THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 265, No. 11, Issue of April 15, pp. 6112-6117, 1990
Printed in U.S.A.

Synthesis and Antiretroviral Activity of Phospholipid Analogs of Azidothymidine and Other Antiviral Nucleosides*

(Received for publication, June 27, 1989)

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Treatment of acquired immunodeficiency syndrome with azidothymidine (AZT, zidovudine) reduces p24 antigenemia, increases CD4 lymphocyte counts, reduces the frequency and severity of opportunistic infections and prolongs life. However, AZT and other dideoxynucleosides do not diminish the ability to isolate human immunodeficiency virus (HIV) from peripheral blood mononuclear cells. Failure to clear infectious virus may be due to inadequate inhibition of virus production by macrophages, a major reservoir of HIV infection.

Cells of the macrophage lineage take up large amounts of parenterally administered liposomal material. To direct larger proportions of antiretroviral nucleosides to this important HIV reservoir, we synthesized phosphatidylAZT, AZT diphosphate dipalmitin, phosphatidylddC and phosphatidylddT, novel phospholipid prodrugs which are readily incorporated into phospholipid bilayers. These liposomal liponucleotides were shown to have antiretroviral activity in HIV-infected U937 and CEM cells.

***In vivo*, it is anticipated that liposomes containing the antiretroviral liponucleotides will be taken up in large proportion by macrophages. This property would appear to make phosphatidylAZT and the related compounds promising candidate agents with a special potential to target drug to the macrophage reservoir of HIV infection, thereby reducing the toxicity of the antiviral nucleosides to other cells.**

Human immunodeficiency virus (HIV)[1] is the etiologic agent of acquired immunodeficiency syndrome (AIDS) (1-3). 3′-Azido-3′-deoxythymidine (AZT) and dideoxynucleosides have been shown to inhibit HIV replication *in vitro* by inhibiting the viral reverse transcriptase after anabolic phosphorylation to the triphosphate by the host cell (4-6). AZT prolongs life and reduces morbidity in patients with advanced HIV infection (7). Both AZT and 2′,3′-dideoxycytidine have been shown to have antiviral activity *in vivo* as indicated by reduction of p24 antigenemia, but neither drug appears to reduce isolation rates of virus from peripheral blood mononuclear cells (7-9) and in addition, both drugs have significant toxicities (8, 10).

In addition to the CD4 subset of T lymphocytes, cells of the monocyte/macrophage lineage are also important host cells of infection with HIV (11-14). It has been proposed that macrophages infected with the LAV strain of HIV may be resistant to dideoxynucleosides due to low levels of nucleoside kinases (15). Perno *et al.* (16), using another strain of HIV which is monocytotropic (Ba-L), found that dideoxynucleosides suppressed HIV replication substantially in spite of the poor rates of phosphorylation found with these compounds. Monocyte and macrophages represent a significant reservoir of HIV infection in the human host (17-21).

Macrophages take up the bulk of parenterally administered liposomes and this property has been utilized in the design of drugs for diseases involving these cells. For example, in leishmaniasis the studies of Black *et al.* (22) and Alving and coworkers (23) established that antimony compounds delivered in liposomes could be 10-100-fold more effective than the free drug in suppressing the disease in experimental animals.

To direct larger proportions of antiretroviral nucleosides to macrophages, we synthesized several phospholipids having AZT or a dideoxynucleoside as the polar head group. These compounds form lipid bilayers and may be incorporated into liposomes. The methods are general, allowing many antiretroviral nucleosides to be similarly modified to permit incorporation into liposomes, thereby providing enhanced delivery to macrophages *in vivo*.

Potential advantages of these liponucleotide prodrugs when incorporated into appropriate liposomes include: greater *in vivo* efficacy and lower toxicity due to a proportionately greater delivery to macrophages, the potential ability to bypass the transport and initial anabolic phosphorylation of the nucleoside analog, a variety of formulation advantages over water-soluble phosphate esters of antiviral nucleosides incorporated in liposomes, and the prospect of improved pharmacokinetics and prolonged intracellular persistence of antiviral compound.

EXPERIMENTAL PROCEDURES[2]

RESULTS AND DISCUSSION

PhosphatidylAZT—Phosphatidic acid is a convenient starting material for the synthesis of phospholipid analogs having

* This work was supported by National Institutes of Health Grants GM 24979 (to K. Y. H.) and AI 52578 and HB 67019 (to D. D. R.), by a grant from Vical Inc., San Diego, CA, and by the Veterans Administration. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: HIV, human immunodeficiency virus; AZT, 3′-azido-3′-deoxythymidine; ddC, 2′,3′-dideoxycytidine; ddT, 3′-deoxythymidine.

[2] The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

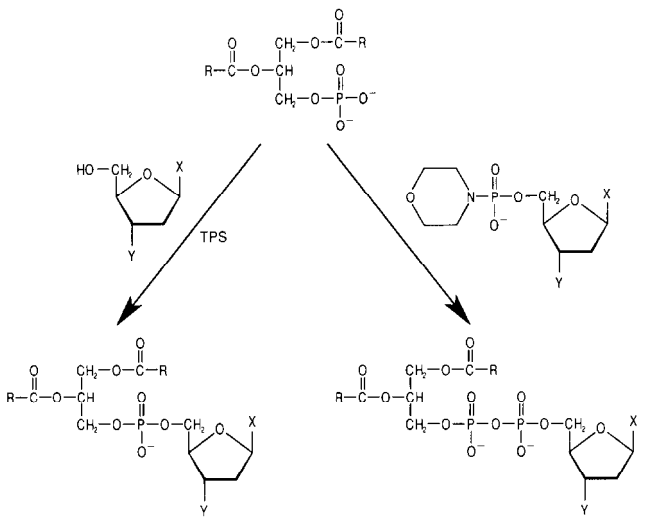

FIG. 1. **Chemical synthesis of nucleoside phospholipid prodrugs.** Monophosphates (*left*): compound 1: X = thymine; $Y = N_3$; $R = C_{13}H_{27}$; compound 2: X = thymine, Y = H; $R = CH_{13}H_{27}$. Diphosphate (*right*): compound 4: X = thymine; $Y = N_3$; $R = C_{15}H_{31}$. *TPS*, triisopropylbenzenesulfonyl chloride.

a nucleoside moiety as polar head group (Fig. 1). Direct esterification of phosphatidic acid with alcohols in the presence of triisopropylbenzenesulfonyl chloride as condensing agent has been used previously for synthesis of phospholipids such as phosphatidylcholine (24, 25). We adapted this procedure to couple phosphatidic acid to the 5′-hydroxyl of AZT (compound 1), ddT (compound 2), and ddC (compound 3). The details of the synthesis, purification, and characterization of these compounds are given under "Experimental Procedures" (Miniprint).

By analogy to natural glycerophospholipids, it is expected that these liponucleotides will be metabolized intracellularly by sequential deacylation catalyzed by phospholipases A and lysophospholipase (26). To verify this point, we treated phosphatidylAZT (compound 1) with either pancreatic phospholipase A_2 or *Crotalus adamanteus* phospholipase A_2, and found that a lysophospholipid was formed having a mobility on silica gel G of 0.36 *versus* 0.52 for the parent compound using chloroform/methanol/water (65:35:4) as developing solvent. This demonstrates in principle that this class of compounds can be degraded by phospholipases A, allowing them to enter the deacylation pathways.

Sequential deacylation is phosphatidylAZT leads to glycero-3-phospho-5′-AZT which may be metabolized by cellular phosphodiesterases to release the nucleoside moiety in either the free form (*e.g.* AZT) or as the 5′-phosphorylated form (AZT monophosphate). The latter cleavage would be preferred since it would bypass thymidine kinase, leading to activity in situations where this enzyme is deficient such as in monocyte/macrophage activation of AZT and in thymidine kinase-deficient herpes viruses. Phosphodiesterase cleavage of glycerophosphocholine was previously thought to give glycerophosphate and choline (27), but more recent data obtained in studies of the catabolism of phosphatidylcholine in fibroblasts (28) suggested that phosphocholine may also be formed. The intracellular metabolism of the glycerophosphodideoxynucleoside has not yet been studied. An alternative pathway leading to direct formation of the 5′-phosphodideoxynucleoside from compounds 1–3 would be phospholipase C cleavage to diglyceride and the respective nucleoside monophosphate. This mechanism has recently been reported for phosphatidylcholine in cultured endothelial cells (29).

AZT Diphosphate Diglyceride—Phosphatidic acid can also serve as a starting material for the chemical synthesis of nucleoside diphosphate diglycerides by coupling to activated nucleoside 5′-monophosphate morpholidate (Fig. 1) as first described for cytidine diphosphate diglyceride (CDP-diglyceride) by Agranoff and Suomi (30). We have used this procedure previously to prepare deoxycytidine dephosphate diglycerides (31), the nucleoside diphosphate diglycerides of adenine, uridine, and guanosine (32), and here to prepare the liponucleotide derivative of AZT (compound 4). Several groups have prepared nucleoside diphosphate diglycerides of anticancer nucleosides using this approach (33–35).

CDP-diglycerides act as normal intermediates in the biosynthesis of anionic glycerophospholipid in mammalian cells by donating its phosphatidic acid moiety to either myoinositol, glycerol 3-phosphate, or phosphatidylglycerol, resulting in the synthesis of phosphatidylinositol, phosphatidylglycerol, and cardiolipin (36, 37). Cytidine 5′-monophosphate is released in these enzymatic reactions. Interestingly, the enzymes involved in the transfer of phosphatidic acid to myoinositol, glycerol 3-phosphate and phosphatidylglycerol, lack absolute specificity for CDP-diglyceride. We have shown previously that these enzymes can also utilize deoxyCDP-diglyceride (31), UDP-diglyceride, ADP-diglyceride, and GDP-diglyceride (32). Furthermore, CDP-diglyceride can give rise to cytidine monophosphate directly when it is degraded by a cellular pyrophosphatase to phosphatidic acid and cytidine monophosphate (38, 39). We synthesized AZTdiphosphate diglyceride (compound 4) with the possibility in mind that this compound might also be metabolized by these pathways releasing AZT monophosphate directly. The details of the synthesis, purification, and characterization of AZT diphosphate diglyceride are given under "Experimental Procedures."

Biological Activity of Antiretroviral Liponucleotides in HIV-infected Cells—To evaluate the efficacy of the new compounds against HIV-infected cells *in vitro*, we prepared liposomes containing dioleoylphosphatidylcholine, dioleoylphosphatidylglycerol, cholesterol, and the various liponucleotides in a molar ratio of 5:1:3:1. Control liposomes were prepared without liponucleotide. After hydration of the thin film with acetate buffer, the suspension was sonicated resulting in the incorporation of essentially all of the liponucleotide into the lipid bilayer. The resulting prosomes were added to U937 or CEM cells infected with the LAV-1_{BRU} strain of HIV at the indicated concentrations of liponucleotide. Control liposomes were also incubated at matched lipid concentrations. After a 3-day incubation, HIV p24 antigen in the supernatant was measured by enzyme-linked immunosorbent assay.

The liponucleotide prodrugs inhibited p24 production by HIV-infected U937 cells *in vitro* as shown in Fig. 2. The order of activity was phosphatidylddC > phosphatidylAZT ≫ AZT

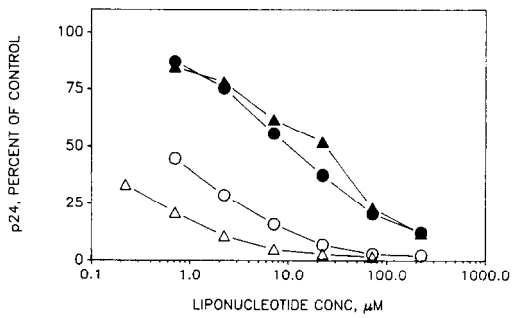

FIG. 2. **Effect of liponucleotides on p24 production by LAV-1_{BRU}-infected U937 cells *in vitro*.** *Open circles*, phosphatidyl AZT; *closed circles*, phosphatidylddT; *open triangles*, phosphatidyl ddC; *closed triangles*, AZT diphosphate dipalmitoylglycerol. The p24 level in the supernatant of no drug control was 0.61 ± 0.06 μg/ml (n = 4).

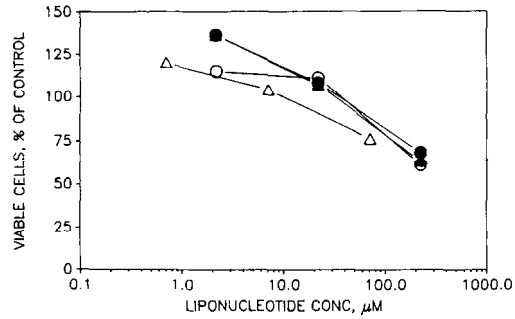

FIG. 3. **Effect of liponucleotides on viable cell number in LAV-1_{BRU}-infected U937 cells.** Symbols as in Fig. 2.

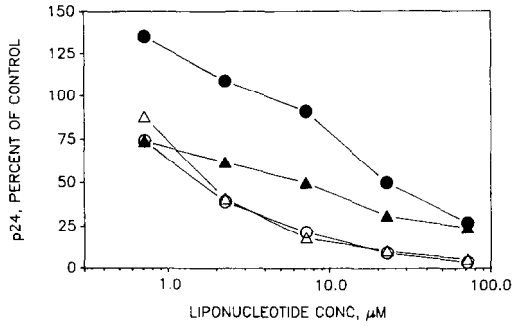

FIG. 4. **Liponucleotide effect on p24 production by LAV-1_{BRU} infected CEM cells *in vitro*.** Symbols as in Fig. 2. The p24 level in the no drug control supernatant was 2.4 ± 0.5 μg/ml (n = 8).

TABLE I

Effect of liponucleotides on HIV replication in U937 and CEM cells in vitro

U937 cells and CEM-CCRF cells were obtained from American Type Culture Collection. IC_{50} is the concentration of inhibitor required to decrease p24 production by 50%. pAZT, dimyristoylphosphatidylAZT; AZTdpdg, AZT diphosphate dipalmitoylglycerol; pddT, dimyristoylphosphatidylddT; pddC, dimyristoylphosphatidylddC; AZT, 3′-azido,3′-deoxythymidine; ddT, 3′-deoxythymidine; ddC, 2′,3′-dideoxycytidine.

Compound	IC_{50}	
	U937	CEM
	μM	
pAZT	<0.7	1.7
AZTdpdg	12.0	7.0
pddT	10.0	22.0
pddC	<0.2	1.8
AZT	0.2	0.2
ddT	>100.0	>31.6
ddC	0.04	0.06

diphosphate diglyceride = phosphatidylddT. Liposomes without liponucleotide were ineffective (data not shown). PhosphatidylAZT and phosphatidylddC had IC_{50} values below 1 μM, while phosphatidylddT and AZT diphosphate dipalmitoylglycerol required larger concentrations to reduce p24 production by 50% (10–12 μM).

To verify that the p24 reduction observed in HIV-infected U937 cells is a true antiviral effect we determined the number of viable cells (Fig. 3). After the incubations with liponucleotides, the cells were counted and cell viability was determined by trypan blue exclusion. In U937 cells, phosphatidylAZT, phosphatidylddT, and AZT diphosphate diglyceride had no effect on viable cell number between 2.2 and 22 μM; between 22 and 220 μM liponucleotide, viable cell number declined only slightly to about 70% of control. PhosphatidylddC had no effect on the viable cell number at 0.71 and 7.1 μM; at 71.4 μM viable cell number declined to 76% of control. Thus, the p24 reduction observed in HIV-infected U937 cells at concentrations of liponucleotide in the 0.2–10 μM range is clearly not due to cytotoxicity and represents a true antiretroviral effect.

CEM cells infected with HIV were also used to assess the efficacy of the liponucleotides as shown in Fig. 4. In these cells phosphatidylAZT and phosphatidylddC were about equally effective (IC_{50} = 1.7–1.8 μM) followed by AZT diphosphate dipalmitin (7 μM) and phosphatidylddT (22 μM). Equivalent amounts of liposomal control lipid had no effect on p24 production (data not shown). In addition, the distribution between cell free and cell associated p24 antigen was not altered by either liponucleotide or free drug, despite the reduction in total synthesis of p24 antigen (data not shown). In control CEM cells infected with HIV, viable cell number did not decrease significantly until liponucleotide concentrations of 100–200 μM were reached (data not shown) demonstrating that p24 reduction is a true antiviral effect not due to toxicity. The viability of normal CEM cells in response to the liponucleotides is similar to HIV-infected cells.

The results are summarized in Table I which also contains the IC_{50} data for the free nucleosides. With AZT and ddC the *in vitro* efficacy of the free nucleoside is greater than that of lipid prodrugs. This is probably due to the fact that U937 and CEM cells are not highly phagocytic which favors free nucleoside over the liponucleotide. Interestingly, phosphatidylddT was much more effective than its free nucleoside counterpart. The explanation for this observation is not certain but it is possible that phosphatidylddT is metabolized directly to ddT monophosphate by the metabolic pathways suggested above, bypassing poor phosphorylation of ddT by thymidine kinase. This possibility is currently being investigated with mutant CEM cells which lack thymidine kinase. DimyristoylphosphatidylAZT was more effective than AZT diphosphate dipalmitoylglycerol as summarized in Table I. This may be due to differences in the respective rates of cellular metabolism of the compounds or to the fatty acid composition of these two analogs. To properly assess their relative antiviral activity, the dimyristoyl and dipalmitoyl species of the two compounds should be directly compared. We are currently synthesizing the diC12:0, diC14:0, diC16:0, and diC18:0 derivatives of AZT diphosphate diglyceride and phosphatidylAZT for this purpose. Although these agents may be less active *in vitro* than the corresponding free nucleosides, liponucleotides incorporated into liposomes will allow for a much larger proportion of the antiretroviral agent to be delivered to HIV-infected macrophages because of the well known tendency of liposomal materials to be take up by macrophages (22, 23). Lipid-based formulations of liponucleotides exhibit much greater tissue retention over time than the free nucleosides. Furthermore, antiretroviral liponucleotides are bilayer-forming and appear to persist in the cells for long periods of time, relative to the nucleoside, providing a depot of antiviral drug which is released slowly by cellular metabolism.[3]

In conclusion, we have synthesized, purified, and characterized several liponucleotide prodrugs of AZT and other dideoxynucleosides. The novel phospholipid prodrugs are active *in vitro* as antiretroviral agents and appear to represent potentially important candidate agents for treatment of the macrophage reservoir of HIV infection.

Acknowledgments—The technical assistance of Judy M. Tsuji, Richard C. Border, Pat Ley, Sandra Rasad, and Sara Albanil is

[3] K. Y. Hostetler and D. D. Richman, unpublished observations.

gratefully acknowledged. Dr. Philip L. Felgner and Anna Abai of Vical Inc. prepared liposome formulations of the antiretroviral liponucleotides.

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SUPPLEMENTAL MATERIAL TO

Synthesis and Antiretroviral Activity of Phospholipid Analogs of Azidothymidine and other Antiviral Nucleosides

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EXPERIMENTAL PROCEDURES

A. Analytical Methods

Proton NMR spectra were obtained with a General Electric QE-300 or a Bruker AM-500 (Compound 4) spectrometer, using tetramethylsilane as internal standard (key: s=singlet, d=doublet, t=triplet, q=quartet, dd=doublet of doublets, m=multiplets, b=broad), UV spectra were recorded on Shimadzu UV-160, spectrophotometer. Fast atom bombardment mass spectra were determined by Mass Spectrometry Service Laboratory, University of Minnesota. Elemental analyses were determined by Galbraith Laboratories, Knoxville, TN. and Schawarzkopf Microanalytical Laboratory, N.Y. Moisture analyses were done with an EM Science Aquastar C 2000 titrator. melting points were obtained with a Fisher-Johns Melting apparatus. Column chromatography was carried out on Merck silica gel 60 (70-230 mesh). Rf values were obtained with 10 x 10 cm HPTLC plates, Kieselgel 60 (EM Reagents, Elmsford, NY). Anhydrous pyridine, 2,4,6-triisopropylbenzenesulfonyl chloride (TPS), and 3'-azido-3'-deoxythymidine (AZT) were purchased from Aldrich (Milwaukee, WI). 2',3'-Dideoxycytidine and 3'-deoxythymidine were obtained from Sigma Chemical, St. Louis, MO. Dimyristoylphosphatidic acid disodium salt was purchased from Avanti Polar Lipids (Pelham, AL).

B. Synthesis of Liponucleotides

1. 1,2-Dimyristoylglycero-3-phospho-5'-(3'-azido-3'-deoxy)thymidine monosodium salt (compound 1).

Preparation of dimyristoylphosphatidic acid (DMPA-H): In a separatory funnel (500 ml), dimyristoylphosphatidic acid disodium salt (1 g, 1.57 mmol) was first dissolved in chloroform:methanol (2:1 by volume, 250 ml). 50 ml of distilled water was added and the pH was adjusted to 1 with concentrated hydrochloric acid. After mixing the chloroform layer was removed and backwashed with 80 ml of methanol:water (1:1 by volume) and the chloroform removed under reduced pressure to yield dimyristoylphosphatidic acid (DMPA-H) as a white foam which was dissolved in 10 ml of cyclohexane and lyophilized to obtain the product as a white powder (850 mg) and stored in a dessicator at -20¡C.

Coupling reaction: DMPA-H (250 mg, 0.42 mmol) was dissolved in 10 ml of cyclohexane in a round bottom flask and lypholized overnight. 315 mg of 2,4,6-triisopropylbenzenesulfonyl chloride (1.04 mmol), 85 mg of dry 3'-azido-3'-deoxythymidine (AZT) (0.31 mmol) and anhydrous pyridine (2 ml) were added to the flask, which was sealed with a rubber serum stopper under argon. The reaction mixture was stirred at room temperature for 18 hours. Water (1 ml) was added to the crude product to destroy excess catalyst and the solvent was evaporated under reduced pressure at 40¡C to yield a yellow gum which was then redissolved in a small volume of methanol:chloroform (1:9 by volume). The solution was applied to a column of silica gel (45 g, Kieselgel 60, E. Merck, Darmstadt, West Germany). The column was eluted with 8% methanol in chloroform. After a forerun (rejected), 1,2-dimyristoylglycero-3-phospho-5'-(-3'-azido-3'-deoxy)thymidine (DMPA-AZT) was obtained. The fractions containing the product were identified by thin layer chromatography, combined and the solvent was removed in vacuo. The product was redissolved in 5 ml of cyclohexane and lyophilized to yield pure DMPA-AZT (270 mg, 0.29 mmol, 95%).

Conversion to monosodium salt: The dried DMPA-AZT was redissolved in 30 ml of chloroform:methanol (2:1 by volume), and 6 ml of distilled water was added. The pH of the aqueous phase was adjusted to 1 and the chloroform layer was collected and 10 ml of methanol:water (1:1) was added. The pH of the aqueous layer was adjusted to 6.8 with 0.1N methanolic NaOH. The mixture was evaporated under reduced pressure and the residue was redissolved in 2 ml of chloroform: methanol (2:1 by volume); acetone was added to precipitate DMPA-AZT monosodium salt which was taken up in 5 ml of cyclohexane and lypholized to yield a white powder (220 mg, 0.26 mmol, 78% yield based on AZT). The melting point was 230¡C; R_f value on silica gel G thin layer plates was 0.32 (chloroform:methanol:water:ammonia; 80:20:1:1), R_f 0.58 (chloroform:methanol:water:ammonia; 70:30:3:2), R_f 0.31 (chloroform:methanol:water; 65:25:4); UV absorption maximum 266 nm (e 10,800); Analysis Calculated for $C_{41}N_5O_{11}P_1H_{72}$. 1 H_2O:C, 57.24; H, 8.44; P, 3.61; Found: C, 56.80; H, 8.83; P, 3.52. MS, m/z 864.60 (MH^+).

Proton NMR: (CDCL3) 0.88 (6H, bt, J=6.9Hz, acyl CH3), 1.26 (40H, s, acyl CH2), 1.60 (4H, bs, Á acyl CH2), 1.94 (3H, s, thymine CH3), 2.31 (4H, m, acyl CH2), 2.39 (2H, m, ribose 2'H), 3.38 (2H, bd, J=12.6 Hz, ribose 5'H), 3.78 (2H, m, sn-3 CH_2 glycerol), 4.00 (1H, dd, J1=12 Hz, J2=6 Hz, sn-1 CH_2 glycerol, 4.07 (1H, m, ribose 3'H), 4.41 (1H, m, ribose 4'H), 5.24 (1H, m, sn-2 CH glycerol), 7.62 (1H, s, thymine 6H), 6.21 (1H, t, J=6 Hz, ribose 1'H). The peak area ratio of phosphatidic acid to AZT is 1.

2. **1,2-Dimyristoylglycero-3-phospho-5'-(3'-deoxy)thymidine, monosodium salt (Compound 2).**

This analogue was synthesized using the same method and reaction proportions described above. Melting point 235¡C, R_f on silica gel G, 0.25 (chloroform:methanol:water:ammonia; 80:20:1:1); 0.57 (chloroform:methanol:ammonia:water; 70:30:3:2); 0.24 (chloroform:methanol:water; 64:25:4); UV absorption maximum 269 nm (e 8,400); Analysis: Calculated for $C_{41}N_2O_{11}P_1H_{72}Na_1$. 1$H_2O$: C, 58.53; H, 8.87; P, 3.69; Found: C, 58.75; H, 9.33; P, 3.58. MS, m/z 823.00 (MH^+).

Proton NMR: (CDCL3) 0.91 (6H, bt, J=6.8 Hz, acyl CH3), 1.23 (4H, bs, acyl CH2), 1.26 (4H, bs, acyl CH2), 1.28 (32H, bs, acyl CH2), 1.62 (4H, m, Á acyl CH2), 1.97 (3H, s, thymine CH3), 2.05 (2H, m, ribose 2'H), 2.35 (4H, m, acyl CH2), 3.39 (2H, bs, ribose 5'H), 3.90 (2H, m, sn-3 CH_2 glycerol), 4.16 (1H, m, sn-1 CH_2 glycerol) 4.24 (1H, m, sn-1 CH_2 glycerol), 4.38 (1H, m, ribose 4'H), 5.23 (1H, m, sn-2 CH glycerol), 6.10 (1H, bt, ribose 1'H), 7.68 (1H, s, thymine 6H). The peak area ratio of phosphatidic acid to 3'-dideoxythmidine is 1.

3. **1,2-Dimyristoylglycero-3-phosph›-5'-(2',3'-dideoxy)cytidine (Compound 3).**

Preparation of 4-acetyl-2',3'-dideoxycytidine: To a stirred, refluxing solution of 2',3'-dideoxycytidine (ddC) (400 mg, 1.89 mmol) in anhydrous ethanol (35 ml, dried first with Lindy type 4x molecular sieve, and twice distilled over magnesium turnings) was added acetic anhydride (0.4 ml, 5.4 mmol). During the course of a 3 hour refluxing period, four additional 0.4 ml portions of acetic anhydride were added at 30 minute intervals. After the final addition, the solution was refluxed for 1 more hour. The reaction mixture was cooled and solvent was evaporated in vacuo. The residue was redissolved in 8% methanol in chloroform (5 ml) and chromatographed on silica gel column (2.2 cm x 30 cm, Kiselgel 60, 70-230 mesh, EM Science, 45g). The column was eluted with 8% methanol to yield pure 4-acetyl-2',3'-dideoxycytidine (ddC-NAc) in an 80% yield.

Coupling reaction: ddC-NAc (85 mg, 0.33 mmol) was coupled to 250 mg DMPA-H (0.42 mmol) in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (315 mg, 1.04 mmol) and anhydrous pyridine (2 ml) and purified as noted above. The product was lypholized from cyclohexane to yield pure DMPA-ddC-NAc (210 mg, 0.21 mmol, in 70% yield). The product was analyzed by thin layer chromatograph on 20 x 20 cm plates of silica gel GF (Analtech); its R_f value was 0.40 when developed with chloroform:methanol:water:ammonia; (80:20:1:1 by volume).

Deblocking with 9N NH4OH: ddC-NAc-DMPA (40 mg, 0.04 mmol) was dissolved in 2 ml of chloroform:methanol (1:1), and 10 drops of 9N NH4OH was added at once. The solution was stirred at room temperature for 15 minutes and neutralized with glacial acetic acid. The solvent was removed under reduced pressure to yield 1,2-dimyristoylglycero-3-phospho-5'-(2',3'a-dideoxy)cytidine (DMPA-ddC, 35 mg, 0.037 mmol). Melting point: DMPA-ddC decomposed at 240¡C. On thin layer chromatography of silica gel GF plates the R_f values were: 0.11 (chloroform:methanol:water: ammonia; 80:20:1:1); 0.38 (chloroform:methanol:ammonia:water; 70:30:3:2) and 0.15 (chloroform:methanol:water; 65:25:4); UV absorption maximum 273 nm (e 5,800). MS, m/z 786.50 (MH^+).

NMR: (CDCL3) 0.86 (6H, bt, acyl CH3), 1.24 (40H, bs, acyl CH2), 1.57 (4H, m, Á acyl CH2), 2.28 (4H, m, acyl CH2), 3.36 (2H, m, ribose 5'H), 3.94 (2H, bs, sn-3 CH_2 glycerol), 4.19 (1H, m, sn-1 CH_2 glycerol), 4.29 (1H, m, sn-1 CH_2 glycerol), 4.40 (1H, bs, ribose 4'H), 5.19 (1H, m, sn-2 glycerol CH glycerol), 5.89 (1H, m, thymine 5-H), 7.44 (1H, bs, thymine NH_2), 7.94 (1H, bs, thymine NH_2). The peak area ratio of phosphatidic acid to 2',3'-dideoxycytidine was 1.

4. **(3'-Azido-3'-deoxy)thymidine-5'-diphosphate-sn-3-(1,2-dipalmitoyl)glycerol (Compound 4).**

Synthesis of AZT-monophosphate morpholidate: This compound was synthesized following the method of Agranoff and Suomi (30). AZT-monophosphate was converted into the acidic form by passing a solution in water through a column of Dowex 50W (50x2-200, 100-200 mesh, Sigma Chemicals, St. Louis, MO). A solution of 117 mg AZT-monophosphate (0.3 mmols) in 3 ml of water was transferred to a two neck round bottom flask. Then 3 ml of t-butanol and 0.106 ml of freshly distilled morpholine (1.20 mmoles) were added and the mixture was placed in a oil bath at 90¡C. Dicyclohexylcarbodiimide (249 mg, 1.20 mmol) in 4.5 ml of t-butanol was added dropwise. The reaction was monitored by thin layer chromatography on silica gel 60/F 254 plates (E Merck, Darmstadt) developed with chloroform:methanol:acetic acid:water (50:25:3:7 by volume). The reaction was noted to be complete after 3 hours. The mixture was cooled, and after addition of 4.5 ml of water, was extracted four times with 15 ml of diethylether. The aqueous layer was removed under reduced pressure and dried in vacuo over P_2O_5. The product was obtained (199 mg, 100% yield) and used for coupling to phosphatidic acid without further purification.

Coupling of AZT-monophosphate morpholidate to dipalmitoylphosphatidic acid: Dipalmitoylphosphatidic acid, disodium salt (DPPA) was converted to the free acid by extracting the material from chloroform by the method of Bligh and Dyer (40) using 0.1N HCl as the aqueous phase. The chloroform layer was evaporated to dryness in vacuo and the DPPA (196 mg, 0.3 millimoles) was redissolved in chloroform and transferred to the vessel containing the AZT-monophosphate morpholidate. After the chloroform was removed in vacuo, the mixture was dried by addition and evaporation of benzene and finally dried in vacuo over P_2O_5. The reaction was started by addition of 30 ml of anhydrous pyridine and the clear mixture was stirred at room temperature. The reaction was monitored with thin layer chromatography as noted above with chloroform:methanol ammonia:water (70:38:82 by volume) as developing solvent. The R_f values of DPPA, AZT-monophosphate morpholidate and AZT-diphosphate dipalmitoylglycerol were 0.11, 0.50, and 0.91, respectively. After 70 hours, the pyridine was removed in vacuo and the product was extracted into chloroform after addition of 15 ml of water, 30 ml of methanol, 22 ml chloroform and sufficient 1N formic acid to adjust the pH to 4.0. After two extractions, the chloroform layers were combined, evaporated to dryness and the residue was dissolved in chloroform:methanol:ammonia:water, 70:38:8:2. The product was purified by silica gel column chromatography in this solvent applying an air pressure equivalent to one meter of water. Fractions not completely pure were further purified by HPLC using a Vydac C18 reverse phase column with water:methanol (8:2 by volume) and methanol as the mobile phase. FRactions containing the product were identified by thin layer chromatography, combined and evaporated to dryness giving 132 mg of a white solid (44% yield) which gave a single spot by thin layer chromatography with silica gel g plates developed with chloroform:methanol:ammonia:water, 70:38:8:2 (R_f 0.35) and chloroform: methanol:water, 65:35:4 (R_f 0.54).

500 MHz NMR: ($CDCl_3$) - 0.88 (3H, t, J=6.93 Hz, sn-2-acyl CH_3), 0.92 (3H t, J=7.48 Hz, sn-1-acyl chain CH_3 1.25 (s, 48H, s, CH_2 acyl chains), 1.55 (4H, bs Á CH_2 acyl chains), 1.83 (3H, s, CH_3 thymine), 2.25 (2H, t, t,J=6.97 Hz, CH_2 sn-2-acyl chain); 2.27 (2H, t, J=7.79 Hz, CH_2 sn-1-acyl chain), 2.44 (4H, bs, 2' and 5' H ribose), 3.78 (1H, dd, J=1.68, 5.51 Hz, 3'H ribose), 3.95 (2H, bs, 3H, sn-3 CH_2 glycerol), 4.07 (1H, bs, H_a/H_b sn-1 CH_2 glycerol, 5.21 (1H, bs sn-2 CH glycerol), 5.66 (1H, bs, 1'H ribose), 7.14 (1H, d, J=6.25 Hz, 6H thymine). The ratio of acyl chains: glycerol: ribose: thymine as deduced from appropriate resonances amounted to 2.12: 0.93: 0.98: 1.00. **IR** (KbR, disk) showed 2105 (azido), 1745 (c=o ester) and 1705 (c=o thymine) as identifiable bands.

C. Methods: in vitro assessment of anti-HIV activity

Preparation of liposomes with antiretroviral liponucleotides: 6.42 micromoles of dioleoylphosphatidylcholine, 3.85 micromoles f cholesterol, 1.28 micromoles of dioleoylphosphatidylclycerol and 1.28 micromoles of dimyristoylphosphatidylazidothymidine were mixed in a sterile 2.0 ml glass vial and the solvent was removed invacuo in a rotary evaporator. In some experiments, dimyristoylphosphatidylAZT was replaced with either dimyristoylphosphatidylddT, dimyristoylphosphatidylddC or AZT diphosphate dipalmitoylglycerol; control liposomes were prepared by omitting the antiviral liponucleotide. The dried film was placed under high vacuum overnight at room temperateure to remove traces of solvent. The lipid film was hydrated at 30 degrees with 0.3 ml of sterile 10 mM sodium acetate buffer (pH 5.0) containing isotonic dextrose and the ampula was sealed. The mixture was vortexed intermittently for 10 minutes followed by sonication using a Heat Systems Ultrasonics sonicator with a cup horn generator (431B) at output control setting #9 for 90 to 120 minutes at which time the sample is clarified. This sonicated preparation was diluted with sterile RPMI buffer and added to the tissue culture wells at the concentration indicated.

Cells and viral infection: The human promonocytic cell line, U937 and the lymphoblastoid cell line CEM-CCRF (American Type Culture Collection, Rockville, MD), were grown in RPMI 1640 medium containing 100 U/ml penicillin G, 100 ug/ml streptomycin, 2 mM glutamine and 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah). Cells were infected with the LAV-1_{BRU} strain (L. Montagnier, Paris, France) at a multiplicity of infection of one 50% tissue culture infectious dose ($TCID_{50}$)/cell for 60 minutes at 37° in medium containing 1% polybrene. U937 or CEM cells were infected in suspension at 6 x 10^6 cells/ml, washed three times by centrifugation and resuspension and then distributed in 96 well plates at 6 x 10^4 cells/well before addition of medium containing the liposomal antiretroviral liponucleotide drugs.

With these conditions of infection, viral p24 antigen production logarithmically increased to a peak at 3 to 4 days after infection. Peak p24 antigen values in untreated control cells and culture supernatant attained levels approximately one thousandfold over residual inoculum levels with 2.4 to 4 ug/ml or greater in the culture supernatant and approximately 0.65 ng of cell associated antigen per 10^5 cells.

HIV p24 Assay: Antiviral activity was assayed after 3 days by the inhibition of the production of HIV p24 (gag) antigen in the cell free culture medium of the infected cells exposed to different concentrations of drug; p24 antigen was measured by ELISA (Abbott Laboratories, Chicago, IL) according to the manufacturer's instructions. The data are the average of two determinations and are expressed as percentage of a control incubated in the absence of drugs.