mL of cell suspension. The thymidine kinase-deficient CEM cell cultures were also infected with HIV-2. Then, 100 µL of the infected cell suspensions were transferred into 96-well microtiter plate wells and mixed with 100 µL of the appropriate dilutions of the test compounds. After 4–5 days, giant cell formation was recorded microscopically in the HIV-infected cell cultures. The EC_{50} was defined as the compound concentration required to inhibit virus-induced cytopathicity by 50%. The CC_{50} was defined as the compound concentration required to inhibit CEM cell proliferation by 50%.

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