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Lipid prodrugs of phosphonoacids: greatly enhanced antiviral activity of 1-*O*-octadecyl-*sn*-glycero-3-phosphonoformate in HIV-1, HSV-1 and HCMV-infected cells, in vitro

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Abstract

Phosphonoformate (PFA) effectively inhibits viral polymerases but is relatively ineffective in virus-infected cells in tissue culture. A lipid prodrug of phosphonoformate was synthesized by coupling the phosphonate residue of phosphonoformate to the *sn*-3 hydroxyl of 1-*O*-octadecyl-*sn*-glycerol. This prodrug, 1-*O*-octadecyl-*sn*-glycero-3-phosphonoformate (ODG-PFA), was 93-fold more active than phosphonoformate in cells infected with the AD169 strain of cytomegalovirus (CMV), and 111–147-fold more active in cells infected with three human clinical isolates of CMV. The compound was also 44-fold more active in human immunodeficiency virus-1 (HIV-1) infected cells and 43-fold more active in cells infected with herpes simplex virus (HSV). Studies of the mechanisms of increased antiviral activity indicate that 1-*O*-octadecyl-*sn*-glycero-3-[^{14}C]phosphonoformate is taken up more extensively than the free drug by the host MRC-5 human lung fibroblasts. Intracellular enzymes convert 1-*O*-octadecyl-*sn*-glycero-3-phosphonoformate to phosphonoformate. This conversion does not occur in the tissue culture medium containing fetal bovine serum (FBS) or in MRC-5-conditioned medium. In view of its greatly increased in vitro potency and selectivity, 1-*O*-octadecyl-*sn*-glycero-3-phosphonoformate may be useful in treating viral diseases.

Keywords: Phosphonoacids; ODG-PFA; ODG-PAA; Antiviral activity

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1. Introduction

The phosphonoacids, phosphonoacetate (PAA) and phosphonoformate (PFA, foscarnet), were first synthesized in 1924 (Nylén, 1924). Subsequently, these compounds were found to inhibit a variety of viral polymerases including those of herpes simplex virus (HSV) (Helgstrand et al., 1978; Reno et al., 1978), cytomegalovirus (CMV) (Huang, 1975; Overall et al., 1976) and retroviruses (Sundquist and Öberg, 1979) including the human immunodeficiency virus (HIV-1) (Sandstrom et al., 1985). Although foscarnet is very active as an inhibitor of viral polymerases, much higher concentrations of the drug are required to inhibit viral replication in virus-infected cells in vitro. For example, the concentration of foscarnet required to inhibit the activity of the HCMV DNA polymerase by 50% is 0.3 μM while the concentration of foscarnet required to inhibit replication of HCMV in infected cells ranges from published values of 25 to 8000 mM (reviewed in Öberg (1983) and Chrisp and Clissold (1991)). Foscarnet is approved for treating cytomegalovirus retinitis in AIDS but must be given intravenously and has significant toxicities (Chrisp and Clissold, 1991).

It would be useful to identify prodrugs of foscarnet with increased antiviral activity, acceptable toxicity and oral bioavailabity. To evaluate lipid analogs of foscarnet and phosphonoacetate, we synthesized the 1-*O*-octadecyl-*sn*-glycerol (batyl alcohol) analogs of foscarnet and phosphonoacetate and evaluated their antiviral activity and cytotoxicity in human cells infected with HIV-1, HSV-1 and HCMV. To evaluate mechanism of action, we synthesized 1-*O*-octadecyl-*sn*-glycero-3-[^{14}C]PFA and compared its cellular uptake and conversion to PFA with the cellular uptake of [^{14}C]PFA in vitro.

2. Methods

2.1. Analytical

Thin layer chromatography (TLC) was done with Analtech Silica Gel GF 250 micron plates. Developing solvents used were chloroform/methanol/water/conc. NH_4OH in volume ratios as follows: Solvent A: 85:15:0.25:0.25; Solvent B: 80:20:1:1; Solvent C: 64:36:5:5; Solvent D: 70:58:8:8. Proton NMR spectra, recorded on a 300-MHz Nicolet spectrometer, were consistent with structure for all compounds. Combustion analyses were performed for certain selected compounds by Oneida Research Services, Inc., Whitesboro, NY.

2.2. Synthesis of 1-O-octadecyl-sn-glycero-3-phosphonoformate (ODG-PFA)

2.2.1. Synthesis of 1-O-octadecyl-2-O-benzyl-sn-glycero-3-phosphonoformate ethyl ester

Ethyl (dichlorophosphonyl)formate (Vaghefi et al., 1986) was synthesized by chlorination of bis(trimethylsilyl) ethylphosphonoformate (Vaghefi et al., 1986) with PCl_5, according to the method of Morita et al. (1980). A solution of 1-*O*-octadecyl-2-*O*-benzyl-*sn*-glycerol (9.9 g, 22.8 mmol, Bachem Bioscience, King of Prussia, PA) in dry pyridine (20 ml) and $CHCl_3$ (25 ml) was added dropwise to a cold (0°C) mixture of ethyl (dichlorophosphonyl)formate (7.0 g, 36 mmol) in $CHCl_3$ (25 ml). After 3 h the reaction mixture was allowed to warm to room temperature, where it was kept for an additional 12 h after which it was concentrated and the residue flash chromatographed on silica gel ($CHCl_3$/MeOH, 9:1), yielding 1-*O*-octadecyl-2-*O*-benzyl-*sn*-glycero-3-phosphonoformate ethyl ester (5.08 g, 47%).

2.2.2. 1-O-octadecyl-sn-glycero-3-phosphonoformate ethyl ester

1-*O*-Octadecyl-2-*O*-benzyl-*sn*-glycero-3-phosphonoformate ethyl ester (5.08 g, 8.9 mmol) was dissolved in ethyl acetate (2.5 ml) and 5% Pd/C (50 mg) was added. A hydrogen-filled balloon was attached to the flask and the mixture was stirred for 7 h at room temperature. After removing the catalyst by filtration, the solvent was evaporated in vacuo and the crude product purified by flash column chromatography using silica gel ($CHCl_3$/

MeOH, 9:1). Fractions containing product were combined and concentrated to afford 2.59 g (61%) of a white solid. $R_F = 0.29$ (solvent B).

2.2.3. 1-Octadecyl-sn-glycero-3-phosphonoformate

To a solution of 1-*O*-octadecyl-*sn*-glycero-3-phosphonoformate ethyl ester (160 mg, 0.325 mmol) in ethanol (0.5 ml) was added ethanolic sodium hydroxide (0.1 N NaOH/ethanol 1:1, 0.5 ml). The mixture was sonicated for 5 min to solubilization. The flask and its contents were placed in an oil bath preheated to 65–70°C. After 30 min the flask was allowed to cool to room temperature and placed in ice-water for 30 min. The precipitate was collected by centrifugation and the pellet washed with cold ethanol (3 × 2 ml). The residual solvent was removed and the residue dissolved in water and lyophilized to afford 60 mg (38%) of ODG-PFA, sodium as a white solid. $R_F = 0.39$ (solvent C). CHN analysis calculated for $C_{22}H_{43}O_7P \cdot Na_2 1.5H_2O$: %C 50.47; %H 8.86. Found: %C 50.15; %H 8.62.

2.3. Synthesis of 1-O-octadecyl-sn-glycero-3-phosphonoacetate (ODG-PAA)

2.3.1. Phosphonoacetate ethyl ester

A solution of phosphonoacetic acid (14.0 g, 100 mmol) was added to ethanolic HCl (1.0 M, 30 ml) and the resulting solution was kept at 70°C for 6 h, then allowed to stir at room temperature overnight. The mixture was concentrated to dryness in vacuo and the residue was dissolved in chloroform (150 ml). The solution was extracted with water (2 × 50 ml), dried over anhydrous magnesium sulfate and concentrated in vacuo. The resulting ethyl phosphonoacetate, obtained in quantitative yield, was used directly for the next step.

2.3.2. 1-O-Octadecyl-2-O-benzyl-sn-glycero-3-phosphonoacetate ethyl ester

To a solution of ethyl phosphonoacetate (0.268 g, 1.6 mmol) and 1-*O*-octadecyl-2-*O*-benzyl-*sn*-glycerol, (0.653 g 1.5 mmol) in pyridine (6 ml) was added a solution of 1,3-dicyclohexylcarbodiimide (0.93 g, 4.5 mmol) in pyridine (60 ml) and the resulting mixture was stirred at 60°C overnight. The mixture was allowed to cool to room temperature, filtered and concentrated. The crude product was purified by silica gel column chromatography to afford 600 mg (70%) of the product as a single homogeneous spot on TLC. $R_F = 0.31$ (solvent A).

2.3.3. 1-O-Octadecyl-sn-glycero-3-phosphonoacetate ethyl ester

The product from the previous step was dissolved in ethanol (40 ml) and the solution was degassed three times. Catalyst (5% Pd/C, 270 mg) was added all at once and a hydrogen-filled balloon was attached to the flask. Upon completion of the reaction as judged by TLC the catalyst was removed by filtration and the filtrate was concentrated. The crude product was purified by silica gel column chromatography ($CHCl_3$/MeOH, 9:1) to afford 1.0 g (100%) of the product as a white solid. $R_F = 0.2$ (solvent B).

2.3.4. 1-O-Octadecyl-sn-glycero-3-phosphonoacetate

To a solution 1-*O*-octadecyl-*sn*-glycero-3-phosphonoacetate ethyl ester (400 mg, 0.83 mmol) in ethanol (5 ml) was added a solution of 0.1 N NaOH (16.6 ml) and the reaction mixture was placed in a preheated oil bath maintained at 60°C with stirring. After 3 h the reaction mixture was cooled to room temperature, diluted with ethanol and placed in ice-water for 30 min. The solid was collected by centrifugation and purified by column chromatography using silica gel (60 micron, Analtech, Neward, DE) and chloroform/methanol/water/conc. NH_4OH (70:58:8:8) as eluting solvent. The fractions containing the product were combined and concentrated to give 200 mg of 1-*O*-octadecyl-*sn*-glycero-3-phosphonoacetate (ODG-PAA), ammonium as a white solid. $R_F =$ 0.38 (solvent C). Analysis calculated for $C_{23}H_{45}O_7P \cdot 1.5NH_3 \cdot 1.5H_2O$: %C 53.42; %H 10.23; %N 4.06. Found: %C 53.58; %H 10.27; %N 4.05.

2.4. Antiviral assays

2.4.1. HCMV antiviral assay

HCMV (AD169) was obtained from American Type Culture Collection, Rockville, MD. Human clinical isolates of CMV obtained form biopsies of gastric, esophageal or rectal mucosa from AIDS patients (X505, X706 and X755) were provided by Dr. Douglas Richman and assayed for drug sensitivity as noted above. Antiviral assays for HCMV DNA were carried out by DNA hybridization as reported by Dankner et al. (1990). Briefly, subconfluent MRC-5 cells in 24-well culture dishes were pretreated for 24 h with various concentrations of drug in Eagle's minimun essential medium (E-MEM) containing 2% FBS and antibiotics. The medium was removed and HCMV strains added at a dilution that will result in a 3–4+ cytopathic effect (CPE) in the no-drug wells in 5 days. The virus was absorbed for 1 h at 37°C, aspirated and replaced with the drug dilutions. After 5 days of incubation HCMV DNA was quantified in triplicate by nucleic acid hybridization using a CMV Antiviral Susceptibility Test Kit from Diagnostic Hybrids, Inc. (Athens, OH). The medium was removed and cells lysed according to the manufacturer's instructions. After absorption of the lysate, the Hybriwix™ filters were hybridized overnight at 60°C. The Hybriwix™ were washed for 30 min at 73°C and counted in a gamma counter. The results are expressed as a percentage of the untreated HCMV-infected control cells.

2.4.2. HSV-1 antiviral assay

Subconfluent MRC-5 cells in 24-well culture dishes were inoculated by removing the medium and adding HSV-1 virus at a dilution that will result in a 3–4+ CPE in the no-drug well in 20–24 h. This was absorbed for 1 h at 37°C, aspirated and replaced with various concentrations of drugs in E-MEM containing 2% FBS and antibiotics. After approximately 24 h of incubation, HSV DNA was quantified in triplicate by nucleic acid hybridization using a HSV Antiviral Susceptibility Test Kit from Diagnostic Hybrids, Inc. (Athens, OH). The medium was removed and cells lysed according to the manufacturer's instructions. After absorption of the lysate, the Hybriwix™ filters were hybridized overnight at 60°C. The Hybriwix™ were washed for 30 min at 73°C and counted in a gamma counter. The results are expressed as a percentage of the untreated HSV-infected control cells.

2.4.3. HIV-1 antiviral assay

CD4-expressing HeLa 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 (Larder et al., 1990). Briefly, monolayers of HT4-6C cells were infected with 100–300 plaque forming units (PFU) of virus per well in 24-well microdilution plates. Various concentrations of drug were added to the culture medium, Dulbecco's modified Eagle medium containing 5% FBS and antibiotics, as noted above. After 3 days at 37°C, the monolayers were fixed with 10% formaldehyde solution in phosphate-buffered saline (PBS) and stained with 0.25% crystal violet to visualize virus plaques. Antiviral activity was assessed as the percentage of control plaques measured in drug-treated samples.

2.5. Cytotoxicity of test compounds in vitro

MRC-5 cells: Subconfluent human lung fibroblast cells (MRC-5, American Type Culture Collection, Rockville, MD) in 24-well plates were treated with drugs diluted in E-MEM (Gibco BRL, Grand Island, NY) supplemented with 2% fetal bovine serum and antibiotics. After 5 days of incubation at 37°C, the cell monolayer was visually inspected under magnification and the concentration of drug which caused a 50% reduction in cell number was estimated.

CEM cells: Human T-lymphoblast cells (CEM, American Type Culture Collection) in 96-well plates were treated with drugs diluted in RPMI-1640 medium plus 10% fetal bovine serum and antibiotics and incubated for 3 days at 37°C. An aliquot was removed to determine the cell number with a Coulter counter (Coulter Corp., Hialeah, FL) and the remaining cells were removed to determine the viability by flow cytometry. Twenty ml of a 50 mg/ml propidium iodide solution was

added to the cells and analyzed after 5 min in a Coulter Elite flow cytometer (Dangl et al., 1982).

2.6. Synthesis of 1-O-octadecyl-sn-glycero-[^{14}C]phosphonoformate

Ethoxy[^{14}C]carbonylphosphodichloridate (New England Nuclear, Boston, MA, specific activity 30 μCi/mmol), 167 mmol in 0.5 ml chloroform, was hydrolysed by dropwise addition into 1.1 ml of ice-cold pyridine/water (9:1). After 1 h, 0.1 ml of water was added and the resulting phosphonoformic acid ethyl ester was dried under a nitrogen stream. Coupling to 1-*O*-octadecyl-*sn*-glycerol was done by the addition of 50 mg dicyclohexylcarbodiimide in 0.5 ml chloroform to 2 ml of dry pyridine containing 167 μmol of phosphono[^{14}C]formic ethyl ester and 200 μmol of 1-*O*-octadecyl-*sn*-glycerol. 1-*O*-Octadecyl-*sn*-glycero-[^{14}C]phosphonoformate ethyl ester was purified by application to a 10-g silica column and elution with C/M/NH_3/W (80:20:1:1). The radioactive fractions were analyzed by HPLC and TLC and pure fractions were pooled for deblocking. 1-*O*-Octadecyl-*sn*-glycero[^{14}C]phosphonoformate (sodium) was obtained by the addition of 2.2 molar equivalents (meq) of NaOH to the ethyl ester in ethanol. This step was monitored by TLC until reaction was completed. ODG-[^{14}C]PFA was purified by three washes with 1 ml ethanol followed by purification on a small (1 g) silica column in which the sample was loaded in Solvent B and eluted with Solvent D. Column fractions were monitored for radioactivity by HPLC and TLC. The pure fractions were pooled and the sodium salt reformed by the addition of 2.2 meq NaOH followed by washing with ethanol to remove excess NaOH. The final product was dissolved in chloroform/methanol/water (2:3:1) and stored at -20°C.

2.7. Metabolism of 1-O-octadecyl-sn-glycero-3-[^{14}C]PFA in MRC-5 human lung fibroblasts

2.7.1. Cellular uptake of [^{14}C]-labeled PFA and ODG-PFA

The culture medium used for cell uptake and metabolism studies was MEM containing 2% fetal bovine serum and drug. Since alkylglycerol conjugates of drugs are generally not soluble in water, for convenience, we generally exposed virus-infected cells to drugs incorporated into liposomes. Alternatively, drug was added in DMSO to a final DMSO concentration of 1%. [^{14}C]PFA or 1-*O*-octadecyl-*sn*-glycero-3-[^{14}C]PFA were formulated in liposomes containing DOPC/DOPG/cholesterol/ODG-PFA in a molar ratio of 50:10:30:10. The liposome preparations in the culture medium were filtered through a sterile 0.45 μm filter before use. Tissue culture medium containing 1 μM [^{14}C]PFA (specific activity 115 DPM/pmol) or 1 μM 1-*O*-octadecyl-*sn*-glycero-3-[^{14}C]PFA (specific activity 66 DPM/pmol) were added to MRC-5 cells grown to 90% confluence in 24-well plates. At the indicated time points the medium was removed and discarded. The cells were washed with ice-cold PBS and 0.2 ml of 0.5M NaOH was added to lyse cells. The cell monolayers in all wells were scraped and the lysates transferred to scintillation vials to which 10 ml of Ecolite (ICN Biochemicals) was added before counting.

2.7.2. Metabolism of ODG-[^{14}C]PFA

Metabolism experiments were done in T-75 flasks containing MRC-5 cells grown to near confluence in tissue culture medium containing 1 mM ODG-[^{14}C]PFA as noted above. After 24 h the medium was removed and the cell monolayer was washed with 2 ml of cold PBS. Two ml of water was added and the flask was frozen and thawed twice followed by a 5-min sonication in a bath sonicator to lyse cells. The flasks were scraped and 0.05 ml of the lysate containing cell debris was counted in triplicate as noted above. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. One ml of the remaining cell lysate was extracted by the method of Folch et al. (1957) and the lower phase was dried and spotted at the origin of a silica gel G-plate and developed with Solvent D to evaluate the presence of ^{14}C-labeled native drug and metabolites.

In other experiments, a control 24-well plate containing complete medium and ^{14}C-drug was

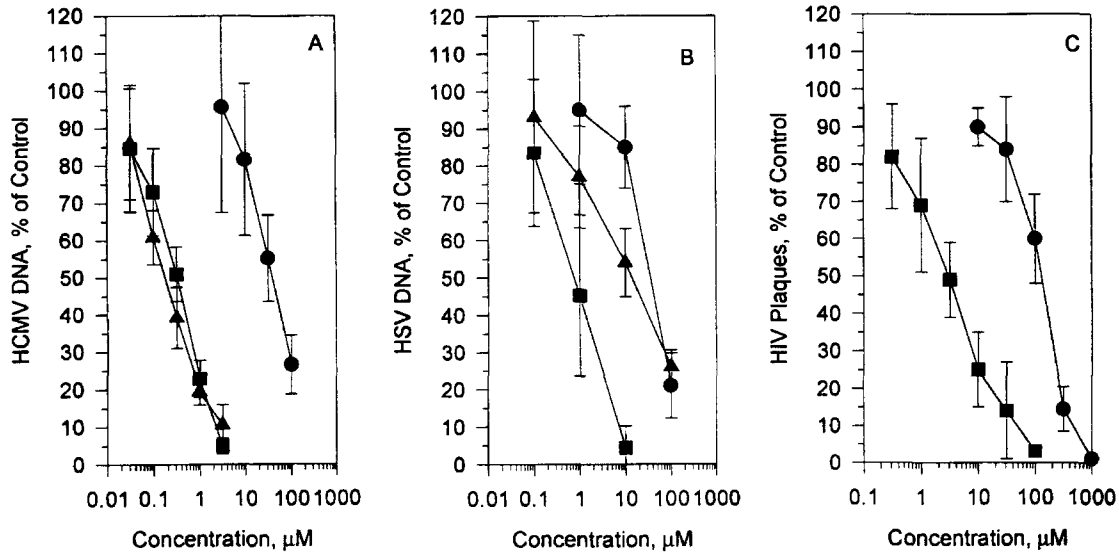

Fig. 1. Antiviral activity of phosphonoacid prodrugs and foscarnet in vitro. Panel A, HCMV DNA retention assay in MRC-5 human lung fibroblasts; Panel B, HSV-1 DNA reduction assay in MRC-5 human lung fibroblasts; Panel C, HIV-1 plaque reduction assay in HT4-6C cells. ●, PFA; ■, 1-*O*-octadecyl-*sn*-glycero-3-PFA; ▲, 1-*O*-octadecyl-*sn*-glycero-3-PAA.

analyzed at time zero. Additional control plates without cells but containing 1 μM ODG-[^{14}C] PFA and complete tissue culture medium were incubated for 1 and 5 days and the medium was analyzed by spotting at the origin of a silica gel plate for TLC as noted above. Similar experiments were done using conditioned medium obtained after 4 days of incubation with MRC-5 cells. The plate was scanned and the percentage of ^{14}C-label in ODG-[^{14}C]PFA and [^{14}C]PFA was determined.

3. Results

The effects of ODG-PFA and ODG-PAA on HCMV replication were evaluated in MRC-5 human lung fibroblasts infected with the AD169 strain of HCMV (Fig. 1A). ODG-PFA and ODG-PAA were both substantially more active than PFA in inhibiting HCMV DNA production by MRC-5 cells. The IC_{50} values for ODG-PFA and ODG-PAA in liposome-formulation were 0.43 μM and 0.2 μM versus 45 μM for free PFA, representing increases in antiviral activity of 93-fold and 182-fold, respectively (Table 1). Identical results were obtained when ODG-PFA and ODG-PAA were added to the cell monolayer directly dissolved in 1% DMSO, indicating that liposomes play no essential role in the antiviral activity. Furthermore, lipid controls consisting of blank liposomes without drug had no effect on HCMV DNA to concentrations equivalent to 100 μM drug and 100 μM 1-*O*-octadecyl-glycerol itself had no antiviral activity (data not shown). Both ODG-PFA and ODG-PAA exhibited more antiviral activity against HCMV than ganciclovir and acyclovir (Table 1). In the host MRC-5 cell, toxicity with ODG-PFA was not substantial with a TC_{50} of >1000 μM. ODG-PAA was more toxic with a TC_{50} of 32–100 μM. However, in rapidly dividing CEM cells the TC_{50} values for ODG-PFA and ODG-PAA were 114 μM and 68 μM, respectively (Table 1). Cytotoxicity was due almost entirely to decreases in cell number; cell viability remained high as assessed by propidium iodide staining and analysis by fluorescence activated cell sorting (Dangl et al., 1982).

Table 1
Effect of phosphonoacids and lipid prodrug phosphonoacids on viral replication in vitro

Compound	IC_{50} (μM)			TC_{50} (μM)	
	HCMV[a]	HSV-1[b]	HIV-1[c]	MRC-5	CEM
PFA	40 ± 16 (6)	47 ± 20 (6)	133 ± 54 (8)	>1000	800
ODG-PFA	0.43 ± 0.27 (9)	1.1 ± 1.1 (5)	2.8 ± 1.6 (5)	>1000	114
ODG-PAA	0.22 ± 0.8 (8)	20 ± 16 (5)	N.D.	32–100	68
GCV	1.5 ± 0.7 (3)	N.D.	N.D.	N.D.	N.D.
ACV	20 ± 8 (6)	0.03 ± 0.02 (4)	N.D.	N.D.	N.D.

Abbreviations: PFA, phosphonoformate acid; ODG-PFA, 1-*O*-octadecyl-*sn*-glycero-3-phosphonoformate; ODG-PAA, 1-*O*-octadecyl-*sn*-glycero-3-phosphonoacetate; GCV, ganciclovir; N.D., not determined; IC_{50}, 50% inhibitory concentration; and TC_{50}, 50% toxic concentration.
[a] HCMV DNA probe assay.
[b] HSV DNA probe assay.
[c] Plaque reduction assay in HT4-6C cells.

ODG-PFA was also more active than PFA in MRC-5 cells infected with HSV-1, as shown in Fig. 1B. PFA inhibited viral replication by 50% at 47 mM versus 1.1 mM with ODG-PFA, representing a 43-fold increase in antiviral activity. ODG-PAA was less active than ODG-PFA against HSV-1 (IC_{50} 20 mM)(Table 1).

ODG-PFA was substantially more active than PFA in HT4-6C cells infected with HIV-1 (Fig. 1C). By HIV-1 plaque reduction assay, the IC_{50} for PFA was 133 mM versus 2.8 mM for ODG-PFA (Table 1), representing a 44-fold increase in antiviral activity.

The sensitivity of three clinical isolates from AIDS patients with CMV disease was determined with PFA, ODG-PFA and ODG-PAA (Table 2). Clinical isolates were 2.7–4.7 times less sensitive to PFA than the AD169 laboratory strain (Table 2). However, only minor losses of sensitivity were noted in human CMV isolates with ODG-PFA and ODG-PAA. Compared with PFA, ODG-PFA and ODG-PAA had 111–147 and 110–271 times more anitviral activity in vitro.

To evaluate the biological basis for the increased antiviral activity against HCMV, HSV and HIV-1, we synthesized ODG-PFA labeled with ^{14}C in the carboxyl carbon of PFA and compared its cellular uptake with that of [^{14}C]PFA (Fig. 2). Cellular uptake of PFA was maximal at the earliest time point and did not increase further during 24 h at 37°. In contrast, ODG-[^{14}C]PFA uptake was progressive from 30 min to 24 h. At 24 h the level of ^{14}C-drug was 9-fold higher with ODG-PFA than with PFA (Fig. 2). At 24 h the cells were washed and analyzed by lipid extraction and TLC; 25% of the cellular radioactivity was due to intact ODG-PFA while 75% of the cellular ^{14}C was recovered as PFA.

We also examined the radioactivity in the culture medium after 0, 1 and 5 days of incubation with complete medium containing ODG-

Table 2
Antiviral activity of ODG-PFA and ODG-PAA in HCMV strain Ad169 and HCMV clinical isolates from AIDS patients

Virus strain	IC_{50} (μM)		
	ODG-PFA	ODG-PAA	PFA
Laboratory strain:			
AD169	0.43 ± 0.27 (9)	0.22 ± 0.08 (8)	40 ± 16 (6)
Human clinical isolates:			
X505	0.95 ± 0.38 (3)	0.96 ± 0.61 (3)	106 ± 68 (3)
X706	1.28 ± 0.52 (3)	0.69 ± 0.52 (3)	187 ± 25 (3)
X766	0.93 ± 0.60 (3)	0.91 ± 0.63 (3)	137 ± 38 (3)

Results are expressed as mean ± standard deviation. The number in parenthesis indicates the number of replicates.

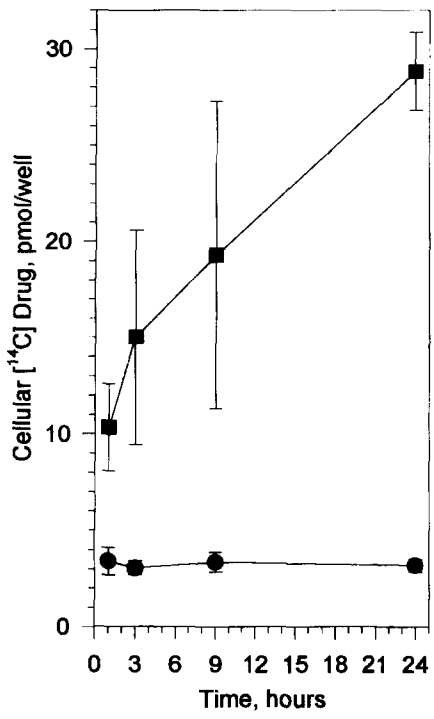

Fig. 2. Uptake of [^{14}C] phosphonoformate and 1-*O*-octadecyl-*sn*-glycero-3-[^{14}C] phosphonoformate in MRC-5 cells, in vitro. ●, [^{14}C]PFA; ■, 1-*O*-octadecyl-*sn*-glycero-3-PFA.

[^{14}C]PFA. At time zero, all of the ^{14}C radioactivity in the medium was due to intact ODG-PFA. After 1 and 5 days of incubation with complete media containing 2% fetal bovine serum, no metabolites of ODG-PFA were detected. Similar results were obtained when MRC-5-conditioned medium was incubated with ODG-[^{14}C]PFA (data not shown).

4. Discussion

Previously many prodrugs of PFA have been synthesized and their antiviral activity ranged from poor to equivalent to PFA in vitro or in vivo (Boezi, 1979; Herrin et al., 1977; Eriksson et al., 1980, 1982; Norén et al., 1983; Öberg, 1983). A palmityl phosphonate ester of PFA has been reported but it was not more active than free PFA in HIV-infected cells in vitro (Neto et al., 1990). In contrast, our results with a lipid prodrug consisting of 1-*O*-octadecyl-*sn*-glycerol esterified to the phosphonate of PFA indicate clearly that the antiviral activity of the conjugate is 40–90-fold greater than that of the free drug. The marked increase in antiviral activity was observed with all three of the viruses tested, HCMV, HSV-1 and HIV-1 (Table 1).

Studies of the mechanism of enhanced antiviral action using [^{14}C]PFA and ODG-[^{14}C]PFA show that the latter was taken up progressively by MRC-5 cells for at least 24 h while [^{14}C]PFA uptake was less pronounced and did not increase further from 1 to 24 h. The ^{14}C drug level in the cell monolayer at 24 h was about 9-fold higher with ODG-[^{14}C]PFA than with the free [^{14}C]PFA. Analysis of the cellular radioactivity at 24 h indicated that ODG-[^{14}C]PFA had been extensively converted to [^{14}C]PFA. When ODG-[^{14}C]PFA was incubated in medium containing fetal bovine serum in the absence of cells, no metabolic conversion was noted, indicating that intracellular conversion to PFA is likely to be the mechanism of action.

We conclude that enhanced cell uptake of ODG-PFA and subsequent intracellular conversion to PFA is responsible for its greater antiviral activity. However, cell uptake is only 9-fold greater at 24 h and the antiviral effects are 43–93 times greater. The reason for this is not clear at present but differences in intracellular compartmentation of PFA versus ODG-PFA and its intracellular metabolites may be important. The effect of ODG-PFA on HCMV DNA polymerase activity needs to be evaluated. Further studies are needed to evaluate these possibilities.

In conclusion, 1-*O*-octadecyl-*sn*-glycero-3-PFA, a lipid prodrug, has been synthesized and found to exhibit 43–93-fold greater antiviral activity than free PFA in human cells infected in vitro with HCMV, HSV-1 or HIV-1. Although the cytotoxicity of ODG-PFA is greater than PFA in rapidly dividing cells (Table 1) its in vitro selectivity appears to be greater because its potency is much higher. ODG-PFA is the first PFA prodrug to exhibit substantially greater levels of antiviral activity than free PFA in vitro. Compounds of this type may be useful in treating viral diseases.

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