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SIMPLE MONO-DERIVATISATION OF THE ARYL MOIETY OF D4A AND DDA-BASED PHOSPHORAMIDATE PRODRUGS SIGNIFICANTLY ENHANCES THEIR ANTI-HIV POTENCY IN CELL CULTURE.

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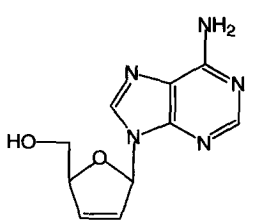
Abstract: Simple mono-derivatisation of the aryl moiety of some phosphoramidate pronucleotide derivatives of d4A and ddA served to increase the lipophilicity of these membrane-soluble prodrugs. A concomitant and significant enhancement of potency against HIV-1 and HIV-2 *in vitro* was observed for the ddA- and d4A-based prodrugs compared to the original underivatised prodrugs.

The antiviral purine nucleoside analogues d4A and ddA (**1** and **2**, Figure 1) are dependent upon conversion to their triphosphorylated forms (d4ATP and ddATP, respectively) for the expression of their antiviral activity. The intracellular conversion of ddA to the corresponding monophosphate (ddAMP) by kinase-mediated phosphorylation, is hampered by significant deamination of the purine base to its hypoxanthine analogue ddI (Figure 2).[1,2] Progression to the ddA monophosphate must then proceed via conversion to the ddI monophosphate (ddIMP) followed by two further enzyme-catalysed steps. Additionally, ddI is highly susceptible to purine nucleoside phosphorylase, degrading it to hypoxanthine. A similar metabolic profile may also be applicable to d4A. Circumvention of this non-trivial metabolic route could potentially be achieved by the direct delivery of the nucleoside monophosphate. However, the charged, highly polar phosphate group would prohibit efficient membrane permeation. Consequently we[3,4] and others[1,5] have proposed the application of masked phosphate prodrug delivery to by-pass both the nucleoside kinase- and adenosine deaminase-catalysed steps. Previously, we reported the successful application of our aryloxyphosphoramidate monophosphate prodrug strategy applied to both nucleoside analogues d4A and ddA.[3,4] Derivatives **4** and **7** (Figure 3) were both found to exhibit a marked elevation of anti-HIV activity in a broad range of cell lines compared to the parent nucleoside analogues, and, particularly in the case of the d4A based prodrug, a substantially improved selectivity index.



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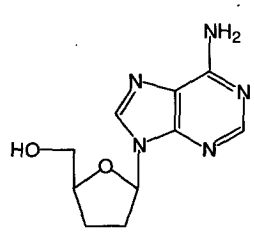

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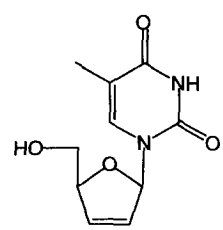

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Figure 1. Some antiviral nucleosides.

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A comprehensive structure-activity relationship (SAR) has been conducted on the phosphoramidate moiety applied to the pyrimidine based antiviral nucleoside analogue d4T (**3**, Figure 1). These studies established L-alanine and, in particular, the methyl and benzyl esters to be the structures consistent with optimal prodrug activity.[6,7] In recent work, we have conducted a comprehensive SAR on the aryl moiety of the d4T-based phosphoramidate.[8] This study revealed that a marked elevation in prodrug activity could be achieved by simple monoderivatisation of the aromatic ring. A direct (and quantifiable[9]) correlation was found to exist between *in vitro* antiviral activity and compound lipophilicity (determined by 1-octanol/water partition coefficient, P).[10] Interpretation of these results is consistent with improved cellular uptake by passive diffusion through increased compound lipophilicity and, accordingly, an increased intracellular level of the prodrug. The range of logP values over which the phosphoramidates expressed optimal activitity was found to lie between logP = 1.4 - 2.0. Furthermore, no direct correlations were seen between antiviral activity and substituent Hammet σ value or substituent sterric bulk or for the positioning of the particular substituent in either a *meta* or *para* position. Among the most active compounds were the aryl phosphoramidates monohalogenated at the aryl moiety.

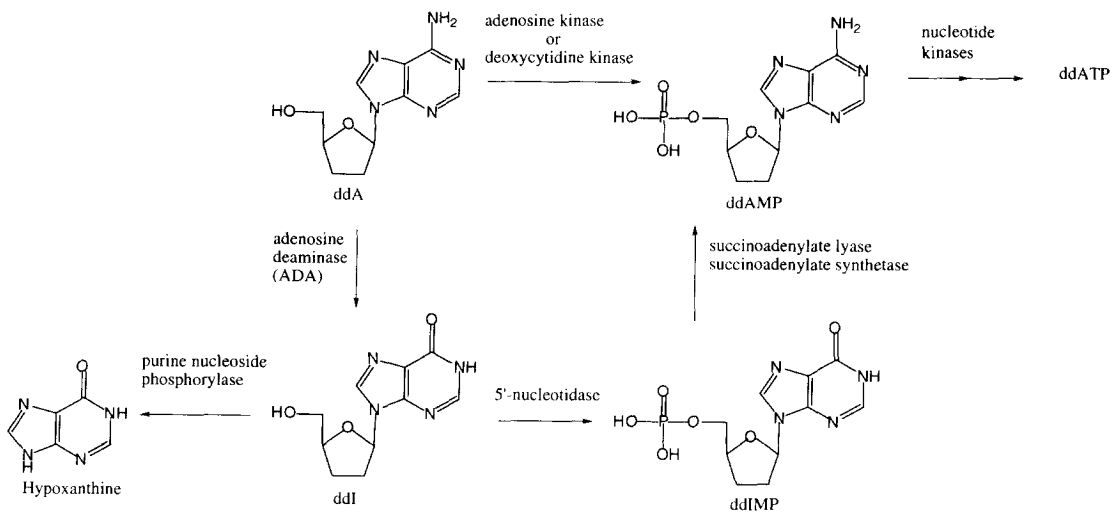

Figure 2 Metabolic conversion of ddA to ddATP.

We sought to apply these findings to other antiviral nucleoside prodrugs, in particular those based on d4A and ddA, in an effort to determine the potential application of simple aryl derivatisation as a mechanism to increase the antiviral potency of phosphoramidate prodrugs for nucleoside analogues other than d4T. Consequently, the *para*-iodo and *para*-bromo analogs of d4A (**5** and **6**) and the *meta*-iodo and *meta*-bromo analogs of ddA (**8** and **9**) were synthesized. Compounds **5**, **6**, **8** and **9** were synthesized by coupling the appropriate substituted-phenyl methoxyalaninyl phosphorochloridate with the desired nucleoside as described earlier.[3,4] The substituted-phenyl dichlorophosphates were synthesized by the addition of the appropriately substituted phenol to phosphorus oxychloride in the presence of triethylamine, by a method described elsewhere.[8] The compounds **5**, **6**, **8** and **9** were isolated as diastereomeric mixtures resulting from mixed stereochemistry about the phosphorus center, with the diastereomeric ratio typically of the order 3:1, as observed by both ^{31}P NMR and HPLC. Diastereomeric

splittings were observed in the ^{1}H, ^{13}C and ^{31}P NMR spectra and splitting patterns from phosphorus coupling, where appropriate, were seen in the ^{13}C and ^{1}H NMR spectra.[10]

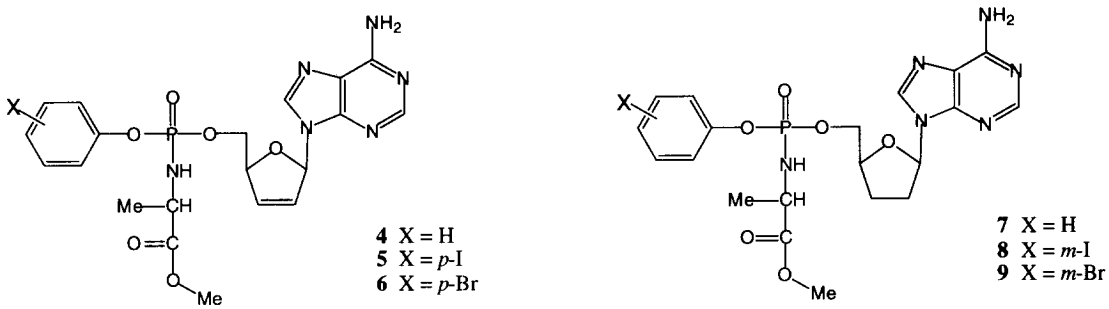

Figure 3. Masked-phosphate nucleotide prodrugs.

The lipophilicity of compounds **5**, **6**, **8** and **9** and the underivatised structures **4** and **7** was determined by measuring their partition coefficients, between 1-octanol and water (phosphate buffer, pH 7.0) (Table 1). The results show the derivatised compounds **5**, **6**, **8** and **9** to have significantly higher lipophilicities than the parent structures (5 to 15 times greater, on the basis of their 1-octanol/water partition coefficients). Furthermore the experimental values agree well with values generated algorithmically by a computer-based predictive program.[11]

Compounds were evaluated as inhibitors of HIV-1 and HIV-2 in CEM cell culture (Table 2). The data revealed an elevation in potency for all four derivatives compared to the less lipophilic parent compounds. Significant enhancements were observed for the *meta*-iodo and *meta*-bromo ddA derivatives (**8** and **9**), both showing a 7- to 13-fold elevation in potency compared to the underivatised parent compound (**7**) against HIV-1 and HIV-2. Against HIV-1, compounds **8** and **9** exceeded approximately 2000-fold the inhibitory activity expressed by ddA. The *para*-iodo derivative of d4A (**5**) and the *para*-bromo derivative (**6**) exhibited respectively a 10-fold and 7-fold improvement in potency against HIV-2.

Compound	log P calculated	log P measured	PLE $t_{1/2}$ (hr)
4	0.87	0.94	301
5	2.14	2.11	62
6	1.88	1.72	100
7	0.83	0.89	301
8	2.09	2.07	62
9	1.83	1.71	75
ddA	-0.88	-0.22[a]	[b]
d4A	-0.83	-0.36[a]	[b]

[a] quoted values.[11] [b] not determined

Table 1. Log P values and esterase half-lives of compounds **4** to **9**, ddA and d4A.

The toxicity of the derivatives was also elevated with respect to the underivatised parent compounds. However, a comparison between the selectivity indices (SI, Table 2) reveals only a marginal difference between the underivatised parent compounds **4** and **7** and their respective monohalogenated derivatives **5**, **6**, **8** and **9**. The elevation in toxicity observed for the derivatives possibly reflects the increased intracellular concentration of the variously phosphorylated forms of the nucleoside. The potential importance of generating an increased prodrug potency without substantially altering the selectivity index lies in the context of the clinical treatment of AIDS. Current HIV Highly Active AntiRetroviral Therapies (HAART) are applied over an indefinite time-scale and switching between multi-drug treatment regimens is often necessitated by the development of resistance and/or the expression of deleterious side-effects.[13] If the concentration of prodrug required to produce a clinical effect can be dramatically reduced, this may lead to a reduction in clinically observed side-effects and an increase in cost-effectiveness.

Compound	$EC_{50}(\mu M)$ CEM (HIV-1)	$EC_{50}(\mu M)$ CEM (HIV-2)	$CC_{50}(\mu M)$ CEM	SI (CC_{50}/EC_{50}) (HIV-1)	SI (CC_{50}/EC_{50}) (HIV-2)
4	0.0055 ± 0.0007	0.018 ± 0.003	3.8 ± 0.32	691	211
5	0.0023 ± 0.0008	0.0017 ± 0.0002	0.86 ± 0.01	374	506
6	0.0032 ± 0.0	0.0027 ± 0.0008	0.84 ± 0.26	263	311
7	0.016 ± 0.0	0.035 ± 0.007	2.57 ± 0.64	161	73
8	0.0022 ± 0.0009	0.0027 ± 0.0008	0.43 ± 0.08	195	159
9	0.0023 ± 0.0008	0.0033 ± 0.0016	0.56 ± 0.28	243	170
ddA	4	8	>100	>25	>13
d4A	20	20	91	4.6	4.6

Table 2. Antiviral activity of compounds **4** to **9**, ddA and d4A (standard deviations shown).[12]

The mechanism by which these phosphoramidates are believed to be degraded to their respective monophosphates occurs via initial esterase-mediated carboxyl ester hydrolysis (Figure 4). Then follows phosphate ester hydrolysis resulting in the loss of the aromatic moiety, mediated by an internal nucleophilc addition of the carboxyl group to the phosphorus atom. Finally, progression to the monophosphate is thought to be enzymatically driven - possibly by a cellular phosphoramidase. An assay using Pig Liver Esterase (PLE) was used to model the initial bio-activation step and formation of intermediate **10**. The formation of this intermediate (or its analogue, depending on the specific nucleoside in question) under the conditions of the esterase assay has been taken as a necessary but not sufficient condition to predict biological activity.[7] The results (Table 1) demonstrated that both the underivatised parent phosphoramidates (**4** and **7**) and all of the monohalogenated derivatives (**5**, **6**, **8** and **9**) were efficiently converted to **10** as determined by ^{31}P NMR. Interestingly, the half-lives of the monohalogenated derivatives **5**, **6**, **8** and **9** are all lower compared to their corresponding parent compounds. Thus, although on the basis of the corresponding study of d4T-based phosphoramidates, the elevations in antiviral activity observed for the halogenated d4A and ddA phosphoramidates would appear to correlate with an increased compound lipophilicity, a contribution to their enhanced activity from an enhanced rate of enzymatic metabolism cannot be ruled out pending a comprehensive SAR study. It is notable that the half-lives for the parent d4A and ddA

phosphoramidates (**4** and **7**) were identical to the half-life of the analogous d4T-based phosphoramidate under the same assay conditions (data[8] not shown). This apparantly suggests that while modifications to the chemical structures constituting the phosphoramidate clearly do effect the rate of enzymatically induced carboxy ester hydrolysis, the nature of the specific nucleoside base may not be influential.

In conclusion, we have shown that increasing the lipophilicity of the aryl phosphoramidate prodrugs of ddA and d4A, by monohalogenation of the aryl moiety leads to a significantly enhanced *in vitro* antiviral potency. Since a concomitant increase in toxicity is also observed, the selectivity indices are not substantially altered. These results suggest the scope to increase the *in vitro* antiviral activity of arylphosphoramidate prodrugs based on a wide range of nucleosides by simple mono-derivatisation of the aryl ring. They also suggest that the phenomenon is not specifically applicable to phosphoramidates based on d4T alone. Further work is currently underway to design lipophilic phosphoramidate prodrugs without compromising aqueous solubility - an important factor for further pre-clinical development.

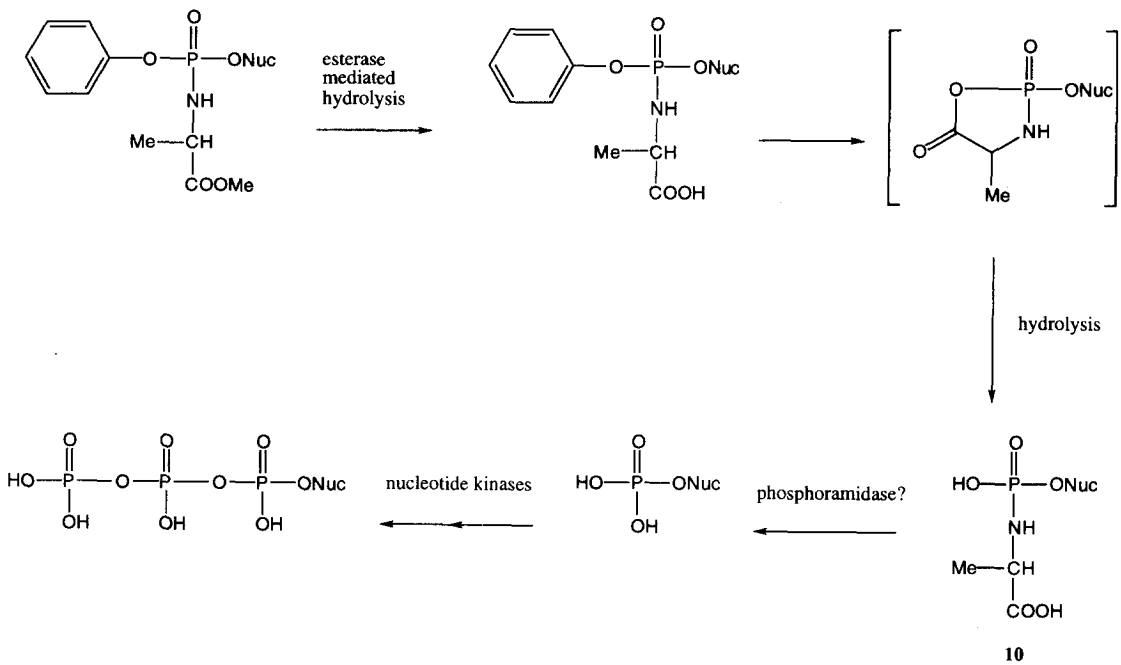

Figure 4 Intracellular bio-activation of phosphoramidate prodrugs (Nuc = ddA or d4A).

Acknowledgments

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9. Unpublished results.

10. Selected data for **6** (2',3'-Didehydro-2',3'-dideoxyadenosine-5'-(4-bromophenyl methoxyalaninyl phosphate)): $\delta_P(CDCl_3)$ 4.15, 4.03 (3:1); $\delta_H(CDCl_3)$ 8.42 (1H, s, H8), 8.03 (1H, s, H2), 7.61 (2H, *meta*-H), 7.13 (1H, s, H1'), 6.99 (2H, m, *ortho*-H), 6.42 (1H, m, H3'), 6.20 (3H, m, H2', NH_2), 5.20 (1H, m, H4'), 4.35 (2H, m, H5'), 4.04 (1H, m, NH-ala), 3.72 (4H, m, OMe, CH-ala), 1.28 (3H, 2d, CH_3-ala); $\delta_C(CDCl_3)$ 174.22, 174.13 (CO-ala) 155.75 (C6), 153.22 (C2), 150.02, 149.94 (Ar-*ipso*, C4), 139.54 (C8), 133.66, 133.55 (C-3'), 133.01 (Ar-*meta*), 126.86, 126.74 (Ar-*ortho*), 122.32, 122.29 (C2'), 118.26 (C5), 88.82, 88.58 (C1'), 85.90, 85.79 (C4'), 67.53, 66.81 (d, J 5.6, J 4.4, C5'), 52.95 (Ome), 50.40, 50.53 (CH-ala), 21.40, 21.06 (CH_3-ala); FAB m/e 553.0596 (MH^+, $C_{20}H_{23}N_6O_6PBr$ requires 553.0600); HPLC t_R 30.37, 30.69 min (gradient I); t_R 32.34, 32.72 min (gradient II). HPLC (Shimadzu) was conducted on an SSODS2 reverse phase column using a water/acetonitrile (Fisher: HPLC grade) eluent ; gradient I (standard gradient): 0-80% CH_3CN (0-60 min), 80-0% CH_3CN (60-65 min), flow rate: 1 ml/min, UV detection at 265 nm; gradient II: 0-10% CH_3CN (0-5 min), 10-70% CH_3CN (5-55 min), 70-0% CH_3CN (55-60 min), flow rate: 1 ml/min, UV detection at 265 nm.

11. ClogP version 1.0.0; Biobyte, P.O. Box 517, Claremont, CA 91711 USA.

12. EC_{50} is the 50% effective compound concentration required to protect CEM cells against the cytopathicity of HIV by 50%. Data are the mean of two to four independent experiments. CC_{50} is the 50% cytotoxic compound concentration required to inhibit CEM cell proliferation by 50%. PLE experiments were conducted at pH 7.6 and 37 °C, using 19 units/mg PLE (full experimental details given in reference 8).

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