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LIPID CONJUGATES OF ANTIRETROVIRAL AGENTS. II. DISODIUM PALMITYL PHOSPHONOFORMATE: ANTI-HIV ACTIVITY, PHYSICAL PROPERTIES, AND INTERACTION WITH PLASMA PROTEINS

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Disodium palmityl phosphonoformate, a novel lipid phosphoester of the anti HIV agent phosphonoformate (foscarnet), inhibits HIV replication in H9 cells and syncytia formation in MOLT-3 cells as effectively as foscarnet itself, as shown by dose-response data from assays for expression of p17 and p24 viral antigens and syncytia formation. Protein binding studies indicate that in serum, the derivative exists bound to albumin and the lipoproteins, and would therefore be likely to exhibit improved serum lifetime *in vivo*.

Trisodium phosphonoformate(foscarnet), a selective inhibitor of viral DNA polymerase, is under clinical investigation for the treatment of HIV infection and associated viral opportunistic infections (cytomegalovirus, retinitus and colitis, acyclovir-resistant herpes) (1-4). The clinical use of foscarnet has proved to be less than ideal, partly because it is so rapidly removed from the bloodstream that continuous IV infusion is required for optimal activity. If foscarnet is to be considered for broader clinical use as a single agent or in combination therapy, its pharmacokinetic properties must be altered and improved.

One approach might be to modify the compound to resemble fatty acids by conjugation to fatty alcohols. Like fatty acids, the foscarnet derivatives would be expected to be orally absorbed and to bind to serum proteins and hence be lost less rapidly by renal filtration

A phosphatidic acid conjugate of the anticancer nucleotide cytosine arabinoside-5'-phosphate, for example, has been shown to exhibit significantly improved pharmacokinetics and *in vivo* efficacy in tumor-bearing mice, together with reduced toxicity compared with the parent nucleoside (5-12). Glycerophospholipid conjugates of the anti-HIV nucleosides azidothymidine (AZT), dideoxycytidine (ddC), and dideoxythymidine (ddT) have been reported to exhibit activity against HIV in cell cultures of T-lymphocytes and macrophages (13, 14). Conjugates of small molecules derived from physiologic molecular species of phospholipids, lysophospholipids or other appropriate classes of lipids generally offer the prospect of manipulation for

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Abbreviations: HDL = high-density lipoprotein, LDL = low-density lipoprotein, HIV = human immunodeficiency virus, PBS = phosphate-buffered saline, BSA = bovine serum albumin.

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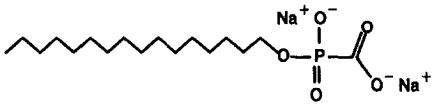

Figure 1. Disodium palmityl phosphonoformate.

enhancement of oral absorption, increased plasma half lives, protection against destructive metabolism of the active moiety, and a degree of selective biodistribution and targeting (5, 7).

In order to evaluate its potential effectiveness as an anti-HIV agent, we have investigated the physical properties and protein-binding affinities of a novel fatty acid analog derived from foscarnet: disodium palmityl phosphonoformate (Figure 1). We have taken advantage of these properties to formulate this compound for *in vitro* testing of anti-HIV activity.

MATERIALS AND METHODS

Palmityl phosphonoformate ethyl ester was synthesized (15) by condensation (pyridine/chloroform) of palmityl alcohol with ethoxycarboxyphosphonochloridic acid methyl ester, followed by cleavage (EtOH-free chloroform, 0°C) with trimethylsilyl bromide. Its purity was verified by thin-layer chromatography on silica gel and by elemental analysis; and its structure by ^1H NMR and FTIR (KBr pellet; strong 1577 cm-1 carbonyl stretch). The ester was hydrolyzed to the disodium salt, a form suitable for administration in cell culture, by dissolving it using brief sonication in 1:1 ethanol/H_2O containing 0.1 M NaOH, then heating at 50°C for 30 minutes. Chilling at 0°C precipitated the salt as a white solid, which was washed several times with ice-cold ethanol until the washings were no longer basic, then dried under vacuum. Trisodium foscarnet hexahydrate was purchased from Fairfield Chemical Co.

All physical and protein binding studies were performed in 0.145 M saline buffered to pH 7.4 with 5 mM phosphate, with 0.02% NaN_3 added as a preservative. Samples for testing in cell culture were prepared in 0.125 M saline buffered with 50 mM phosphate (Dulbecco's PBS 1X, Advanced Biotechnologies Inc.).

Binding of disodium palmityl foscarnet to bovine serum albumin (BSA) and to human high-density and low-density serum lipoproteins was determined by monitoring turbidity. The compound was prepared as a stable turbid suspension in PBS by heating above the Kraft temperature of 78°C (determined by calorimetry) until dissolved, then slowly cooling to re-aggregate it. Various dilutions were stirred together for 15 minutes with a fixed concentration of protein in PBS, pH 7.4, at room temperature. The absorbance at 350 nm was plotted against drug concentration; saturation of protein with drug occurs when the solution no longer clears. High and low-density lipoproteins were isolated from fresh human plasma by ultracentrifugation (16), using NaBr to adjust densities, followed by exhaustive dialysis in PBS containing 2mM EDTA and 0.02% NaN_3. The LDL fraction contained lipoproteins with densities in the range of 1.019 to 1.063 g/ml; HDL contained the range between 1.063 to 1.21 g/ml. The purity of the two classes of protein was verified by chromatography on Sephacryl S300-HR by monitoring the effluent at 280 nm. Fatty-acid-free and Fraction V BSA were purchased from Sigma Chemical Co.

To determine whether disodium palmityl foscarnet is hydrolyzed by enzymes present in blood serum, the drug at a concentration of 2.5 mM (1 mg/ml) was incubated for 48 hours at 37°C in 95% human serum / 5% PBS, then analyzed for the presence of free foscarnet by using ion-pair reversed-phase HPLC and coulometric detection (17). Controls of 95% serum and 95% serum containing 2.5 mM trisodium foscarnet hexahydrate were treated the same way.

Samples containing disodium palmityl foscarnet prepared either free or bound to various concentrations of bovine serum albumin to solubilize the drug were tested for anti-HIV activity in cell culture. The samples were prepared as follows: 2 mg/ml disodium palmityl foscarnet in PBS was heated to above 78°C to dissolve it, then slowly cooled to room temperature, accompanied by slow aggregation to give a turbid suspension. This suspension was mixed with an equal volume of buffer alone or buffer containing fatty-acid-free bovine serum albumin

(Sigma) dissolved in the same buffer at a concentration to give either 2.5% or 5% BSA after mixing. After mixing at 37°C for 15 minutes, clearing was observed as all drug bound to the albumin. The resulting solutions containing 1 mg/ml disodium palmityl foscarnet were sterilized by filtration through 0.2 µm filters into autoclaved Wheaton vials. For assay, these stock solutions were diluted with culture medium. For preparations of the drug not bound to albumin, it was necessary to heat the suspension of disodium palmityl foscarnet to above 78°C to dissolve and to filter the hot solution through sterile 0.2 µm filters.

The anti-HIV activity of these preparations was determined in HIV-infected cells by counting syncytia formation in MOLT-3 cells, viewed under the microscope, and measuring p17 and p24 marker protein expression in H9 cells by immunofluorescence assay. The growth medium was RPMI 1640 containing 10% fetal calf serum. Activity is reported as the percentage inhibition compared to syncytia formation, and p17 and p24 expression in infected but untreated cells. The assay methods are described in the literature (1,18). A range of doses of disodium palmityl foscarnet between 1 and 200 µg/ml was employed. Equimolar concentrations of the parent drug, trisodium foscarnet hexahydrate, and the appropriate controls of buffer alone or serum albumin in buffer were also tested under the same conditions.

RESULTS

A turbidimetric titration of Fraction V BSA is shown in Figure 2a, where the absorbance at 350 nm at a fixed protein concentration is plotted against increasing concentrations of drug. Since the unbound drug produces a stable turbid suspension which clears upon binding, the drug concentration needed to saturate the albumin binding sites is marked by a departure from

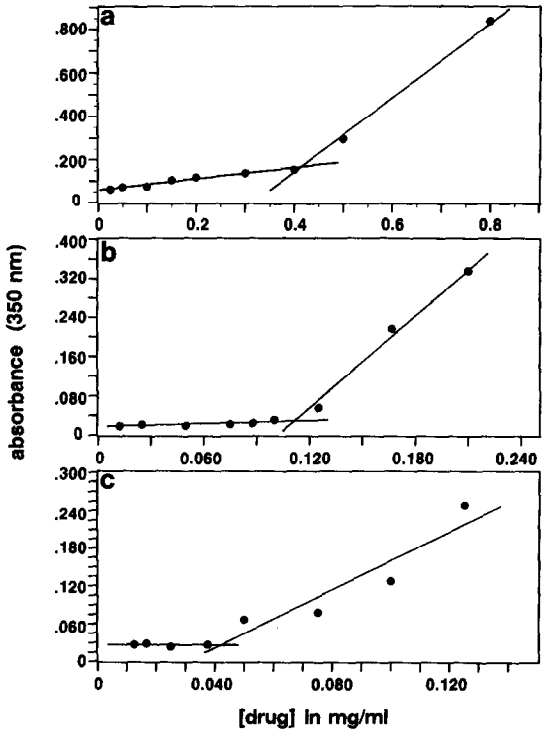

Figure 2. Turbidimetric determination of the binding of palmityl foscarnet in phosphate buffered saline, pH 7.4 to plasma proteins. (a) 10 mg/ml Fraction V bovine serum albumin, (b) 0.5 mg/ml high-density lipoproteins, (c) 1.5 mg/ml low-density lipoproteins. The change in slope in each plot designates the appearance of unbound drug and is a measure of the saturation binding capacity of the protein.

Table 1. Saturation concentrations for binding of disodium palmityl foscarnet to bovine serum albumin and high and low density lipoproteins

Protein	mg drug per mg protein	Molecules drug per average protein molecule	Drug bound (mg) per ml plasma
1. bovine serum albumin (MW = 69,000)	0.04	7	1.6
2. HDL (avg. MW= 265,000)	0.22	145	0.8
3. LDL (avg. MW= 2.5 X 10^6)	0.027	160	0.10

linearity due to the onset of turbidity. For 10 mg/ml albumin, saturation occurs at about 0.4 mg/ml drug. The same result was obtained with fatty-acid-free albumin. Turbidimetric titrations of HDL at 0.5 mg/ml and LDL at 1.5 mg/ml are presented in Figures 2b and 2c. For HDL, saturation occurred above 0.11 mg/ml drug; LDL was saturated above 0.040 mg/ml drug.

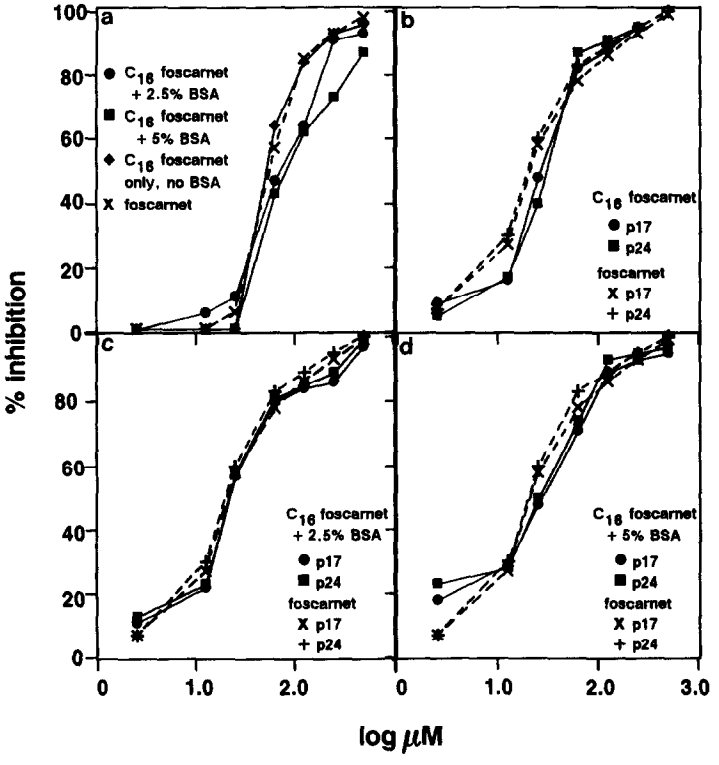

Figure 3. Dose-response curves showing: (a) activity of disodium palmityl foscarnet against syncytia formation in HIV-infected MOLT-3 cells, and (b)-(d) activity of disodium palmityl foscarnet against HIV replication in HIV-infected H9 cells expressed as % inhibition of the production of p17 and p24 marker proteins. Concentrations of BSA refer to the amount of BSA used to prepare a stock solution of 1 mg/ml drug in PBS, not to the final concentration of BSA in the assay medium. Samples were made up (a) both with and without BSA, (b) with no BSA, (c) with 2.5% BSA, (d) with 5% BSA. The stock solutions were diluted with growth medium to the proper dose concentrations. A control of free trisodium foscarnet in PBS is shown in each case.

Table 1 lists mg of bound drug per mg of protein, together with binding capacity on a molecular basis, calculated by assuming a molecular weight of 69,000 for BSA and mean molecular weights of 265,000 for HDL and 2.5 X 10^6 for LDL. In the same table are given estimates of the total binding capacity of human plasma, based upon average human plasma concentrations of 40 mg/ml albumin, 3.8 mg/ml HDL, and 3.8 mg/ml LDL (16). Human and bovine albumins are assumed to have identical binding capacities. The total binding capacity of the plasma for the drug would be 2.5 mg/ml, well above concentrations to be encountered *in vivo*. In the bloodstream, all would be bound and most would be transported as a serum albumin complex. Note also that the 10% fetal calf serum included in the culture medium used for the activity assays is more than enough to completely bind the highest concentrations of drug tested. Palmityl foscarnet is not hemolytic when incubated with washed human red cells in PBS.

The activities of disodium palmityl foscarnet and trisodium foscarnet against syncytia formation by MOLT-3 cells are plotted in Figure 3a. The curves for palmityl foscarnet, prepared without albumin, and for free foscarnet are nearly identical, with IC_{50} values in the neighborhood of 50 μm (see Table 2). Preparing the palmityl foscarnet bound to BSA before diluting it with growth medium for bioassay appears to increase the IC_{50} slightly, but this protein effect is not obtained consistently. Dose-response curves for inhibition of HIV replication in H9 cells as monitored by p17 and p24 viral marker proteins are displayed in Figures 3b-d. The responses to free foscarnet and palmityl foscarnet initially bound to 2.5% albumin are identical (Figure 3c) although the IC_{50} value of 22 μm is significantly lower than for syncytia inhibition in MOLT-3 cells. When prepared in 5% albumin (Figure 3d), the derivative appears to be slightly less active than free foscarnet. Similarly, the derivative prepared as a suspension in buffer without albumin (Figure 3b) appears slightly less active in H9 than free foscarnet.

We are inclined to view the small differences in IC_{50} values, particularly in the p17 and p24 data, to lack statistical significance, and conclude that the activities of palmityl foscarnet and free foscarnet are essentially the same. In no case was toxicity observed, as judged by viable cell count, and in all cases controls of buffer and BSA were inactive.

The most trivial explanation for the identical or nearly identical activities of derivative and free foscarnet is hydrolysis of palmityl foscarnet to release the foscarnet moiety into the culture medium. This is apparently not the case, since incubation of palmityl foscarnet for two days in 95% fresh human serum for two days released no foscarnet detectable above 0.1%, the limit of sensitivity of this method.

Table 2. Activities of disodium palmityl foscarnet and foscarnet against p17 and p24 expression in H9 cells and syncytia formation by MOLT-3 cells
IC_{50} is the concentration of drug needed to reduce HIV antigen expression or syncytia formation by 50% compared to infected but untreated cells

	IC_{50} (μM)		
Preparation	syncytia	p17	p24
1. palmityl foscarnet, no BSA	51	27	31
2. palmityl foscarnet + 2.5% BSA	71	22	22
3. palmityl foscarnet + 5% BSA	81	28	25
4. foscarnet	56	20	21

DISCUSSION

The results for *in vitro* activity of disodium palmityl foscarnet against HIV suggest that the palmityl conjugate is as effective as trisodium foscarnet in inhibiting virus replication and syncytia formation in the cell lines tested. Furthermore, the affinity of this amphiphilic species for both serum albumin and lipoproteins, the presence of fetal calf serum in the culture medium, and the absence of hydrolysis in serum ensure that the drug is expressing its activity from a protein-bound form. The bound drug would also likely be the circulatory transport form *in vivo*.

The binding of the drug to albumin and lipoproteins is likely to prove pharmacokinetically advantageous in terms of increasing serum lifetime, since it would be expected to be transported as a protein complex, and would escape rapid filtration by the kidneys. An anticancer lysophosphatidylcholine analog has been shown to be orally absorbed and to have a serum half-life of 27 hours in rats (19), and a renin-inhibitory lysophosphatidyl-ethanolamine analog has been found to exhibit pronounced anti-hypertensive activity when administered once daily to Goldblatt hypertensive rats (20). Liponucleotide derivatives of cytosine arabinoside, which show greatly improved in vivo efficacy over the parent nucleoside, also readily bind to HDL and LDL in large quantities (21, 22). The similarities of palmityl foscarnet to fatty acids in terms of molecular structure and physical properties, including micelle formation and affinity for serum albumin suggest that, like a free fatty acleid, it may diffuse across the intestinal mucosa and hence be a good candidate for oral administration. We are presently examining plasma lifetime and oral uptake in mice.

Binding of disodium palmityl foscarnet to proteins at room temperature is slow, taking up to 15 minutes to complete. Administration of the drug *in vivo* as an albumin complex would eliminate problems possibly associated with particles being present in an unbound form. However, slow binding kinetics is attributable to its high Kraft temperature of 78°C, below which the compound exists as a microcrystalline suspension. The velocity of binding would be greatly increased and the need to prepare the drug as an albumin-bound complex could be eliminated by lowering the Kraft temperature to below 37°C, so that at physiological temperature it exists as micelles. This can be accomplished by shortening the chain length or introducing unsaturation. Another advantage to using shorter-chain versions is that smaller binding energies between the drug and protein may make the drug more easily available to target cells and perhaps more active than free foscarnet.

Since no extracellular hydrolysis of palmityl phosphonoformate has been demonstrated, presumably initial interaction with target cells occurs by exchange directly from the protein-bound form. The mechanism of inhibition is unknown, but it is reasonable to expect that the phosphonofatty acid functions as an inhibitor of viral DNA polymerase via intracellular release of carboxyphosphate. As the development of this class of experimental anti-AIDS drugs progresses, more detailed studies on mechanism of action will be undertaken on the disposition of intact molecules prior to and upon entry into target cells.

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