Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 3121–3126

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

ENHANCING EFFECTS OF A MONO-BROMO SUBSTITUTION AT THE *PARA* POSITION OF THE PHENYL MOIETY ON THE METABOLISM AND ANTI-HIV ACTIVITY OF D4T-PHENYL METHOXYALANINYL PHOSPHATE DERIVATIVES

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Received 6 February 1998; accepted 17 September 1998

Abstract: d4T-5′-[*p*-Bromophenyl methoxyalaninyl phosphate] (d4T-pBPMAP), a novel phenyl phosphate derivative of 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) that has an enhanced ability to undergo hydrolysis due to the electron withdrawing properties of its single bromo substituent at the *para*-position of the phenyl moiety, was found to yield substantially more of the key metabolite alaninyl d4T monophosphate (A-d4T-MP) than the unsubstituted d4T-5′-phenyl methoxyalaninyl phosphate or *para*-methoxy substituted d4T-5′-phenyl methoxyalaninyl phosphate. d4T-pBPMAP was tested for its anti-HIV-1 activity in peripheral blood mononuclear cells (PBMNC) and thymidine kinase (TK)-deficient CEM T-cells. d4T-pBPMAP was 12.6-fold more potent than the parent compound d4T in inhibiting p24 production (IC_{50} values: 44 nM vs 556 nM) and 41.3-fold more potent than d4T in inhibiting the reverse transcriptase (RT) activity (IC_{50} values: 57 nM vs 2355 nM) in HIV-1-infected TK-deficient CEM cells. Similarly, d4T-pBPMAP was more potent than the unsubstituted or *para*-methoxy substituted phenyl methoxyalaninyl phosphate derivatives of d4T. d4T-pBPMAP did not exhibit any detectable cytotoxicity to PBMNC or CEM cells at concentrations as high as 10,000 nM. Notably, d4T-pBPMAP was capable of inhibiting the replication of a zidovudine (ZDV/AZT)-resistant HIV-1 strain as well as HIV-2 in PBMNC at nanomolar concentrations. To our knowledge, this is the first demonstration that the potency of the d4T-aryl-phosphate derivatives can be substantially enhanced by introducing a single *para*-bromo substituent in the aryl moiety.

The 5′-triphosphates of 2′,3′-dideoxynucleoside (ddN) analogues, which are generated intracellularly by the action of nucleoside and nucleotide kinases, are potent inhibitors of HIV-RT.[1-3] The rate-limiting step for the conversion of AZT to its bioactive metabolite AZT-triphosphate seems to be the conversion of the monophosphate derivative to diphosphate derivative, whereas the rate-limiting step for the intracellular generation of the bioactive d4T metabolite d4T-triphosphate was reported to be the conversion of the nucleoside to its monophosphate derivative.[2,4] In an attempt to overcome the dependence of ddN analogues on intracellular nucleoside kinase activation, McGuigan et al. have prepared aryl methoxyalaninyl phosphate derivatives of 3′-azido-3′-deoxythymidine (AZT)[5,6] and 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T).[4-7] As illustrated in Figure 1, such compounds have been shown to undergo intracellular hydrolysis to yield monophosphate derivatives that are further phosphorylated by thymidylate kinase to give the bioactive triphosphate derivatives in a TK-independent fashion.[4,8] However, all attempts to date to further improve the potency of the aryl phosphate derivatives of d4T by various substitutions of the aryl moiety without concomitantly enhancing their cytotoxicity have failed.[4] Here, we extend these earlier studies by reporting the identification of a new *para*-bromo substituted phenyl methoxyalaninyl phosphate derivative of d4T as an active anti-HIV agent, which potently inhibits HIV replication in TK-deficient CEM T-cells without any detectable cytotoxicity.

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00547-2

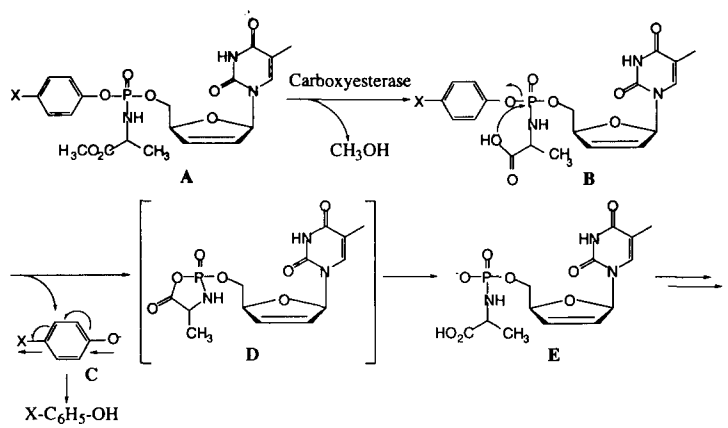
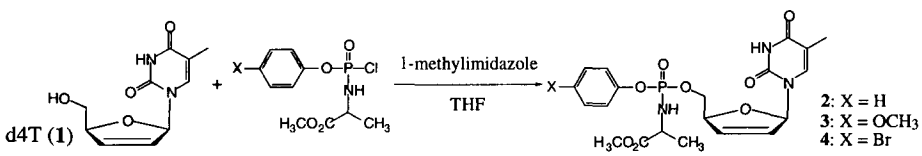


Figure 1. *Literature proposed metabolic pathway of aryl phosphate derivatives of d4T.*[8]

d4T **1** was prepared from thymidine following the literature procedure.[9] Appropriately substituted phenyl methoxyalaninyl phosphorochloridates were also prepared according to the method reported by McGuigan et al.[6] and compounds **2–4** were synthesized as outlined in Scheme 1.



Scheme 1. *Synthesis*: Phenyl methoxyalaninyl phosphorochloridate was added to a solution of d4T in anhydrous THF containing 1-methylimidazole and the contents were stirred at room temperature for 5–6 h. Workup of the reaction mixture furnished the required derivatives in good yields. Column chromatography was applied to obtain analytically pure compounds as distereoisomers due to the phosphate center.

The presence of an electron withdrawing substituent at the *para* position of the phenyl moiety is likely to increase the hydrolysis rates of the phenoxy group in the metabolite precursor **B** (Figure 2A and 2B) generated by the carboxyesterase-dependent first step (**A** to **B** in Figure 1) of the metabolic pathway of phenyl phosphate derivatives of d4T. We assumed that a single bromo substitution at the *para* position of the phenyl ring would not interfere with the recognition and hydrolysis of this compound by the carboxyesterase (Step **A** to **B** in Figure 1). We further postulated that the electronic effect induced by the electron-withdrawing *para*-bromo substituent would result in enhanced hydrolysis of phenoxy group **C** yielding **D** and subsequently **E**, the precursors of the key metabolite A-d4T-MP. In order to test this hypothesis, we compared the unsubstituted compound **2**, *para*-methoxy (OCH_3) substituted compound **3**, and *para*-bromo substituted compound **4** (=d4T-5′-[*p*-bromo-phenyl methoxyalaninyl phosphate] or d4T-pBPMAP), for their rate of chemical hydrolysis after treatment with 0.002 N NaOH by measuring the generation of alaninyl-d4T-monophosphate (**A-d4T-MP**). As shown in Figure 2C, compound **4** with a *para*-bromo substitent showed a much faster hydrolysis rate than the unsubstituted compound **2**, whereas compound **3** with the electron donating substituent -OCH_3 at *para* position had a slower hydrolysis rate than either of those two compounds. Similarly, the lead compound **4** was more sensitive to enzymatic hydrolysis by porcine liver esterase than compound **2** (Figure 2D).

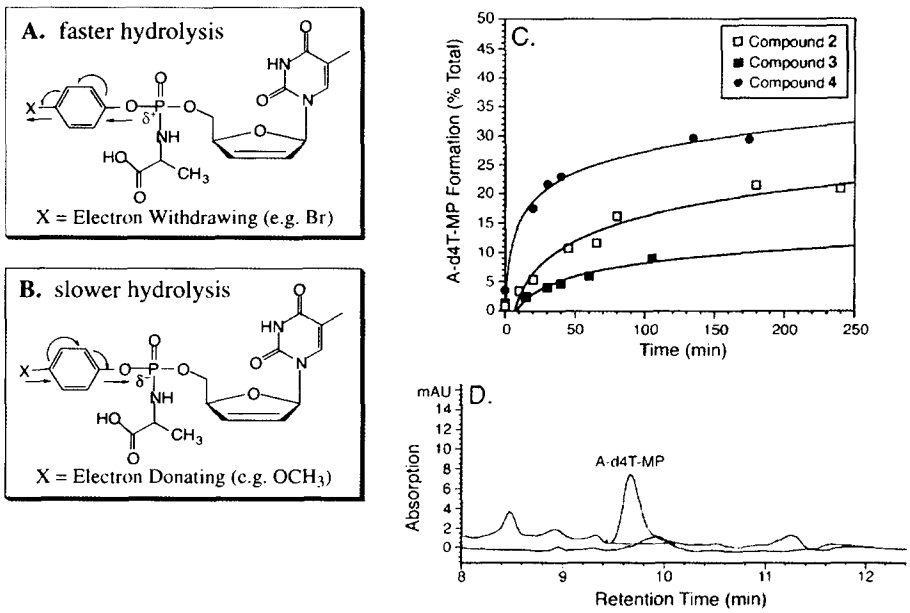


Figure 2. *Susceptibility of compounds 2–4 to hydrolysis.* (A and B) Schematic representation of the electronic effects of the *para* substituent in the phenyl ring of metabolite precursor **B** (see Fig. 1). (C) Compounds **2**–**4** were dissolved in methanol and then treated with 0.002 N NaOH. The concentrations were kept constant and the generation of the hydrolysis product **A-d4T-MP** was monitored using HPLC. A Lichrospher column (C18) was used for the HPLC runs. The column was eluted under isocratic conditions using the solvent mixture 70/30 water/acetonitrile. (D) Compounds **2** and **4** (1 mM in Tris-HCl) were incubated with 100 U of porcine liver esterase (Sigma) in Tris-HCl buffer (pH 7.4) for 2 h at 37 °C. Reaction was stopped by adding acetone and chilling the reaction mixture. Following centrifugation at 15,000 × *g*, 0.1 mL aliquots of the reaction mixture were examined for the presence of the active metabolite **A-d4T-MP** by using a quantitative analytical HPLC method capable of detecting 50 pmols of the metabolite. The 0.1 mL aliquot of the reaction product of compound **4** contained 1.4 nmols of **A-d4T-MP**, wheras no metabolite was detected in the reaction product of compound **2**.

We also incubated 1×10^6 CEM cells with compounds **2**–**4** (100 μM) for 3 h and subsequently examined the formation of the recently reported partially hydrolyzed phosphate diester metabolite, alaninyl d4T monophosphate[4] by HPLC. Notably, the amount of this metabolite in CEM cells treated with compound **4** was substantially greater than in CEM cells treated with compound **2** or **3** (680 pmol/10^6 cells vs <50 pmol/10^6 cells; Figure 3). While this significant difference is likely due to enhanced intracellular hydrolysis of compound **4**, it is also possible that it is owing to a better passive penetration of compound **4**, as compared to compounds **2** and **3**.

Because of its enhanced susceptibility to hydrolysis yielding substantially greater amounts of A-d4T-MP (the key precursor of the active d4T-TP metabolite), compound **4** was postulated to be a more potent anti-HIV agent than the other compounds. Compounds **2**–**4**, as well as the parent compound d4T (**1**), were tested for their ability to inhibit HIV replication in peripheral blood mononuclear cells and TK-deficient CEM T-cells using previously described procedures.[10] Percent inhibition of viral replication was calculated by comparing the p24 and RT activity values from the test substance-treated infected cells with those from untreated infected cells. In parallel, the cytotoxicity of the compounds was examined using a microculture tetrazolium assay (MTA) of cell proliferation, as described.[10] As evidenced by the similarity of the IC_{50} values for inhibition of HIV-1 replication

shown in Table 1, the d4T-aryl phosphate derivatives were not more potent than the parent compound d4T when tested in HIV-1-infected peripheral blood mononuclear cells. In accord with previous reports, the ability of d4T to inhibit HIV-1 replication was substantially reduced in TK-deficient CEM cells. Whereas the IC_{50} value for inhibition of p24 production by d4T was 18 nM in peripheral blood mononuclear cells, it was 556 nM in TK-deficient CEM cells. Similarly, the IC_{50} value for inhibition of RT activity increased from 40 nM to 2355 nM (Table 1). While all 3 aryl phosphate derivatives were more potent than d4T in TK-deficient CEM cells, compound **4** (= d4T-5′-[*p*-bromo phenyl methoxyalaninyl phosphate]) with a *para*-bromo substituent in the aryl moiety was 12.6-fold more potent in inhibiting p24 production (IC_{50} values: 44 nM vs 556 nM) and 41.3-fold more potent in inhibiting the RT activity (IC_{50} values: 57 nM vs 2355 nM) than d4T (Table 1).

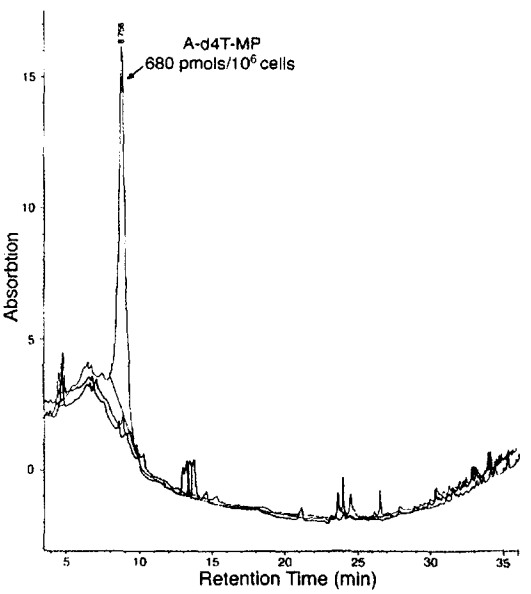
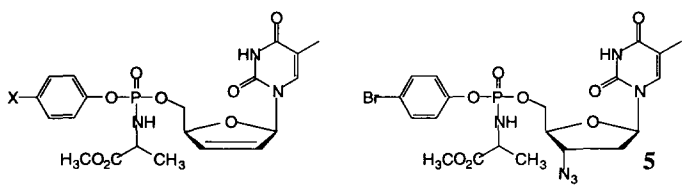


Figure 3. *Intracellular metabolism of compounds 2–4 in TK-deficient CEM cells.* CEM cells were cultured in a medium composed of RPMI, 10% fetal bovine serum, and 1% penicillin/streptomycin. Ten million cells at a density of 10^6 cells/mL were incubated with 100 μM of these compounds for 3 h at 37 °C. After incubation, cells were washed twice with ice-cold PBS, and extracted by addition of 0.5 mL of 60% methanol. Cell lysates were kept at −20 °C overnight, after which lysates were centrifuged at 15000 × *g* for 10 min to remove the cell debris. One hundred μL aliquots of these lysates were injected directly to HPLC. The HPLC system consisted of a Hewlett–Packard 1100 series equipped with a quarternary pump, an auto sampler, an electronic degasser, a diodearray detector, and a computer with a chemstation software program for data analysis. The samples were eluted on a 250 × 4.6 mm Sulpelco LC-DB C18 column. A solvent gradient was utilized to resolve the metabolite from the parent compound, which consisted of a mixture of methanol and 10 mM ammonium phosphate (pH 3.7). The gradient ran at a flow rate of 1 mL/min from 5 to 35% methanol for the first 10 min, kept at 35% methanol for 5 min, and finished with a linear gradient from 35 to 100% methanol in the next 20 min. The detection wavelength was set at 270 nm. A metabolite peak with a retention time of 8.7 min corresponding to 680 pmols of **A-d4T-MP** was detected only in aliquots from CEM cell lysates incubated with compound **4**.

Compound **5**, AZT-5′-(*para*-bromophenyl methoxyalaninyl phosphate),[12] was used as the corresponding control AZT derivative. None of the compounds exhibited any detectable cytotoxicity to peripheral blood mononuclear cells or CEM cells at concentrations as high as 10,000 nM, as determined by MTA. Intriguingly, compound **3** with a *para*-methoxy substituent in the aryl moiety was 5.6-fold less effective than compound **4** in inhibiting the RT activity in HIV-infected TK-deficient CEM cells (IC_{50} values: 320 nM vs 57 nM) although these two compounds showed similar activity in peripheral blood mononuclear cells (IC_{50} values: 33 nM vs 42 nM). Thus, the identity of the *para*-substituent appears to affect the anti-HIV activity of the aryl phosphate derivatives of d4T in TK-deficient cells. Similarly, compound **5** was highly active against HIV-1 in both PBMNC and CEM T-cells. Compound **4** had potent antiviral activity against RTMDR-1 and moderate activity against HIV-2.

However, the corresponding *para*-bromo substituted phenyl methoxyalaninyl phosphate derivative of AZT (compound **5**) and AZT were not effective against the AZT resistant RTMDR-1 or against HIV-2. To our knowledge, this is the first demonstration that the potency as well as the selectivity index of the d4T-aryl-phosphate derivatives can be substantially enhanced by introducing a single *para*-bromo substituent in the aryl moiety. This previously unknown structure–activity relationship determined by the aryl moiety of the phosphate derivatives of d4T may provide the basis for the design of potentially more potent d4T analogues.

Table 1. *Anti-HIV Activity of d4T-5′(p-bromo-phenyl methoxyalaninyl phosphate) (4) in normal peripheral blood mononuclear cells and TK-deficient CEM T-cells.* All data are in μM and represent concentrations required to inhibit viral replication, as measured by assays of p24 production as well as RT activity, by 50% (IC_{50} [p24] and IC_{50}[RT])[10] or the 50% cytotoxic concentration, as measured by MTA (IC_{50}[MTA]).[10] (A) PBMNC or CEM cells were infected with the $HTLV_{IIIB}$ strain of HIV-1. (B) PBMNC were infected with the multidrug-resistant HIV-2 or RTMDR-1 strain of HIV-1 (catalog # 252a, NIH AIDS Research and Reagent Program) which is known to contain mutations in the RT amino acid residues 74V, 41L, 106A and 215Y rendering the virus resistant to AZT, ddI and non-nucleoside RT inhibitors such as nevirapine.



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A.

Compound	X	PBMNC IC_{50} [p24]	PBMNC IC_{50} [RT]	PBMNC IC_{50} [MTA]	CEM IC_{50} [p24]	CEM IC_{50} [RT]	CEM IC_{50} [MTA]
1 (= d4T)		0.02	0.04	>10	0.6	2.4	>10
2	H	ND	ND	>10	0.1	0.1	>10
3	$-OCH_3$	0.03	0.03	>10	0.1	0.3	>10
4	Br	0.02	0.04	>10	0.04	0.06	>10
5		ND	0.004	>10	ND	0.04	>10

B.

Compound	HIV-2 IC_{50} [RT]	RTMDR-1 IC_{50} [RT]
4	0.4	0.02
5	3.9	1.5
AZT	2.4	2.0

References and Notes

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11. Analytical data: (a) compound **2**: yield: 81%; IR (Neat): 3222, 2985, 2954, 1743, 1693, 1593, 1491, 1456, 1213, 1153, 1039, 931, 769 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.30 (br s, 1H), 7.30–7.10 (m, 6H), 6.85–6.82 (m, 1H), 6.36–6.26 (m, 1H), 5.91–5.85 (m, 1H), 5.00 (br m, 1H), 4.19–3.68 (m, 4H), 3.72, 3.71 (s, 3H), 1.83, 1.80 (d, 3H), 1.38–1.25 (m, 3H); ^{13}C NMR ($CDCl_3$, starred peaks are split due to diastereoisomers) δ 173.9, 163.7, 150.7, 149.7, 135.7*, 133.2*, 129.6*, 127.3*, 125.0*, 120.0, 111.1, 89.6*, 84.5*, 66.9*, 52.5*, 50.0*, 20.9 and 12.3; ^{31}P NMR ($CDCl_3$) δ 2.66 , 3.20 (two peaks with a ratio of intensity = 1/1 due to the diastereomeric isomers arising from the phosphorus stereogenic center.); MS (MALDI-TOF) *m/z* 487.9 (M^+ + Na); HPLC retention time (C18 4 × 250 mm LiChrospher column, 1 mL/min, 70/30 water/acetonitrile): 5.54 and 5.85 min; (b) Compound **3**: yield: 92%; IR (Neat): 3223, 3072, 2999, 2953, 2837, 1743, 1693, 1506,1443, 1207, 1153, 1111, 1034, 937, 837 and 756 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.40 (br s, 1H), 7.30–7.00 (m, 5H), 6.83–6.81 (m, 1H), 6.37–6.27 (m, 1H), 5.91–5.86 (m, 1H), 5.00 (br m, 1H), 4.40–4.30 (m, 2H), 4.20–4.10 (m, 2H), 3.95–3.93 (s, 3H), 3.82–3.80 (s, 3H), 1.85–1.81 (s, 3H) and 1.39–1.29 (m, 3H); ^{13}C NMR ($CDCl_3$, starred peaks are split due to diastereoisomers) δ 174.0, 163.9, 156.6 , 150.8, 143.5, 135.8*, 133.3*, 127.4*, 121.2*, 114.5, 111.2, 89.7*, 84.5, 66.9*, 55.5, 52.5, 50.6*, 20.9, and 12.3; ^{31}P NMR($CDCl_3$) δ 3.82 , 3.20 (two peaks with a ratio of intensity = 1/1 due to the diastereomeric isomers arising from the phosphorus stereogenic center); MS (MALDI-TOF) *m/z* 518.2 (M^+ + Na); HPLC retention time (C18 4 × 250 mm LiChrospher column, 1 mL/min, 70:30 water/acetonitrile): 5.83 and 6.26 min; (c) Compound **4**: yield: 83%; IR (Neat): 3203, 3070, 2954, 2887, 2248, 1743, 1693, 1485, 1221, 1153, 1038, 912, 835, 733 cm^{-1}; ^1H NMR($CDCl_3$) δ 9.60–9.58 (br s, 1H), 7.45–7.42 (m, 2H), 7.30–7.09 (m, 4H), 6.37–6.27 (m, 1H), 5.93–5.88 (m, 1H), 5.04–5.01 (m, 1H), 4.35–4.33 (m, 2H), 4.27–3.98 (m, 2H), 3.71–3.70 (s, 3H) , 1.85–1.81 (s, 3H), 1.37–1.31 (m, 3H); ^{13}C NMR ($CDCl_3$, starred peaks are split due to diastereoisomers) δ 173.7, 163.8, 150.8, 149.7*, 135.6*, 133.1*, 127.4*, 121.9*, 118.0, 111.2*, 89.7*, 84.4*, 67.8*, 52.5, 50.0*, 20.7, and 12.3; ^{31}P NMR ($CDCl_3$) δ 3.41, 2.78 (two peaks with a ratio of intensity = 1/1 due to the diastereomeric isomers arising from the phosphorus stereogenic center); MS (MALDI-TOF) *m/z* 567.1 (M^+ + Na); HPLC retention time (C18 4 × 250 mm LiChrospher column, 1 mL/min, 70/30 water/acetonitrile): 12.04 and 12.72 min.
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