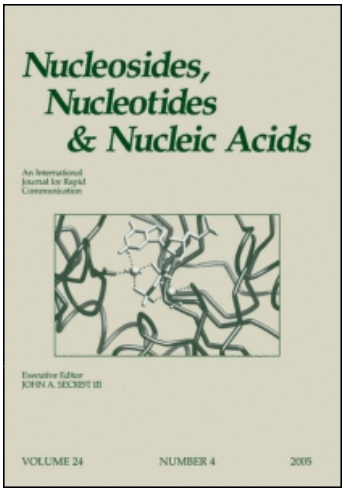

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SYNTHESIS AND ANTIVIRAL EVALUATION OF 1-O-HEXADECYLPROPANEDIOL-3-P-ACYCLOVIR: EFFICACY AGAINST HSV-1 INFECTION IN MICE

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Dedicated to the memory of Dr. Gertrude B. Elion

ABSTRACT: We synthesized, 1-O-hexadecylpropanediol-3-P-acyclovir, an orally bioavailable lipid prodrug of acyclovir and evaluated it for *in vitro* and *in vivo* activity against herpes simplex virus infections. Although 1-O-hexadecylpropanediol-3-P-acyclovir was less active *in vitro* than acyclovir, on a molar basis it was 2.4 times more active orally in preventing mortality from acute HSV-1 infection in mice. *In vitro*, 1-O-hexadecylpropanediol-3-P-acyclovir was also more active than acyclovir in a thymidine kinase negative mutant strain of HSV-1 (DM21) and had somewhat higher activity in cytomegalovirus infection *in vitro* due to it's ability to bypass thymidine kinase.

INTRODUCTION

Over twenty years ago, Gertrude Elion, Howard Schaeffer and coworkers established that 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV) is remarkably effective against herpes simplex virus (HSV) infection[1,2]. Acyclovir is phosphorylated by a HSV-coded thymidine kinase[3] and subsequently converted to ACV-triphosphate by cellular enzymes[4]. Acyclovir triphosphate inhibits the DNA polymerase of HSV[5] and may be incorporated into viral DNA causing chain termination because ACV lacks the equivalent of a 3'-hydroxyl group.

Acyclovir is not well absorbed from the gastrointestinal tract and oral absorption in man is between 10 and 20% [6]. To increase oral bioavailability of acyclovir, we synthesized an alkyl ether lipid prodrug, 1-O-octadecyl-*sn*-glycero-3-P-acyclovir (ODG-P-ACV), which was found to be 100% orally absorbed in mice[7]. ODG-P-ACV and closely related analog, 1-O-hexadecyl-propanediol-3-P-acyclovir (HDP-P-ACV) , had substantial antiviral activity against hepatitis B virus replicating in 2.2.15 cells because of direct cellular metabolism of these compounds to acyclovir monophosphate, bypassing thymidine kinase[7]. HDP-P-ACV was also orally active in woodchucks with woodchuck hepatitis virus infection while ACV itself had no significant activity[8]. In this paper, we report the activity of oral HDP-P-ACV in HSV-infected cells and in mice with HSV-1 encephalitis.

MATERIALS AND METHODS

CHEMISTRY:

Thin-layer chromatography was performed on Analtech 250 μm silica gel GF Uniplates visualized by UV, phospray (Supelco, Bellafonte, PA, USA) and charring. Chromatographic purification on silica gel was done by the flash method using Merck silica gel 60, 240-400 mesh. Elemental analyses were performed by Oneida Research Services (Whitesboro, NY, USA). ^{1}H and ^{31}P NMR spectra were recorded at 300 MHz on a Varian HG-300 spectrophotometer with tetramethylsilane (internal) or 85% D_3PO_4 in D_2O (external) as ^{1}H and ^{31}P references (0.00 ppm), respectively.

Acyclovir was obtained from Interchem (North Paramus, NJ, USA) and was dried 24 h in a vacuum oven at 70°C prior to use. 3-Hexadecyloxy-propan-1-ol was prepared as previously described by Kini et al[9]. All other reagents were obtained from Aldrich Chemical Co. unless otherwise noted.

N^2-(p-Anisyldiphenylmethyl)-9-[(2-hydroxyethoxy)methyl]guanine (1)

To acyclovir (9.7 g, 43 mmol) suspended in 250 mL of dry pyridine was added chlorotrimethylsilane (10.5 g, 97 mmol). After the mixture was stirred for 15 minutes, monomethoxytrityl chloride (Fluka, 15.0 g, 50 mmol) and N, N-dimethylaminopyridine (0.35 g, 2.9 mmol) were added and the reaction was maintained at room temperature overnight. The mixture was then cooled in an ice bath and cold (4°C) water (20 mL) was added. After 10 minutes, 29% aqueous NH_4OH (25 mL) was added and the mixture was stirred 30 min.

and then allowed to warm to room temperature. The reaction was filtered and the filtrate evaporated. The residue was purified by flash chromatography on silica gel using 1:5 ethanol/ethyl acetate to elute 16.9 g (79% yield) of pure **1** as a foamy solid. Stirring the solid in toluene while heating gave crystalline **1**. The ^{1}H NMR spectrum was consistent with that previously reported by Martin et al[10]. ^{1}H NMR (DMSO-d_6) δ 10.75 (br s, 1H), 7.78 (br s, 1H), 7.62 (s, 1H), 7.24-7.13 (m, 12 H), 6.80 (d, 2H), 4.89 (s, 2H), 4.46 (t, 1H), 3.65 (s, 3H), 3.29 (m, 2H), 2.87 (t, 2H).

N^2-(p-Anisyldiphenylmethyl)-9-[(2-hydroxyethoxy)methyl]guanine 2-chlorophenyl (3-hexadecyloxypropyl) phosphate (2)

To a solution of 1,2,4-triazole (1.10 g, 16 mmol) and triethylamine (1.62 g, 16 mmol) in anhydrous tetrahydrofuran (THF, 10 mL) was added a solution of 2-chlorophenyl dichlorophosphate (1.96 g, 8 mmol) in THF (10 mL). The mixture was stirred 30 min. then filtered. To the filtrate was added sequentially, additional THF (30 mL), compound **1** (2.98 g, 6 mmol) and 1-methylimidazole (0.66 g, 8 mmol). After 1 hour 3-hexadecyl-1-propanol (1.8 g, 6 mmol) was added. The mixture was stirred overnight at room temperature, then the solvent was evaporated. The residue was purified by flash chromatography on silica gel using a hexane/ethyl acetate gradient to elute byproducts and finally 5% EtOH/ethyl acetate to elute the fully protected phosphotriester **2** as a thick oil (4.2g, 72%). ^{1}H NMR ($CDCl_3$) δ 8.20 (s, 1H), 7.38-7.15 (m, 16H), 6.75 (d, 2H), 4.92 (s, 2H), 4.22 (q, 2H), 4.12 (q. 2H), 3.98 (m, 2H), 3.75 (s, 3H), 3.48 (t, 2H), 3.37 (t, 2H), 3.19 (m, 2H), 1.92 (p, 2H), 1.51 (m, 2H), 1.25 (br s, 26H), 0.87 (t, 3H).

9-[(2-hydroxyethoxy)methyl]guanine (3-hexadecyloxypropyl) phosphate, sodium salt (HDP-P-ACV)

Compound **2** (4.0 g, 4 mmol) was suspended in 80% aqueous acetic acid, stirred and heated to 60°C for 5 hours. The mixture was then cooled slowly to 4°C. The detritylated product, 9-[(2-hydroxyethoxy)methyl]guanine 2-chlorophenyl (3-hexadecyloxypropyl) phosphate was collected by filtration, washed with ether and dried in vacuo.

Without further purification, 9-[(2-hydroxyethoxy)methyl]guanine 2-chlorophenyl (3-hexadecyloxypropyl) phosphate was added to 0.4 N NaOH, stirred and heated to 60°C. After 4 hours, 50 % aqueous acetic acid was added dropwise to adjust the pH to approximately 8. The precipitate was collected by filtration, washed with water and dried under vacuum. The

crude product was recrystallized (2:1 1,4-dioxane/water) to provide 1.87 g of analytically pure HDP-P-ACV (78% yield). ^{1}H NMR ($CDCl_3$ + DMSO-d_6) δ 10.90 (s, 1H), 7.70 (s, 1H), 6.65 (s, 2H), 5.30 (s, 2H), 3.80 (m, 2H), 3.72 (q, 2H), 3.54 (m, 2H), 3.31 (t, 2H), 3.23 (t, 2H), 1.67 (t, 2H), 1.38 (m, 2H), 1.15 (br s, 26H), 0.8 (t, 3H) ^{31}P NMR ($CDCl_3$ + DMSO-d_6) δ -0.61. Calculated for $C_{22}H_{49}N_5O_7PNa \cdot 1.15\ H_2O$: %C, 51.44; %H, 8.20; %N, 11.11. Found: %C, 51.19; %H, 8.15; %N, 11.07.

ANTIVIRAL EVALUATION *IN VITRO*:

Liposomes consisting of dioleoylphosphatidylcholine/dioleoylphosphatidylglycerol/cholesterol/HDP-P-ACV in a molar ratio of 50:10:30:10 were prepared by sonication in a buffer containing 250 mM sorbitol/20 mM sodium acetate, pH 5.4, as previously described[7] to obtain a stock solution with drug concentration of 5 mM.

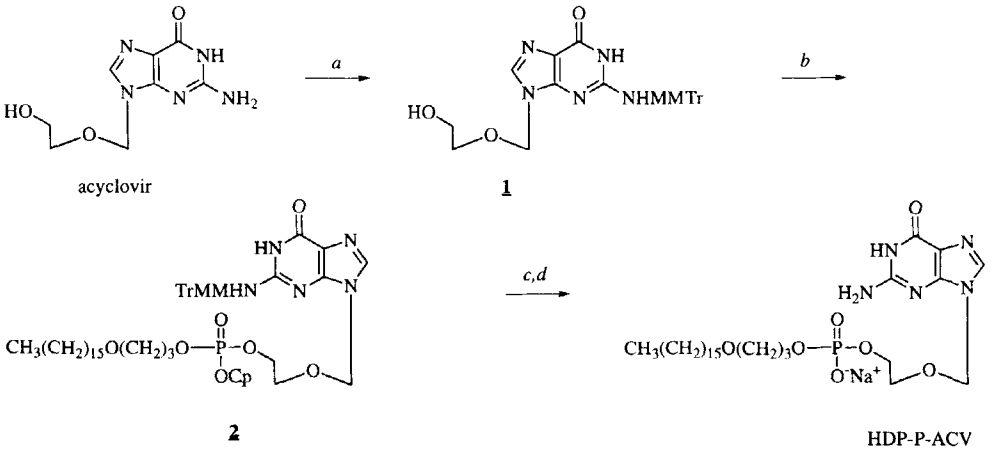
The effect of HDP-P-ACV on HSV-1 and HCMV replication *in vitro* was determined using Hybriwix filters (Diagnostic Hybrids, Athens, OH) as previously described[11]. The results are expressed as a percentage of the untreated HSV-infected or HCMV-infected control. Control liposomes without drug had no effect on HSV-1 or HCMV replication up to 1000 μM liposomal lipid (data not shown). Cytotoxicity was assessed by visual grading after exposure of the cells to trypan blue[11]. The thymidine kinase negative mutant HSV-1 strain, DM21, was the generous gift of Graham Darby, Glaxo Wellcome, Inc.

ANTIVIRAL EVALUATION IN HSV-1 INFECTED MICE:

Three week old female BALB/c mice were obtained from Charles River Breeding Laboratories, Raleigh, NC. Mice were inoculated intranasally with 1.0 x 10^5 plaque forming units (pfu) of HSV-1 strain E-377, in a volume of 0.04 ml (0.02 ml in each nostril). ACV and HDP-P-ACV were administered orally at doses of 25, 50 and 100 mg/kg twice daily for seven days in 0.1 ml doses beginning 24 hours after viral inoculation. Animals were monitored daily for 21 days. Therapeutic efficacy in HSV-1 infected mice was determined by comparing placebo treatment with ACV or HDP-P-ACV treatment. Final mortality rates and mean day of death (MDD) values were compared using Fisher's exact test and the Mann-Whitney U Sum Rank test, respectively. A p-value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

HDP-P-ACV was synthesized using the phosphotriester approach shown in Figure 1. The exocyclic amino function of acyclovir was protected using the transient hydroxyl



Reagents and conditions: a) (1) chlorotrimethylsilane, pyridine 0 °C, 30 min, (2) MMTr-Cl, DMAP, rt, 18h, (3) H_2O; (b) (1) 2-chlorophenyldichlorophosphate, 1.2.4-triazole, triethylamine, 1-methylimidazole, THF, (2) 3-hexadecyloxy-1-propanol; c) 80% aq acetic acid, 60 °C, 5h; d) 0.4 N NaOH, 60 °C, 4h.

FIGURE 1. Synthesis of 1-*O*-Hexadecylpropanediol-3-phospho-acyclovir

protection method developed by Ti et al[12]. Thus, acyclovir was suspended in dry pyridine and treated with chlorotrimethylsilane, then monomethoxytrityl chloride. After addition of water to hydrolyse the trimethylsilyl ether, N-MMTr-ACV (**1**) was isolated (82% yield) by flash column chromatography.

Phosphorylation of **1** with 2-chlorophenyl-phosphoro-bis-(1,2,4-triazolide) followed by treatment with 3-hexadecyloxy-1-propanol gave the fully protected phosphotriester **2** which was isolated by flash column chromatography in 72% yield. Heating **2** in 80% aqueous acetic acid removed the monomethoxytrityl group. Basic hydrolysis of the triester in 0.4 N NaOH yielded the phosphodiester, HDP-P-ACV. HDP-P-ACV was recrystallized from 1,4-dioxane/water (2:1) to give the target compound in 46% overall yield from ACV. This process was quite convenient for preparation of up to 50 gram batches of HDP-P-ACV.

Acyclovir reduced HSV-1 DNA by 50% at 0.04 μM versus 0.32 μM for HDP-P-ACV (Table 1). However, in the thymidine kinase negative, DM21 mutant strain of HSV-1, HDP-P-ACV was substantially more active than ACV. The EC_{50} for ACV was 32.5 versus 6.8 μM for HDP-P-ACV. This is consistent with the demonstrated ability of HDP-P-ACV to bypass thymidine kinase as shown previously in 2.2.15 cells and HepG2 cells[7]. Previously, we

TABLE 1.
Antiviral Activity of ACV and 1-O-Hexadecylpropanediol-3-P-ACV
in HSV-1 and HCMV Infected Cells

Virus	Compound	EC_{50} (μM)	TC_{50} (μM)	Selectivity
HSV-1 (wt)	ACV	0.04 ± 0.03 (3)	>1000	>25,000
	HDP-P-ACV	0.32 ± 0.22 (14)	660	2062
HSV-1 (DM21)	ACV	32.5 ± 12.0 (4)	>1000	>31
	HDP-P-ACV	6.8 ± 2.0 (3)	660	97
HCMV (AD-169)	ACV	24.2 ± 7.5 (5)	>1000	>41
	HDP-P-ACV	14.2 ± 8.6 (4)	660	46

Data are mean ± SD. The number of replicates is indicated in parenthesis. Abbreviations: ACV, acyclovir; HDP-P-ACV, 1-O-hexadecylpropanediol-3-P-ACV; EC_{50}, the μM concentration of drug which reduces viral DNA by 50%; TC_{50}, the μM concentration which reduces viable cell number by 50%. Selectivity = TC_{50}/EC_{50}.

showed that ACV diphosphate dimyristoyl glycerol, another type of lipid prodrug, was highly active against the DM21 thymidine kinase deficient mutant of HSV-1 by virtue of cellular metabolism to acyclovir monophosphate[13]. Human cytomegalovirus (HCMV) lacks a viral thymidine kinase. As might be predicted, HDP-P-ACV was also more active than ACV in HCMV-infected cells, EC_{50} 14.2 versus 24.2 μM (Table 1). As predicted, HDP-P-ACV was more cytotoxic than ACV in MRC-5 cells, TC_{30} 660 versus >1000 μM. In rapidly dividing CEM human T-lymphoblastic cells, the TC_{30} was 363 versus >1000 μM for ACV.

We treated mice which had been infected with HSV-1 intranasally with oral ACV or HDP-P-ACV for 7 days and observed the animals for 21 days. Both HDP-P-ACV and ACV were orally active against HSV-1 infection at all dosages (Table 2). Mortality rates of mice treated with HDP-P-ACV ranged from 14% at 100 mg/kg b.i.d. to 36% at the low dose of 25 mg/kg b.i.d. The percent mortality with ACV treatment was similar with 13% mortality at 100 mg/kg b.i.d. to 27% at 25 mg/kg b.i.d. Insignificant toxicity was observed in both ACV and HDP-P-ACV treatment groups at a total daily dose of 200 mg/kg for 7 days based on the appearance and body weight of the mice.

The molecular weight of ACV is 225 versus 630 for HDP-P-ACV. To compare the efficacy of these two compounds on a molar basis, we plotted the HSV-1 mortality rates

TABLE 2.
Effect of Oral Treatment with ACV or HDP-P-ACV on Mortality of CD-1 Mice Inoculated Intranasally with HSV-1

Treatment	Dose (mg/kg)	Mortality Number	Mortality Percent	P-value	MDD[a]
Placebo	None	13/15	87	-	5.9
Acyclovir	100	2/15	13	<0.001	7.5
	50	3/15	20	<0.001	8.3
	25	4/15	27	<0.01	10.3
HDP-P-ACV	100	2/14	14	<0.001	3.5
	50	3/15	20	<0.001	9.7
	25	5/15	36	<0.05	7.2
Uninfected	None	1/15	7	-	4.0

[a] - MDD = mean day of death
Abbreviations as in Table 1

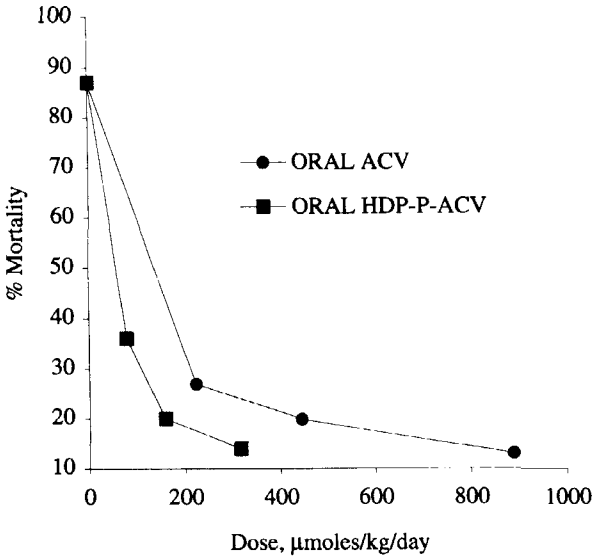


FIGURE 2. Effect of oral acyclovir and 1-O-hexadecyl-3-P-acyclovir on HSV-1 Encephalitis in Mice.

versus the total daily dose of compound in micromoles (Figure 2). On a molar basis, HDP-P-ACV is about 2.4 times more active than ACV in mice with HSV-1 infection. Although one might expect that kinase bypass might lead to greater toxicity, no significant toxic effects were observed with HDP-P-ACV even at total daily doses of 200 mg/kg. This is probably due to the fact that human DNA polymerase alpha has significant selectivity, 30 to 50 fold, for dGTP versus ACV triphosphate[14]. The greater activity of HDP-P-ACV on a molar basis may be due to the compound's greater oral bioavailability since the molar efficacy of the two compounds was nearly identical when they were administered parenterally, ED_{50} 64 to 72 μmoles/kg/day. These data suggest that HDP-P-ACV may be useful against HSV infection in humans.

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