Aryloxy Phosphoramidate Triesters as Pro-Tides

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Abstract: We herein describe the development of aryloxy phosphoramidate triesters as an effective pro-tide motif for the intracellular delivery of charged bio-active antiviral nucleoside monophosphates. The review covers the discovery of such aryl phosphoramidates, their mechanism of action and structure-activity relationships. The application of this strategy to a range of antiviral nucleosides is highlighted.

Keywords: Nucleotide, Pro-drug, Phosphoramidate.

SCOPE OF THIS WORK

We will describe the discovery, *in vitro* evaluation, Structure-Activity Relationships (SARs) and Mechanism of Action (MoA) of phosphoramidate triesters of a range of antiviral nucleosides. We describe these compounds as triesters to emphasise the fact that all of the charges on the phosphate nucleus are blocked and to distinguish our compounds from the phosphoramidate diesters described by Wagner and Coworkers [1]. These latter compounds have been well reviewed [1,2], operated by a quite separate mechanism, and displayed distinct SARs to those compounds we will describe here. Therefore we will not include them below. We will thus describe the development of fully blocked phosphoramidates, culminating in our lead series, the aryloxy phosphoramidates.

ALKYL AND HALOALKYL PHOSPHATE TRIESTERS

Early work from our laboratories indicated that simple alkyl triesters of the antiviral agent araA (vidarabine) and the anti-neoplastic agent araC (cytarabine), of general formulas **1** and **2**, (Fig. **1**) respectively displayed significant biological activity in tissue culture [3,4]. However, analogous dialkyl phosphate triesters of AZT (**3**) were devoid of significant anti-HIV activity, in marked contrast to the parent nucleoside analogue [5]. Similarly, whilst haloalkyl phosphate triesters of araA and araC (**4, 5**) had enhanced biological activity [6], the corresponding AZT derivatives (**6**) and also those of 2',3'-dideoxycytidine (ddC) (**7**) were in general poorly active [7].

Thus, although the bis(trifluoroethyl) analogue (**6**) was active at 0.4μM, and thus >200 times more active than compound (**3**), it was still 100-fold less potent than AZT itself [7]. Attempts to boost the potency of these haloalkyl phosphate triesters by changing the degree of halogenation were in general not successful [8].

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ALKYLOXY PHOSPHORAMIDATES

The original rationale for preparing phosphoramidate-based pro-tides was the possibility that HIV aspartate protease [9] might cleave a suitable oligo-peptide from the phosphate moiety of a blocked nucleotide phosphoramidate. Simple model mono-amino acyl analogues were prepared and evaluated in the first instance and were of sufficient interest to pursue in their own right. Thus, a series of simple alkyloxy phosphoramidates of AZT were prepared with a small family of methyl esterified aminoacids (**8**) [10]. By comparison to earlier dialkyl phosphates of AZT (**3, 6**) the alkyloxy phosphoramidates (**8**) showed significant anti-HIV activity. A notable dependence of the antiviral activity on the aminoacid side-chain began to emerge; with alanine being most efficacious, and with leucine and, particularly, isoleucine being less active [10,11]. By contrast, the alkyl phosphate chain could be varied from C_1 to C_6 with no significant change in activity [11].

In a subsequent study [12], α−aminoacids were compared to their β,γ derivatives etc (**9**). Anti-HIV activity was maximal for the parent α system (glycine) and diminished with increasing alkyl spacer length, being 10-fold less active for n=3 as compared to n=1 [12].

Given the earlier improvements in antiviral activity noted for the haloalkyl phosphate parents, we wondered whether haloalkyl phosphoramidates might also be more potent. Therefore, a small series of compounds (**10**) was prepared [13]. For each of the aminoacids glycine, alanine and valine, the alkyl chain either was ethyl, trifluoroethyl or trichloroethyl. However, by contrast to earlier observations, we herein noted no enhancement in antiviral potency compared with the haloalkyl compounds, with one striking exception. The trichloroethyl alanine compound (**10**, X=Cl, R=Me) was active at 0.08μM and thus 50 times more potent than either the ethyl or trifuoroethyl analogues. Interestingly, this enhancement was only seen for the alanine series, and not for the glycine and valine systems [13]. Thus, alanine emerged as a preferred aminoacid, although the mechanistic origins of this preference were, and still largely remain, unknown. Much of the preceding literature from our labs and others has utilized alanine as the empirical aminoacid of choice, although as we will note below there are other aminoacids, which may usefully substitute for it.

PHOSPHORODIAMIDATES

Given the promising activity of alkyl phosphoramidates, particularly those related to alanine, we wondered whether diamidates might also be efficacious. Thus, several methyl esterified amino acyl phosphorodiamidates (**11**) were prepared and tested [14]. Non-amino acyl phosphoro-diamidates derived from simple primary and secondary amines were also prepared. Structure-activity relationships were noted that indicated a strong preference for aminoacids such as phenylalanine [14]. Thus a different aminoacid SAR emerged for these diamidates as compared to the earlier alkyl phosphoramidates. It is intriguing to note that this preference for aromatic side-chains was also seen by Wagner for the rather un-related phosphoramidate diesters [1]. However, in general the diamidates appeared to offer no biological advantage over the amidates, and the chemical yields of the diamidates were significantly lower; hence they were not further pursued.

LACTYL DERIVED SYSTEMS

In an effort to establish the importance of the bridging aminoacid nitrogen atom for the biological activity of the phosphoramidates a small family of isosteric O-linked analogues derived from lactic and glycolic acid was prepared (**12**) [15]. In each case, lengthening of the alkyl phosphate chain (R") leads to a reduction in potency. It was also notable that glycolyl systems (R'=H) were more active than lactyl (R'=Me) by a factor of ca. 20. This is in contrast to the earlier work on phosphoramidates noted above where alanine was preferred over glycine [10,11]. A brief hydrolytic stability study was undertaken on compounds **12**, which revealed liberation of polar compounds and traces of AZT in biological media, but not in DMSO/water. Thus, enzyme-mediated activation was possible. However, since the anti-HIV activity of even the most active compound in the series was significantly (>10-fold) lower than AZT itself, these compounds were not further pursued.

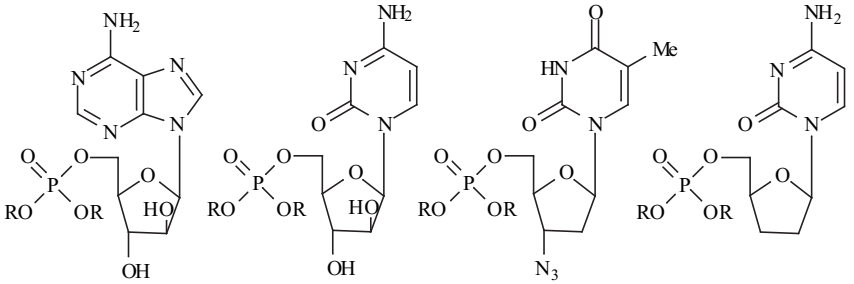
DIARYL PHOSPHATES OF AZT

One of our major breakthroughs in phosphoramidate pro-tide research was made in 1992, when we noted the efficacy of aryloxy phosphates and phosphoramidates [16]. Thus, diaryl phosphates (**13**, Fig. **2**) were prepared from AZT using simple phosphorochloridate chemistry. For the first time, the anti-HIV activity of these phosphate derivatives of AZT exceeded that of the parent nucleoside in some cases. Thus, the bis (p-nitrophenyl) phosphate was ca. 3-fold more potent than AZT vs. HIV-1 in C8166 cells, with an EC_{50} of 3nM [16]. Moreover, whilst AZT was almost inactive (EC_{50} 100μM) in the JM cell line, the substituted diaryl phosphate was 10-times more active (EC_{50} 10μM). At the time, it was considered that JM was AZT – insensitive due to poor phosphorylation [17]. It later emerged that an AZT-efflux pump was the source of this poor AZT sensitivity [18]. However, the conclusion remains valid that the diaryl phosphate was *more* able to retain activity in the JM cell line, and that this may imply a (small) degree of intracellular phosphate delivery. The nitro group was implicated as vital to this activity, as the parent diphenyl phosphate was ca. 100-fold less active (C8166 cells). The electron-withdrawing aryl leaving group ability were suggested as the major driving force of this SAR [16].

Thus, a series of analogues of (**13**) were prepared, with various alternative para substituents (CN, SMe, CF_3, I, OMe, H) [19]. A very clear correlation emerged between electron-withdrawing power of the para substituent and antiviral potency; the nitro and cyano substituted compounds being the most potent, the parent phenyl substituted compound intermediate in activity and the methoxy analogue least active, being 500-fold less active than the nitro compound. The effect of location of the electron withdrawing nitro group on the aryl rings was also briefly pursued, with symmetrical bis-ortho nitro and bis-meta nitro analogues being prepared [20]. In a study of both HIV-1 and HIV-2 in several cell lines it was found that the location of the nitro group had little effect on activity. However, for the first time we were able to assess the activity of the phosphate pro-drugs in the 'true' kinase-deficient cell line CEM-TK^-. This was a clear but disappointing result, with all of the diaryl phosphates losing almost all their activity, alongside AZT, in the TK^- cell line. This most likely implied poor intracellular phosphate delivery and that the diaryl phosphates were acting largely, if not entirely, as AZT pro-drugs, not as AZTMP pro-drugs as intended [20]. However, the earlier work using JM cells on phosphoramidates [16] had indicated that aryloxy phosphoramides may offer a chance for true phosphate delivery, and this became the main focus of our work.

ARYLOXY PHOSPHORAMIDATES OF AZT

Thus, a series of aryloxy phosphoramidates of AZT was prepared (**14**) with various p-aryl substituents and several aminoacids [21]. Compounds were only studied in the AZT-resistant JM cell line to probe potential (implied) AZTMP release, and the alanine phosphoramidate emerged as strikingly effective. In HIV-1 infected JM all cultures, AZT was inhibitory at 100μM, whilst the phenyl methoxy alaninyl phosphoramidate (**14**, R=Me, Ar=Ph) was active at 0.8μM. This was taken as the first evidence of a successful nucleotide delivery. As had been noted by us previously in other series there was a marked preference for alanine over leucine (10-fold) and glycine (>100-fold). Moreover, whilst electron-withdrawing aryl substitution had been noted to be very effective in the diaryl systems [19], it was detrimental here. Para fluoro substitution had a slight adventitious effect, but not significantly so, whilst para-nitro substitution led to a 100-fold loss of activity. In a subsequent study [22] the range of aryl substituents was extended and compounds studied in true TK^+ and TK^- cell lines. None of the phosphoramidates retained the high (2-4 nM) potency of AZT in TK competent cell lines (CEM and MT-4) against either HIV-1 or HIV-2 [22]. However, whilst AZT lost all of its activity in the TK^- deficient cell line CEM/TK^-, most of the phosphoramidates retained antiviral activity, thus being ca >10-35-fold more active than AZT in this assay. Again, alanine emerged as an important component, with the glycine analogue being inactive in HIV-infected CEM/TK^- all cultures. In this assay, leucine and phenylalanine were as effective as alanine, although they were less so in CEM/TK^+ assays. Thus, the parent phenyl methoxy alanyl phosphoramidate emerged as an important lead compound

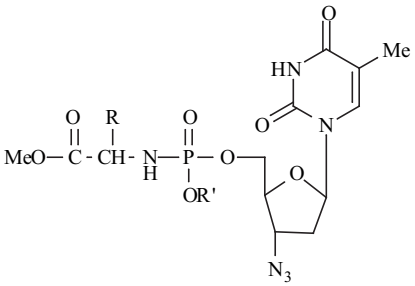


(1) R = Me, Et, Pr
(4) R = CCl_3CH_2, CF_3CH_2

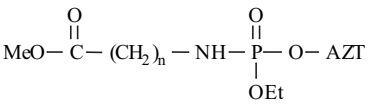
(2) R = Me, Et, Pr
(5) R = CCl_3CH_2, CF_3CH_2

(3) R = Me, Et, Pr
(6) R = CCl_3CH_2, CF_3CH_2

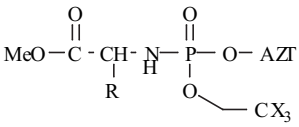
(7) R = CCl_3CH_2, CF_3CH_2



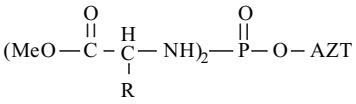
(8) R' = Me, Et, Pr, Bu, Hex
R = H, Me, iPr, CH_2iPr, CHMeEt, Bn



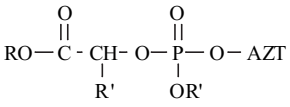
(9) n = 1-6



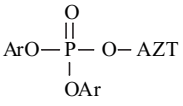
(10) X = H, F, Cl
R = H, Me, iPr



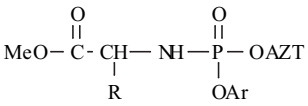
(11) R = H, Me iPr, CH_2Ph, CH_2CO_2Me



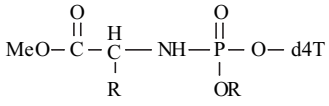
(12) R = Me, Et; R' = H, Me;
R'' = Me, nPr, $nC_{12}H_{25}$



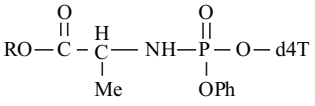
(13) Ar = Ph, p-XPh
X = various



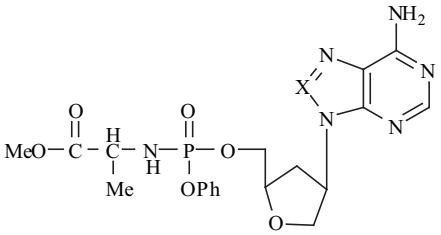
(14) R = H, Me, CH_2iPr, CH_2Ph
Ar = p-X-Ph
X = H, F, NO_2, Me, Et, nPr, nPnt, MeO



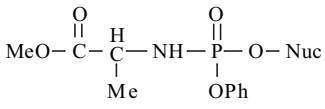
(15) R = nPr, CCl_3CH_2



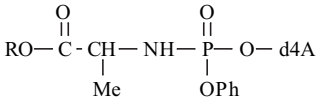
(16) R = Me, Bn



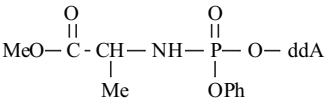
(17) X = N
(18) X = CH



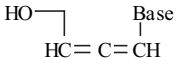
(19) Nuc = ddU
(20) Nuc = 3TC



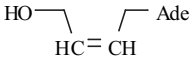
(21) R = Me, Bn



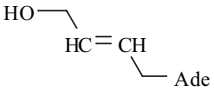
(22)



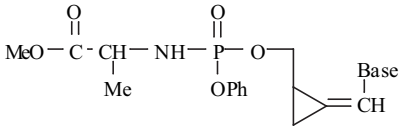
(23) Base = adenine
(24) Base = hypoxanthine



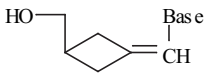
(25)



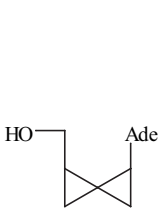
(26)



(27) Base = various



(28) Base = Ade, Gua



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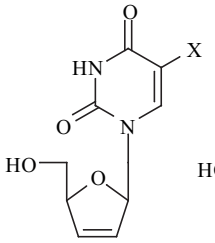
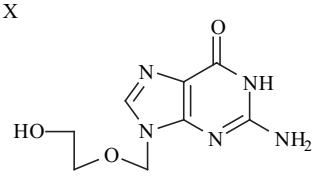


(30) X = F, Cl, I



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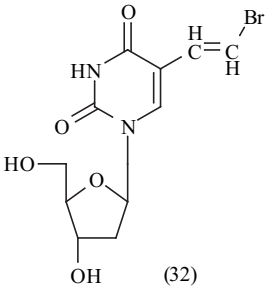

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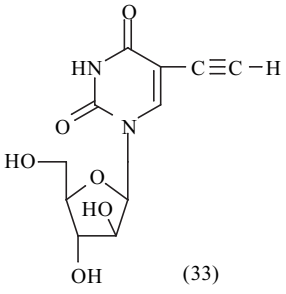


(33)

Fig. (1). Structures of some nucleosides and nucleotides. All nucleotides are 5' linked.

APPLICATION TO OTHER NUCLEOSIDES

As with other research groups reported in this compilation, we had sought to find a universal phosphate delivery motif that could be applied to a range of nucleosides. Indeed as early as 1993 we suggested that the phenyl alanyl phosphoramidate approach might be successful on a range of nucleosides (ddC, d4T) and phosphonates (PMEA) [22]. This has subsequently been confirmed to be the case with extensive application of the technology by others and us.

Stavudine (d4T) was an early application of ours [23]. This was a rational choice based on the known kinetics of phosphorylation of d4T. Thus, whilst the 2nd phosphorylation (AZTMP to AZTDP) but not the first phosphorylation (AZT to AZTMP) is regarded as rate limiting for AZT, the first step (d4T to d4TMP) is thought in general to be the slow step for d4T [24]. Thus, an intracellular (mono) nucleotide delivery should have maximal impact for d4T and similar nucleosides. In the first instance (halo)alkyloxy phosphoramidates of d4T (**15**) were prepared [23] and found to retain activity in d4T-resistant JM cells. The activity was dependent on the haloalkyl group; the parent propyl system being poorly active. Subsequent studies in HIV-infected CEM/TK$^-$ cell cultures [25] revealed the aryloxy phosphoramidates of d4T (**16**) to be highly effective and, notably, to retain their full activity in CEM/TK$^-$ cells. In this study the benzyl ester emerged as slightly more potent than the parent methyl compound, being almost 10-times more active than d4T in CEM/TK$^+$ assays and thus ca 300-500 fold more active than d4T, in CEM/TK$^-$ assays. Extensive studies followed on these promising d4T derivatives [26,27] which we will discuss later.

In 1994 Franchetti and coworkers [28] applied the aryl phosphoramidate technology to 8-aza-isoddA (**17**) and isoddA (**18**). Very significant boosts in the antiviral potency of the parent nucleosides were noted; >25-fold for (**17**) and 350-800 fold for (**18**). This was important an work, which demonstrated the power of the aryloxy phosphoramidate approach to greatly improve the biological profiles of poorly active nucleosides. Thus, (**18**) was transformed from 32μM activity versus HIV-2, to 40nM activity, on phosphoramidate formation. Subsequent analysis of these compounds by the Montpellier team [29] leads to the clear conclusion that they function as efficient intracellular phosphate delivery forms.

To a large extent this could be regarded as an example of what in 1990 we termed 'kinase bypass', wherein an inactive, or moderately active and poorly phosphorylated nucleoside could be 'activated' or potentiated by suitable pro-tide modification [5,30]. A further example of this has emerged in our labs on application of the technology to ddU [31]. Thus, whilst dideoxyuridine (ddU) is almost inactive (EC_{50} 200μM) vs. HIV-1 in C8166 cells, the phosphoramidate (**19**) was noted to be active at low μM levels and to retain potency in the AZT-resistant JM cell line. This activity was specific to the aryloxy phosphoramidate both with our lab [31] and the Montpellier group [32] noting poor activity for the alkyloxy phosphoramidates.

Given the success of the phosphoramidate approach by the Franchetti lab when applied to iso nucleosides [28], we were interested to pursue other sugar modifications. Therefore, we applied the approach to 2',3'-dideoxy-3'-thiacytidine (3TC) [33]. In fact, compound **20** was found to be less effective than 3-TC in deoxycytidine (dCyd) kinase competent HIV-1 and –2 infected cell assays, but assay in dCK deficient cells indicated far less of an impact on potency for the phosphoramidate than the parent CEM cells 3TC (ca. 20-fold vs 2000-fold). Interestingly, both compounds were equally effective versus hepatitis B virus in hepatoma G2 cells indicating efficient pro-tide activation in these cells but not in the CEM cells used for the HIV assay [33]. This was amongst the first indications that the (relative) efficacy of phosphoramidates might be cell-line dependent.

One of the most remarkable demonstrations of the effectiveness of the aryloxy phosphoramidate approach came from our application of the technology to the dideoxydidehydro purine d4A [34]. Compounds of the type **21** were found to be exquisitely potent inhibitors of HIV-1 and 2. Both the methyl and benzyl esters displayed EC_{50} values of ca. 6-18nM thus being 1000-4000 times more potent than the parent nucleoside analogue d4A. Although the phosphoramidates (**21**) are more cytotoxic than d4A (ca. 30-fold), their extraordinary potency enhancements still leave them with enhanced selectivities (50-150 fold) [34], and they are taken as a good example of nucleoside'kinase' (in this case adenosine) bypass. Subsequent application of the technology to dideoxyadenosine ddA (**22**) revealed a similar outcome; a >100-fold potency boost, with some increase in cytotoxicity [35].

The Detroit-based lab a Jiri Zemlicka has pioneered the synthesis of highly modified nucleosides with alkene, alkyne, alkene, methylenecyclopropane, methylenecyclobutane and spiropentane modifications and successfully applied our phosphoramidate technology. Indeed, they have recently reviewed these efforts [36].

Thus, phenyl methoxy alaninyl phosphoramidates of the anti-HIV active adenallene (**23**) and the inactive hypoxallene (**24**) were prepared [37].

A 10-20 fold boost in anti-HIV potency was noted on phosphoramidate formation from (**23**). Alkenyl adenine nucleosides such as (**25**) and (**26**) were similarly studied [38,39]. In these cases, both the Z (**25**) and E (**26**) nucleosides were inactive, whilst the phosphoramidate of (**25**) was active in the 1-10 μM range and non-toxic; the isomeric phosphoramidate (**26**) remained inactive. The hypoxanthine analogue of (**25**) was also poorly active [38,39].

A study of methylenecyclopropane nucleoside phosphoramidates (**27**) was conducted by the Zemlicka group [40-43]. Besides these active Z-isomers, the inactive E-series were also phosphorylated and compounds evaluated against a very wide range of viruses (HCMV, HSV-1, HSV-2, HHV-6, EBV, VZV, HBV, HIV-1 and HIV-2). Amongst the conclusions were the following:

- The Z-adenine compound is a potent inhibitor of a variety of viruses, but is cytotoxic.

- The Z-guanine analogue is active against HCMV, HBV, EBV and VZV and is non-cytotoxic.
- The Z-diaminopurine is highly active against HBV and HIV-1, with lower activity against other viruses and is non-cytotoxic. This compound emerged as the best candidate for further development. Again, whilst the E-isomers of the parent nucleosides were either inactive or very poorly active, their phosphoramidates emerged as potent and selective antivirals, particularly the adenine compound.

The Zemlicka group has also applied the phosphoramidate technology to methylene cyclobutane (**28**) [44] and spiropentane (**29**) [45] nucleosides with varying degrees of efficacy.

Further pursuing the kinase bypass approach we prepared some inactive novel d4T derivatives with 5-halo substituents in place of the 5-methyl group (**30**) and converted them to their phosphoramidates [46]. Whilst all compounds (**30**) were poorly active/inactive at >10-50 μM, the phosphoramidates were all active. The phenyl methoxy alaninyl phosphoramidate of the 5-chloro compound (**30**, X = Cl) was active at sub μM concentrations, being >100-fold more potent than its nucleoside parent, and was also non-cytotoxic [46].

Inspired by Zemlicka, who had shown that phosphoramidate formation could broaden the spectrum of activity of nucleoside analogues, we sought its application to a variety of nucleosides with different therapeutic targets. Thus, the anti-herpetic agents acyclovir (**31**), BVDU (**32**) and netivudine (**33**) were all converted to their phosphoramidates [47-49].

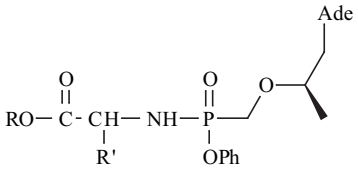
In general terms, the approach failed for acyclovir (**31**), where the phosphoramidate was significantly less active than the parent versus HSV-2, and slightly more active versus HCMV [47]. Similarly, with BVDU (**32**) we noted a reduction in anti-VZV activity (5-25 fold) for the phosphoramidates, as well and very poor activity in TK^- assays [48]. This was taken as evidence of a low degree of kinase bypass resulting from inefficient pro-tide activation in this case or, alternatively, fact dephosphorylation of the released nucleoside monophosphate. It is therefore surprising to note the apparent efficacy of these phosphoramidates of BVDU in anti-cancer assays reported by the NewBiotics Group [50]. It may be that the necessary pro-tide activating enzymes (see below) are absent in the cell lines used in our antiviral assays, but present in the (tumour) cell lines used by NewBiotics, or that the apparent anti-cancer activity of BVDU phosphoramidates does not arise via monophosphate release. However, the apparent clinical progression of these agents [51] would suggest that the anti-cancer arena may well be a fruitful area for future phosphoramidate studies.

Finally, our application of the technology to the potent anti-VZV agent netivudine (**33**) [49] was again disappointing, with little activity being noted.

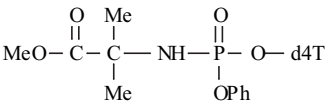
Thus, in conclusion others and we have demonstrated that the phenol methoxyalaninyl group may significantly enhance the potency, selectivity and activity of spectrum of a range of nucleosides and by-pass their dependence on nucleoside-kinase mediated activation. The approach is very successful for the Zemlicka agents with highly modified sugar regions, and for ddA and d4A. It is also effective for d4T and AZT in nucleoside kinase deficient cells. However, it is clearly dependent on the nature of the parent nucleoside and the cell line/target studied. The example of BVDU highlights this final point [48,50].

APPLICATION TO ACYCLIC NUCLEOSIDE PHOSPHONATES

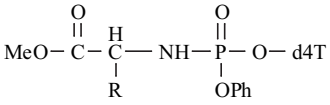
Until recently all of the published work on phosphoramidate pro-tides was on nucleoside analogues, as noted above. However, recently there have been a few reports of application of the technology to phosphonates, and in particular acyclic nucleoside phosphonates (ANPs). The Gilead group who has been active in the commercialization of ANPs have reported [52] that aryloxy phosphonamidates (**34**, Fig. **2**) of PMPA (tenofovir) are highly active anti-retrovirals. We reached the same conclusion for PMPA and the closely related PMEA [53]. Boosts in antiviral potency of 30-100 fold were noted for PMPA and PMEA, with the usual preference for alanine as the aminoacid component.



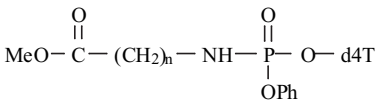
(34) R = Me, iPr; R' = H, Me, Bn



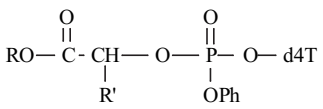
(35)



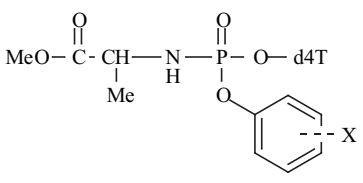
(36) R = Et, nPr, nBu, Ph



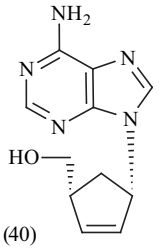
(37) n = 1-6



(38) R = Me, Et; R' = H, Me



(39) X = various



(40)

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