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PHOSPHOLIPID PRODRUG INHIBITORS OF THE HIV PROTEASE

ANTIVIRAL ACTIVITY AND PHARMACOKINETICS IN RATS

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Abstract—The aspartyl protease of the human immunodeficiency virus (HIV) is an important target for chemotherapeutic intervention because of its key role in cleaving the HIV *gag-pol* polyprotein during viral assembly and budding. Short peptides and peptidomimetics, which bind to the active site of the HIV aspartyl protease and inhibit processing of the polyprotein, have been synthesized. These compounds are active against HIV *in vitro*, but many face substantial development problems because of their rapid elimination from the body in bile and urine. Refinement of these agents appears to be necessary if they are to become useful clinically. Recently, we developed a novel chemical strategy for increasing plasma levels of HIV protease inhibitory peptides, which involves the attachment of a biodegradable phospholipid group to the C-terminus of a pentapeptide, iBOC-[L-Phe]-[D-β-Nal]-Pip-[α-(OH)-Leu]-Val (7194). We coupled phosphatidylethanolamine to the C-terminal valine of 7194 to make a phospholipid prodrug (7196). *In vitro* assays in HT4-6C cells infected with HIV-1 showed that the antiviral activity of the C-terminal phospholipid prodrug, 7196, was equal to that of the free peptide, 7194. Similar results were obtained *in vitro* when a related pentapeptide (7140) was derivatized at the N-terminal with dipalmitoylphosphatidylethanolamine-succinic acid (7172). Tritium-labeled 7194 and 7196 were prepared and injected intravenously into rats at 3 μmol/kg; then the plasma was assayed for native compound and metabolites by HPLC radioactivity flow detection. The peak plasma level of the tritium-labeled lipid prodrug (7196) was 36 μM versus 1.6 μM for the free protease inhibitor pentapeptide (7194). The area under the curve of the phospholipid prodrug (7196) was 48-fold greater and its mean residence time was increased 43-fold versus the free peptide (7194). Phospholipid prodrugs appear to offer an alternative approach to optimizing *in vivo* performance of HIV protease inhibitors and other small peptides.

Key words: antiviral agents; HIV; HIV protease inhibitors; prodrugs; phospholipids

Antiretroviral therapy for AIDS†† has progressed in the past 6 years to the point that AZT and other dideoxynucleosides have been found to alter favorably the natural history of the disease, prolonging survival, slowing progression of asymptomatic infection to active disease, and improving quality of life [1]. However, resistance to AZT has been demonstrated in 90% of patients with AIDS after a year of therapy [1]. Combination therapies with AZT, dideoxycytidine and dideoxyinosine are being investigated as alternative ways to eliminate or reduce viral resistance. Clearly, therapeutic approaches targeted to other steps in the viral life cycle will be required to control HIV infection more effectively. Late events in the viral life cycle are especially interesting because of the need to limit the production of infectious virus by chronically HIV-infected cells and macrophages that may produce virus for prolonged periods of time.

HIV produces long *gag* and *gag-pol* polyproteins that are cleaved during viral budding into smaller proteins, which have various viral functions [2]. This proteolytic cleavage is catalyzed by a specific HIV aspartyl protease, and cleavage must occur for production of infectious virions [3]. Small peptide fragments, which conformationally resemble the protease binding site but which have the p1 site replaced by non-hydrolyzable structures, act as inhibitors of the HIV protease.

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†† Abbreviations: AZT, zidovudine; AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; DPPE, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine; DPPE-Suc, 1,2-dipalmitoyl-sn-glycero-3-phospho-*O*-(*N*-succinyl)-ethanolamine; DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; Peptides: 7140 = iBOC-[D-Phe]-[D-α-Nal]-Pip-[α-(OH)-Leu]-Val-amide; 7172 = dipalmitoylphosphatidyl-succinyl-[D-Phe]-[D-α-Nal]-Pip-[α-(OH)-Leu]-Val-amide; 7194 = iBOC-[L-Phe]-[D-β-Nal]-Pip-[α-(OH)-Leu]-Val; and 7196 = iBOC-[L-Phe]-[D-β-Nal]-Pip-[α-(OH)-Leu]-Val-dipalmitoylphosphatidylethanolamine; Pip, 4-piperidinecarboxylic acid; and Nal, naphthylalanine.

Small peptidomimetic inhibitors of the HIV protease are being developed as AIDS therapeutics [4–9; reviewed in 10–14], but some of these agents have important drawbacks as drug candidates. These include short half-life, low plasma levels (due to rapid biliary and renal excretion), poor cell membrane penetration and low oral bioavailability. Nevertheless, HIV protease inhibitors are particularly promising because they can inhibit production of infectious HIV virions in chronically infected cells in contrast to dideoxynucleosides. Dideoxynucleosides can only prevent new infection of cells by blocking HIV reverse transcription.

To attempt to overcome some of these generic problems, we synthesized C-terminal and N-terminal phospholipid prodrugs of HIV protease inhibitor peptides to evaluate their *in vitro* activity, plasma half-life, mean residence time, and area under the curve.

MATERIALS AND METHODS

Peptides. The protease inhibitor peptides 7140 (iBOC-[D-Phe]-[D-α-Nal]-Pip-[α-(OH)-Leu]-Val-amide) and 7194 (iBOC-[L-Phe]-[D-β-Nal]-[α-(OH)Leu]-Val) were synthesized on a Beckman 990B Peptide Synthesizer (Beckman Instruments, Palo Alto, CA) using *t*-butyloxycarbonyl-protected amino acids by the Merrifield solid phase method [15]. The peptides containing a carboxamide function at the C-terminus were prepared on 4-methyl-benzhydralamine resin [16], and the peptides with a free carboxyl group at the C-terminus were synthesized on tBoc-Val-phenylacetamidomethyl resin obtained from Applied Biosystems Inc. (Foster City, CA). The peptide-resins were then cleaved by reaction with anhydrous hydrogen fluoride for 45 min at −10° in an HF apparatus from Peninsula Laboratories, Inc. (Belmont, CA). Following evaporation of the HF, the peptides were extracted from the resin with dioxane. The resulting crude peptides were purified to >95% by preparative reverse-phase HPLC using a Vydac (Hesperia, CA) C-4 column and a buffer gradient of 0.1% TFA in 100% water to 0.1% TFA in 100% acetonitrile. Peptide purity was determined by analytical HPLC, fast atom bombardment mass spectrometry, and amino acid analysis.

DPPE-succinic acid. The lipid-linker material DPPE-Suc was prepared by reacting one equivalent of DPPE (Avanti Polar Lipids, Birmingham, AL) and one equivalent of triethylamine with three equivalents of succinic anhydride (Sigma Chemical Co., St. Louis, MO) in chloroform. The reaction was stirred at room temperature under a dry nitrogen atmosphere for 16 hr. Upon completion of the reaction, the product material was purified by silica gel chromatography using a solvent gradient of 100% chloroform to 15% methanol in chloroform.

Lipid-peptide derivatives. The N-terminal lipid-derivatized protease inhibitor was prepared by reacting one equivalent of DPPE-Suc in DMF with one equivalent of 7140 peptide containing a free amine function at the N-terminus and three equivalents of DCC in dichloromethane. The reaction was stirred for 24 hr at room temperature under dry conditions, after which time, the product (7172) was purified by silica gel chromatography using a solvent gradient of increasing methanol in chloroform.

The C-terminally-derivatized inhibitor 7196 was prepared by reacting one equivalent of DPPE-tributylamine salt in chloroform with one equivalent of 7194 peptide in DMF with two equivalents each of DCC and 1-hydroxybenzotriazole. The reaction was stirred for 16 hr at room temperature under dry conditions, and the target compound was then purified by preparative thin-layer chromatography using a solvent system of chloroform:methanol:acetic acid (85:10:5 by vol.) on 1000 μm 20 × 20 cm silica gel GF plates (Analtech Inc., Newark, DE).

The purified lipid derivatives were subsequently characterized by HPLC, amino acid analysis, fast atom bombardment mass spectrometry and gas chromatography fatty acid analysis; the results confirmed the expected structure.

HT4-6C cells and HIV plaque reduction assay. HeLa cells expressing CD4 (HT4-6C cells) were obtained from Bruce Chesebro, Hamilton, MT. The effect of antiviral compounds on HIV replication was measured by a plaque reduction assay described by Larder *et al.* [17]. Briefly, monolayers of HT4-6C cells were infected with 100–300 PFU of HIV-1_{BRU} virus per well in 24-well microdilution plates. Various concentrations of drug were added to the culture medium, Dulbecco's Modified Eagle's Medium containing 5% fetal bovine serum and antibiotics. After 3 days at 37°, the monolayers were fixed with 10% formaldehyde solution in PBS and stained with 0.25% crystal violet to visualize syncytial foci (plaques). Antiviral activity was assessed as the percentage of control plaques measured in drug-treated samples.

Radiolabeled compounds. The protease inhibitor peptide 7194 was prepared as described above, but with L-phenylalanine replaced with L-(4-bromo)-phenylalanine (Bachem Bioscience Inc., Philadelphia, PA). This peptide was coupled subsequently with dipalmitoylphosphatidylethanolamine as described above for 7196, and both peptide and lipid derivative were subjected to radiolabeling by reductive dehalogenation with tritium gas (Moravek Biochemicals, Brea, CA). The tritiated 7194 and 7196 (sp. act. 11 and 14 Ci/mmol, respectively) were employed in the pharmacokinetics studies in rats.

Formulation for intravenous pharmacokinetics experiment. The ^{3}H-labeled protease inhibitors, 7194 and 7196, were formulated for 3.0 μmol/kg dosing in the pharmacokinetics study. Unlabeled compounds first were dissolved in Tween 20 while mixing at 37°, followed by addition of the radiolabeled compounds in ethanol. Finally, isotonic sorbitol was mixed slowly into the [^{3}H]peptide solutions while vortexing at 37° to yield a final concentration for both compounds of 1.2 μmol/mL in a final formulation of 10% ethanol:10% Tween 20:80% isotonic sorbitol. The final specific activities were 0.081 Ci/mmol for [^{3}H]7194 and 0.145 Ci/mmol for [^{3}H]7196.

Intravenous administration to rats. Male rats of the Sprague–Dawley strain, weighing about 200 g, were fasted overnight and injected intravenously with 3 μmol/kg of either ^{3}H-labeled 7194 or 7196.

At 5, 10 and 30 min and 1, 4, 12 and 24 hr following the dose, three rats were anaesthetized with ketamine:xylazine (7:1, v/v) and euthanized by cardiac puncture. The blood was collected in sterile EDTA tubes and centrifuged at 4° to obtain plasma, which was stored frozen at −18° in 1.0-mL aliquots until assay.

HPLC assay in plasma of 7194 *and* 7196. An HPLC assay was developed for 7194, 7196 and phenylalanine. Plasma samples were prepared by centrifugation at 10,000 *g* prior to injection on HPLC. Two SSI high pressure column prefilters (Alltech, Deerfield, IL) and a 10 × 4.6 mm C4 guard cartridge (Alltech) were placed between the injector and the analytical column to remove any additional contaminants or precipitates. The filters were back-flushed at the end of each day and replaced when excessive back pressure was noted. In addition, the guard cartridge was replaced after 30 injections, and a reverse-phase standard solution (RP-MIX-D, Alltech) was analyzed before and after each series of samples (<10 injections) to assure consistent, reproducible performance of the analytical column. Separations were done on a Beckman model 344 HPLC using a 5 μm Macrosphere 300, C4 column (Alltech). Mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. The flow rate was 1 mL/min. A gradient from 50–100% B in 5 min was started 2 min after the injection of 5–50 μL of plasma and held for 13 min. Detection was with a Radiomatic Flo-1-Beta radioactivity flow detector model A-100 (Packard Instrument Co., Meriden, CT) using a 750-μL flow cell with a final flow (HPLC + scintillation fluid) of 5 mL/min. The retention times for 7194, 7196 and phenylalanine were 8.60, 12.60 and 4.40 min, respectively.

HPLC recovery of 7196 was determined by injecting 25 μL of a spiked plasma sample and comparing the counts recovered from the HPLC radiodetector with those attained by direct liquid scintillation counting of 25 μL from the same spiked sample. The recovery was 99.6%. Recovery of metabolites of 7196 from HPLC was achieved in the same way by counting and injecting an aliquot of the same plasma sample from an actual analysis. Recovery was 100%. Minimum detectable levels achieved were 17.2 pmol for 7196 and 3.1 pmol for 7194. The minimum quantifiable level (signal/noise >4) for 7196 was 186 pmol (10-μL injections = 18.6 nmol/mL) and for 7194 55 pmol (25-μL injections = 2.2 nmol/mL).

Data analysis. The raw data from the plasma determinations of [^{3}H]7194 and [^{3}H]7196 were subjected to regression analysis. The regressions for polyexponential curve fits of the data were performed using RStrip statistical software (MicroMath Scientific Software, Salt Lake City, UT).

RESULTS

The pentapeptide 7140 inhibited HIV-1 plaque formation in HT4-6C cells *in vitro* (Fig. 1). A similar degree of inhibition was noted with DPPE-succinate linked to the N-terminal phenylalanine of the 7140 pentapeptide (7172). The concentrations required to reduce plaque formation by 50% (IC_{50}) were

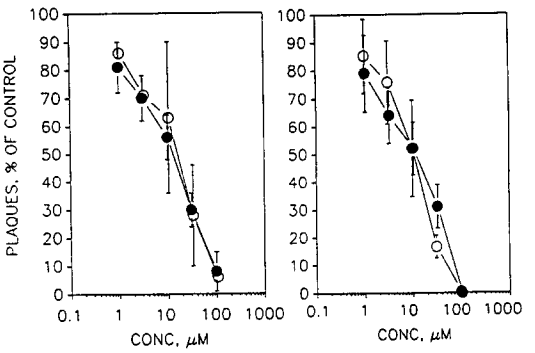

Fig. 1. N-terminal and C-terminal derivatization of HIV protease inhibitor peptides. Left panel: Effects of derivatization of 7140 peptide N-terminus with phosphatidylethanolamine succinic acid (7172) on plaque formation by HIV-infected HT4-6C cells. Key: (●) 7140; and (○) 7172. Right panel: Effect of an HIV protease inhibitor peptide (7194) and its C-terminal phosphatidylethanolamine conjugate (7196) on plaque formation in HIV-infected HT4-6C cells. Key: (●) 7194; and (○) 7196. Data are means ± SD, N = 3–6. Control plaque numbers ranged from 80 to 120/well.

Table 1. Inhibition of plaque formation in HIV-1 infected HT4-6C cells by free, N-terminal- or C-terminal phospholipid-conjugated HIV protease inhibitor peptides

Peptide*	Type of compound†	IC_{50} (μM)
7140	Free peptide	14.0 ± 4.6 (6)
7172	N-terminal DDPE-succinate-	15.3 ± 8.1 (3)
7194	Free peptide	11.5 ± 6.2 (5)
7196	C-terminal DPPE-	9.6 ± 4.6 (5)

Values are means ± SD; numbers in parentheses are the number of replicate experiments. IC_{50} is the drug concentration that reduces plaque number by 50%.

* Peptides: 7140 = iBOC-[D-Phe]-[D-α-Nal]-Pip-[α-(OH)-Leu]-Val-amide; 7172 = dipalmitoylphosphatidyl-succinyl-[D-Phe]-[D-α-Nal]-Pip-[α-(OH)-Leu]-Val-amide; 7194 = iBOC-[L-Phe]-[D-β-Nal]-Pip-[α-(OH)-Leu]-Val; and 7196 = iBOC-[L-Phe]-[D-β-Nal]-Pip-[α-(OH)-Leu]-Val-dipalmitoylphosphatidylethanolamine.

† Abbreviation: DPPE, dipalmitoylphosphatidylethanolamine.

14.0 μM for 7140 versus 15.3 μM for 7172. The differences were not statistically significant (Table 1).

The free pentapeptide 7194 inhibited HIV-1 plaque formation in HT4-6C cells by 50% at 13.4 μM (Fig. 1). The C-terminally-derivatized pentapeptide 7196 was equally active with an IC_{50} of 12.7 μM. Thus, derivatization at the C-terminal does not cause a statistically significant reduction in the antiviral activity of the peptide (Table 1). 7194 and 7196 were also active in HT4-6C cells infected with several of the clinical isolates of HIV, which are AZT-resistant [16], as would be expected (data not shown).

The radiolabeled protease inhibitors 7194 and

Table 2. Summary of pharmacokinetic parameters for compounds 7194 and 7196*

Pharmacokinetic parameters†	[^{3}H]7194	[^{3}H]7196
Dose (μmol)	0.640	0.685
A (nmol/mL)	7.033	0.368
k_α (hr^{-1})	17.498	0.094
B (nmol/mL)	—	35.535
k_β (hr^{-1})	—	2.317
$T_{1/2\alpha}$ (hr)	0.040	0.299
$T_{1/2\beta}$ (hr)	—	7.402
AUC ($nmol \cdot hr \cdot mL^{-1}$)	0.402	19.261
Cl (mL/hr)	1.592	0.036
V_c (L)	0.091	0.019
V_β (L)	0.091	0.380
V_{SS} (L)	0.091	0.090
MRT (hr)	0.057	2.521

* Best fits were monoexponential for [^{3}H]7194 and biexponential for [^{3}H]7196.

† Parameter abbreviations: A and B are the weighting factors, the sum of which equals the t = 0 plasma levels; k_α and k_β are the early and late phase elimination rate constants. $T_{1/2\alpha}$ and $T_{1/2\beta}$ are the corresponding early and late phase half-lives; AUC is the area under the curve ($\int$Cdt); Cl is the total body clearance; V_c, V_β and V_{SS} are, respectively, the apparent volume of distribution for the central compartment, the terminal apparent volume of distribution, and the apparent volume of distribution at steady state; and MRT is the mean residence time, the mean time a molecule of the drug remains in the plasma.

7196 were given intravenously to rats at a dose of 3.0 μmol/kg (equivalent to 1.6 mg/kg of the free peptide 7194). The peak level of free [^{3}H]peptide (7194) 5 min after administration was 1.6 μM versus 36 μM for the phospholipid conjugate (7196); after 30 min, intact 7194 was undetectable while 7196 was detected for 12 hr (Fig. 2). The free peptide was removed much more rapidly from plasma with clearance of 1.59 mL/hr versus 0.036 mL/hr for the lipid conjugate. The free peptide left the plasma in a monoexponential decay with an apparent $T_{1/2\alpha}$ of 0.040 hr versus biexponential decay observed with the lipid prodrug which had a $T_{1/2\alpha}$ of 0.299 hr and a $T_{1/2\beta}$ of 7.40 hr (Table 2).

In terms of total exposure, the AUC($0 \rightarrow \infty$) for the lipid prodrug was 19.2 $nmol \cdot hr \cdot mL^{-1}$ versus 0.4 $nmol \cdot hr \cdot mL^{-1}$ for the free peptide. The plasma mean residence time for the lipid prodrug (7196) was markedly longer (2.52 hr) than for the free peptide (0.057 hr). The lipid prodrug was converted to a major metabolite *in vivo* in an apparent precursor–product relationship; the identity of the compound is unknown (retention time 10.6 min). After an accumulation phase (peaking at 16.5 min), the unknown metabolite exhibited a biexponential decline. The unknown appears to have both of the lipid prodrug fatty acid chains because hydrolysis of 7196 to its monoacylated analog by phospholipase A_2 treatment resulted in the formation of a compound with a retention time of 4.9 min, shorter than that of the unknown compound (10.6 min). Several other minor metabolites were also noted. However, in plasma, conversion of 7196 to 7194 was not detected. Comparison of the plasma profiles of the intact drug versus total plasma tritium for each compound (Fig. 2) indicates that 7194 was rapidly metabolized to [^{3}H]phenylalanine, whereas 7196 underwent substantially less metabolism *in vivo*.

These results appear to represent an important proof of principle indicating that a phospholipid-conjugated HIV protease has a much longer plasma half-life, mean residence time and greater AUC and provides greater potential exposure of HIV-infected tissues to drug.

DISCUSSION

We found that C-terminal or N-terminal derivatization of a pentapeptide HIV protease inhibitor with phosphatidylethanolamine or phosphatidylethanolamine-succinate did not appear to interfere with the *in vitro* activity of the inhibitor compounds. These results are surprising in view of the presence of the bulky lipophilic groups, which would likely hinder the precise interactions required for the inhibitor peptide to bind to the catalytic site of HIV protease. A possible sequence of events leading to inhibition of HIV replication by the 7196 lipid prodrug would be binding of 7196 to the cell by insertion of the lipid chains into the cell membrane and entry of the lipid prodrug into the cell during endocytosis and other membrane cycling events. Subsequently, cellular phospholipases A and/or C and phosphodiesterases may catalyze breakdown of the lipid portion of the molecule, freeing the inhibitor peptide to interact with the protease. Alternatively, the lipid conjugated protease inhibitors might inhibit the HIV protease while still attached to the plasma membrane where viral assembly and budding are occurring. We showed previously that a liponucleotide prodrug, phosphatidyl-AZT, was degraded in CEM cells by phospholipase A and lysophospholipase to glycero-3-phospho-5′-AZT, which was converted subsequently to AZT or AZT-monophosphate by cellular phosphodiesterases [18]. Further studies will be required to establish the mechanisms involved in the antiviral action of phospholipid prodrug inhibitors of the HIV protease.

The pharmacokinetic results indicated a greater plasma AUC value and slower removal from the circulation for the phospholipid derivative relative to the free peptide. This suggests that the phospholipid derivatives may have therapeutic advantages by providing higher levels of material in the circulation available for potential uptake by infected tissues. Thus, phospholipid derivatives of HIV protease inhibitors may produce advantageous alterations of *in vivo* biodistribution.

The use of metabolizable phospholipid groups to reduce rapid elimination of the HIV protease inhibitor represents a new approach to optimizing tissue exposure to this class of drugs. The approach may be generally applicable to protease inhibitors provided that appropriate linking functionalities are present.

The protease inhibitor peptides utilized in this demonstration are weak inhibitors of HIV replication *in vitro*, 100 to 1000 times less potent than compounds

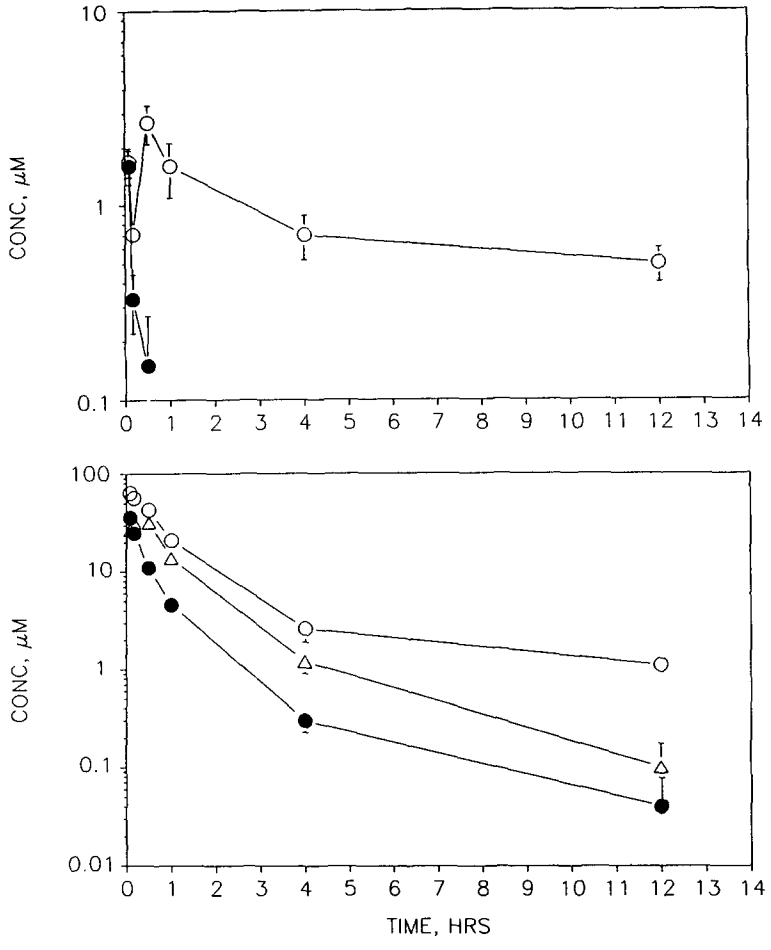

Fig. 2. Effect of C-terminal phosphatidylethanolamine conjugation on the plasma levels of [^{3}H]7194 (upper panel) and [^{3}H]7196 (lower panel) after intravenous administration to rats. Key: (●) intact drug (7194 or 7196); (○) total tritium; and (△) metabolite of 7196. Data are means ± SD, N = 3.

currently under investigation elsewhere [4–14]. They were designed to assess the effect of phospholipid derivatization on antiviral activity and pharmacokinetics. Future studies should utilize more potent HIV protease inhibitors that have isosteric residues not subject to hydrolysis by proteases [4–14]. Although the present compound requires parenteral administration, it is conceivable that functional groups may be identified which can promote or enhance absorption of orally administered compounds of this class.

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