Application of Phosphoramidate ProTide Technology Significantly Improves Antiviral Potency of Carbocyclic Adenosine Derivatives

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We report the application of phosphoramidate pronucleotide (ProTide) technology to the antiviral agent carbocyclic L-d4A (L-Cd4A). The phenyl methyl alaninyl parent ProTide of L-Cd4A was prepared by Grignard-mediated phosphorochloridate reaction and resulted in a compound with significantly improved anti-HIV (2600-fold) and HBV activity. We describe modifications of the aryl, ester, and amino acid regions of the ProTide and how these changes affect antiviral activity and metabolic stability. Separate and distinct SARs were noted for HIV and HBV. Additionally, ProTides were prepared from the D-nucleoside D-Cd4A and the dideoxy analogues L-CddA and D-CddA. These compounds showed more modest potency improvements over the parent drug. In conclusion, the ProTide approach is highly successful when applied to L-Cd4A with potency improvements in vitro as high as 9000-fold against HIV. With a view to preclinical candidate selection we carried out metabolic stability studies using cynomolgus monkey liver and intestinal S9 fractions.

Introduction

Nucleoside analogues continue to dominate antiviral therapy and also make a significant contribution to the chemotherapy of cancer, particularly leukemia. Without exception, nucleoside analogues with such activity require phosphorylation in vivo to their active nucleotide forms. In the case of antiviral nucleosides this is almost always the 5′-triphosphate. Poor phosphorylation can be a major cause of poor activity, with several examples now known where nucleoside analogues are inactive, despite the corresponding triphosphates being inhibitors at their enzyme (polymerase, reverse transcriptase) target.[1,2] The triphosphates themselves cannot be considered to be useful drugs due to their inherent hydrolytic instability and poor membrane permeation. However, it appears that in most cases the first phosphorylation to the 5′-monophosphate is the rate-limiting step,[3] leading to the consideration of the monophosphates as chemotherapeutic agents. In fact, nucleoside monophosphates suffer from similar qualitative problems as triphosphates; instability (in this case to phosphatases and nucleotidases) and poor membrane permeation. Given these problems, and the perceived advantage of bypassing the nucleoside kinase dependence of nucleoside analogues, many groups have worked on phosphate prodrug ("ProTide") strategies.[4−6] Since 1990, we have developed a phosphoramidate strategy; initial work was on anti-retroviral AZT[a] derivatized with alkyl phosphates carrying an esterified amino acid.[7] Alanine quickly emerged as a most effective amino acid. Subsequently, we discovered aryl phosphate analogues as potent, nucleoside kinase-independent antiretrovirals.[8,9] Thus, phenyl methyl alanine phosphoramidates have emerged as general nucleotide delivery forms, known as aryloxy phosphoramidate ProTides. We have applied this motif successfully to d4T,[10] ddU,[11] 3TC,[12] ddA,[13] and d4A.[14] In the case of d4A, a 100−4000-fold boost in vitro antiviral activity was noted on application of phosphoramidate ProTide technology. Other labs have also utilized this methodology, notably Franchetti and co-workers[15] on isoddA and 8-azaisoddA and Zemlicka et al.[16] on alkene and related nucleosides. Applying our methodology to anti-herpetic BVDU gives unusual results; we found a decrease in antiviral action,[17] while the NewBiotics group reported promising anticancer action for the same compounds.[18] We have recently reported the enhancement of the in vitro profile of these agents by modifications in the phosphoramidate structure.[19]

A further issue surrounding nucleosides as drugs is the lability of the glycoside (base-sugar) bond toward phosphorylase-induced cleavage. This frequently leads to inactivation of nucleoside drugs. Moreover, as in the case of 5-fluorouracil and *E*-5-(2-bromovinyl)arabinofuranosyluracil, for example, coadministration can lead to serious toxic events.[20] Efforts to address this problem have largely led to carbocyclic nucleosides. The first of these to enter clinical use is the carbocyclic purine analogue abacavir (ABC) (**1**, Figure 1).[21−23] We have recently reported the application of phosphoramidate ProTide methods to **1** and noted a ca. 50-fold boost in anti-HIV potency and correlated this directly with a similar increase in the intracellular levels of the bioactive carbovir triphosphate.[24] A 10−20-fold boost was also noted in antihepatitis B activity for ProTides of **1**.

Given the very high ProTide potentiation noted for adenines such as d4A,[14] we were interested to examine the effect on carbocyclic adenines and particularly Cd4A. In fact, the "natural" D-form D-Cd4A (**2**) is approximately 3-fold less potent than (**1**) versus HIV.[25] The enantiomer, L-Cd4A (**3**), has modest activity versus HBV (ca. 1 μM) but is poorly active versus HIV

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[a] Abbreviations: HIV, human immunodeficiency virus; HBV, hepatitis B virus; AZT, 3′-azido-3′-deoxythymidine; d4T/d4A, 2′,3′-dideoxy-2′,3′-didehydrothymidine/adenosine; ddU/ddA, 2′,3′-dideoxyuridine/adenosine; 3TC, L-3′-thia-2′,3′-dideoxycytidine; BVDU, *E*-5-(2-bromovinyl)-2′-deoxyuridine; L-Cd4A, (1*R*,*cis*)-4-(6-amino-9*H*-purin-9-yl)-2-cyclopentene-1-methanol.

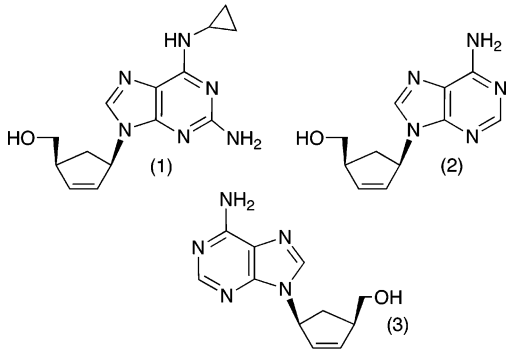


Figure 1. Structures of some antiviral carbocyclic nucleoside analogues.

(ca. 80 μM).[26] We wondered to what extent this difference might reflect the relative efficiency of phosphorylation, which might be bypassed by ProTide methodologies. This paper describes our initial attempts in this regard.

Results and Discussion

Chemistry. D-Cd4A (**2**) and L-Cd4A (**3**) were prepared as outlined in Scheme 1 following published procedures. Briefly, 4-amino-2-cyclopentene-1-methanol (**D**) was prepared from commercially available azabicyclo[2.2.2]hept-5-en-3-one (**A**) as peviously described in the literature.[27] Condensation of this 4-amino-2-cyclopentene-1-methanol (**D**) and 5-amino-4,6-dichloropyrimidine (**E**) in butanol at elevated temperature resulted in the formation of 4-[(5-amino-6-chloro-4-pyrimidinyl)amino]-2-cyclopentene-1-methanol (**F**).[28−29]

The carbocyclic chloropurine (**G**) was formed by treatment of **F** with triethylorthoformate in the presence of acid. Finally, treatment with liquid ammonia in a Parr bomb gave the desired carbocyclic L-Cd4A (**3**). Carbobocyclic L-ddA (**6**) was synthesized by reducing the cyclopentene using 5% Pd/C under 40 psi of hydrogen.

The D-analogues **2** and **7** were prepared in a similar manner as described for the L-analogues.

We followed the standard phosphorochloridate approach to the synthesis of ProTides that we developed in the 1990s.[9] This involved the preparation of an aryloxy phosphorodichloridate by reaction of an appropriate phenol with phosphoryl chloride, followed by condensation with an esterfied amino acid hydrochloride to give the key phosphorochloridate reagent. Reaction of these phosphorochloridates with nucleosides such as Cd4A has two challenges. The first is poor solubility, and the second is regiochemistry. It is important to restrict the phosphorylation to the 5′-hydroxyl group and eliminate any base (amino) phosphorylation. This was addressed very successfully by Uchiyama[30] using Grignard reagents of strong bases to generate the 5′-alkoxide, which gives preferential reaction with electrophiles. We have noted the efficacy of the Uchiyama method on abacavir.[24] Thus, we employed the same general method here (Scheme 2).

In the first instance, L-Cd4A (**3**) was converted into its phenyl methylalaninyl phosphoramidate (**4a**) in 81% yield. As noted for almost every nucleoside phosphoramidate ProTide, this was isolated as a roughly 1:1 mixture of phosphate diastereomers, as evidenced by two closely spaced ^{31}P NMR signals (δ_P 3.8, 4.1). The isomeric mixture was also evident in the ^{1}H NMR (e.g., OMe δ_H 3.70, 3.72) and the ^{13}C NMR (e.g., CH_3-Ala, δ_C 19.8, 20.0). Similarly prepared were the alanine analogues with the effect of ester modification on the antiviral potency of phosphoramidate ProTides, with a clear preference for benzyl.[31−2] Indeed, our recent work on BVDU ProTides versus cancer indicated a >100-fold improvement of in vitro potency on replacement of the methyl ester present in NewBiotics' lead thymectacin[18] by a benzyl ester.[19] Similarly, we have reported extensive SAR studies on the amino acid region, including natural amino acid variation,[33] un-natural α,α-dialkyls,[34] stereochemical variation,[35] amino acid extensions,[36] and replacements.[37] In general, alanine and the un-natural amino acid α,α-dimethylglycine emerged as the amino acids of choice. Indeed, we recently noted that dimethylglycine was a particularly efficacious motif with regard to anti-HBV activity when applied to abacavir (**1**).[24] Thus, using similar methods (Scheme 2) we prepared the glycine (**4g**), valine (**4h**), leucine (**4i**), isoleucine (**4j**), methionine (**4k**), methyl aspartate (**4l**), phenylalanine (**4m**), proline (**4n**), lysine (**4o**), tyrosine-*O*-*tert*-butyl ether (**4p**), and dimethylglycine (**4r**) analogues, each as the methyl ester. The tyrosine compound (**4q**) was prepared via TFA-mediated hydrolysis of (**4p**), it being notable that the phosphoramidate was stable to these conditions. As noted above, we have previously found D-alanine to be less effective than L-alanine.[35] However, this has not been extended to other amino acids and not on L-nucleosides. Thus, we prepared a small panel of D-amino acid analogues: D-alanine (**4s**), D-phenylalanine (**4t**), D-leucine (**4u**), D-valine (**4v**), D-tryptophan (**4w**), D-methyl aspartate (**4x**), D-proline (**4y**), and D-methionine (**4z**).

As long ago as 1992 we noted the effects on in vitro potency of aryl substitution in phosphoramidate ProTides.[8,9] We identified *p*-halogen systems as particularly effective, including the *p*-chloro.[38,39] Indeed, we subsequently published a rigorous QSAR analysis of this effect.[40] The group of Uckun have very actively pursued the *p*-bromo derivative on d4T ("stampidine").[41]

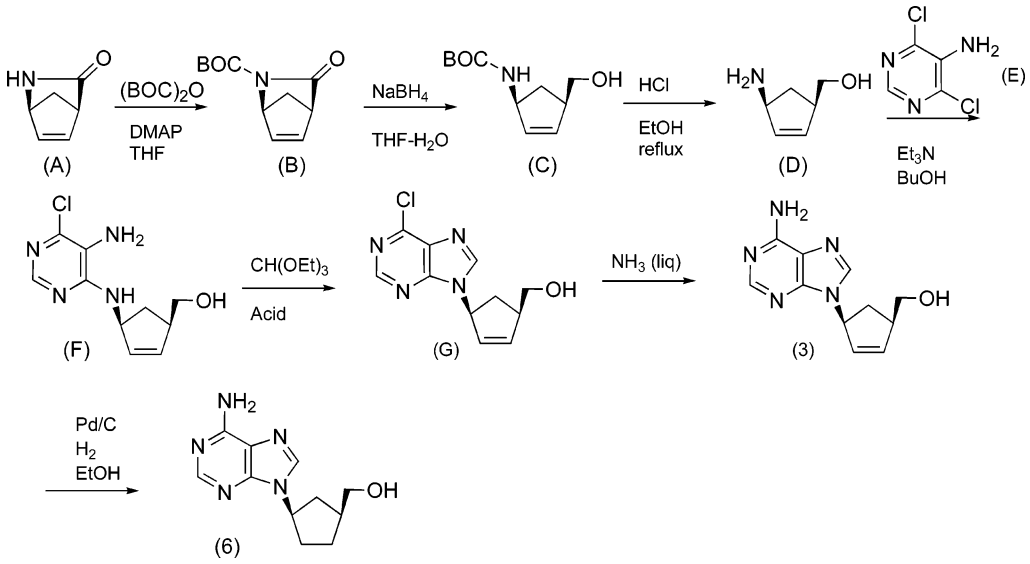
Thus, by the above methodologies, and preparing the aryloxy phosphorochloridate from the appropriate phenol where it was not commercially available, we prepared methyl alanine analogues with aryl substitution as follows: *p*-chloro (**4aa**), *p*-nitro (**4ab**), *p*-CF_3 (**4ac**), *m*-CF_3 (**4ad**), 3,4-dichloro (**4ae**), *p*-CO_2Me (**4af**), *m*-CO_2Et (**4ag**), and *o*-CO_2Et (**4ah**).

Finally, for purposes of comparison, the parent phenyl methyl alanine derivatives were prepared from enantiomeric D-Cd4A-(**2**) and the corresponding L-CddA (**6**) and D-CddA(**7**) (compounds **5**, **8**, and **9**, respectively).

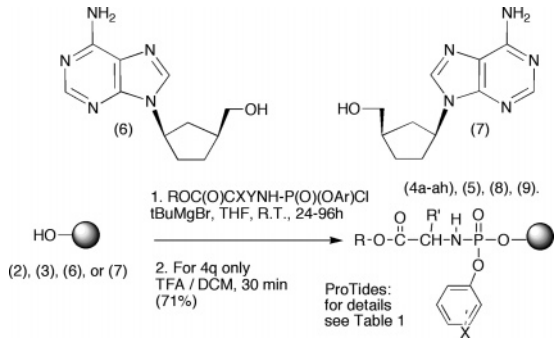
Antiviral Activity. All of the phosphoramidates described above (**4a**−**ah**), **5**, **8**, and **9** were tested in vitro against HIV-1, HIV-2, and HBV, with nucleosides **2**, **3**, **6**, and **7** as controls. Cytotoxicity was also evaluated in MT4 and CEM cells. All of the data are presented in Tables 1 and 2 (in μM). Thus, the parent phenyl methylalaninyl ProTide of L-Cd4A (**4a**) displayed a ca. 2700-fold boost in anti-HIV potency, being active at 30 nM, vs 80 μM for the parent. The ProTide was ca. >15 times more cytotoxic than the parent but still displays a selectivity index (SI = CC_{50}/EC_{50}) of >200. As expected, no significant differences in potency were noted for HIV-2 vs HIV-1 and for MT4 vs CEM cells. Versus hepatitis-B virus (HBV), where **3** is already quite active (EC_{50} ca. 1 μM), **4a** is ca. 60-times more potent at 17 nM and shows little toxicity (CC_{50} 1280 μM; SI ca. 75 000).

As the ester was lengthened from methyl to ethyl (**4b**), there was no significant change in antiviral potency, while the pattern was variable for the secondary, ispropyl ester (**4c**) and tertiary

Scheme 1. The Synthetic Route to L-Cd4A (3) and L-CddA (6)



Scheme 2. The Synthetic Route to ProTides of Carbocylic Nucleoside Analogues 2, 3, 6, and 7[a]



[a] For details of the structures, see Table 1.

been ascribed to the relative stability of tertiary esters to enzyme-mediated hydrolysis. However, the isopropyl showed a slight increase in potency versus HBV and variable results versus HIV dependent on the cell line and assay. The extended system with a $tBuCH_2$ ester (**4e**) showed high activity versus both HIV and HBV, being ca. 6−12-fold more potent than **4a**. Finally, regarding esters, the benzyl analogue **4f** emerged as the most potent ester versus HIV, being active at 9 nM and thus ca. 9000 times more active than **3**. It was also rather nontoxic and displayed an SI of >28 000. It was also highly active versus HBV, with an EC_{50} of ca. 7 nM, although some cytotoxicity was noted in this assay (at 5 μM).

Turning now to amino acid variations, leaving the ester as methyl, we have previously noted a 60−70-fold reduction in anti-HIV potency for d4T ProTides on alanine to glycine replacement[29] and a 20−40-fold reduction for the corresponding abacavir ProTides.[24] In this study with L-Cd4A we again note a significant drop in anti-HIV potency on this substitution (**4g**), giving, depending on the cell line, a 10−200-fold reduction. However, HBV activity displays a different trend, showing only a modest 5-fold drop in potency, thus retaining a log more potency than the parent **3**. Similarly, the valine compound **4h** showed a 10−20-fold reduction in anti-HIV potency as compared to **4a** but was equipotent to **4a** against HBV, at 20 nM. The data for the isoleucine analogue **4j** parallel that of the valine compound, as might be expected from their similar structure, proximately a log more active in each assay and thus rather similar to alanine. The methionine (**4k**) and methyl aspartate (**4l**) analogues were rather similar to the leucine compound, while the phenylalanine analogue **4m** was slightly more active, particularly versus HBV, where it was the most potent amino acid to date at 4.5 nM. The proline compound **4n** was the least active of the amino acids to date, an observation that we have made previously of this rather unique amino acid.[29] We report in this paper our first successful ProTide example with lysine as the amino acid. This was isolated and tested as its TFA salt (**4o**) and found to be rather poorly active; in fact, it is rather similar to parent **3** in several assays and 2−10-fold less active than the proline analogue **4n**. It is interesting to compare the methionine (**4k**) and lysine (**4o**) cases, as they have side chains with similar geometries. The lysine case is ca. 50−100 fold less potent versus both HIV and HBV. Partly, this may correspond with the higher polarity of the lysine compound (particularly when protonated) and diminished membrane permeability. The calculated ClogP values of **4k** and **4o** are 0.9 and 0.46 (Chemdraw Ultra 9.0), but these figures may not fully reflect the likely protonation of the lysine side chain at physiological pH, further diminishing its lipophilicity.

The tyrosine analogue **4q** was prepared via its protected *tert*-butyl ether (**4p**), so we evaluated both the free and protected versions in vitro. In fact, they were both rather similar in antiviral profile and similar to valine. The boost in anti-HBV activity seen for the Phe analogue (**4m**) was not seen for the Tyr compounds.

The dimethylglycine compound (**4r**) was not available for evaluation versus the whole panel of assays, but initial data indicate a slight reduction in potency vs HIV and slight increase in potency against HBV. Thus, **4r** emerged as the most potent member of this series against HBV with activity at 2.5 nM, thus being almost 400 times more potent than the parent nucleoside.

We have previously found D-alanine to be significantly less effective than natural L-alanine in phosphoramidate ProTides of d4T.[35] We recently noted the same trend for activity of abacavir ProTides against HIV.[24] In the present case the data are clear and marked; the D-alanine system (**4s**) is ca. 100-fold less potent than the L-alanine parent (**4a**) against HIV, but D

Table 1. Anti-HIV Activity and Cytotoxicity Data for Nucleoside and Nucleotide Analogues

				MT4/μM Rega			CEM/μM Rega			MT4/μM GSK	
compd	Ar	ester	AA	HIV-1	HIV-2	CC_{50}	HIV-1	HIV-2	CC_{50}	HIV-1	CC_{50}
4a	Ph	Me	Ala	0.045	0.043	16.4	0.13	0.09	18.1	0.03	6.4
4b	Ph	Et	Ala	ND	ND	ND	ND	ND	ND	0.017	>25
4c	Ph	iPr	Ala	0.15	0.22	91.4	0.3	0.25	84	0.425	100
4d	Ph	tBu	Ala	8.19	3.08	84.9	2.93	3.67	130	<0.26	<25
4e	Ph	$tBuCH_2$	Ala	0.043	0.03	20.2	0.067	0.083	19.5	>4	>10
4f	Ph	Bn	Ala	ND	ND	ND	ND	ND	ND	0.009	255
4g	Ph	Me	Gly	4.35	5.73	>250	13.5	20	≥250	7.3	≫100
4h	Ph	Me	Val	1.09	1.05	152	1	4.5	167	0.64	<25
4i	Ph	Me	Leu	0.19	0.13	23.2	0.42	0.56	70	0.64	>5
4j	Ph	Me	Ile	0.96	1.13	99.6	2.1	3.5	118	1.3	>5
4k	Ph	Me	Met	0.16	0.21	34.7	0.53	1	85.8	1.4	>5
4l	Ph	Me	MeAsp	0.32	0.26	53.5	1.1	3.5	>50	>1	>16
4m	Ph	Me	Phe	ND	ND	ND	ND	ND	ND	0.1	16
4n	Ph	Me	Pro	5.28	21	106	3.5	7.5	92.1	10	≫10
4o	Ph	Me	Lys(TFA)	15.2	27.2	>250	25	150	>250	25	≫25
4p	Ph	Me	Tyr(OtBu)	0.11	0.13	19.8	3	5.5	79.7	≪1	<40
4q	Ph	Me	Tyr	0.58	0.72	26.9	5.33	7	110	0.95	>32
4r	Ph	Me	Me_2Gly	ND	ND	ND	ND	ND	ND	0.14	20
4s	Ph	Me	D-Ala	ND	ND	ND	ND	ND	ND	2.05	>100
4t	Ph	Me	D-Phe	13.3	5.93	71	9	8.67	108	2.8	>40
4u	Ph	Me	D-Leu	1.24	0.93	77.1	1.6	3.5	88.5	<1	<16
4v	Ph	Me	D-Val	6.93	14.2	101	7.5	10	230	2.3	>16
4w	Ph	Me	D-Trp	13.3	21.9	66.9	20	25	93.4	.16	>40
4x	Ph	Me	D-Asp(OMe)	3.9	3.71	≥250	5	15	≥250	2.1	≫10
4y	Ph	Me	D-Pro	117	105	227	110	105	≥250	ND	ND
4z	Ph	Me	D-Met	4.07	3.48	133	7.67	7.87	211	2.5	>25
4aa	*p*-ClPh	Me	Ala	0.009	0.018	3.43	0.015	0.047	3.11	0.013	2
4ab	*p*-NO_2Ph	Me	Ala	0.12	0.17	21.6	0.1	0.1	17.4	0.18	10
4ac	*p*-CF_3Ph	Me	Ala	0.035	0.046	5.87	0.06	0.06	6.4	ND	ND
4ad	*m*-CF_3Ph	Me	Ala	0.044	0.04	22.4	0.15	0.1	16.3	0.06	4
4ae	*m,p*-Cl_2Ph	Me	Ala	0.12	0.076	15.6	0.09	0.11	13	0.077	>4
4af	*p*-CO_2MePh	Me	Ala	0.14	0.056	11.5	0.13	0.13	12.9	0.12	>4
4ag	*m*-CO_2EtPh	Me	Ala	0.053	0.033	4.39	0.065	0.08	8.39	–	–
4ah	*o*-CO_2EtPh	Me	Ala	ND	ND	ND	0.053	0.057	4.17	<0.26	<4
3	–	–	–	ND	ND	ND	ND	ND	ND	80	>100
2	–	–	–	ND	ND	ND	ND	ND	ND	15	>500
5	Ph	Me	Ala	ND	ND	ND	ND	ND	ND	0.3	3.7
6	–	–	–	ND	ND	ND	ND	ND	ND	>10	>10
8	Ph	Me	Ala	ND	ND	ND	ND	ND	ND	1.8	>12.5
7	–	–	–	ND	ND	ND	ND	ND	ND	50	>125
9	Ph	Me	Ala	ND	ND	ND	ND	ND	ND	0.20	6.5

The D-alanine analogue is ca. 10-fold less potent versus both HIV and HBV. However, it is also less cytotoxic (>5 to >20 fold), leaving its selectivity index similar or slightly better.

Comparing the potency of the alanine (**4a**), D-alanine (**4s**), and dimethylglycine (**4r**) systems indicates that substitution on the "D-face" of the amino acid is beneficial for HBV and detrimental for HIV. This may reflect cell to cell differences in processing of the different phosphoramidates. Similar L to D trends were observed for D-Phe (**4t**), D-Leu (**4u**), D-Val (**4v**), D-Trp (**4w**), D-MeOAsp (**4x**), D-Pro (**4y**), and D-Met (**4z**), giving reductions in anti-HIV potency for D-systems of 5−50-fold and retention or only slight reduction for HBV. Exceptions were the Val (**4v**) and Met (**4z**) cases, which did show significant reductions in anti-HBV potency. Thus, versus HIV, in conclusion alanine remained the most effective amino acid, although dimethylglycine was of similar potency, as were Met and Leu in some assays, and Val, Ile, and Tyr were also reasonably effective. Glycine, proline, and lysine were poorly effective, as were the D-amino acids in most cases. The amino acid could be varied considerably with little reduction in potency against HBV, and several amino acids were either equipotent or more potent than alanine, notably dimethylglycine and D-alanine but also phenylalanine.

Finally, on the SAR of the L-Cd4A ProTides, we probed boosted in d4T phosphoramidates[39] and this has later been picked up by the group of Uckun with their development of stampidine, the *p*-bromo species.[41] Thus, in the present case we first prepared the *p*-chloro analogue, which we favor over the *p*-bromo for toxicological reasons, given the mole for mole release of *p*-halophenol on ProTide activation. Indeed, the *p*-chlorophenyl methyl alanine compound (**4aa**) emerged as the most potent methyl alanine to date with a ca. 3−10-fold potency improvement against HIV as compared to **4a**, with some cell to cell variation, and a 5-fold greater potency against HBV than **4a**. We have previously noted the poor anti-HIV efficacy of ProTides with phenyl groups containing strongly electron withdrawing substitution,[40] and to some extent we found the same to be the case here, with the *p*-nitro analogue (**4ab**) being 10−20 fold less active than the *p*-chloro lead (**4aa**). However, it was not significantly less active than the unsubstituted phenyl parent (**4a**) versus HIV. Moreover, **4ab** was rather potent vs HBV, being equipotent with **4aa** and thus slightly more active than **4a**. This is in contrast to previous experience with d4T ProTides and HIV.[40] The CF_3 group is not as electron withdrawing as nitro and is more lipophilic; we previously noted it to be more effective in the case of d4T ProTides vs HIV,[40] and we noted the same trend here. Thus, **4ac** was more active than **4ab**, and in general than **4a** also, versus HIV and HBV.

Table 2. Anti-HBV Activity, Cytotoxicity, and Stability Data for Nucleoside and Nucleotide Analogues

	HepG2-2.2.15 GSK		% S9 remaining	
compd	HBV	CC_{50}	int	liver
4a	0.017	1280	74	24
4b	0.02	22	39	15
4c	0.006	>2	90	8
4d	0.25	170	96	1
4e	0.003	13	57	0
4f	0.0075	5	0	0
4g	0.086	>2	85	46
4h	0.02	120	91	2
4i	0.01	20	75	0
4j	0.06	76	69	0
4k	0.03	16	66	0
4l	0.05	>2	8	4
4m	0.0045	9	–	–
4n	0.51	>2	–	–
4o	2.0	≫100	17	95
4p	0.026	>2	–	–
4q	0.05	>2	0	0
4r	0.0025	10	70	16
4s	0.0265	>200	94	41
4t	0.7	200	–	–
4u	0.13	>2	–	–
4v	0.6	>200	–	–
4w	0.2	>2	–	–
4x	0.02	>2	–	–
4y	>2	>2	–	–
4z	0.22	>2	–	–
4aa	0.003	>2	–	
4ab	0.007	>2	–	
4ac	<0.0032	0.72	–	
4ad	<2	>2	92	45
4ae	0.005	>2	–	–
4af	0.004	>2	48	7
4ag	0.004	>2	42	19
4ah	0.004	12	80	25
3	0.98	>200	–	–
2	52	>200	–	–
5	4	5.4	–	–
6	61	>200	–	–
8	0.60	>2	–	–
7	33	>200	–	–
9	5.0	38	–	–

it is also notably toxic in the HBV assay, being the only ProTide in the family that is toxic at submicromolar concentrations. Interestingly, the meta analogue **4ad** is rather less cytotoxic but also apparently slightly less active. Several other aryl substituted compounds (**4ae**−**4ah**) are also noted in Table 2. The meta-substituted ester is the most active against HIV, while several compounds are highly active vs HBV. As noted above, the anti-HBV activity appears less sensitive to aryl substitution than the anti-HIV activity.

The D-enantiomer of **3**, D-Cd4A (**2**), is slightly more active than **3** against HIV and rather poorly active vs HBV. It was interesting to see whether ProTides would have a similar impact here and we report in Table 1 data on the parent phenyl methyl alanine parent (**5**). Thus, a 50-fold boost in anti-HIV potency is noted. This is much less of an improvement (ca. 2600-fold) than noted above for the analogous compounds in the L-series (**3** and **4a**), and thus the D-ProTide (**5**) is about a log less potent than the L-ProTide analogue (**4a**). Similarly, versus HBV, the D-ProTide (**5**) is only 13-fold more potent than the nucleoside, whereas in the L-family the boost was 60-fold. More dramatic, the D-ProTide (**5**) is cytotoxic at its effective concentration, with a SI barely above unity, while the L-compound (**4a**) has an anti-HBV SI of >75 000.

preparation of the ProTides **8** and **9**. Thus, unlike several other dideoxynucleosides, the carbocyclic analogues **6** and **7** are both rather poorly active. Application of ProTides did give interesting boosts in potency in both cases. Indeed, while the L-system (**6**) was primarily enhanced (100-fold) versus HBV, the D-compound (**7**) was only slightly enhanced vs HBV but significantly so versus HIV (250-fold).

In conclusion, ProTide methods have been shown to significantly enhance the antiviral profile of a series of carbocyclic nucleosides, primarily L-Cd4A but also its D-analogue, and the dd analogues in both L- and D-series. Very significant differences were noted for HIV and HBV, with separate leads emerging for each virus, with quite separate and distinct SARs noted for each. Effects were noted for variations in the ester, amino acid, and aryl regions. In general, the HBV system was more tolerant of structural modifications.

Several nanomolar compounds emerged, representing an almost 4-log improvement in potency of several ProTides versus the parent L-Cd4A nucleoside. With this background we were keen to seek to perform some preclinical analysis of the most promising compounds that would allow rational choices regarding further evaluation. In particular, the stability of the ProTides to metabolic deactivation prior to reaching their target site was of particular interest, given our experience with abacavir ProTides.[24] Thus, we employed a cynomolgus monkey liver and intestinal S9 stability assay to gain an initial understanding of the stability issues in this family and to probe any stability−structure correlations. Data are reported for selected compounds in Table 2. Under the conditions of the assay **4a** underwent some decomposition (ca. 25% over 1 h) in the intestinal fraction, but the majority of ProTide remained. The position was reversed in the liver fraction, where only 30% remained. The most striking of the ester variations is the benzyl (**4f**), which showed complete disappearance in both assays over 1 h. Thus, although **4f** was rather potent in the in vitro antiviral assays, the S9 data may be predictive of a limited in vivo exposure. Interestingly, the tyrosine compound (**4q**) revealed a similar instability.

In terms of ester variation; the ethyl ester (**4b**) was less stable than the methyl parent (**4a**) in both media, whereas branching to isopropyl (**4c**) and tBu (**4d**) stabilized it in intestine but destabilized it in liver. This would suggest that such esters may be beneficial to consider for delivery to the liver, in hepatitis or liver cancer.

Several amino acid substitutions for alanine gave a similar profile of increased or maintained intestinal stability but diminished liver stability, e.g., Val (**4h**), Leu (**4i**), Ile (**4j**), and dimethylglycine (**4r**). The only compounds with enhanced liver stability over the parent **4a** were the glycine (**4g**), Lys (**4o**) and D-alanine (**4s**) amino acid variants and the *m*-CF_3 aryl substitution (**4ad**). Interestingly, the lysine stabilization in liver was uniquely accompanied by a significant labilization in intestine.

In terms of overall maximal stability, a property which might be anticipated to be useful with regard to systemic drug delivery, the D-alanine and glycine compounds emerge as the most notable. Both retained good antiviral potency in the HBV assays but were only moderate in the HIV assay. The *m*-CF_3 compound (**4ad**) also looked rather stable in both S9 assays and showed good anti-HIV potency. This indicates the potential merit of extensive aryl modification to tune stability and improve pharmacokinetic properties; however, the relevance of the S9

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