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], $\alpha-$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.

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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 phosphorodiamidates 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 power of the p-nitro groups and putative enhancements in 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 [22].

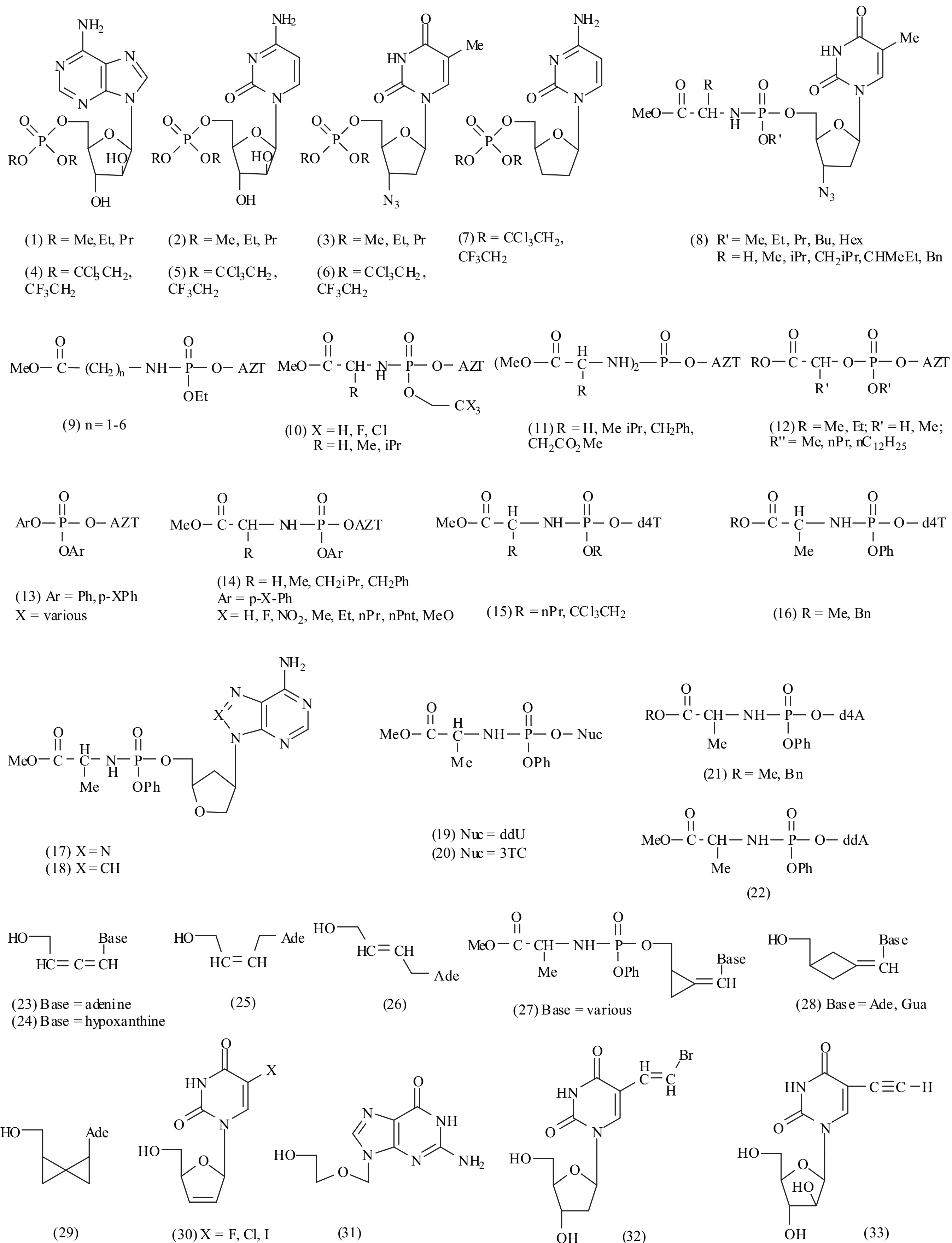


Fig. (1). Structures of some nucleosides and nucleotides. All nucleotides are 5'-linked.

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, methylenecyclo-butane 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.

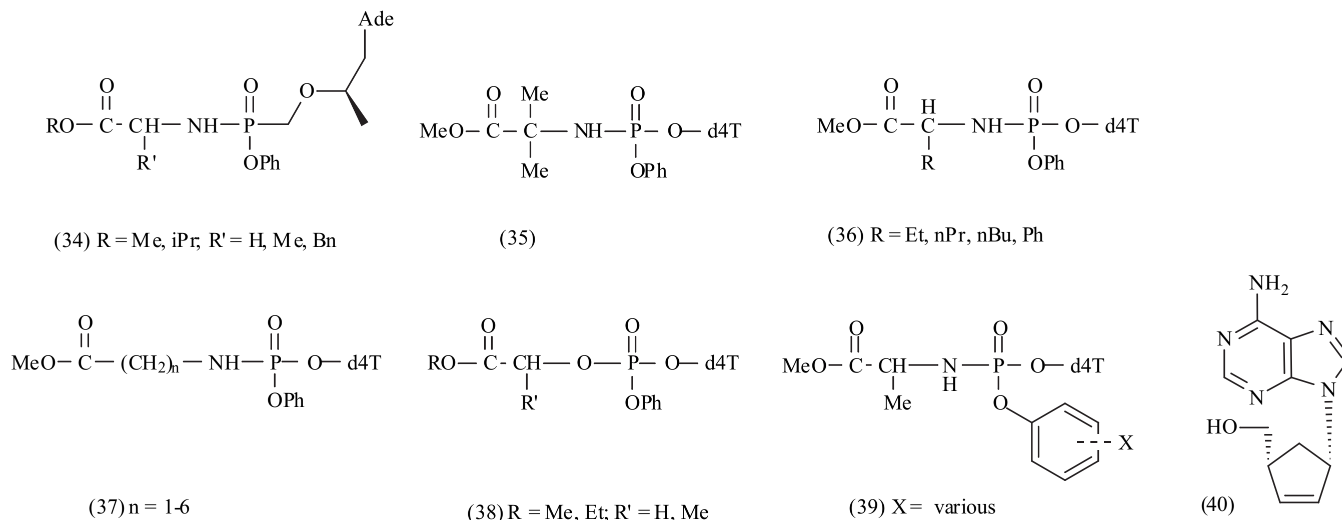


Fig. (2). Structures of some nucleotides.

Interestingly, we noted a significant preference for L-alanine over D-alanine (5-60 fold), whereas Gilead observed a preference for one phosphonate diastereoisomer over the other, observations we will further discuss later under 'stereochemistry' matters. Gilead commenced clinical trials on their amidate GS7340 in 2002, most notably progressing with one phosphate diastereoisomer, afforded by an efficient large-scale synthesis and isomer separation [54].

D4T Aryloxy Phosphoramidate SARs

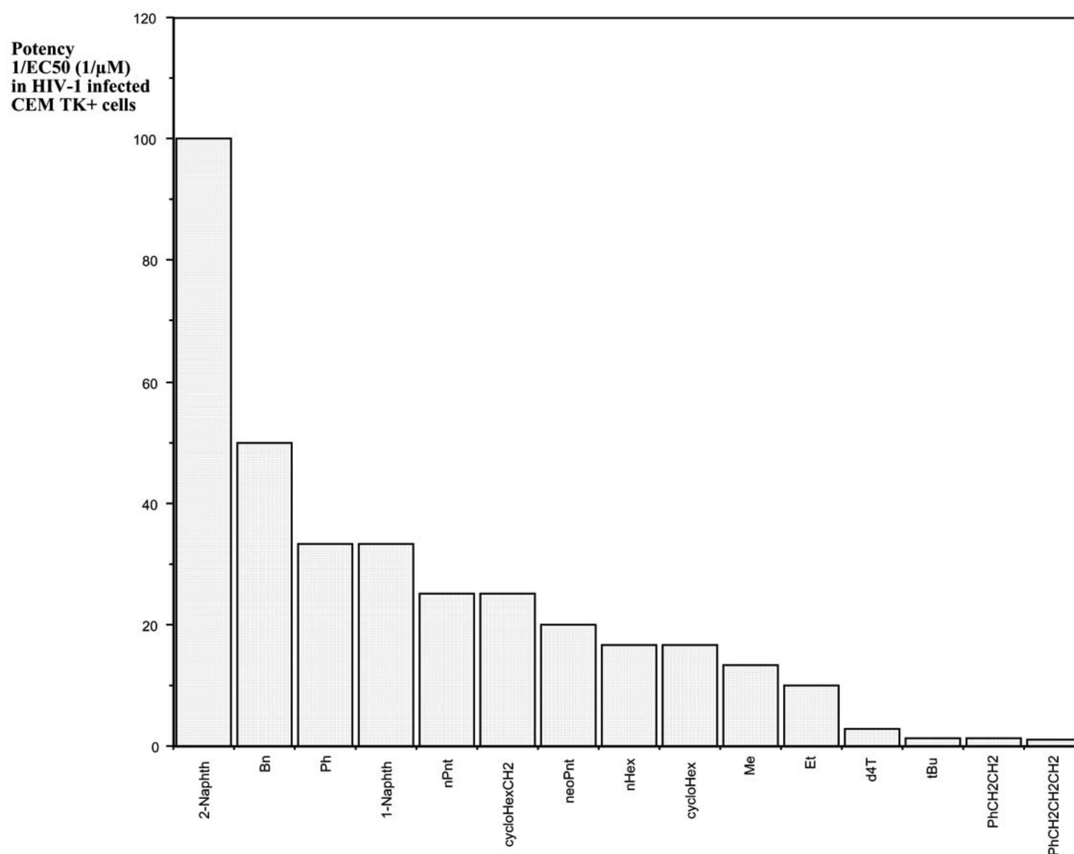
We have conducted fairly extensive structure-activity relationship studies of various regions of the phosphoramidate unit when attached to d4T, with over 250 analogues prepared to date [26,27]. These will be discussed by the respective region of the phosphoramidate:

Ester Region

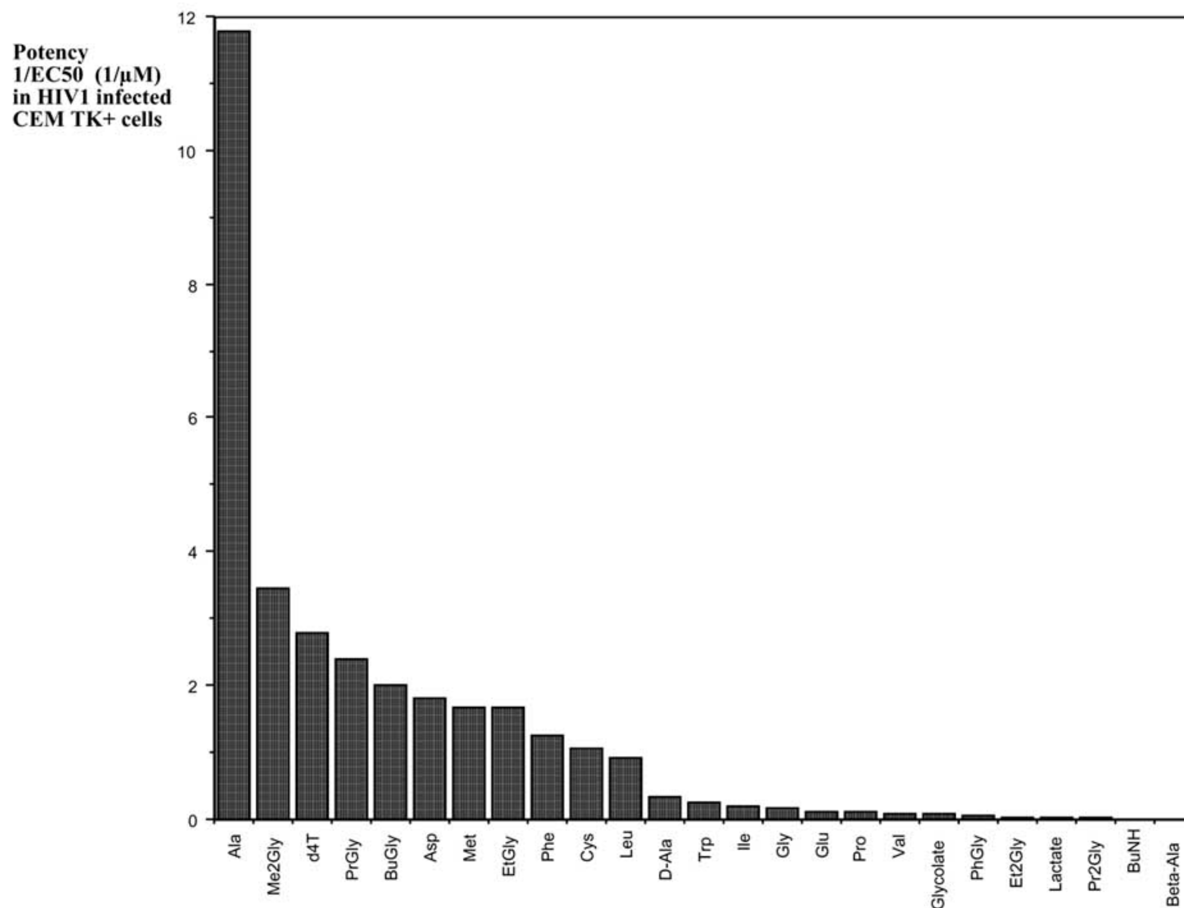
Some early work on AZT alkyloxy phosphoramidates revealed the importance of the carboxyl ester region for anti-HIV activity [55], which was subsequently confirmed for d4T aryloxy analogues [56]. Thus, a range of primary, secondary, tertiary, alkyl, benzyl and linear and branched esters related to (**16**) were prepared and evaluated against HIV-1 and –2 in thymidine kinase – competent and –deficient cell lines. Data are presented in Graph 1 as plot of potency ($1/EC_{50}$) for a range of esters, versus HIV-1 in CEM TK^+ cells, with d4T as control. A number of esters lead to potent activity, comparable with, or slightly more potent than, the methyl parent with compound. The benzyl, and naphthyl esters in particular were noted to be highly potent. The t-butyl ester analogue on the other hand was >10-times less potent than the methyl ester parent compound. As we will note below under Mechanism of Action studies, this correlates well with the poor esterase susceptibility of this particular ester. We later conducted a QSAR analysis using calculated physical properties (TSAR^{TM}) for 15 esters related to (**16**), which showed a good degree of correlation between predicted and measured activity, with a clear dependence on the shape and electronic distribution of the ester [57]. In particular, there emerged a strong dependence on the directional component of the ester group lipophilicity (the 'lipole moment'), indicating that electron withdrawing groups in the ester, but removed from the ester bond, should boost potency.

Amino Acid

As noted above, alanine had arisen as the aminoacid of choice based on limited studies with alkyloxy phosphoramidates of AZT. We now compared 13 aminoacids related to (**16**, R=Me) [58]. Very clear SARs emerged from this study as presented in Graph 2, along with other highly amino-modified systems will be described later. Thus, versus HIV-1 in CEM TK^+ cells, alanine remained the most effective aminoacid. However, several other aminoacids led to potencies, which were not significantly reduced, notably the un-natural, achiral α,α-dimethylglycine compound (**35**), which was slightly more potent than the alanine compound in CEM / TK^- cells. In fact, all of the phosphoramidates retained full potency in the nucleoside



Graph 1. The effect of ester changes on antiviral potency.



Graph 2. The effect of amino acid changes on antiviral potency.

kinase deficient CEM cell assay, indicating action via intracellular d4TMP release. However, some aminoacids were less effective; proline in particular, leads to a compound 20-100 times less potent than (**16**); whether this relates to the importance of a free aminoacid NH, or steric or conformational issues relating particularly to proline is not entirely clear. However, valine and isoleucine were also poorly effective, indicating some steric restriction issues related to the side-chain. On the contrary, however, glycine is a striking example; the simple loss of the alanine methyl group resulting in a ca 60-70 fold reduction in potency.

The potency of (**35**) was an important discovery, since for the first time it indicated that 'un-natural' (or less common) aminoacids could be utilized in the phosphoramidate approach. Indeed, we subsequently studied the α,α-diethyl- and -dipropyl analogues of (**35**), but found that these were poorly active ($\geq$100-fold less potent than the dimethyl parent) [59]. On the other hand, considerable tolerance was allowed for un-natural, non-alkyl glycines, of the type (**36**). Thus, whilst α-ethylglycine was 10-fold less effective than alanine, the n-propyl and n-butyl analogues showed no subsequent losses in potency [59].

These compounds are therefore all substantially more potent than the 'natural' glycine system. Replacement of the side-chain in (**36**) by a phenyl did however lead to a further loss of activity, to yield a material similar in potency to the glycine compound [58,59].

Amino Acid Stereochemistry

Given the importance of the aminoacid side-chain it became interesting to probe its stereochemical requirements. Thus, we prepared the isomeric, D- compound related to (**16**, R=Me) and found it to be 20-30 fold less effective than the L-parent [60]. Despite this reduction in potency, the D-compound did retain full potency in TK-deficient CEM assays, indicating its functioning entirely as a d4TMP delivery form, with little or no free d4T release. It is further interesting to note that the D-alanine compound is of similar (slightly higher) potency as the glycine analogue [58,60]. This implies that a methyl group on the L-face of the aminoacid (as in L-alanine and α,α-dimethylglycine) contributes about a log in potency to the baseline of glycine, that the D-face-methyl group of D-alanine cannot substitute, and that the Pro-D-methyl group of α,α-dimethylglycine is neither advantageous nor detrimental to potency.

Amino Acid Replacement

In this section we briefly describe some large-scale aminoacid changes, which have a significant (largely negative) impact on potency. Thus, replacement of the aminoacid in (**16**) by a family of non-aminoacyl simple n-

alkylamines (C3 to C12) leads to a complete removal of activity [61]. The same result was observed for simple alkylamine analogues of the AZT phosphoramidates (**14**) [61]. Thus, an aminoacid appears a pre-requisite for a successful aryloxy phosphoramidate approach. This is in marked contrast to recent data from the Montpellier group who has prepared phosphoramidate – SATE hybrids of AZT, and found them to be highly potent and not to depend on an aminoacid type structure for potency [62]. As noted by Peyrottes *et al.* in this Compilation, this indicates a different mechanism of action for SATE – phosphoramidates as compared to aryloxy phosphoramidates (see below).

We had previously noted that chain extended aminoacid-related alkyloxy phosphoramidates of type (**9**) were poorly effective [12]. It was of interest to extend this study to analogous beta- and other aminoacids, when applied to aryloxy derivatives of d4T. Thus, compounds (**37**) were prepared and found to be very poorly active; all extended compounds were ca 40-times less potent than the glycine parent compound [63]. Interestingly the β-alanine compound (**37**, n=2) was thus ca 2500 times less potent than its structural isomer, the alanine lead compound (**16**, R=Me). It is striking therefore, that the beta- and further extended aminoacids are no more effective as aryloxy phosphoramidate motifs than simple n-alkylamines [61,63] (Graph 2) and that the substantial potency benefit offered by aminoacids such as alanine does not extend to their beta- and longer isomers. We will discuss this SAR further below under Mechanism of Action.

Finally in this section, we note that the bridging aminoacid nitrogen atom is vital for the antiviral potency, since its replacement by a bridging oxygen atom, as in (**12**), but now for aryloxy phosphoramidates of d4T (**38**), leads to a very significant loss (ca.600-fold) of activity [64].

Aryl Substitution

We had previously noted the effect of aryl substitution on aryloxy phosphoramidates of AZT (**14**) [21,22]. We now extended this study to d4T with a variety of substituents ranging from electron donating to electron withdrawing (**39**) [65]. It was notable that strongly electron withdrawing groups (p-CN, p-NO_2) lead to slight reductions in potency, whilst several substituents, such as the p-COOMe and, particularly p-Cl groups showed significant potency boosts. The p-chloro compound emerges as a new lead structure, being 14-times more potent than the phenyl parent compound, with an EC_{50} versus HIV-1 in wild-type nucleoside kinase competent CEM cells of 5nM, and being fully active in the CEM/TK^- assays. The chloro compound was also the most lipophilic studied, and logP measurements indicated a correlation (r=0.9) between measured logP and antiviral activity. However, this may be an artefact of the small sample size, and logP may not in itself correlate with activity in wider compound series. In general, this study concluded that lipophilic substituents which were mildly electron-withdrawing (σ 0.15 - 0.48) were preferred [65]. Indeed, a subsequent more rigorous QSAR analysis of 21 compounds of this type confirmed lipophilicity as an important factor, with steric and electronic factors of more secondary importance [66].

Uckun *et al.* also noted the efficacy of compounds of the type (**39**), and in particular with a para-bromo substitutent [67-69]. This group later went on to suggest these agents as virucidals [70,71].

Phosphate Stereochemistry

One of the notable structural features of all of the phosphoramidate triesters, excluding some phosphorodiamidates [14], is the presence of a chiral centre at the phosphate. Due to the chirality of the nucleoside all of the compounds prepared are thus isolated as a pair of diastereoisomers. In this regard the phosphoramidate approach is similar to the cylcoSAL approach of Meier reported in this compilation. As early as 1990 we had started to partly separate the diastereoisomers [10]. Thus, some alkyoxy phosphoramidates of AZT (**8**) were partly separated by flash silica chromatography and fractions enriched in the more lipophilic ('fast') and less lipophilic ('slow') isomer were separately evaluated. We found a small, ca 3-fold, difference in potency, with the 'fast' isomer being less potent. A subsequent study on some mixed haloalkyl triesters of the type (**6**) indicated a 10-fold difference, with the 'fast' isomer being more potent [8]. In neither case was any link made between the absolute chirality at the phosphorus atom and the relative lipophilicity/potency; such work was only to emerge later. Thus, working with alanine phosphonamidates of PMEA (**34**), researchers at Gilead found a 10-fold difference in potency, with the S-phosphate isomer being more potent [52]. Their recent disclosure of a large scale synthesis and purification of the most active isomer clears the way towards clinical evaluation of single isomers of phosphoramidate triesters [54].

MECHANISM OF ACTION STUDIES

The putative first activation step for these phosphoramidates is esterase mediated carboxyl ester cleavage. In an effort to model this in a predictive sense, we exposed various esters of type (**16**) to pig liver esterase, and followed the P-31 NMR signal [56]. The parent compound always shows two closely spaced signals, due to the phosphate diastereoisomers. Upon esterase treatment these signals collapsed to give a downfield singlet (δ_P ca 8.2ppm). This was characterised as the amino acyl phosphate monoester (Fig. **3**, A). Whilst the rate of cleavage of various esters did not readily correlate with antiviral activity, we did note that esterase lability was a necessary (but not sufficient) condition for high biological potency; the t-butyl ester in particular was not hydrolyed, and that phosphoramidate was the least potent assayed in cell culture [56].

As noted above, subsequent studies on compounds with chain-extended aminoacids of type (**37**) [63] showed the importance of the α-aminoacid for activity. Indeed, the esterase study on these compounds, each of which had a methyl ester, showed that they were all well processed. However, only the α-compound, derived from glycine, proceeded to form the intermediate of type A (Fig. **3**). The beta- and longer homologues were processed only to the carboxylate B (Fig. **3**). It appears that for entropic, or steric, reasons the loss of the phenyl group from these systems does not proceed under the conditions of the assay, diesters of type A do not arise, and the antiviral activity is poor. We surmise that an alpha aminoacid (or similar) is required for neighboring group assistance to displace the phenyl group,

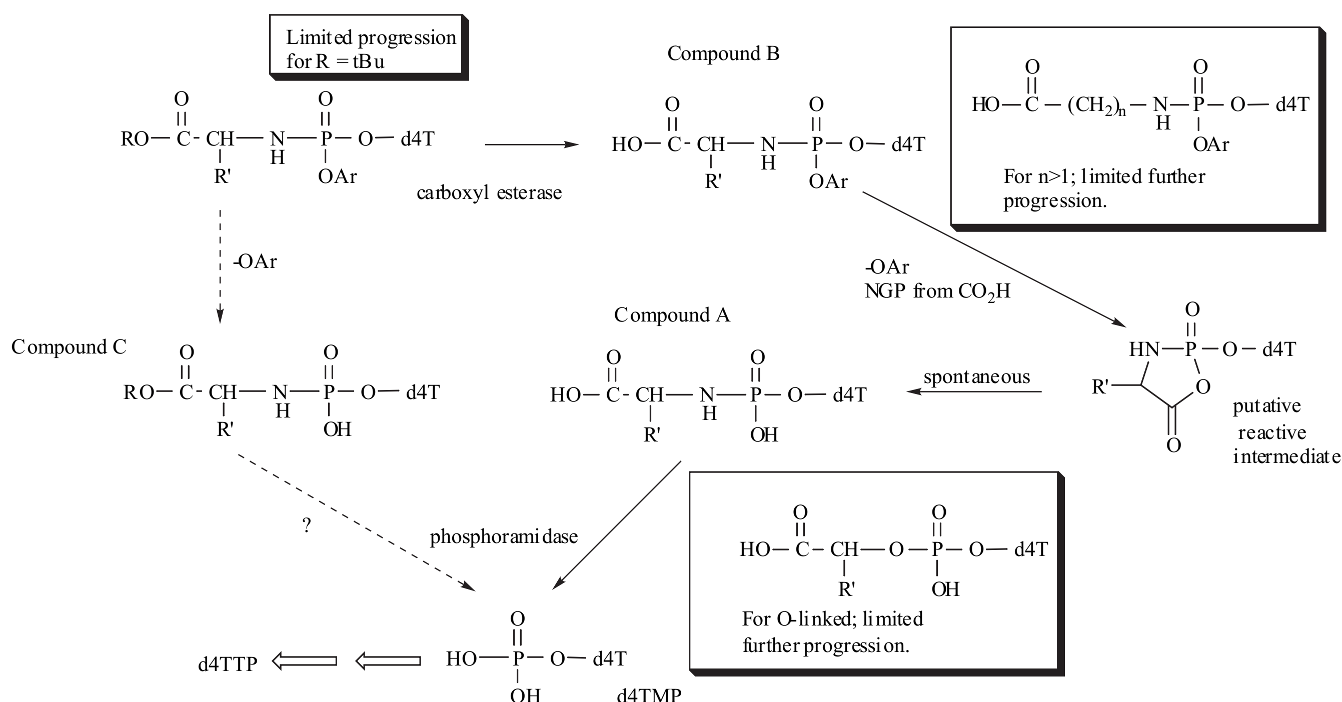


Fig. (3). Proposed mechanism of activation of aryloxy phosphoramidates of d4T. The pathway via compound A is considered to predominate, unless the aryl groups bears a strongly electron withdrawing group and esterase is limiting. The putative reactive intermediate between B and A has not been observed, but is surmised.

and that in its absence phenyl loss does not take place, and moreover, that in the absence of such a displacement the phenyl group is not lost *in vitro*, and thus the antiviral action is limited.

Similar mechanistic conclusions were derived by the Montpellier group [72] on the isoddA compounds of Franchetti (**18**) using an elegant online ISRP-cleaning HPLC method they had earlier reported for POM esters [73]. However, in contrast to our work, under some conditions this group noted an alternative pathway involving loss of the aryl group prior to ester hydrolysis, to give compounds of the type C (Fig. **3**) [72]. We believe this to be specific to compounds with strong electron withdrawing aryl substituents (e.g. nitro) and/or under conditions of reduced esterase activity, and may be of limited *in vitro* significance for un-substituted phenyl systems.

We confirmed our general view using radio labelled compound (**16**, R=Me) incubated with a range of cell lines used in the antiviral assays and then studied by HPLC [74]. In these studies high levels of metabolite A were noted, which varied with the cell line, but in general corresponded with high anti-retroviral activity. Thus, compound 'A' was suggested as an important intracellular depot form for the free nucleotide [74]. This study was subsequently extended to include a wide range of compounds of the general type (**36**), with varying aminoacids and esters, and also chain extended compounds (**37**), and AZT analogues (**14**) [75]. Again, the generation of intermediates of the general type 'A' was seen as a necessary condition for high antiviral potency. For the first time, this study rationalised many of the SARs noted previously, such as the strong aminoacid preferences we had seen [58]. For example, valine had been noted to be a poorly effective aminoacid, and its phosphoramidate was now of to be poorly processed by carboxy esterase, human serum, and CEM cell extracts to the type 'A' compound [75].

In this study, the final step in conversion of metabolites 'A' to the free nucleotide was also studied, namely the cleavage of the aminoacid moiety. This represents a phosphoramidase activity [EC 3.9.1.1] as originally defined by Dixon and Webb [76]. However, a variety of phosphoramidase preparations have been reported from a range of sources, and "pure" enzyme has proved elusive [77-81]. Using a rat liver cytosolic preparation we were able to partially purify a fraction with the ability to hydrolyse compounds of type A to generate d4TMP. The fraction was distinct from creatine kinase, alkaline phosphatase and phosphodiesterase on the basis of its lack of inhibition by known reagents (enzyme inhibitions), and may truly be described as a phosphoramidase [81]. It appears to have a molecular weight in the range of 50-100KDa, has a pH optimum of 7.4, and is inhibited by the phosphoramidase inhibitor iodobenzene [82].

Lastly, when exposed to carboxyl esterase hydrolysis the poorly active lactate and glycolate compounds (**38**) [64] gave a rapid hydrolysis to give amino acyl intermediates related to 'A' (but O-bridged). Thus, we surmise that such lactates are poorly effective as antivirals due to poor onward processing of this intermediate by phosphodiesterase under the conditions of the assay, and that such analogues are not substrates for the putative phosphoramidase that hydrolyses 'A'.

CONCLUSIONS

The aryloxy phosphoramidate approach has emerged, along with SATE, CycloSAL, aryl phosphoramidate diester and others [1,2] as a viable method for the intracellular

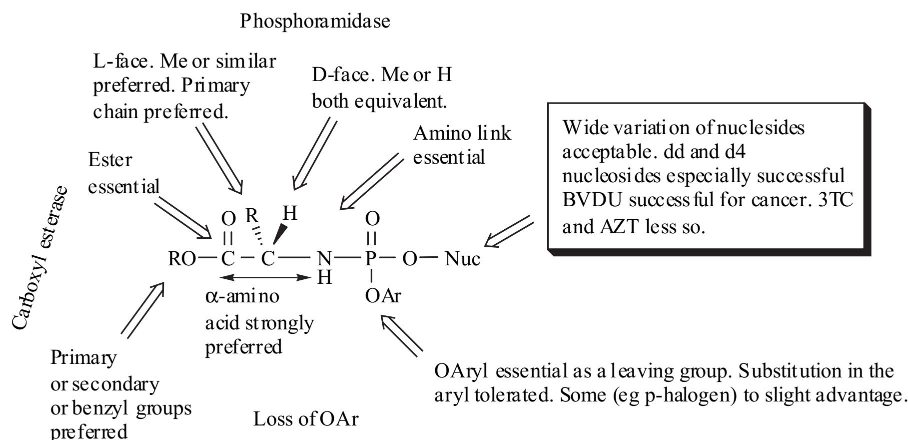


Fig. (4). Preferred moieties for a successful phosphoramidate triester outcome. Possible major origins of key SARs are highlighted with the appropriate activation step.

delivery of free monophosphates of a range of nucleoside analogues. The approach works poorly for AZT, where the second phosphorylation is rate limiting [24] but well for d4T and a range of dd and d4 nucleosides. D4A is a particularly dramatic example with >1,000 fold boosts in potency on phosphoramidate formation. Indeed, recent work from our labs in collaboration with researchers at GlaxoSmithKline on the carbocylic L- d4A (**40**) has shown potency enhancements of almost 10,000 on phosphoramidate formation [83]. We believe that this has reached the levels that we originally described over 10 years ago as 'kinase bypass' [5], wherein an 'inactive' nucleoside is activated by phosphate pro-drug formation. This suggests the prudency of pro-tide synthesis on a range of nucleoside analogues, and particularly not just those selected as active in initial screens; the inactivity of other structures may simply correspond to poor initial phosphorylation, which may now be by-passed with pro-tides. The activity of the phosphoramidates of the highly modified Zemlicka nucleosides highlights this point well [36].

In terms of SARs, it has emerged that an alpha aminoacid is essential for this approach, by contrast to phosphoramidate-SATE hybrids of the Montpellier group. Alanine remains a good choice of aminoacid, although the achiral α,α–dimethylglycine is a good alternative. The ester and aryl moieties can be varied considerably, provided the ester can be cleaved by esterase, and the aryl is a reasonable leaving group. A P-N bond is also essential for activity, with lactate isosteres being poorly active. This appears to correlate with putative phosphoramidase mediated cleavage of the key amino acyl intermediate (A, Fig. **3**). Summary SARs and their mechanistic origins are collected together in (Fig. **4**).

Finally, the issue of phosphate stereochemistry is worth considering. All of the current syntheses of phosphoramidate triesters currently give mixtures, often approximately 1:1, of the phosphate diastereoisomers. These can be separated by chromatography, and often have 5-10 fold potency differences. Whilst it may be possible for mixtures to be progressed towards the clinic, such progression faces substantial regulatory hurdles, and separation and clinical evaluation of pure isomers seem more likely. The recent example of Gilead's GS-7340 supports this view [54]. On the Kilo scale, Gilead is using moving bed chromatography to separate the unwanted diastereomer. On a smaller scale, we have reported the novel use of molecular imprinted polymers (MIPs), which may be a useful tool in the research lab in this regard [84].

As *in vivo* data begin to emerge on aryloxy phosphoramidates [85] and with clinical evaluation proceeding, the next few years will confirm whether or not they have a role to play in future drug development for viruses and beyond.

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