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LIPID CONJUGATES OF ANTIRETROVIRAL AGENTS. I. AZIDOTHYMIDINE-MONOPHOSPHATE-DIGLYCERIDE: ANTI-HIV ACTIVITY, PHYSICAL PROPERTIES, AND INTERACTION WITH PLASMA PROTEINS

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3'-Azido-3'-deoxythymidine-5'-phosphate diglyceride (16:0/18:1ω9), a phosphatic acid conjugate of AZT, is active against HIV replication in H9 cells and syncytia formation in MOLT-3 cells. The activities rank as AZT > pure conjugate > conjugate in mixed liposomes, with the pure conjugate having about one-third the activity of free AZT. The compound binds very rapidly to serum lipoproteins, but not to serum albumin, α and β globulins, or red cells. Pancreatic phospholipase A_2 hydrolyzes it to the lysophosphatidic acid conjugate.

Glycerophospholipid conjugates of pharmaceutical agents can provide a means of improving drug efficacy, as has been demonstrated by investigations of the anti-cancer liponucleotide araC diphosphate diglyceride (1-4). With the goal of targeting toward macrophages, others have investigated the activities of the monophosphate dimyristin conjugates of azidothymidine, dideoxythymidine, and dideoxycytidine against HIV replication in U937 and CEM cells (5). The conjugates were formulated as mixed liposomes containing natural glycerophospholipids and cholesterol.

Although the development of dideoxynucleoside phospholipid conjugates for formulation as liposomes for targeted delivery to macrophages is one possible goal, they possess a number of other attributes which suggest that they may have a much wider range of applicability.

We have synthesized a monophosphate diglyceride conjugate of AZT containing esterified saturated palmitoyl and unsaturated oleoyl fatty acids, a composition which more closely mimics the natural state (AZTMPDG, Figure 1). We report here its activity against

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ABBREVIATIONS: AZT = 3'-azido-3'-deoxythymidine; AZTMPDG = 3'-azido-3'-deoxythymidine-5'-phosphate diglyceride (16:0.18:1w9, sn-1/sn-2); PC = phosphatidylcholine; HDL and LDL = high- and low-density lipoproteins; PBS = phosphate-buffered saline.

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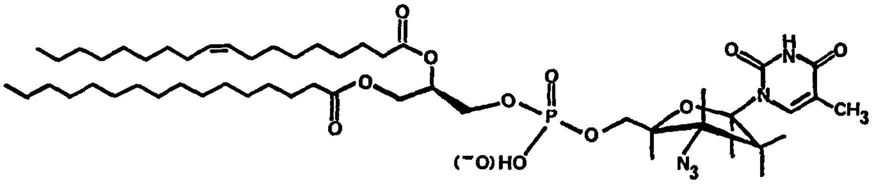

Figure 1. The structure of 3'-azido-3'-deoxythymidine-5'-phosphate diglyceride (C16:0/C18:1ω9, sn-1/sn-2), abbreviated in the text as AZTMPDG.

HIV replication in H9 cells and against syncytia formation by MOLT-3 cells, its interaction with blood components, and its physical state in plasma.

MATERIALS AND METHODS

AZTMPDG, Na^+ salt, was synthesized by condensation (dcc/pyridine) of sn-1-palmitoyl-sn-2-oleoyl phosphatidic acid (Avanti) and AZT by a method similar to one previously described (6). Purity was verified by TLC on silicic acid using $CHCl_3/CH_3OH/H_2O$ 65/25/4 v/v and by elemental analysis (C, H, N, P); structure was established by ^1H NMR, ^{13}C NMR, and FTIR. The Na^+ salt was used only to prepare mixed liposomes for biological testing; all other experiments were done with the pure ammonium salt. It was prepared by extracting the compound from 0.5 M HCl into $CHCl_3$, washing with water, equilibration with 2 M NH_3, a second wash, and finally evaporation of the $CHCl_3$ phase to dryness. Cholesterol and other phospholipids were purchased from Sigma Chemical Co.

Vesicles of AZTMPDG, either alone or mixed with other lipids, were made by dissolving the sample in $CHCl_3$, removing the solvent under vacuum, and re-hydrating the resulting thin lipid film with buffer. Extrusion four times through 0.1 µm Nuclepore filters (7) produced unilamellar vesicles averaging about 0.1 µm in diameter. Their structure and size were verified by negative stain and freeze-fracture electron microscopy. For bioassays, stock liposome preparations containing 1.0 mg/ml of drug were made in 0.125 M NaCl buffered to pH 7.4 with 50 mM phosphate, and sterilized by filtration through 0.22 µm filters into autoclaved Wheaton vials. For other studies, the buffer was 0.145 M NaCl buffered to pH 7.4 with 5 mM phosphate, containing 0.02% NaN_3. Liponucleotide concentrations were determined from absorbance at 265 nm, after adding 5% deoxycholate.

Binding to isolated plasma proteins was studied at room temperature by chromatography on Sephacryl S-300-HR (Pharmacia) with continuous monitoring of the effluant at 280 nm. Various concentrations of pure AZTMPDG vesicles were stirred for 20-30 minutes with a fixed concentration (5 mg/ml) of human HDL or LDL, then applied to a 1.6 x 30 cm column and eluted at 20 ml/hr. In the case of HDL, when all the drug bound the vesicles vanished and the drug-HDL complex produced a single peak; at drug concentrations beyond the binding capacity of the lipoprotein the excess unbound vesicles emerged separately in the void volume. The difference between the areas of the peaks produced by the saturated complex and by HDL containing no drug is a measure of the drug bound at saturation. Binding to fatty-acid-free bovine serum albumin (Sigma) and to human α and β globulins (Cohn Fraction IV, Sigma) was studied in the same way, except that the drug was held constant while the protein concentration was varied. In the case of LDL, both the vesicles and the lipoprotein eluted in the void volume. Attempts to separate the two by using other column packings, reducing vesicle size by sonication, or centrifuging in NaBr solutions were unsuccessful. Although a direct measurement of binding by LDL was not achieved, competitive binding by HDL and LDL was measured by stirring a mixture of HDL and LDL (5 mg/ml each) with an amount of liponucleotide lower than needed to saturate HDL alone. The LDL/drug complex emerged in the void volume, followed by the HDL/drug complex.

Lipoproteins were prepared from fresh human plasma by centrifugation at densities adjusted with NaBr (8). After removal of chylomicrons and VLDL by centrifuging the plasma, the LDL wre floated at 1.063 g/ml and the HDL at 1.216 g/ml. They were dialyzed in PBS and stored at 4°C in PBS containing 2 mM EDTA and 0.02% NaN_3. Stock concentrations were determined by dialyzing aliquots against distilled water and drying in vacuo over P_2O_5. The binding capacity of whole plasma for pure AZTMPDG was titrated by taking advantage of the fact that binding clears turbid suspensions of vesicles. Fresh human plasma and drug vesicles at various concentrations were mixed for 30 minutes, and the absorbance at 350 nm plotted against concentration; a sudden increase indicates the appearance of unbound vesicles. The rate of clearance was used to measure the kinetics of binding. Suspensions of extruded AZTMPDG liposomes were injected at room temperature into a stirred curvette in the spectrophotometer, and absorbance at 350 nm was monitored against time.

Hemolytic activity was investigated by incubating extruded vesicles of AZTMPDG with human red cells, centrifuging to remove cells, and measuring the absorbance at 412 nm. The cell concentration was adjusted to give an absorbance of 1.8 after lysis by 2.5% deoxycholate. Binding of drug to human red cells was investigated by room temperature incubation of 0.120 mg/ml of vesicles with cells at a hematocrit of 50%. After 1 hour, the cells were centrifuged down and the absorbances of the supernatant solution at 265 nm and 412 nm measured. Corrections for hemoglobin at 265 nm and for interstitial volume allowed the amount of unbound drug to be calculated.

Anti-HIV activities were determined in HIV-infected cells by counting syncytia formation in MOLT-3 cells and measuring p17 and p24 expression in H9 cells by immunofluorescence assay (9, 10). The growth medium used was RPMI 1640 in the presence of 10% fetal calf serum.

Potential hydrolysis of the drug in serum was checked by incubating extruded vesicles of AZTMPDG at a concentration of 0.5 mg/ml in 75% fresh human serum for 3 days at 37°C. After incubation, the samples were analyzed for free AZT by chromatography on Sephadex G-25, which separated free AZT from free or protein-bound AZTMPDG emerging in the void volume. Incubated controls were run of 75% serum, AZT in 75% serum, and AZTMPDG in buffer.

RESULTS

The sodium salt of AZTMPDG is a white solid powder, soluble in chloroform. It will not form liposomes at temperatures as high as 100°C, and cannot be extruded. When mixed with egg phosphatidylcholine or cholesterol, it forms liposomes at room temperature. Extruded unilamellar vesicles having the following molar compositions were prepared from the Na^+ salt: 20% AZTMPDG/80% egg PC; 80% AZTMPDG/20% cholesterol; 18% AZTMPDG/35% cholesterol/47% egg PC. In contrast, the pure ammonium salt spontaneously forms extrudable liposomes at room temperature; extruded unilamellar vesicles of pure ammonium AZTMPDG were chosen for all subsequent biophysical studies.

AZTMPDG does not bind to bovine serum albumin or α and β human globulins. It does not bind to human red cells in the presence or absence of the lipoproteins, and is not hemolytic. Appreciable quantities of the drug bind to human serum lipoproteins. The binding capacity of HDL is approximately 0.11 mg per mg of lipoprotein, or about 30 molecules per average HDL particle (8). In mixtures containing equal concentrations of LDL and HDL, the liponucleotide partitions between the two, with LDL binding 2.4 times the amount bound by HDL. Binding to whole fresh human plasma is consistent with the binding capacities of the separate proteins. The determination of binding capacity of 50% plasma for pure AZTMPDG is shown in Figure 2a. At

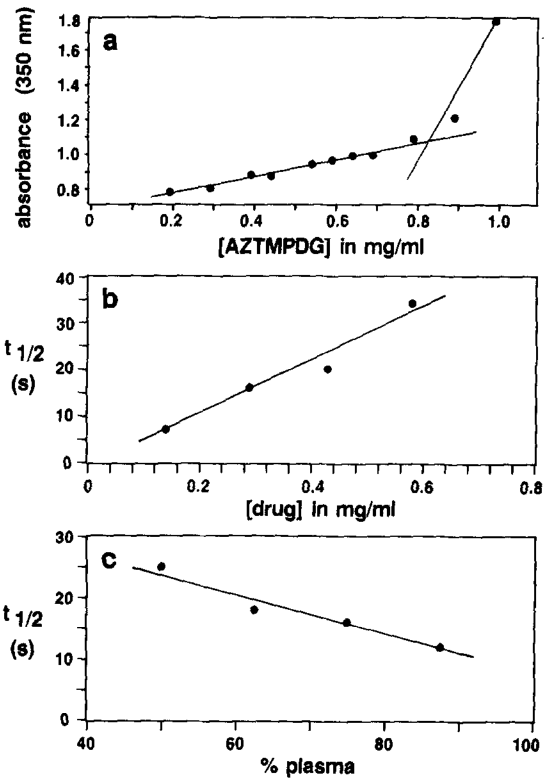

Figure 2. The binding of unilamellar vesicles of AZTMPDG in human plasma. (a) Turbidimetric determination of binding capacity of 50% plasma. The drug is lipoprotein-bound at concentrations below 0.8 mg/ml; free vesicles are present above that concentration. (b) Half-time for clearance of turbid suspensions of vesicles as a function of drug concentration at a constant plasma concentration of 75%. (c) Half-time for clearance as a function of plasma concentration at a constant drug concentration of 0.29 mg/ml.

saturation the capacity is approximately 0.80 mg/ml, or 1.6 mg/ml of whole serum, an amount well beyond any to be encountered pharmacologically. HDL would account for about one-fourth of this amount, LDL for the rest. Mixed vesicles of the liponucleotide and/or cholesterol apparently are not stable in plasma, since turbidity changes rapidly when a suspension of vesicles is mixed with the plasma; this phenomenon was not investigated in detail. Binding of AZTMPDG (ammonium salt) vesicles in plasma is very rapid. Half-times for clearance are plotted against drug concentration at constant plasma concentration in Figure 2b, and against plasma concentration at constant drug concentration in Figure 2c. The rate increases with decreasing drug concentration and increasing plasma concentration; under physiological conditions, the process would be essentially instantaneous.

Dose-response curves for pure AZTMPDG formulated as extruded vesicles are presented in Figure 3a. The liponucleotide was active against HIV-1 replication in H9 cells (p17 and p24 markers) and syncytia formation by MOLT-3 cells, but less active than the parent AZT. Relative to free AZT, the IC$_{50}$'s for inhibition of p17 and p24 differ by a factor of about 3; for inhibition of syncytia formation the factor is greater than 15. However, the relatively low activity against

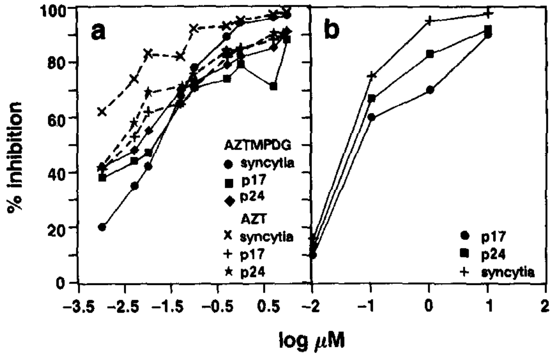

Figure 3. Dose-response curves for inhibition of HIV replication in H9 cells and syncytia formation by MOLT-3 cells. (a) Pure AZTMPDG. (b) Mixed liposomes consisting of 18 mole% AZTMPDG/35 mole% cholesterol/47 mole% egg phosphatidylcholine. A control of AZT is also shown in (a).

syncytia formation may be anomalous. In two other similar preparations, both the liponucleotide and free AZT produced IC$_{50}$'s for syncytia inhibition that were lower than those for p17 and p24 production. Similar dose-response curves were obtained for the three mixed liposome preparations listed at the beginning of this section: the conjugate formulated as mixed liposomes is less active than free AZT on a molar basis, and in fact appears to be less active than pure AZTMPDG. A set of representative curves are shown in Figure 3b. Table 1 lists the IC$_{50}$'s of the various formulations of the drug. These numbers are calculated from straight-line interpolations such as those pictured in Figure 3 , so that little or no significance should be attached to the second digit. Nevertheless, a trend is apparent: in decreasing order, the relative activities are AZT > pure AZTMPDG > AZTMPDG in mixed liposomes. Neither the pure conjugate nor the mixed liposome preparations were toxic as judged by viable cell count.

The activity of the conjugate does not arise from free AZT released by enzymes in the serum present in the growth medium, since no free AZT was released upon incubation for 3 days

Table 1. Activities of AZTMPDG against p17 and p24 production in H9 cells and syncytia formation by MOLT-3 cells. IC$_{50}$ is the concentration of drug needed to reduce HIV antigen expression / syncytia formation by 50% compared to infected but untreated cells

	IC$_{50}$(mM)		
Formulation	p17	p24	syncytia
pure AZTMPDG	0.014	0.0066	0.016
20% AZTMPDG/ 80% PC	0.052	0.055	0.060
80% AZTMPDG/ 20% cholesterol	0.19	0.19	----
18% AZTMPDG/ 47% PC/ 35% cholesterol	0.070	0.053	0.041
AZT	0.0038	0.0023	< 0.001

at 37°C in fresh human serum (detection limit 0.1% hydrolysis). The compound was rapidly hydrolyzed to the corresponding lysoglycerophospholipid by pancreatic phospholipase A_2, but was resistant to phospholipase C (Bacillus subtilus) and phospholipase D (cabbage).

DISCUSSION

Introduced into the bloodstream of an animal or into a culture medium containing serum, vesicles of pure AZTMPDG would vanish within a few seconds as the drug binds almost exclusively to the serum lipoproteins. In vivo, it would be transported and biodistributed as the lipoprotein complex, and may undergo much the same metabolic transformations and catabolic fate as natural lipoprotein glycerophospholipids. Since, unlike AZT, glucuronidation of AZTMPDG would not occur and the bound drug would not undergo filtration in the kidneys, a significant increase in plasma lifetime may result. The susceptibility of the compound to pancreatic lipase may bode well for oral administration (11). For example, an anticancer thioalkyl lysophosphatidylcholine analog has been shown to be orally absorbed with a plasma half-life of 27 hr in rats (12).

Highly selective targeting to macrophages is also an attractive goal, and would be possible if AZTMPDG or similar conjugates can be formulated with other lipids as sufficiently stable liposomes. As judged by rapid changes in turbidity when mixed with human plasma, the mixed liposomes reported here appear to be rather unstable; the drug may be quickly removed and bound to lipoproteins in the blood or in the culture medium used for in vitro assays (3).

The conjugate is somewhat less active than AZT against HIV-1 replication in H9 cells and syncytia formation by HIV-1 infected MOLT-3 cells. A similar relative reduction of activity was noted for HIV replication in U937 and CEM cells. However, relative activities in the test tube often do not parallel those in the animal. The anticancer liponucleotide araC diphosphate diglyceride is less active than araC in cell culture, but far more efficacious in tumor-bearing mice (2,3). Extracellularly, AZTMPDG is best viewed as a drug in and of itself, rather than simply a prodrug.

The mode of delivery to the infected cell is unknown, but presumably involves exchange from lipoproteins since the growth medium contains more than enough serum to bind the drug. Lack of toxicity, as judged by viable cell count, indicates a specific antiviral effect, and there is no evidence that the activity is not due to reverse transcriptase inhibition.

In view of their molecular structures, binding, and other biophysical and biochemical properties, AZTMPDG and most likely other anti-HIV dideoxynucleoside conjugates of glycerophospholipids (and/or analogs thereof) present remarkable opportunities for drug development in the treatment of AIDS not possible with the parent clinical anti-AIDS drugs. An even stronger case may evolve upon further development and evaluation of lysoglycerophospholipid type anti-HIV conjugates.

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REFERENCES

1. Raetz, C.H.R., Chu, M.Y., Srivastava, S.P., Turcotte, J.G. (1977) Science 196, 303-305.
2. Turcotte, J.G., Srivastava, S.P., Steim, J.M., Calabresi, P., Tibbetts, L.M., and Chu, M.Y. (1980) Biochim. Biophys. Acta 619, 619-631.
3. Bernaki, R., Rustum, Y., Steim, J. M., Turcotte, J. G., unpublished data.
4. Ryu, E.K., Ross, R.J., Matsushita, T., and MacCoss, M. (1982) J. Med. Chem. 25, 1322-1329.
5. Hostetler, K.Y., Stuhmiller, L.M., Lenting, H.B.M., van den Bosch, H., and Richman, D.D. (1990) J. Biol. Chem. 265, 6112-6117.
6. Turcotte, J.G., Srivastava, S.P., Meresak, W.A., Rizkalla, B.H., Wunz, T.P. (1980) Biochim. Biophys. Acta 619, 604-618.
7. Hope, M.J., Bally, M.B., Weble, G., and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55-65.
8. Schumaker, V.N., and Puppione, D.L. (1986) Methods in Enzymology 128, 155-169.
9. Sarin, P.S., Taguchi, Y., Sun, D., Thornton, A., Gallo, R.C., and Oberg, B. (1985) Biochem. Pharm. 34, 4075-4079.
10. Sarin, P.S., Agrawal, S., Civeira, M.P., Goodchild, J., Ikeuchi, T., and Zamecnik, P.C. (1988) Proc. Natl. Acad. Sci. USA 85, 7448-7451.
11. Patton, J.S. (1981) Physiology of the Gastrointestinal Tract (L.R. Johnson, Ed), pp 1123-1146.
12. Herrmann, D.J., Besenfelder, E., Bicker, U., Pahlke, W., and Bohm, E. (1987) Lipids 22, 952-966.