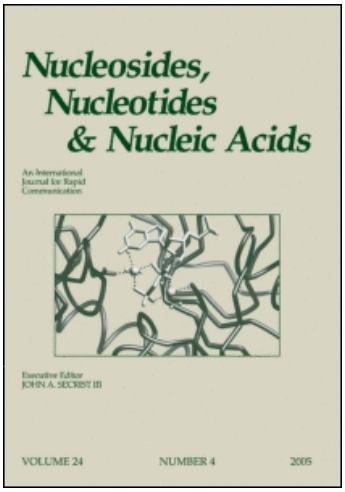

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(8), 1571–1582 (2001)

COMPARISON OF THE ANTIVIRAL ACTIVITY OF HYDROPHOBIC AMINO ACID PHOSPHORAMIDATE MONOESTERS OF 2′,3′-DIDEOXYADENOSINE (DDA) AND 3′-AZIDO-3′-DEOXYTHYMIDINE (AZT)

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ABSTRACT

A series of hydrophobic, water soluble and non-toxic amino acid phosphoramidate monoesters of dideoxyadenosine (ddA) and 3′-azido-3′-deoxythymidine were shown to inhibit the replication of HIV-1 in human peripheral blood mononuclear cells (PBMC) from two donors. The tryptophan methyl ester phosphoramidates of AZT and ddA were equally potent ($EC_{50}S = 0.3-0.4\,\mu M$), while the phenyl methyl ester of ddA was 40- to 100- fold more potent than the AZT derivatives. The alaninyl methyl ester of AZT was found to be 70- fold more potent than the ddA derivative. The methyl amide derivatives were found to be 5–20 fold less active than the methyl esters for the ddA series, while for AZT the derivatives were found to be of similar potency or 60- to 166- fold more potent than the methylesters.

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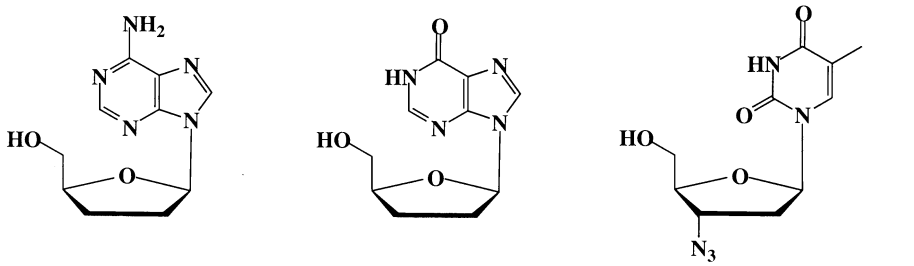
INTRODUCTION

Nucleoside analogs, such as AZT, d4T, 3TC and ddC, exhibit potent anti-HIV-1 activity and are currently used to control HIV-1 infection. In addition to the pyrimidine based nucleosides, ddI (didanosine, 2′,3′-dideoxyinosine) (Figure 1) and abacavir, are currently the only clinically approved purine nucleoside RT inhibitors[1–3].

Didanosine (ddI), the deamination product of ddA, inhibits HIV-1 replication *in vitro* at nM concentrations and is effective for the treatment of HIV-1 infection in a variety of clinical situations with diverse patient populations.[1] The antiviral activity of ddI is dependent on the rate limiting intracellular conversion to ddA-MP, which is followed by conversion to ddA-TP by a series of enzymatic reactions[4–7]. The triphosphate of ddA is a potent inhibitor of HIV-RT DNA chain elongation[4,5,8].

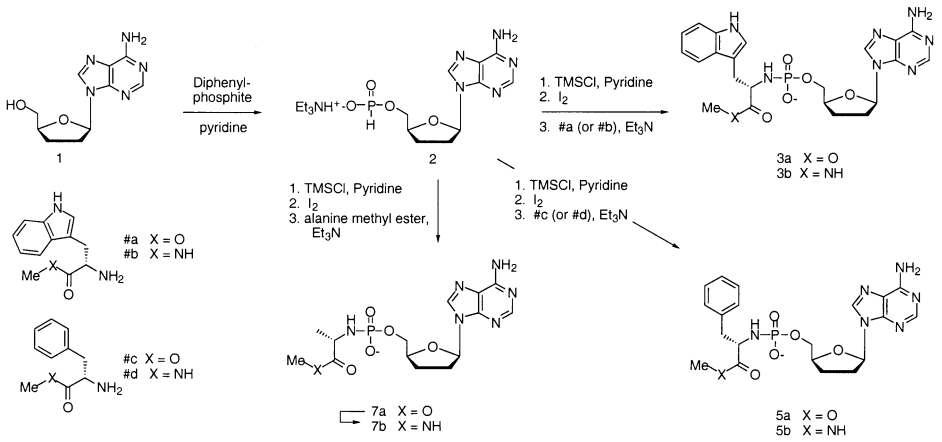
Although the efficacy of ddI as a first-line treatment for patients suffering HIV-1 infection is now well established, several clinical limitations have been recognized with ddI therapy. Pharmacokinetic studies revealed that acid lability of the N-glycosyl bond ($t_{1/2} = 30$ sec at pH 2) significantly complicates the oral administration of ddA and ddI.[2] Bone marrow cytotoxicity, humoral immunity suppression, as well as other toxic side effects were also observed with the administration of ddI[9–11]. Furthermore, ddI is hydrolyzed by purine nucleoside phosphorylase (PNP) to hypoxanthine which then enters the purine metabolic pool or is degraded by xanthine oxidase to uric acid[6]. *In vivo,* approximately 30 to 60% of i.v. administered ddI is recovered unmetabolized in the urine within 8 hr[12]. ddI is also rapidly eliminated from the plasma ($t_{1/2} = 0.6$ to 1.6 hr)[1]. Due to the poor pharmacokinetic properties, toxic side effects, and the lengthy activation steps, attempts have been made to increase the therapeutic index of ddI by intracellular delivery of ddAMP with neutral, lipophilic pronucleotides.

Several pronucleotide approaches have been described for the delivery of ddA monophosphate[13–20]. For example, the bis(SDTE)- and bis(SATE)-nucleotides approaches[13–17] developed by Imbach and Gosselin *et al.*, the



ddA (2',3'-deoxyadenosine) **ddI** (2',3'-dideoxyinosine) **AZT** (3'-azido-3'-deoxythymidine)

Figure 1. Structure of ddA, ddI and AZT.



Scheme 1.

aryloxy phosphoramidate approach[18] developed by McGuigan *et al*. and the cyclo*Sal*-nucleotide approach[19,20] developed by Meier *et al*. have all successfully delivered ddAMP into CEM and MT-4 cells by either chemical or enzymatic hydrolysis. However, *in vivo* pharmacokinetic or antiviral data for the ddA pronucleotides has not been reported.

Previously, we have demonstrated that AZT phosphoramidate derivatives exhibited anti-HIV-1 activity at nanomolar concentrations in PBMCs and CEM cells with no observable cytotoxicity at concentrations up to 100 μM[21–23]. In addition, we have shown that these compounds have plasma half lives at least 5 times longer and volumes of distribution at least 10 times greater than observed for AZT in rats. Consequently, we sought to investigate the potential for phosphoramidate monoesters of ddA to serve as pronucleotides for ddAMP. A series of ddA phosphoramidate monoester derivatives with different amino acid moieties was synthesized and the antiviral potencies determined. Antiviral potency exceeding that of ddA would suggest ddA-MP delivery, since the conversion of ddA to ddA-MP has been shown to predominantly proceed through ddI-MP[20].

RESULTS AND DISCUSSION

Chemistry

We have recently used H-phosphonate chemistry, similar to that developed by Stawinski and coworkers[24], to prepare phosphoramidates of AZT[25]. We found that the same approach could be applied to adenine containing nucleosides without protection of the amino group at position 4. (Scheme 1) First, ddA (**1**) was treated with diphenyl phosphite in pyridine to

yield the H-phosphonate intermediate (**2**). This was treated with excess trimethylsilyl chloride followed by oxidation with iodine. Next, tryptophan methylester was added with triethyl amine and the desired phosphoramidate **3a** was isolated in 28% yield. Similar reactions with tryptophan methyl amide, phenylalanine methyl ester, phenylalanine methyl amide and alanine methyl ester gave **3b**, **5a**, **5b** and **7a** in 40%, 51%, 47% and 40% yields, respectively. The conversion of the methyl ester of the alanine derivative to the methyl amide was achieved by treating **7a** with 10 M methylamine in methanol to give **7b** in 61% yield.

Biological Results

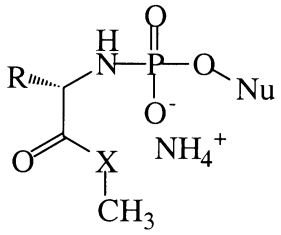
In addition to studies with AZT, acyclovir, FUdR and FLT, our approach of using phosphoramidates bearing single amino acids for the delivery of nucleoside monophosphates was also applied to the purine nucleoside analog of ddA[21,22,25–27]. As seen in Table 1, all compounds exhibited anti-HIV-1 activity in PBMCs from donors 1 and 2 and with no appreciable cytotoxicity up to a concentration of 100 μM. Donor variability in potency ranged from 1.3- to 5.3-fold. The antiviral potency associated with compounds **3a** and **5a** appeared to be comparable or better than the parent nucleoside ddA. According to the structure activity relationships of the AZT derivatives, the alanine based phosphoramidate, **8a**, was found to be more potent than phosphoramidates composed of aromatic amino acids, **4a** and **6a**. The same effect, however, was not observed with the ddA derivatives. Conversely, changing the amino acid from alanine (**7a**) to the bulky phenylalanine (**5a**) and tryptophan (**3a**) led to a 4.4- to 14.7-fold increase in potency, suggesting that the size of the amino acid does play a major role in the antiviral activity of ddA phosphoramidates. Replacement of the methylester with methylamide led to a 0.8- to 20-fold reduction in antiviral potency (**3b, 5b**).

The role of amino acid esterification on antiviral potency was also investigated. For the tryptophan and phenylalanine phosphoramidates of ddA, the results were similar to results obtained for AZT tryptophan phosphoramidates (**4a, 4b**), but not AZT phenylalanine phosphoramidates (**6a,6b**). None of the compounds exhibited inhibitory effects on RT, again suggesting that intracellular activation is required for antiviral activity (Data not shown).

CONCLUSIONS

Taken together, these results demonstrate that phosphoramidate monoesters of ddA and AZT are potent antiviral agents in PBMCs with moderate donor variability. Unlike previous ddA pronucleotides, the ddA

Table 1. The Effect of ddA Phosphoramidate Monoester Derivatives on HIV-1 Replication and their Cytotoxicity in PBMCs[a]



Cpd[b]	R	X	Nu	EC_{50}[c,d] Donor 1	EC_{50}[c,d] Donor 2	CC50
3a	3-indolyl-methyl	O	ddA	0.4	0.3	>100
4a	3-indolyl-methyl	O	AZT	0.3	0.35	>100
3b	3-indolyl-methyl	NH	ddA	2	2.5	>100
4b	3-indolyl-methyl	NH	AZT	0.7	1.8	>100
5a	benzyl	O	ddA	0.8	0.15	>100
6a	benzyl	O	AZT	30	15	>100
5b	benzyl	NH	ddA	1	3	>100
6b	benzyl	NH	AZT	0.18	0.25	>100
7a	methyl	O	ddA	3.5	2.2	>100
8a	methyl	O	AZT	0.05	0.03	>100
7b	methyl	NH	ddA	3.5	1	>100
ddA	–	–	–	0.58	0.60	>100
AZT	–	–	–	0.003	0.010	>100

[a]Values are in μM.
[b]Compounds.
[c]Effective concentration required to inhibit the replication of HIV-1 by 50%.
[d]IC_{50} for all compounds concentration required to kill 50% of the cells as compared to the untreated cultures.
[e]Values represent an average of three experiments. The variance for the EC_{50}, and IC_{50} was less than $\pm 20\%$.

phosphoramidates did not exhibit enhanced toxicity[13–20]. However, the structure activity relationships differ from those observed for AZT phosphoramidates, especially with regard to the phenylalanine and alanine derivatives. Although more derivatives with other amino acids should be studied to broaden the conclusions, with exception of the alanine derivative, the antiviral activity of the methylesters exceeded that of the methylamides. Whether the activation of the ddA and AZT phosphoramidates proceeds through direct P-N bond cleavage remains to be determined, since the most potent ddA compounds were only slightly more potent than ddA in PBMCs. However, given the stability of nucleoside amino acid phosphoramidate monoesters (i.e., $t_{1/2} \geq 6$ days)[22,25,26] and potent antiviral activity of the free

nucleoside, it is unlikely that the phosphoramidates are rapidly converted extracellularly to the parent nucleosides. Differences in the potencies between the parent nucleosides and corresponding phosphoramidates may be a reflection, not of their inability to deliver nucleoside monophosphate, but of their relative rates of conversion to monophosphate. Consequently, an explanation for the idiosyncratic nature of the antiviral potency of the phosphoramidates will depend on the elucidation of the intracellular mechanism of activation. Nevertheless, given the low toxicity of these compounds and the enhanced pharmacokinetic parameters observed for nucleoside amino acid phosphoramidates their further study as potential antivirals is warranted.[23]

MATERIALS AND METHODS

Materials

NMR (^{1}H and ^{31}P) spectra were recorded on a Varian VAC-200 and VAC-300 spectrometers in D_2O. An external standard of 85% H_3PO_4 was used for all ^{31}P-NMR spectra. FAB mass spectra were obtained on a VG 7070E-HF mass spectrometer. Analytical TLC was performed on Analtech Silica Gel GHLF (0.25 mm) plates. Column chromatography was performed with grade 62, 60–200 mesh silica gel. Flash chromatography was performed with grade 60, 230–400 mesh Merck silica gel. Anhydrous pyridine was purchased from Aldrich Chemical Co. and was used without further purification. L-amino acid methyl esters (hydrochloride salts), trimethylsilyl chloride, and diphenyl phosphite were also purchased from Aldrich. All other solvents were reagent grade and used as received. Anhydrous methylamine was bubbled through methanol to give approximately a 10 M solution. Concentration under reduced pressure refers to solvent removal on a Buchi rotary evaporator. High vacuum refers to $<10^{-2}$ psi attained with a DuoSeal mechanical pump.

Triethylammonium 2′,3′-dideoxyadenosine 5′-phosphite (2)

A suspension of ddA (325 mg, 1.38 mmol) in 15 mL of dry pyridine was treated with diphenyl phosphite (270 μL, 1.41 mmol under an Argon atmosphere. After stirring for 2 h, Et_3N (3 mL) and H_2O (3 mL) were added and stirring was continued for 30 min. The reaction mixture was then concentrated under reduced pressure. The resulting residue was dissolved in H_2O and extracted with CH_2Cl_2 (4×). The aqueous portion was then concentrated under reduced pressure. The resulting solid was then purified by flash chromatography (SiO_2, 5 : 2.5 : 0.37 $CHCl_3$/MeOH/H_2O containing 0.5% NH_4OH) to give the desired product (420 mg) as a colorless solid.

^{1}H NMR (D_2O, 300 MHz) δ 8.193 (s, 1H), 8.006 (s, 1H), 6.423 (d, $J = 638$ Hz, 1H), 6.135 (m, 1H), 4.282 (m, 1H), 3.920 (ddd, J = 2.9, 7.1, 11.6 Hz, 1H), 3.766 (ddd, J = 4.6, 8.0, 11.6 Hz, 1H), 3.019 (q, J = 7.3 Hz, 6H), 2.479–2.356 (m, 2H), 2.107–1.964 (m, 2H), 1.097 (t, J = 7.3 Hz, 9H); ^{31}PNMR (D_2O, 121 MHz) δ 7.107.

2*S*-(Hydroxy-(2,3-deoxyadenosyl)-phosphorylamino)-3-(3-indolyl)-propionic acid methyl ester (3a)

Triethylammonium 2′, 3′-dideoxyadenosine 5′-phosphite (**2**) (125 mg, 0.312 mmol) was dissolved in 5 mL of dry pyridine and treated with TMSCl (118 μL, 0.930 mmol) under Ar. After 5 min, a solution of iodine (118 mg, 0.465 mmol) in 3 mL of pyridine was added dropwise, via cannula, until the reaction color changed from yellow to a reddish-brown color. At this point, addition of the iodine solution was stopped. After 5 min, tryptophan methyl ester (HCI salt, 159 mg, 0.624 mmol) and Et_3N (0.30 mL, 2.2 mmol) were added. After stirring for 30 min, the reaction mixture was concentrated under reduced pressure. The resulting syrup was partitioned between 1N NH_4OH solution and $CHCl_3$. The aqueous portion was then extracted with additional portions of $CHCl_3$ to remove unreacted amino acid methyl ester. The aqueous portion was then concentrated to give crude phosphoramidate which was passed through a small Amberlite (IRP-64) ion exchange column, eluting with H_20, and the desired fractions were concentrated under reduced pressure. Flash chromatography (SiO_2, 5:2:0.25 $CHCl_3$/MeOH/H_2O containing 0.5% conc. NH_4OH) gave the desired product (45 mg, 28%) as a white solid. ^{1}H NMR (D_2O, 300 MHz) δ 8.042 (s, 1H), 7.841 (s, 1H), 7.155 (d, $J = 7.9$ Hz, 1H), 7.100 (d, $J = 8.2$ Hz, 1H), 6.872 (dd, $J = 1.0$, 7.1 Hz, 1H), 6.834 (s, 1H), 6.728 (m, 1H), 5.953 (dd, $J = 2.9$, 7.0 Hz, 1H), 4.063 (m, 1H), 3.719–3.633 (m, 2H), 3.534 (ddd, $J = 4.4$, 4.6, 11.3 Hz, 1H), 3.355 (s, 3H), 2.709 (d, $J = 6.4$ Hz, 2H), 2.372–2.117 (m, 2H), 1.920–1.768 (m, 2H); ^{31}P NMR (D_2O, 121 MHz) δ 6.792; HRMS (FAB), m/z calcd for $C_{22}H_{26}N_7O_6P$ $(M + H)^+$ 516.1760, found 516.1757.

(2-(3-Indolyl)-1*S*-methylcarbamoyl-ethyl)-phosphoramidic acid mono-(2,3-deoxyadenosyl) ester (3b)

Using the method described for the preparation of **3a**, **3b** was prepared from **2** (126 mg, 0.314 mmol) and tryptophan methyl amide 143 mg, 0.659 mmol), and Et_3N (0.22 mL, 1.57 mmol) were added. Flash chromatography (SiO_2, 5:2.5:0.37 $CHCl_3$/MeOH/H_2O containing 0.5% NH_4OH) gave the desired product (65 mg, 40%) as a white solid. ^{1}H NMR (D_2O, 300 MHz) δ 7.921 (s, 1H), 7.732 (s, 1H), 7.142 (d, $J = 7.9$ Hz, 1H), 7.025 (d, $J = 8.2$ Hz, 1H), 6.796 (s, 1H), 6.796 (dd, $J = 8.0$, 8.2 Hz, 1H), 6.677

(dd, $J = 7.9$, 8.0 Hz, 1H), 5.822 (dd, $J = 2.7$, 7.0 Hz, 1H), 3.925 (m, 1H), 3.600 (m, 1H), 3.481–3.353 (m, 2H), 2.705 (d, $J = 6.0$ Hz, 2H), 2.388 (s, 3H), 2.335–2.026 (m, 2H), 1.885–1.602 (m, 2H); ^{31}P NMR (D_2O, 121 MHz) δ 6.513; HRMS (FAB) m/z calcd for $C_{22}H_{27}N_8O_5P$ $(M + H)^+$ 515.1920, found 515.1906.

2*S*-(2,3-Deoxy-3-adenosyl-(hydroxy)-phosphorylamino)-3-phenyl-propionic acid methyl ester (5a)

Using a method described for the preparation of **3a**, **5a** was prepared from **2** (124 mg, 0.309 mmol) and phenylalanine methyl ester (HCl salt, 133 mg, 0.618 mmol). Flash chromatography (SiO_2, 5 : 2 : 0.25 $CHCl_3$/MeOH/H_2O containing 0.5% conc. NH_4OH) gave the desired product (75 mg, 51%) as a white solid. 1H NMR (D_2O, 300 MHz) δ 8.070 (s, 1H), 7.854 (s, 1H), 6.921–6.875 (m, 3H), 6.773–6.742 (m, 2H), 5.968 (dd, $J = 2.9$, 7.0 Hz, 1H), 4.098 (m, 1H), 3.606–3.534 (m, 2H), 3.474 (ddd, $J = 4.6$, 4.8, 11.3 Hz, 1H), 3.362 (s, 3H), 2.520 (d, $J = 6.8$ Hz, 2H), 2.239–2.168 (m, 2H), 1.976–1.821 (m, 2H); ^{31}P NMR (D_2O, 121 MHz) δ 6.546; HRMS (FAB) m/z calcd for $C_{20}H_{25}N_6O_6P$ $(M + H)^+$ 477.1651, found 477.1615.

(2-Phenyl-1S-methylcarbamoyl-ethyl)-phosphoramidic acid mono-(2,3-deoxyadenosyl)ester (5b)

Using a method described for the preparation of **3a**, **5b** was prepared from **2** (125 mg, 0.312 mmol) and phenylalanine methyl amide (120 mg, 0.674 mmol). Flash chromatography (SiO_2, 5 : 2.5 : 0.37 $CHCl_3$/MeOH/H_2O containing 0.5% NH_4OH) gave the desired product (70 mg, 47%) as a white solid. ^{1}H NMR (D_2O, 300 MHz) δ 8.121 (s, 1H), 7.912 (s, 1H), 6.958–6.879 (m, 3H), 6.830–6.803 (m, 2H), 6.027 (dd, $J = 2.9$, 7.0 Hz, 1H), 4.097 (m, 1H), 3.559–3.375 (m, 3H), 2.553 (m, 2H), 2.415 (s, 3H), 2.496–2.220 (m, 2H), 2.025–1.740 (m, 2H); ^{31}P NMR (D_2O, 121 MHz) δ 6.193; HRMS (FAB) m/z calcd for $C_{20}H_{26}N_7O_5P$ $(M + H)^+$ 476.1811, found 476.1821.

2*S*-(2,3-Deoxy-adenosyl-(hydroxy)-phosphorylamino)-propionic acid methyl ester (7a)

Using a method described for the preparation of **3a**, **7a** was prepared from **2** (219 mg, 0.546 mmol) and alanine methyl ester (HCl salt, 152 mg, 1.092 mmol). Flash chromatography (SiO_2, 5 : 2 : 0.25 $CHCl_3$/MeOH/H_2O containing 0.5% conc. NH_4OH) gave the desired product (88 mg, 40%) as a white solid. ^{1}H NMR (D_2O, 300 MHz) δ 8.182 (s, 1H), 7.958 (s, 1H), 6.089 (dd, $J = 2.9$, 6.8 Hz, 1H), 4.253 (m, 1H), 3.819 (ddd, $J = 2.5$, 5.3, 11.6 Hz,

1H), 3.640 (ddd, $J = 4.4$, 4.5, 11.6 Hz, 1H), 3.419 (s, 3H), 3.252 (m, 1H), 2.462–2.347 (m, 2H), 2.070–1.982 (m, 2H), 0.921 (d, $J = 7.1$ Hz, 3H); ^{31}P NMR (D_2O, 121 MHz) δ 6.546; HRMS (FAB) m/z calcd for $C_{14}H_{22}N_6O_6P$ $(M + H)^+$ 401.1338, found 401.1320.

(2-Methyl-1*S*-methylcarbamoyl-ethyl)-phosphoramidic acid mono-(2,3-deoxy-adenosyl)ester (7b)

Compound **7a** (88 mg) was dissolved in 5 mL of 10 M methyl amine in methanol and stirred in a sealed vial for 7 d. The reaction mixture was then concentrated under reduced pressure. Flash chromatography (SiO_2, 5 : 2.5 : 0.37 $CHCl_3$/MeOH/H_2O containing 0.5% conc. NH_4OH) gave the desired product (54 mg, 61%) as a white solid. ^{1}H NMR (D_2O, 300 MHz) δ 8.200 (s, 1H), 7.969 (s, 1H), 6.109 (dd, $J = 2.9$, 6.8 Hz, 1H), 4.264 (m, 1H), 3.833 (ddd, $J = 2.7$, 5.1, 11.4 Hz, 1H), 3.666 (ddd, $J = 4.8$, 4.9, 11.4 Hz, 1H), 3.276 (m, 1H), 2.482 (s, 3H), 2.593–2.314 (m, 2H), 2.108–1.934 (m, 2H), 0.949 (d, $J = 7.1$ Hz, 3H); ^{31}P NMR (D_2O, 121 MHz) δ 7.102; HRMS (FAB) m/z calcd for C_{14},$H_{23}N_7O_5P$ $(M + H)^+$ 400.1498, found 400.1508.

Culture of Human PBMCs

Human PBMCs were isolated using Ficoll-Paque (Pharmacia Biotech, NJ) density sedimentation and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 μg/ml)[28]. Cultures were supplemented with phytohemagglutinin (PHA, Sigma, MI) (10 μg/ml) and IL-2 (10 U/ml) (Boehringer Mannheim, IN) where noted. Incubations were done at 37 °C in a 10% CO_2-90% air environment, at a concentration of 10^6 cells/ml. Cultures were supplemented with PHA (10 μg/ml) where noted.

Antiviral Activity Assay

The procedures for the antiviral activity assays in human PBMCs and CEM cells were adapted from previous reports[29]. Briefly, uninfected PHA-stimulated human PBMCs or CEM cells were counted using the trypan blue dye exclusion method and spun down at 1,500 rpm, at room temperature for 10 min. Infection was carried out at 37 °C for 3 hr with 5,000 disintegrations of reverse transcriptase (RT) activity per min per 10^6 cells (DPM/10^6 cells) of LAI/HIV-1 for PBMCs and 10,000 DPM/10^6 cells for CEM cells. The virus inoculum was prepared from infected PBMC cultures and cell free virions were quantitated by measurement of RT in the supernatant 6 days post-infection. At the end of a 3 hr adsorption period, unbound virus was removed and the cells were washed three times with 15 ml of Hank's balanced salt

solution (Gibco/BRL, NY) using centrifugation (1,500 rpm, 10 min, room temperature). The cells were resuspended to 2.5×10^5 cells/ml in RPMI 1640 medium (10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 U/ml IL-2) and cultures were set up in 24-well tissue culture plates, with 1 ml/well. The drugs, prepared in ddH_2O, were then added to duplicate or triplicate cultures. Uninfected and untreated cultures as well as infected untreated cultures were grown at equivalent cell density as controls. The cultures were maintained at 37 °C for 6 days. Samples were then collected from PBMC cultures for the supernatant RT activity and from CEM cultures for supernatant p24 detection using a p24 ELISA (Cellular Product, NY).

The RT assays were performed by modification of the method of Spira[30]. The supernatant from each culture was clarified by centrifugation at 1,500 rpm for 10 min at room temperature. 750 μl of the supernatant was then transferred to a new tube and an equal volume of 20% PEG (polyethyleneglycol) solution was added. The samples were mixed and placed on ice for 1 hr. Virus particles were pelleted by centrifugation at 12,000 rpm for 10 min at room temperature then disrupted with 30 μl of virus solubilization buffer (0.5% Triton X-100, 0.8 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, MI), 20% glycerol, and 50 mM Tris-HCl, pH 7,8). 10 μl samples of solubilized virus were placed into the wells of 96-well flat-bottomed microtiter plates (Costar, MA) containing 90 μl of the reaction mixture (50 mM Tris-HCl, pH 7.8, 9 mM $MgCl_2$, 5 mM DTT, 5 μg/ml poly (rA)d$(T)_{12-18}$, 140 μM dATP and 0.22 μM ^{3}H-TTP (78 Ci/mmol)). The plates were covered and incubated at 37 °C for 2 hr. Then 50 μl of trichloroacetic acid (TCA) solution (10% TCA, 0.45 mM sodium pyrophosphate) was added to each well to precipitate the DNA. After precipitation, the DNA was harvested onto a glass fiber filter using a cell harvester. The glass fiber sheet was dried and disks containing the precipitated DNA were removed and placed into scintillation vials. 4 ml of scintillation fluid (Ecolite$^+$) was added to each vial then the vials were counted in a beta scintillation counter (Beckman Instruments, Inc., CA). Results were expressed as disintegrations per min per 0.25 ml of clarified tissue culture supernatant. The extent of HIV-1 replication inhibition was then determined from a set of six drug concentrations and expressed as 50% effective molar concentration at which 50% HIV-1 production was inhibited (EC_{50}). The amount of $p24^{gag}$ in cell-free CEM culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) (Cellular Products, Inc., NY) using the instructions and standards supplied by the manufacturer.

Cytotoxicity Assay

The compounds were evaluated for their potential toxic effects on uninfected, PHA-stimulated human PBMCs and CEM cells. After cell den-

sity and viability determination (trypan blue dye exclusion method), cells were distributed at a density of 2.5×10^5 cells/well into 96-well tissue culture plates to which diluted drug solutions and medium had been added. The plates were then incubated for 6 days at 37 °C in H_2O-saturated air with 10% CO_2. After incubation, 50 µl of XTT/PMS solution (XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2-H-tetrazolium-5-carboxanilide sodium salt, Sigma, MI; PMS, phenazine methosulfate, Sigma, MI) was added to each well. The XTT/PMS solution was made by first dissolving 0.15 g XTT in 144 ml of RPMI 1640 medium (without phenol red). Then, 1% (w/v) of PMS solution was prepared in PBS (phosphate buffer saline). 6 ml of PMS solution was then added to the XTT solution and mixed well before being applied to each well. The plates were incubated for 4 hr at 37 °C to allow for the PMS-coupled XTT reduction. Cell viability was quantified by measuring the absorbance at 450 nm using 630 nm as a reference wavelength.

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