Synthesis and Evaluation of Novel Ether Lipid Nucleoside Conjugates for Anti-HIV-1 Activity

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Combinations of an amidoalkylphosphocholine, 8, and AZT have been found to cause an apparent synergistic action in suppressing infectious HIV-1 replication. In addition, amidoalkyl, oxyalkyl, and thioalkyl ether lipids have been chemically linked to anti-HIV-1 nucleosides (AZT and DDI) through phosphate and phosphonate linkages. These conjugates have shown promising in vitro anti-HIV-1 activity. Also, the conjugates have a 5–10-fold reduction in cell cytotoxicity compared to AZT alone. The most active compound, an amidoalkyl ether lipid–AZT conjugate, **4A**, was found to have a differential selectivity of 1793 in a syncytial plaque assay. In comparison, AZT alone has a value of 1281.

Introduction

The AIDS pandemic continues to expand at an exponential rate without any immediate therapeutic means for eradicating the etiologic agent of the disease. To date the only clinically approved drug for treating AIDS is 3′-azido-3′-deoxythymidine (AZT). Although AZT is a powerful inhibitor of the reverse transcriptase enzyme[1] and the replication of human immunodeficiency virus (HIV), this drug produces undesirable toxicity in the host (e.g., myelosuppression, neuropathy).[2,3] New strategies are needed to develop compounds that have a selective attack against HIV infection and replication and that target other sites of the viral replication cycle not involving DNA synthesis. Novel anti-HIV agents should also lack the undesirable systemic toxicity associated with many nucleoside analogues currently being used or evaluated.[2,3] The status of such new drug development for AIDS was reviewed[4] and discussed at the recent Second International Conference on Drug Research in Immunologic and Infectious Diseases, Acquired Immune Deficiency Syndrome (AIDS), sponsored by the New York Academy of Sciences and National Institutes of Health.

The overall goal of the present study is to develop and evaluate innovative strategies for selectively attacking the viral etiologic agent of AIDS with ether lipid (EL) analogues that are membrane interactive and have potent anti-HIV-1 activity. These new analogues have the potential to initiate new strategies for the treatment of AIDS, since EL are membrane interactive but not DNA interactive. In addition, EL are not associated with the myelosuppression or neuropathy shown by AZT, on the basis of Phase I/Phase II clinical trials in humans as anticancer drugs.[5,6]

Previous work led to the first report from these laboratories that selected EL have potent activity against infectious HIV-1 replication.[7] The mechanism of action of these EL against HIV-1 involves a shift in virus assembly from the plasma membrane to intracytoplasmic vacuoles and the production of defective virus.[7] EL are lipophilic compounds that accumulate in the plasma membrane[8] of T-lymphocytes and monocyte/macrophages (cells which serve as important reservoirs of latent and active HIV-1 infections)[9,10] and undoubtedly cross the blood–brain barrier in the host.

Since EL target a late step and AZT targets an early step in HIV-1 replication, it was first decided to evaluate combinations of a selected EL, 8, and AZT for anti-HIV-1 activity. The advantages of this approach, since the agents act by different mechanisms of action, are (1) both compounds alone have potent anti-HIV-1 activity,[7] (2) the development of viral resistance[11] to two active drugs most likely would occur at a slower rate than to either compound alone, and (3) the combination of compounds may reduce the toxicity associated with nucleoside analogues like AZT.[2,3] In addition, EL were covalently linked to anti-HIV nucleosides as illustrated in Table I for several reasons. First, EL–AZT conjugates might allow a larger concentration of drug to enter the cell due to the lipophilic nature

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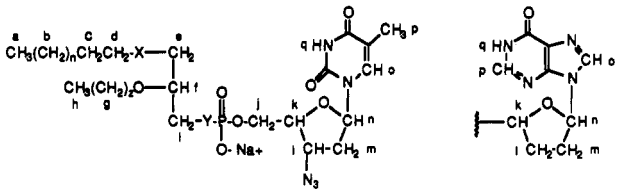
Table I. Analytical and Spectral Data for Compounds 4A–D and 7



4A X= NHC=O, n= 14, z= 1, Y= O
4B X= O, n= 13, z= 1, Y= O
4C X= S, n= 13, z= 0, Y= O
7 X= O, n= 13, z= 0, Y= absent

4D X= NHC=O, n= 14, z= 1, Y= O

assign.	mult	ppm (number of hydrogens) 4a[a]	4B	4C	7	4D[a]
a	t	0.87 (3)	0.87 (3)	0.87 (3)	0.87 (3)	0.87 (3)
b	m	1.1–1.3 (28)	1.1–1.3 (26)	1.1–1.3 (26)	1.1–1.3 (26)	1.1–1.3 (28)
c	m	1.55 (2)	1.5 (2)	1.45 (2)	1.5 (2)	1.55 (2)
d	t	2.1 (2)	3.2–3.5 (2)	2.4 (2)	3.2–3.5 (2)	2.1 (2)
e	m or d	3.2–3.5 (2)	3.2–3.5 (2)	2.6 (2)	3.2–3.5 (2)	3.1–4.1 (2)
f	m	3.2–3.5 (1)	3.2–3.5 (1)	3.5 (1)	3.2–3.5 (1)	3.1–4.1 (1)
g	m	3.2–3.5 (2)	3.2–3.5 (2)			3.1–4.1 (2)
h	m or s[b]	1.1–1.3 (3)	1.1–1.3 (3)	3.3 (3)	3.2–3.5 (3)	1.1–1.3 (3)
i	m	3.75 (2)	3.8 (2)	3.9–4.1 (2)	1.95 (2)	3.1–4.1 (2)
j	m	3.95 (2)	3.95 (2)	3.9–4.1 (2)	3.95 (2)	3.1–4.1 (2)
k	m	3.85 (1)	3.9 (1)	3.9–4.1 (1)	3.9 (1)	3.1–4.1 (1)
l	m	4.35 (1)	4.35 (1)	4.4 (1)	4.4 (1)	2.1 (2)
m	m	2.2 (2)	2.25 (2)	2.25 (2)	2.25 (2)	1.7 (2)
n	m	6.1 (1)	6.1 (1)	6.1 (1)	6.1 (1)	6.1 (1)
o	s	7.4 (1)	7.4 (1)	7.4 (1)	7.4 (1)	7.9 (1)
p	s	1.8 (3)	1.8 (3)	1.8 (3)	1.8 (3)	8.5 (1)
q	s	11.3 (1)	11.3 (1)	11.3 (1)	11.3 (1)	8.0 (1)
			analytical data[c]			
% yield		26	28	23	23	22
mass spectra[d]						
theoretical		759.3795	696.3713	698.3328	688.3426	728.3739
found		759.3810	696.3681	698.3344	688.3437	728.3738
ppm		2.0	4.6	2.2	1.6	0.2

[a] NHC=O: **4A** 6.95 (t, 1 H) and **4D** 7.85 (m, 1 H). [b] Signal h is a multiplet for **4A**, **4B**, and **4D** and a singlet for **4C** and **7**. [c] All of the final analogues were hygroscopic solids, therefore no melting points were determined. [d] $(M + 2\ Na)^+$ for **4A**, **4D** and **7** and $(MH + Na)^+$ for **4B** and **4C**.

of the EL. Second, hydrolysis of the conjugate by cellular enzymes (e.g., phosphatases) might result in the slow release of AZT monophosphate into treated cells, possibly reducing resistance development to AZT[11-13] since the thymidine kinase pathway may not be needed and potentially decreasing the toxicity associated with AZT[2,3] and increasing duration of action. Third, the degradative metabolism of the conjugate might release two active species. The EL portion, either as a dialkyl- or (amidoalkyl)glycerol derivative, can inhibit protein kinase C (PKC).[14] Previous literature reports[15] have shown that inhibitors of PKC decrease the endocytosis of the CD4-HIV complex into T-cells and subsequent virus infection. Therefore, viral entry may be partially inhibited due to the action of these glycerol derivatives on PKC. The released dialkyl or amidoalkyl derivatives may also be converted de novo to their respective phosphocholine congeners, which have been shown to possess anti-HIV-1 activity.[7] The nucleoside portion, either as AZT or its 5′-monophosphate, is advantageous for reasons previously detailed. Finally, it is possible that the conjugate compounds have anti-HIV-1 activity in the absence of any metabolic activation.

It has been reported in the literature that EL have been successfully used in combination or conjugation with a variety of antineoplastic agents and/or therapies.[16-18] Combinations of EL with DNA interactive agents have shown enhanced antitumor activity in vitro.[16] Also, thioether lipid–nucleoside conjugates have shown improved efficacy in tumor-bearing animals.[17] Using isobologram analysis, Fujiwara et al.[18] reported that combinations of EL and heat (44 °C) treatment produce a superadditive effect at low drug concentrations and an additive effect at moderate EL concentrations on suppression of colony formation of human ovarian carcinoma cells in tissue culture.

The synthesis and biological evaluation of prodrugs of anti-HIV-1 nucleosides also have been previously reported. Aggarwal et al.[19] synthesized a series of prodrugs of AZT in order to increase the uptake and half-life of AZT in HIV-1-infected cells. The prodrugs consisted of nucleoside adducts with morpholine, *N*-phenylpiperazine-1-acetic

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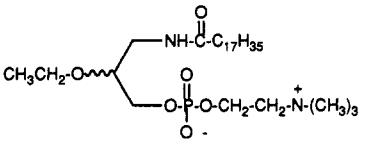
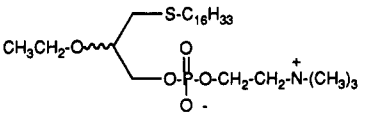
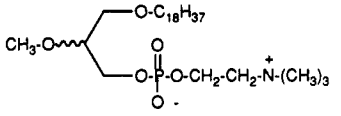
Chart I. Overview of Nucleoside and Ether Lipid Reference Analogues



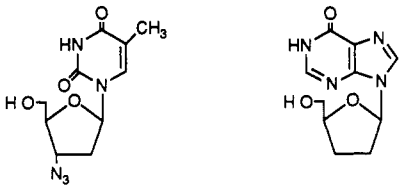
AM-18-OEt (8)



ETS-16-OEt (9)



ET-18-OMe (10)



AZT DDI

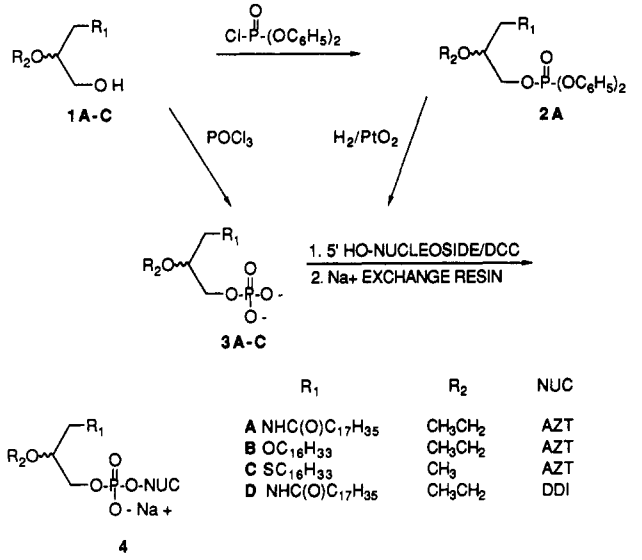
acid, 1,4-dihydro-1-methyl-3-nicotinic acid, retinoic acid, and certain amino acids. The 50% inhibitory concentration (IC_{50}) for HIV-1 induced p24 core antigen synthesis in infected human peripheral blood leukocytes was 0.12 μM for AZT and 0.05–0.2 μM for the prodrugs. The most active prodrug was 1,4-dihydro-1-methyl-3-[(pyridylcarbonyl)oxy] ester and the $T_{1/2}$ based on susceptibility of plasma esterases was >240 min.[19]

Finally, Hostetler et al.[20] synthesized phospholipid analogues of AZT (liponucleotide prodrugs) in attempts to improve AZT therapy in HIV-1-infected human peripheral blood mononuclear cells (macrophages). The prodrugs consisted of phosphatidyl-AZT, AZT diphosphate dipalmitin, phosphatidyl-ddC, and phosphatidyl-ddT. All of the phospholipids used were natural membrane components. The most active prodrug was phosphatidyl-AZT with an IC_{50} of <0.7 and 1.7 μM in HIV-1-infected U937 and CEM cells, respectively, indicating that the prodrug has less efficacy than AZT alone (0.2 μM in both cell lines) in suppressing p24 core antigen production. The differential selectivity (differential selectivity = IC_{50} for cell growth divided by IC_{50} for HIV-1 replication) for the prodrug was about 100.[20]

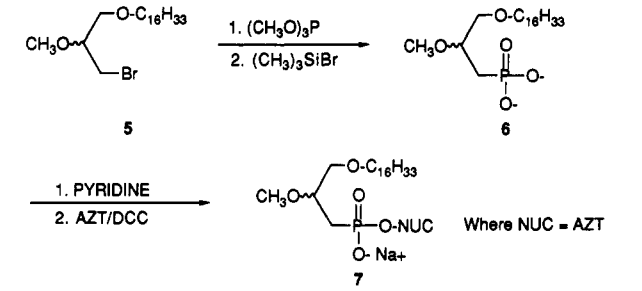
Chemistry

The structures of the ether phospholipids and nucleosides used in this study are shown in Chart I. The syntheses of the EL have been reported previously in the literature.[21,22]

Scheme I. Synthesis of Phospholipid–Nucleoside Conjugates



Scheme II. Synthesis of Phosphonate–Nucleoside Conjugate 7



Phospholipid–nucleoside conjugates **4A–D** were prepared according to Scheme I. Starting alcohols **1A–C** were synthesized as previously described.[21,22] (Amidoalkyl)-glycerol derivative **1A** was phosphorylated with diphenyl chlorophosphate in pyridine to give the corresponding phosphate ester **2A**.[23] The phenyl groups were then removed via hydrogenolysis with PtO_2 to give **3A**.[23] Oxy and thioether derivatives **1B** and **1C**, were phosphorylated by an alternative procedure using phosphorus oxychloride and triethylamine or pyridine.[24,25] The phosphatidic acid derivatives were then conjugated to the 5′-hydroxyl of the appropriate nucleoside via a dicyclohexylcarbodiimide (DCC) condensation, and subsequent conversion to the sodium salt gave the desired products **4A–D**.[26]

The synthesis of phosphonate analogue **7** is shown in Scheme II. By starting with (±)-1-(hexadecyloxy)-2-methoxy-3-bromopropane (**5**),[27] the halide was displaced with trimethyl phosphite to afford the corresponding phosphonate.[28] The protective methyl groups were then cleaved with trimethylsilyl bromide[29] to give the expected

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Table II. Effect of 8 and AZT Combinations on Inhibition of HIV-1 Syncytial Plaque Formation[a]

compound combination	concn, μM	% inhibn of HIV-1 plaque formation	combination index[b]
AZT alone	0.012	93 ± 5[c]	
	0.006	87 ± 6	
	0.003	79 ± 9	
	0.0015	70 ± 4	
	0.0007	53 ± 7	
	0.0003	18[d]	
8 alone	1.0	89 ± 5	
	0.5	74 ± 5	
	0.25	60 ± 6	
	0.12	49 ± 9	
	0.06	34 ± 12	
	0.03	14 ± 7	
AZT + 8	0.012 + 0.005	96	0.70 ± 0.23[c]
	0.006 ± 0.005	91	0.76 ± 0.37
	0.003 + 0.005	91	0.36 ± 0.12
	0.0015 + 0.005	74	0.63 ± 0.23
AZT + 8	0.012 + 0.015	94	0.87 ± 0.39
	0.006 + 0.015	85	1.31 ± 0.42
	0.003 + 0.015	73	1.38 ± 0.75
AZT + 8	0.006 + 0.06	79	2.24 ± 1.83
	0.003 + 0.06	76	1.36 ± 0.46
	0.0015 + 0.06	70	0.96 ± 0.28

[a] See the Experimental Section for the assay procedure. [b] The combination index was calculated by the method of Chou: [diskette (IBM PCa DOC 3.3)] Dose-Effect Analysis with Microcomputers for Two Drug Combinations and Single Drug and with Dose-Reduction Index Calculated; Elsevier-Biosoft: Cambridge, UK, 1989. A combination index of <1 = synergistic (S), 1 = additive (Add), and >1 = antagonistic (Anta). [c] Standard Error of Mean (SEM). [d] Data from single experiment done in triplicate. All other data are from two to six experiments, with each determination done in triplicate.

phosphonic acid **6**. Condensation of the phosphonic acid intermediate with AZT was performed as described for **4A–D** to give **7**.

Results and Discussion

Initial experiments in the present study were designed to evaluate mixtures of our leading anti-HIV-1 EL (AM-18-OEt, **8**) and AZT against HIV-1 syncytial plaque formation. Results indicated that at low concentrations of AM-18-OEt (0.005 μM) and various concentrations of AZT (0.012–0.0015 μM) the combinations of the two compounds produced an apparent synergistic activity against HIV-1 syncytial plaque formation (Table II, see Experimental Section part F for statistical methods). When the concentration of AM-18-OEt was increased (0.015 to 0.06 μM), the interaction of the two compounds against HIV-1 syncytial plaque formation varied from an apparent synergistic to additive activity (Table II).[30] These preliminary data are very encouraging in that subinhibitory concentrations of EL are apparently synergistic with AZT in suppressing infectious HIV-1 replication.

Since low concentrations of amidoalkyl EL may be synergistic in combinations with AZT against HIV-1 replication, conjugates of amidoalkyl, oxyalkyl, and thioalkyl EL with nucleoside analogues (AZT, DDI) were synthesized and tested for activity against HIV-1 syncytial plaque formation. The results (Table III) indicated that all the EL–AZT conjugates tested have an IC_{50} value against HIV-1 syncytial plaque formation ranging from 0.02 to 1.56 μM. The IC_{50} values for cell cytotoxicity ranged from 25.2 to >100 μM. Of interest are data indicating that the differential selectivity for the EL–nucleoside conjugates ranged from >64 to 1793 compared to 1281 for AZT and >59 for DDI alone. The highest differential selectivity (1793) was obtained with the amidoalkyl EL–AZT conjugate **4A**. The apparent increased differential selectivity of this compound (1793) over AZT alone (1281) is accompanied by a 10-fold decrease in cell cytotoxicity of the amidoalkyl EL–AZT conjugate (IC_{50} = 53.8 μM) compared to AZT alone (IC_{50} = 5.1 μM). Interestingly, the amidoalkyl EL–AZT conjugate is about 4-fold less cell cytotoxic than the amidoalkyl EL alone (IC_{50} = 14.3 μM). The differential selectivity of amidoalkyl EL–AZT conjugate **4A** is about 14-fold higher (1793) compared to that of amidoalkyl EL **8** alone (130) (Table III). The variations in AZT IC_{50} value for HIV-1 plaque formation from 0.0008 μM (Table II) to 0.004 μM (Table III) is most likely due to AZT lot differences. The EL–AZT conjugates were synthesized with the same AZT lot number used in the comparative studies presented in Table III. The amidoalkyl compounds with either a hydroxyl (**1A**) or phosphatidic acid (**3A**) at position 3 have no activity against HIV-1 plaque formation or cell growth. In general, the oxyalkyl (**4B**) and thioalkyl (**4C**) ether lipid conjugates of AZT retained the same activity for the inhibition of HIV-1 plaque formation as amidoalkyl conjugate **4A**. However, **4B** and **4C** were more cytotoxic for uninfected CEM-SS cells. The increased cell cytotoxicity could possibly be attributed to a decreased ability of the cells to metabolize the oxy and thioethers,[31] compared to a possible hydrolysis of the amide by cellular amidases. Phosphonate conjugate **7** possessed a similar activity profile to that shown by the phosphate conjugates **4B** and **4C**. This was not anticipated due to the inability of **7** to release AZT as the monophosphate. However, the possibility that conjugate **4A** is acting as an intact molecule with little or no metabolism cannot be excluded. Finally, the amidoalkyl EL conjugate of DDI (**4D**) retained the same activity and relative toxicity of the parent nucleoside. Since both the parent nucleoside and conjugate were innocuous toward infected and uninfected CEM-SS cells, a difference in activity was not observed. These data do suggest that the alkyl EL–AZT conjugates have potent activity in inhibiting infectious HIV-1 replication at concentrations noncytotoxic to uninfected CEM-SS cells. Data indicating reduced cell cytotoxicity of the conjugate compounds in vitro suggest that these compounds might have reduced myelosuppression in vivo compared to AZT alone.

Next, the effect of the leading anti-HIV-1 EL–AZT conjugate (**4A**) on HIV-1 acutely infected H9 cells and H9IIIB persistently infected cells was evaluated. Although both H9 and H9IIIB are human T lymphoblastoid cell lines, the acutely infected H9 cells require reverse transcriptase (RT) to replicate the proviral DNA, whereas the persistently infected H9IIIB cells do not, already having the viral genome incorporated into the host DNA. The results (Table IV) indicated that compound **4A** caused a marked inhibition of both RT and infectious HIV-1 production in continuously treated and acutely infected H9 cells. In persistently infected H9IIIB cells, **4A** had little effect on RT activity but a marked inhibition of infectious HIV-1 production (Table IV). A possible interpretation of these results is that **4A** inhibits reverse transcription

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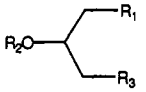
Table III. Anti-HIV-1 Activity of Nucleosides, Ether Lipids, and Ether Lipid-Nucleoside Conjugates



compd	R_1	R_2	R_3	IC_{50}, mM: HIV-1 plaque formation	IC_{50}, mM: CEM-SS cell growth	differential selectivity
AZT				0.004 ± 0.002	5.1 ± 3.0	1281
DDI				1.7	>100	>59
4A	$NHC(O)C_{17}H_{35}$	CH_3CH_2	OPO_3AZT	0.03 ± 0.02	53.8 ± 7.8	1793
4D	$NHC(O)C_{17}H_{35}$	CH_3CH_2	OPO_3DDI	1.56 ± 0.8	>100	>64
4B	$OC_{16}H_{33}$	CH_3CH_2	OPO_3AZT	0.03 ± 0.02	35.0 ± 2.1	1167
4C	$SC_{16}H_{33}$	CH_3	OPO_3AZT	0.02 ± 0.01	29.2 ± 5.7	1465
7	$OC_{16}H_{33}$	CH_3	PO_3AZT	0.02 ± 0.01	25.2 ± 1.1	1260
8	$NHC(O)C_{17}H_{35}$	CH_3CH_2	PC[a]	0.11 ± 0.06	14.3 ± 2.3	130
9	$SC_{16}H_{33}$	CH_3CH_2	PC	0.6 ± 0.3	4.1 ± 0.3	7
10	$OC_{18}H_{37}$	CH_3	PC	1.4 ± 0.4	4.0 ± 0.7	3
1A	$NHC(O)C_{17}H_{35}$	CH_3CH_2	OH	>100	>100	ND[b]
3A	$NHC(O)C_{17}H_{35}$	CH_3CH_2	OPO_3	>100	>100	ND[b]

[a] PC = phosphocholine [$OPO_3CH_2CH_2N(CH_3)_3$]. [b] ND = not determined.

Table IV. Effect of Long-Term Treatment with **4A** on HIV-1 Replication in Acutely Infected H9 Cells and Persistently Infected H9IIIB Cells[a]

condition of cells	RT dpm (% inhibn by **4A**) posttreatment: 7 days	14 days	21 days	28 days	35 days	42 days	50 days
H9 + HIV-1	172	93 173	47 500	33 110	26 979	42 130	46 331
H9 + HIV-1 + **4A**	(100)	(100)	(97)	(92)	(97)	(98)	(67)
H9IIIB + HIV-1	11 045	17 788	22 660	22 056	32 708	46 881	32 237
H9IIIB + HIV-1 + **4A**	(0)	(18)	(33)	(0)	(26)	(28)	(0)
conditions of cells	**syncytial plaque count per mL (% inhibn by 4A) posttreatment: 7 days**	**14 days**	**21 days**	**28 days**	**35 days**	**42 days**	**50 days**
H9 + HIV-1	346	20 640	11 042	6 986	3 894	8 000	ND
H9 + HIV-1 + **4A**	(43)	(98)	(96)	(94)	(95)	(99)	ND
H9IIIB + HIV-1	7 706	6 638	8 960	8 000	12 450	16 000	13 000
H9IIIB + HIV-1 + **4A**	(87)	(91)	(91)	(89)	(95)	(84)	(93)

[a] Cells were subcultured three times a week in fresh RPMI-1640 growth medium containing 10% (v/v) fetal bovine serum and 1 mM **4A**. At 7-day intervals the supernatant fluids were harvested at the time of subculture and assayed for RT and infectious HIV-1 production by syncytial plaque count. Numbers without brackets are RT dpm or syncytial plaque count in infected control cells without **4A** treatment. Numbers with parentheses are percent inhibition of RT dpm or plaque count in infected cells continuously treated with **4A**. ND = not determined.

and replication of provirus DNA and infectious virus production in HIV-1 acutely infected cells. In persistently infected cells that have already integrated provirus DNA, **4A** markedly inhibits infectious virus production. Previous data[7] indicate that EL compounds cause a shift in HIV-1 assembly from the plasma membrane to intracytoplasmic vacuoles, resulting in the production of defective virus.

Monocyte/macrophages represent a major reservoir of HIV-1 in the infected human host.[10] However, these cells tend to be resistant to nucleoside analogues due to low level of kinases needed to activate the parent compound upon hydrolysis of the carrier.[32] It is reasonable to assume that EL-AZT conjugates should allow larger proportions of drug to enter the cell due to the lipophilic nature of the EL component and might result in the slow release of AZT monophosphate and AZT into treated cells due to metabolism by cellular phosphatases. To test this possibility, HIV-1 persistently infected monocyte/macrophage (U1) cells were treated with AZT, AM-18-OEt (**8**), and **4A**, and the effect on HIV-1 replication was measured. The results (Table V) indicated that the compounds did not significantly inhibit HIV-1-induced RT and p24 core antigen production. As expected, AZT alone caused only 13% inhibition of infectious HIV-1 production. However, both AM-18-OEt (**8**) and **4A** inhibited infectious HIV-1 production by 67% and 33%, respectively (Table V). The increase in activity of **4A** compared to that of AZT could be due to direct action of the conjugate or to the release of AZT monophosphate upon hydrolysis of the conjugate, therefore diminishing the resistance associated with decreased kinase activity in this cell line. Concurrently, AM-18-OEt, which does not require activation by cellular kinases, does possess promising activity and may be an effective agent alone or in combination with other anti-HIV-1 agents. These data confirm that the amide EL

Table V. Effect of **8**, AZT, and **4A** Treatment on HIV-1-Induced RT, p24 Core Antigen Synthesis, and Infectious Virus Production in Persistently Infected Monocyte/Macrophage (U1) Cells[a]

compound	% of control: RT dpm	p24 core Ag	PFU[b]
control	(79328)	(94)	(750)
	100	100	100
+AZT	90	101	87
+**8**	147	74	33
+**4A**	121	84	67

[a] U1 cells persistently infected with HIV-1 virus were treated with 1 μM AZT, **4A**, or **8** starting at the time of TPA (1 μM) induction of HIV-1 replication. At 48 h after treatment and induction at 37 °C supernatant fluids were harvested to measure RT, p24 core antigen (pg/mL), and infectious virus production as previously described.[7] [b] PFU = plaque-forming units.

(32) Perno, C.-F.; et al. *J. Exp. Med.* **1988**, *168*, 1111.

alone or the conjugate with AZT is more active than AZT itself in suppressing infectious HIV-1 production in persistently infected monocyte/macrophage cells.

In summary, on the basis of the results of this preliminary study, it appears that EL-AZT conjugates offer a novel therapeutic regimen for treating HIV-1-infected cells and appear promising as a potential new treatment for the AIDS virus.

Experimental Section

Proton nuclear magnetic reasonance spectra were recorded in $CDCl_3$ on either a Bruker 300-MHz or a Varian 400-MHz spectrometer. Chemical shifts are reported in parts per million relative to internal tetramethylsilane. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. The composition of all final analogues (**4A–D** and 7) were verified by high-resolution mass spectrometry as opposed to elemental analysis due in part to the limited quantities available. In addition, because these compounds exist in various hydrated states and contain multiple sodium adducts, elemental analysis would not be entirely conclusive. Mass spectral data was obtained from either a VG7OS or a VG7OS-25OSEQ hybrid mass spectrometer. All reactions were performed under a positive pressure of dry nitrogen with dry solvents. Tetrahydrofuran (THF) was distilled from Na and benzophenone, triethylamine (Et_3N), and pyridine were stored over KOH, and hexanes were placed over molecular sieves. Chromatographic purification was performed with silica gel 60 (230–400 mesh). All final analogues were purified using column chromatography. Thin-layer chromatographic plates were visualized by UV light, iodine vapor, molybdenum phosphate spray, and charring following sulfuric acid spray.

(±)-3-Octadecanamido-2-ethoxypropyl Diphenyl Phosphate (2A). To a three-neck round-bottom flask equipped with a magnetic stir bar, nitrogen inlet, and reflux condenser was added a solution of diphenyl chlorophosphate (0.7 mL, 3.39 mmol) (FLUKA) in 10 mL of anhydrous ether. The solution was cooled to 4 °C, and (amidoalkyl)glycerol **1A**[21] (1.0 g, 2.6 mmol) in 15 mL of pyridine and 5 mL of ether was then added. The solution was warmed to room temperature, and then heated to 52 °C for 3 h. After cooling to room temperature, the reaction mixture was diluted with 50 mL of ether, washed twice with 25-mL portions of distilled water, once with 25 mL of cold 0.5 N HCl, and once with 25 mL of distilled water. The ether layer was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to give a pale yellow oil. Purification by silica gel chromatography (discontinuous gradient of hexane/ethyl acetate 10:1 to 1:1 as eluent) gave 961 mg of pure product (60.1%). ^1H NMR ($CDCl_3$): δ 0.87 (t, 3 H, terminal methyl), 1.1–1.3 [m, 31 H, $(CH_2)_{14}$, CH_3CH_2O], 1.55 (m, 2 H, $NHCCH_2CH_2$), 2.15 (t, 2 H, $NHCCH_2$), 3.3–3.6 (m, 5 H, $CH_3CH_2OCHCH_2NH$), 4.25 (m, 2 H, CH_2OP), 5.9 (t, 1 H, NH), 7.15–7.35 [m, 10 H, $(OC_6H_5)_2$].

(±)-3-Octadecanamido-2-ethoxypropyl Phosphate (3A). Into a Parr hydrogenation bottle was placed 69 mg of PtO_2. A solution of 500 mg of **2A** in 100 mL of absolute ethanol was then added. The reaction mixture was placed under 14.5 psi of hydrogen and was shaken at room temperature. After 100 min, 6 psi had been consumed, and TLC ($CHCl_3/MeOH/H_2O$ 70:35:4) revealed the absence of starting material. The reaction mixture was suction filtered through Celite and the ethanol removed in vacuo. The resulting oil was taken up in 25 mL of pyridine, concentrated in vacuo, and dried under high vacuum to give 352 mg (93.3%) of pure product as a fine powder. ^1H NMR ($CDCl_3$): δ 0.87 (t, 3 H, terminal methyl), 1.1–1.3 [m, 31 H, $(CH_2)_{14}$, CH_3CH_2O], 1.55 (m, 2 H, $NHCCH_2CH_2$), 2.25 (t, 2 H, $NHCCH_2$), 3.3–3.75 (m, 5 H, $CH_3CH_2OCHCH_2NH$), 4.15 (m, 2 H, CH_2OP), 6.7 (t, 1 H, NH).

(±)-3-(Hexadecyloxy)-2-ethoxypropyl Phosphate (3B). To a three-neck round-bottom flask equipped with a magnetic stir bar and nitrogen inlet was added a solution of phosphorus oxychloride (0.62 mL, 6.6 mmol) in 5 mL of THF. The solution was cooled to 0 °C, and a solution of the starting dialkylglycerol **1B**[22] (2.0 g, 5.8 mmol) and pyridine (1.4 mL, 17.3 mmol) in 15 mL of THF was added. The reaction mixture was maintained at 0 °C for 3 h, and then 10 mL of 10% sodium bicarbonate was added. The mixture was stirred an additional 20 min, and poured into 30 mL of ice water. The solution was acidified by the dropwise addition of 2 N HCl, and then extracted twice with 30-mL portions of ether. The ether layer was dried over sodium sulfate, filtered, concentrated in vacuo, taken up in 100 mL of pyridine, concentrated, and dried under vacuum to give 1.5 g (46%) of product as a waxy solid. ^1H NMR ($CDCl_3$): δ 0.87 (t, 3 H, terminal methyl), 1.1–1.3 [m, 29 H, $(CH_2)_{13}$, CH_3CH_2O], 1.4 (m, 2 H, OCH_2CH_2), 3.4–3.7 (m, 7 H, $CH_3CH_2OCHCH_2OCH_2$), 3.85 (m, 2 H, CH_2OP).

(±)-3-(Hexadecylthio)-2-methoxypropyl Phosphate (3C). To a three-neck round-botton flask equipped with a magnetic stir bar, nitrogen inlet, and reflux condenser was added a solution of phosphorus oxychloride (0.6 mL, 7 mmol) in 1 mL of hexane. The solution was cooled to 0 °C, and a solution of triethylamine (1 mL, 10 mmol) in 1 mL of hexane was added dropwise. The starting (thioalkyl)glycerol **1C**[22] (1.6 g, 5 mmol) was azeotropically dried with toluene, and the volume reduced to 10 mL. This was then added dropwise to the $POCl_3/Et_3N$ solution and stirred overnight at room temperature. One milliliter of water was added to the reaction mixture and stirred for 1 h. The reaction mixture was diluted with 20 mL of water, and extracted twice with 25-mL portions of ether. The organic layers were collected, dried over sodium sulfate, filtered, and concentrated in vacuo. In an attempt to form the pyridinium salt, the resulting oil was taken up into 50 mL of pyridine, heated to 50 °C for 2 h, and concentrated in vacuo. Proton NMR indicated that the salt did not form. Silica gel chromatography ($CHCl_3/MeOH/NH_4OH$ 70:35:1 to 70:35:7 as eluent) gave 535 mg of product (24%). ^1H NMR ($CDCl_3$): δ 0.87 (t, 3 H, terminal methyl), 1.2 [bs, 26 H, $(CH_2)_{13}$], 1.4 (m, 2 H, SCH_2CH_2), 2.4 (t, 2 H, SCH_2CH_2), 2.5 (m, 2 H, $CHCH_2S$), 3.4–3.7 (m, 4 H, CH_3OCHCH_2S), 4.0 (bm, 2 H, CH_2OP).

3′-Azido-3′-deoxy-5′-(3-octadecanamido-2-ethoxypropyl)-phosphothymidine (4A). Into a 25-mL round-bottom flask were placed **3A** (100 mg, 0.22 mmol) and AZT (43 mg, 0.16 mmol). The two reactants were then azeotropically dried by in vacuo removal of 3 mL of pyridine three times. To this slury was added dicyclohexylcarbodiimide (DCC, 220 mg, 1.07 mmol), and once again the reactants were azeotropically dried four times with 3-mL portions of pyridine. The solution was then diluted to a final volume of 3 mL and the round-bottom flask stoppered and placed in a desiccator for 4 days. One gram of water was added to the reaction mixture, and the mixture stirred at room temperature for 4 h. The solvents were removed in vacuo, and the resulting crude material was applied to 2 g of silica gel. The column was eluted in a gradient of $CHCl_3/MeOH$ (15:1 to 2:1) in 10-mL portions. Fractions were collected in 1-mL increments. Fractions were pooled that showed UV activity, were phosphorus positive as indicated by molybdenum phosphate spray, charred following sulfuric acid spray, and displayed an R_f of ca. 0.2 in $CHCl_3/MeOH$ (4:1). Purity was also determined by HPLC using a C18 reverse-phase column, with a mobile phase of H_2O/CH_3CN (50:50 to 0:100) as eluent, and at a flow rate of 1 mL per min. In addition, to reaffirm the purity of this analogue and all other final analogues, a typical TLC would first be developed in $CHCl_3$, followed by $CHCl_3/MeOH$ (9:1), and finally $CHCl_3/MeOH$ (4:1). The product was dissolved in 11 mL of $CHCl_3/MeOH/H_2O$ (4:6:1), placed in a round-bottom flask, and stirred with 1.5 g of Whatman preswollen microgranular cation (Na^+) exchange (carboxymethyl)-cellulose resin for 1 h. The resin was filtered, and the filtrate concentrated in vacuo to give 32 mg of product as the sodium salt (26%). NMR and mass spectral data are given in Table I.

Final analogues **4B** and **4C** were synthesized as described above for **4A** from the appropriate phosphatidic acid and AZT. Compound **4D** was prepared in a similar manner from **3A** and dideoxyinosine (DDI). The composition and purity of these analogues was determined as described for **4A**. Physical and spectral data are reported in Table I.

(±)-3-(Hexadecyloxy)-2-methoxypropylphosphonate (6). Into a three-neck round-bottom flask equipped with a magnetic stir bar, nitrogen inlet, and reflux condenser was placed a solution of the starting dialkyl halide **5**[27] (954 mg, 2.4 mmol) in trimethyl phosphite (4.987 g, 30.2 mmol). The solution was heated to 120 °C for 90 h with continuous stirring. The reaction mixture was cooled to room temperature, reduced in vacuo, and purified by silica gel chromatography (discontinuous gradient of petroleum

ether/ether 10:1 to 1:1 as eluent) to give 741 mg of the dimethyl phosphonate product (80%) as a yellow viscous oil. ^{1}H NMR ($CDCl_3$): δ 0.87 (t, 3 H, terminal methyl), 1.1–1.3 [m, 26 H, $(CH_2)_{13}$], 1.57 (m, 2 H, OCH_2CH_2), 2.08 (m, 2 H, CH_2P), 3.4–3.6 (m, 7 H, $CH_3OCHCH_2OCH_2$), 3.75 [m, 7 H, CH_3OCH, $P(OCH_3)_2$].

To a three-neck round-bottom flask equipped with a magnetic stir bar and nitrogen inlet was added a solution of the above dimethyl phosphonate (740 mg, 1.92 mmol) in 10 mL of alcohol-free chloroform.[33] To this solution was added bromotrimethylsilane (1.6 g, 10.6 mmol) dropwise. After 1 h the solvents were removed in vacuo, and the resulting oil was taken up in 25 mL of THF/H_2O (8:2) and stirred overnight at room temperature. The solvents were removed on a rotary evaporator, and the residue was recrystallized from ether/acetonitrile (1:5) to give 577 mg of pure product (85%) as a white solid (mp 59–61 °C). The product was taken up into 50 mL of pyridine; the pyridine was removed in vacuo and then dried under vacuum. ^{1}H NMR ($CDCl_3$): δ 0.87 (t, 3 H, terminal methyl), 1.1–1.3 [m, 26 H, $(CH_2)_{13}$], 1.57 (m, 2 H, OCH_2CH_2), 2.12 (m, 2 H, CH_2P), 3.4–3.6 (m, 7 H, $CH_3OCHCH_2OCH_2$), 3.75 (m, 1 H, CH_3OCH).

3′-Azido-3′-deoxy-5′-*O*-[[3-(hexadecyloxy)-2-methoxypropyl]oxidophosphoryl]thymidine (7) was prepared from compound 6 and AZT by using the procedure described for 4A. The composition and purity of analogue 7 was also determined as described for 4A. Analytical data are given in Table I.

Biological Materials and Methods. A. Cells and Medium. The CEM-SS cell line, a syncytial-sensitive clone was maintained in RPMI-1640 medium supplemented with 20% (v/v) fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and 2 mM/L glutamine (growth medium). The cells were seeded at a density of 5×10^5 cells/mL and subcultured 1:3 three times a week. For use in a plaque assay, the cells were subcultured 1:2 18–24 h prior to seeding as a monolayer in 96-well dishes.[7] The H9IIIB cells were seeded in growth medium and subcultured as described above for CEM-SS cells.

B. Virus. HIV-1 (strain HTLV-III$_B$) was grown and harvested from persistently infected H9IIIB cells.[7] Briefly, 20 mL of a log-phase cell culture containing 7×10^5 cells/mL in a 75-cm^2 flask was incubated at 37 °C for 24 h. A viable cell count was made with trypan blue so that cultures with less than 10% dead cells were used as a source of virus. To harvest HIV-1, the cells were pelleted by centrifugation (400*g*, 5 min) and the supernatant containing infectious HIV-1 was filtered through a 0.45 μm Millipore filter, aliquoted, and stored at −80 °C. The titer of HIV-1 ranged from 3×10^3 to 5×10^3 plaque-forming units/mL.

C. Reverse Transcriptase Activity. Reverse transcriptase activity was measured as previously described by Poiesz et al.[34] Briefly, virus was precipitated from culture fluids with 4 M NaCl/30% polyethylene glycol and disrupted with 0.9% Triton X-100 and 1.5 M KCl. An aliquot of disrupted virus was added to template-primer buffer containing [^{3}H]dTTP (16 Ci/mmol, New England Nuclear, Boston, MA), trichloroacetic acid precipitated DNA was collected and washed on 0.45 μm filters (Millipore Corp.) and disintegrations per minute (dpm) were counted by scintillation spectrometry.

D. Virus Syncytial Plaque Assay and 50% Inhibitory Concentration (IC_{50}) Determinations. To form a monolayer of CEM-SS cells, individual wells of a 96-well plate were treated with 100 μL of poly(L-lysine) solution (50 μg/mL, mol wt 90000; Sigma Chemical Co.). The coated plates were incubated for 30 min at 22 °C and washed three times with 0.01 M phosphate-buffered 0.85% (w/v) sodium chloride (PBS). Aliquots of CEM-SS cells in log-phase growth were washed three times with PBS, suspended at a density of 50000 CEM-SS cells per 50 μL of RPMI-1640 medium without serum, and transferred to each poly(L-lysine) precoated well. After 30 min of incubation at 37 °C in a humidified atmosphere containing 5% CO_2, the cell monolayer was inoculated with 50 μL of HIV-1 diluted in growth medium. After 60 min of virus adsorption, the cell monolayer was overlaid with 100 μL of growth medium with or without test compound. The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 3 days each well received a second 100-μL overlay of growth medium with or without test compound and incubation was continued for an additional 2 days. On day 5, syncytial plaques were microscopically observed to be light refractive, large, multicellular foci (10–25 nuclei per syncytium) that appeared either brown and granular or clear. Plaques were counted microscopically with the aid of a 10× gridded ocular lens. In control experiments the number of HIV-1 plaques correlates with reverse transcriptase activity and p24 core antigen activity (Coulter Laboratories EIA) in the HIV-1 infected cell overlay fluids (L. Kucera and N. Iyer, unpublished data).

E. Combination Assay with AZT and Analogue 8. CEM-SS cell monolayers were infected with HIV-1 (about 100 plaque-forming units). After a 60-min virus-attachment period, the infected cell monolayers were overlaid with medium containing different concentrations of AZT alone, 8 alone, or combinations of AZT and 8. HIV-1 syncytial plaques were counted after 5 days of incubation in the usual manner and compared to the number of syncytial plaques without compound in the overlay medium.

F. Statistical Methods. The primary statistical objective of this investigation was to estimate the dose–response functions for these various compounds alone and in the presence of AZT. Dose levels were chosen that could be reliably measured and would cover a range of inhibition of HIV-1 plaque formation. The concentration required to produce 50% inhibition of plaque formation (IC_{50}) was then estimated from the dose–response curve using a logistic parametric model.[35] The same method was used for dose–response functions of AZT plus compounds and for inhibition of CEM-SS cell growth. To quantify the interaction between AZT and the compounds, the combination index of Chou[36] was calculated for each drug combination treatment from two to four independent experiments. These combination indices were then averaged and their standard error of the mean calculated. To approximate a therapeutic index, the differential selectivity was calculated as the IC_{50} for cell growth divided by the IC_{50} for HIV-1 plaque formation.

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(33) Alcohol-free chloroform was prepared by washing chloroform twice with concentrated sulfuric acid and twice with water, drying over sodium sulfate, filtering, and distilling over phosphorus pentoxide.

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