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Enhanced Oral Absorption and Antiviral Activity of 1-O-Octadecyl-*sn*-glycero-3-phospho-acyclovir and Related Compounds in Hepatitis B Virus Infection, *In Vitro*

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ABSTRACT. Acyclovir (ACV) triphosphate and azidothymidine (AZT) triphosphate inhibit the DNA polymerase of human hepatitis B virus (HBV) by 50% at submicromolar concentrations, but no effects of ACV or AZT treatment have been noted on the clinical manifestations of hepatitis B. We synthesized 1-O-octadecyl-*sn*-glycero-3-phospho-acyclovir (ODG-P-ACV), 1-O-hexadecylpropanediol-3-phospho-acyclovir (HDP-P-ACV), and 1-O-octadecyl-*sn*-glycero-3-phospho-azidothymidine (ODG-P-AZT), and evaluated their antiviral activity in human hepatoma cells that constitutively produce HBV (2.2.15 cells). ACV and AZT up to 100 μM caused only slight inhibition of HBV replication in 2.2.15 cells. However, HDP-P-ACV and ODG-P-ACV inhibited viral replication by 50% at 0.5 and 6.8 μM, respectively. ODG-P-AZT also showed increased antiviral activity, with a 50% reduction in HBV replication at 2.1 μM. Based on the EC_{50}, HDP-P-ACV, ODG-P-ACV, and ODG-P-AZT were > 200, > 14.7, and > 48 times more active than their free nucleosides in reducing HBV replication in 2.2.15 cells. To evaluate the biochemical basis for the increased antiviral activity, we studied the uptake and metabolism of 1-O-octadecyl-*sn*-glycero-3-phospho-[^{3}H]acyclovir (ODG-P-[^{3}H]ACV) in HepG2 cells. Cellular uptake of ODG-P-[^{3}H]ACV was found to be substantially greater than that of [^{3}H]ACV, and cellular levels of ACV-mono-, -di-, and -triphosphate were much higher with ODG-P-ACV. ODG-P-[^{3}H]ACV was well absorbed orally. Based on urinary recovery of tritium after oral or parenteral administration of the radiolabeled compounds, oral absorption of ODG-P-ACV in mice was 100% versus 37% for ACV. ODG-P-ACV plasma area under the curve was more than 7-fold greater than that of ACV. Lipid prodrugs of this type may be useful orally in treating viral diseases. BIOCHEM PHARMACOL 53;12:1815–1822, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. prodrugs; antiviral agents; hepatitis B virus

HBV[‖] is a DNA virus that infects hepatocytes, causing an acute and chronic hepatitis that may eventually lead to liver cirrhosis and hepatocellular carcinoma [1]. Despite the availability of effective vaccines, HBV infection is an important public health problem, with over 300 million chronically infected persons worldwide [2]. The virus DNA replicates through reverse transcription of a pregenomic RNA intermediate catalyzed by HBV polymerase [3, 4]. Numerous studies have shown inhibition of HBV replication *in vitro* by various nucleosides [5–12]. Both (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (lamuvidine, 3TC) and 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine (penciclovir, PCV) are currently in human clinical trials for HBV infection.

ACV triphosphate was shown to effectively inhibit HBV DNA polymerase and woodchuck hepatitis virus DNA polymerase by 50% at 0.9 and 0.7 μM, respectively [13]. Nevertheless, ACV treatment in HBV infection had no additional effect on serum levels of hepatitis BeAg levels in interferon α-treated patients [14]. Similarly, AZT triphosphate inhibited HBV DNA polymerase by 50% at 0.3 μM [6] but had no effect on serum HBV DNA in HBV infection in AIDS patients [15, 16].

We synthesized ODG-P-AZT and the conjugates of ACV (ODG-P-ACV and HDP-P-ACV) and evaluated their *in vitro* activity and metabolism in human hepatoblastoma cells (2.2.15 cells) that constitutively produce HBV. Surprisingly, the alkylglycerol-3-phosphate drug analogs

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‖ *Abbreviations:* HBV, hepatitis B virus; ACV, acyclovir; AZT, 2′,3′-dideoxy-3′-azidothymidine; ODG-P-AVC, 1-O-octadecyl-*sn*-glycero-3-phospho-acyclovir; HDP-P-ACV, 1-O-hexadecylpropanediol-3-phospho-acyclovir; ODG-P-AZT, 1-O-octadecyl-*sn*-glycero-3-phospho-azidothymidine; DMF, dimethylformamide; and TK, thymidine kinase.

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exhibited much greater anti-HBV activity than ACV or AZT *in vitro*. We examined the biochemical basis for this observation in HepG2 cells by determining the uptake and conversion of radiolabeled ACV or the ODG-P-ACV conjugate to ACV phosphates *in vitro*. Finally, oral bioavailability of ODG-P-[^{3}H]ACV was compared with [^{3}H]ACV in mice by two methods based on urinary recovery of drug and plasma levels after oral administration.

MATERIALS AND METHODS

Synthesis of ODG-P-ACV

Thin-layer chromatography was done with Analtech silica gel GF plates developed with chloroform:methanol:ammonia:water (65:35:5:5, by vol.). ^{1}H-NMR spectra consistent with the assigned structures were obtained from all compounds at 300 MHz on a GE QE-300 spectrophotometer. Combustion analyses were performed by Oneida Research Services, Inc., Whitesboro, NY.

N^2-ACETYLACYCLOVIR CYANOETHYLPHOSPHATE. N^2-acetylacyclovir was prepared according to the procedure described by Stimac and Kobe [17]. To a stirred mixture of N^2-acetylacyclovir (3.47 g, 13 mmol) and cyanoethylphosphate (1.96 g, 13 mmol) in DMF (40 mL) was added a solution of 1,3-dicyclohexylcarbodiimide (5.36 g, 26 mmol) in pyridine (20 mL), and the mixture was heated to 40°. After stirring overnight and cooling, water (2.5 mL) was added, then the mixture was filtered, and the filtrate was concentrated *in vacuo* to give a light yellow oil. The oil was applied to a column of silica gel and chromatographed (4:1, CH_2Cl_2:MeOH) to give N^2-acetylacyclovir cyanoethylphosphate. Yield, 3.65 g (70%). R_f = 0.2.

1-O-OCTADECYL-*SN*-GLYCERO-3-CYANOETHYLPHOSPHATE N^2-ACETYLACYCLOVIR. To a stirred mixture of N^2-acetylacyclovir cyanoethylphosphate (1.0 g, 2.5 mmol), 1-O-octadecyl-*sn*-glycerol (1.0 g, 3.0 mmol), and 1-methylimidazole (0.5 g, 6 mmol) in dry pyridine (75 mL) was added a solution of 1-(2-mesitylene sulfonyl)-3-nitro-1,2,4-triazole (1.1 g, 3.75 mmol) in dry pyridine; the mixture was stirred overnight at room temperature and then concentrated *in vacuo*. The residue was chromatographed on a column of silica gel (9:1, CH_2Cl_2:MeOH) to afford 1-O-octadecyl-*sn*-glycero-3-cyanoethylphospho-N^2-acetylacyclovir. Yield, 310 mg (17%). R_f = 0.55.

ODG-P-ACV. 1-O-Octadecyl-*sn*-glycero-3-cyanoethylphospho-N^2-acetylacyclovir (300 mg, 0.4 mmol) was added to concentrated ammonia (25 mL), and the mixture was stirred and heated to 50° for 5 hr. The mixture was cooled to room temperature, then chilled to 0°, and the product was collected by vacuum filtration. Recrystallization (95% EtOH) gave pure ODG-P-ACV. Yield, 250 mg (92%). R_f = 0.35. Calculated for $C_{29}H_{54}N_5O_8P \cdot 0.5\ NH_4OH \cdot 1.0\ H_2O$: %C, 52.20; %H, 8.84; %N, 11.54. Found: %C, 52.04; %H, 8.38; %N, 11.28.

Synthesis of HDP-P-ACV

Full details of an improved method for the synthesis of this compound will be reported elsewhere. Briefly, *n*-monomethoxy trityl ACV was treated first with 2-chlorophenyl-dichlorophosphate/12,4-triazole/1-methylimidazole and then with 1-O-hexadecylpropanediol. The resulting triester was isolated and the protecting groups were removed to yield HDP-P-ACV as the sodium salt. Calculated for $C_{22}H_{49}N_5O_7PNa \cdot 1.15\ H_2O$: %C, 51.44; %H, 8.20; %N, 11.11. Found: 5%C, 51.19; %H, 8.15; %N, 11.07.

Synthesis of ODG-P-[8-^{3}H]ACV

Unlabeled ODG-P-ACV was labeled by catalytic exchange with T_2O by Moravek Biochemicals (Brea, CA). T_2O was removed by coevaporation with methanol, and the product was purified by TLC. The final purity by TLC in chloroform:methanol:ammonia:water (70:38:8:2, by vol.) was 99.5%. This material was used without further purification after dilution with unlabeled ODG-P-ACV to a specific activity of 0.1 mCi/μmol.

Synthesis of ODG-P-AZT

AZT monophosphate ammonium salt (1.52 g, 4 mmol) was converted to the free acid by passing it through a Dowex-50 (hydrogen form) ion-exchange column, and lyophilized to dryness. The residue was co-evaporated with pyridine (2 × 25 mL). To a mixture of the resulting pyridinium salt and 1-O-octadecyl-*sn*-glycerol (1.9 g, 5 mmol) in dry pyridine (25 mL), cooled to 0° in an ice bath, was added a solution of dicyclohexylcarbodiimide (2.5 g, 12 mmol) in dry pyridine (20 mL), dropwise with stirring. The mixture was stirred at 0° for 3 hr, and at room temperature overnight. The mixture was filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by flash chromatography over silica gel with an increasing gradient of methanol in dichloromethane as the eluting solvent. The appropriate fractions were pooled and concentrated *in vacuo* to yield 0.7 g (25%) of ODG-P-AZT as an amorphous solid. Analysis calculated for $C_{31}H_{56}N_5O_8P \cdot 0.6\ H_2O$: %C, 54.23; %H, 8.43; %N, 10.20. Found: %C, 54.47; %H, 8.16; %N, 9.46.

Assay of Drug Effects on HBV Replication in 2.2.15 Cells

ACV, AZT, ODG-P-ACV, and ODG-P-AZT were added to wells containing 2.2.15 cells at the indicated concentrations. The lipid prodrugs were added as a liposomal formulation prepared as described below. Fresh medium was added daily, and after 9 days HBV-specific DNA was assayed using HBV-specific DNA probes as noted previously [9]. Results are expressed as picograms of HBV virion DNA per milliliter of medium. The 50% cytotoxic concentration (CC_{50}) of the respective agents was determined by

neutral red uptake as described previously [9]. A liposome control without added lipid prodrug had no significant effect on HBV replication or cytotoxicity to 1 mM (data not shown).

Uptake of [^{3}H] ACV and ODG-P-[^{3}H]ACV in HepG2 Cells

A 10 μM solution of [8-^{3}H]ACV (1.47 mCi/μmol) was prepared in Minimal Essential Medium (MEM) containing 2% fetal bovine serum. ODG-P-[^{3}H]ACV was incorporated into liposomes containing dioleoylphosphatidylcholine: dioleoylphosphatidylglycerol:cholesterol:ODG-P-ACV (50:10:30:10 molar ratio) by sonication for 1 hr at 37° in 360 μL of sorbitol/acetate buffer, pH 5.4. The sonicated liposomes were diluted to the indicated concentrations with MEM containing 2% fetal bovine serum. The specific activity of ODG-P-[^{3}H]ACV was 0.1 mCi/μmol. Medium (0.5 mL) was added to a confluent monolayer of HepG2 cells in 24-well plates and incubated at 37°. At the indicated times, the supernatant was removed, and cells were lysed with 0.2 mL of 0.5 M NaOH. The contents of the wells were scraped into a liquid scintillation vial and counted with 10 mL Flo-Scint IV (Packard Instruments, Meriden, CT) in a Tracor liquid scintillation counter. In some experiments, medium was added to the HepG2 cells in a 24-well plate and was replaced with fresh medium daily for 9 days; cells were assayed each day by removing the medium, washing the cells, and replacing the medium with 200 μL of 0.5 N NaOH. At the end of the experiment, the wells were scraped into liquid scintillation vials and counted with 10 mL Flo-Scint IV.

Metabolism of [^{3}H]AZT, [^{3}H]ACV, and ODG-P-[^{3}H]ACV in HepG2 Cells In Vitro

MEM containing 2% fetal bovine serum and 10 μM of either [8-^{3}H]ACV (1.47 mCi/μmol), [*methyl*-^{3}H]AZT (1.04 mCi/μmol), or liposomal ODG-P-[8-^{3}H]ACV (0.1 mCi/μmol) (all from Moravek Biochemicals, Brea, CA), was prepared, and the drugs were added to HepG2 cells grown to 70% confluence in T75 flasks. After 24 hr the medium was removed and the cell monolayer was washed briefly with 2 mL of cold PBS. Distilled water (2 mL) was added and the flask was frozen/thawed twice; then the cell suspension was transferred to a 16 × 100 tube, cooled, and cold trichloroacetic acid (TCA) was added to a final concentration of 8%. Tubes were vortexed and centrifuged, and then the TCA supernatant was removed and neutralized with 5 N NaOH for analysis by HPLC using a Whatman Partisil 10 SAX column. Buffer A was 25 mM KH_2PO_4, pH 3.5; buffer B was 5 M KCl in 1 M KH_2PO_4, pH 3.5. A linear gradient from 0 to 100% B was run from 8 to 40 min at 1 mL/min. One-minute fractions were collected from the end of the column and analyzed for radioactivity in 10 mL FloScintIV. Radioactivity was plotted and peaks representing ACV and ACV-mono-, -di-, and -triphosphate were identified by comparing retention times with those of unlabeled standards run under the same conditions. AZT metabolites were assessed by HPLC using a Partisil SAX column, eluted and analyzed as described previously [18].

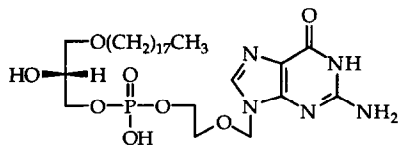
Oral and Intravenous Administration of Tritium-Labeled ACV and ODG-P-ACV

COMPARISON OF URINARY EXCRETION. ODG-P-[8-^{3}H]ACV, specific activity 1.91 μCi/μmol, in normal saline/β-cyclodextrin (Molecusol^R, Pharmatec Inc., Hialeah, FL) and [8-^{3}H]ACV, specific activity 1.40 μCi/μmol, in normal saline/β-cyclodextrin were administered to each of three female Balb-C mice by i.v. injection or orally via a stomach tube in a dose of 44.4 μmol/kg (27.96 mg/kg ODG-P-ACV and 10 mg/kg ACV). The mice were placed in metabolic cages, and the urine was collected for 24 hr. Aliquots of the urine were counted by liquid scintillation spectrometry, and the total disintegrations per minute of ACV metabolites in the urine were determined and expressed as micromole equivalents of ACV/24 hr.

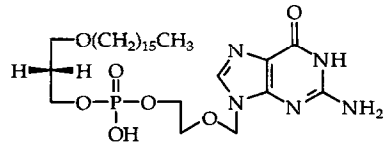
COMPARISON OF PLASMA LEVELS. In other experiments, ODG-P-[^{3}H]ACV, incorporated into liposomes consisting of dioleoylphosphatidylcholine:dioleoylphosphatidylglycerol:cholesterol:ODG-P-ACV at a molar ratio of 40:10:30:20, was administered orally to a group of three female NIH Swiss mice, at a dose of 55.6 μmol/kg (36 mg/kg) and a specific activity of 1.61 μCi/μmol. [2′-^{3}H]ACV (Moravek Biochemicals) in 250 mM sorbitol, 20 mM acetate, pH 5.5, and 8% EtOH was administered orally at a dose of 88.8 μmol/kg (20 mg/kg) and a specific activity of 1.37 μCi/μmol. At various time points, plasma samples were obtained and analyzed by liquid scintillation spectrometry. The total radioactivity of ACV in plasma was determined and expressed as micromoles per liter. Further analysis of the metabolites of ODG-P-[^{3}H]ACV in plasma was performed as follows: Plasma (150 μL; 50 μL pooled from each of the mice representing each time point) was extracted with chloroform:methanol (2:1, v/v) by the method of Folch *et al.* [19]. Aliquots of the upper and lower phases were analyzed by liquid scintillation spectrometry, and the data were expressed as micromolar ACV or ODG-P-ACV. The remainder of the lower phase was concentrated under a nitrogen stream and applied to a silica gel GF TLC plate (Analtech, Newark, DE). The plate was developed in a solvent system of chloroform:methanol:water:ammonia (65:35:5:5, by vol.) and subjected to radioscanning.

RESULTS

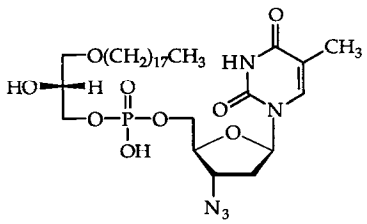
Neither ACV nor AZT was an effective inhibitor of HBV replication, with EC_{50} values > 100 μM. However, liposomal formulations of both the acyclovir prodrugs, HDP-P-ACV and ODG-P-ACV (Fig. 1), were substantially more effective in reducing virion HBV DNA in 2.2.15 cells, with



1-O-octadecyl-sn-glycero-3-phospho-acyclovir (ODG-P-ACV)



1-O-hexadecyl-propanediol-3-phospho-acyclovir (HDP-P-ACV)



1-O-octadecyl-sn-glycero-3-phospho-azidothymidine (ODG-P-AZT)

FIG. 1. Structures of ODG-P-ACV, HDP-P-ACV, and ODG-P-AZT.

EC_{50} values of 0.5 and 6.8 μM and EC_{90} values of 3.9 and 30 μM (Table 1, Fig. 2). The hexadecylpropanediol analog was substantially more active *in vitro* than the octadecylglycerol derivative of ACV-monophosphate with an EC_{50} of 0.5 versus 6.8 μM. AZT up to 100 μM did not inhibit HBV replication, but ODG-P-AZT was effective with an EC_{50} value of 2.1 μM and an EC_{90} of 10 μM (Fig. 2). Blank liposome controls did not significantly affect viral replication to 1 mM (data not shown). The selectivity indexes (CC_{50}/EC_{90}) for HDP-P-ACV, ODG-P-ACV, and ODG-P-AZT were 107, 28, and 40, respectively (Table 1).

TABLE 1. Anti-HBV activity and cytotoxicity of ACV, AZT and their ether lipid conjugates in 2.2.15 cells

Compound	CC_{50} (μM)	EC_{50} (μM)	EC_{90} (μM)	Selectivity index
ACV	684	>100	>100	NM*
HDP-P-ACV	417	0.5	3.9	107
ODG-P-ACV	840	6.8	30	28
AZT	610	>100	>100	NM
ODG-P-AZT	400	2.1	10	40

Data are expressed as the micromolar concentration of drug required to reduce viral replication by 50% (EC_{50}) or 90% (EC_{90}) or to reduce cell viability by 50% (CC_{50}). The selectivity index is the CC_{50}/EC_{90}. Abbreviations: ACV, acyclovir; HDP-P-ACV, 1-O-hexadecylpropanediol-3-phospho-acyclovir; ODG-P-ACV, 1-O-octadecyl-sn-glycero-3-phospho-acyclovir; AZT, 3′-azido-3′-deoxythymidine; and ODG-P-AZT, 1-O-octadecyl-sn-glycero-3-phospho-azidothymidine.

* NM = not meaningful.

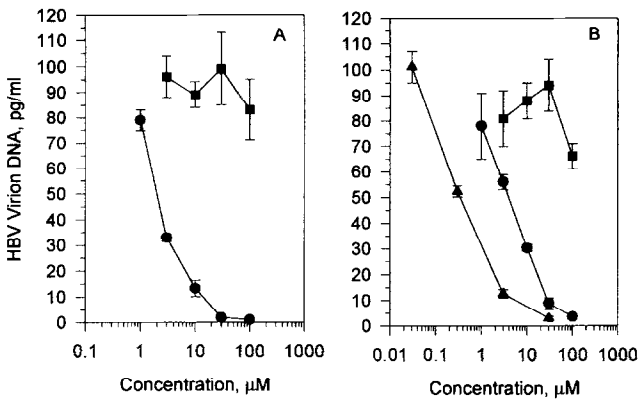


FIG. 2. Effects of ODG-P-AZT and the conjugates of ACV (ODG-P-ACV and HDP-P-ACV) on HBV replication in 2.2.15 cells *in vitro*. Key: (A) AZT (■), and ODG-P-AZT (●); (B) ACV (■), ODG-P-ACV (●), and HDP-P-ACV (▲). Values are means ± SD, N = 4.

To evaluate the biochemical basis for the increased antiviral activity, we incubated a liposomal formulation of radiolabeled ODG-P-ACV with HepG2 cells to assess cellular uptake and intracellular levels of metabolites. [^{3}H]ACV and ODG-P-[^{3}H]ACV were incubated with HepG2 cells in a 24-well plate, and cellular levels of ^{3}H-labeled drug were measured at times from 2 hr to 6 days following daily exposures to drug (Fig. 3). With exposure to ACV, cellular levels of tritium in HepG2 cells reached a plateau at 8 hr, and cellular levels of ACV and its metabolites did not increase further for 6 days (0.07 nmol/well). However, following the initial exposure to ODG-P-[^{3}H]ACV (1.34 nmol/well), the peak level of labeled drug and metabolites in the cell monolayer was 19 times greater than that observed with ACV. Strikingly, when fresh medium containing the respective drugs was added daily, the cellular level of ODG-P-ACV and its metabolites continued to increase progressively in contrast to repeated addition of ^{3}H-labeled ACV. At day 9, the cellular content of ODG-P-ACV and its metabolites represented 3.42 nmol/well versus 0.12 nmol/well for ACV, an increase of 29-fold.

To analyze the intracellular levels of nucleoside phosphates, we incubated 10 μM [^{3}H]ACV or ODG-P-[^{3}H]ACV with monolayers of HepG2 cells in T75 flasks. After 24 hr, cellular metabolites were assessed by HPLC (Table 2). Cellular levels of ACV nucleotides were very low in the HepG2 cells incubated with [^{3}H]ACV. In contrast, the ACV-mono-, -di-, and -triphosphate content of cells incubated with tritium-labeled ODG-P-ACV was 11.6-, 3.2-, and 4.7-fold greater than with ACV. In addition, 5.49 pmol of ODG-P-ACV or a lipid metabolite was present in the cell pellet representing a substantial reservoir of prodrug for eventual conversion to ACV-triphosphate. [^{3}H]AZT (10 μM) was also incubated with HepG2 cells,

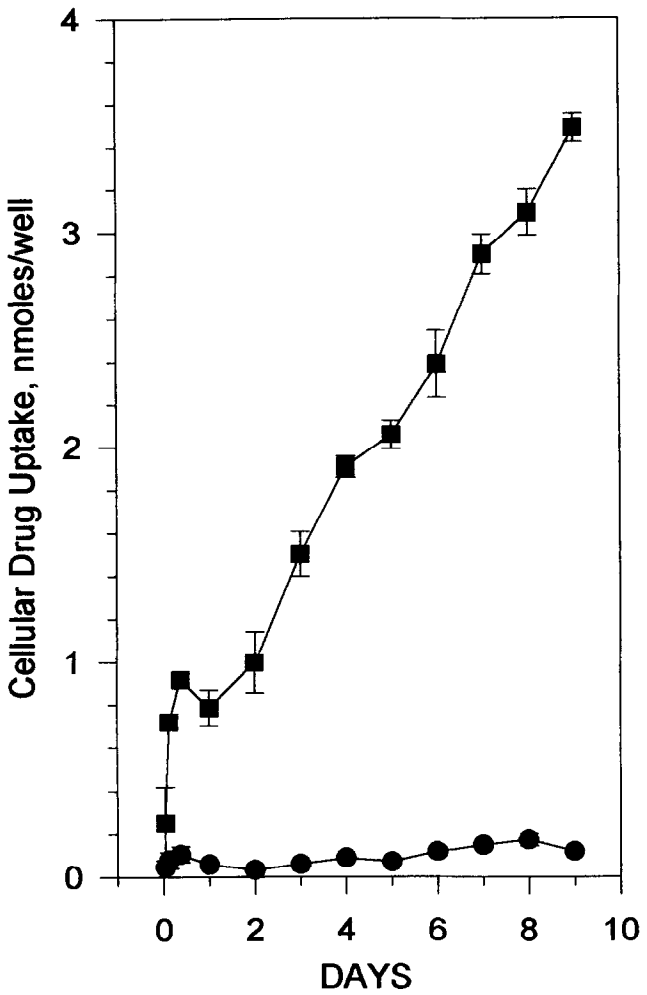


FIG. 3. Cellular levels of ^{3}H-labeled ACV and ODG-P-ACV after daily exposure of hepG2 cells to drug *in vitro*. Key: ACV (■), and ODG-P-ACV (■). Values are means ± SD, N = 3.

and the cellular metabolites were assessed by HPLC. AZT-monophosphate, AZT-glucuronide, and AZT-diphosphate represented 89.8, 8.2, and 2% of the cellular metabolites. AZT-triphosphate could not be detected in HepG2 cells under these conditions, indicating poor conversion of AZT to AZT-triphosphate (data not shown).

TABLE 2. Cellular levels of acyclovir metabolites following 24 hr incubation of [^{3}H]ACV or ODG-P-[^{3}H]ACV with HepG2 Cells *in vitro*

Metabolite	Compound (pmole/T75 flask) ACV	ODG-P-ACV	Fold increase
ACV-monophosphate	0.69 ± 0.15	8.03 ± 1.42	11.6
ACV-diphosphate	0.18 ± 0.04	0.56 ± 0.11	3.2
ACV-triphosphate	0.04 ± 0.02	0.18 ± 0.02	4.7
ODG-P-ACV	0	5.49 ± 3.92	NM*

Results are the means ± SD for four determinations. See legend of Table 1 for definitions of abbreviations.

* NM = not meaningful.

TABLE 3. Urinary excretion of [^{3}H]ACV and ODG-P-[^{3}H]ACV in mice following oral or intravenous administration

Compound	[^{3}H]ACV (μmol/24 hr) Oral	Intravenous	% Oral absorption
[^{3}H]ACV	0.37 ± 0.09	1.00 ± 0.09	37
ODG-P-[^{3}H]ACV	0.48 ± 0.03	0.47 ± 0.04	102

[2'-^{3}H]ACV (10 mg/kg sp. act. 8.4 μCi/μmol) or ODG-P-[8-^{3}H]ACV (27.96 mg/kg, sp. ac. 1.9 μCi/μmol), representing in each case 44.4 μmol/kg, was administered to three mice by either the oral or intravenous route as noted in Materials and Methods. Urine was collected after 24 hr, and tritium was determined by liquid scintillation counting. The results, expressed as μmol drug recovered are means ± SD; N = 3. See Table 1 for definitions of abbreviations.

Oral absorption of ACV and ODG-P-ACV was evaluated in mice by urine recovery of metabolites using tritium-labeled compounds (Table 3). Cyclodextrin formulations of [^{3}H]ACV and ODG-P-[^{3}H]ACV were administered i.v. or orally to mice, and urinary recovery of tritium was determined after 24 hr. Recovery of ODG-P-ACV in the urine was equivalent after oral or i.v. administration, indicating 100% absorption. In contrast, oral absorption of ACV in mice was 37%.

In another experiment, the ODG-P-ACV was present in a liposomal formulation. ODG-P-[^{3}H]ACV and [^{3}H]ACV were administered orally to mice, and plasma levels of tritiated drug were measured (Fig. 4). Aliquots of the plasma from mice receiving ODG-P-ACV at the respective time points were pooled, subjected to lipid extraction [19], and analyzed for ACV and ODG-P-ACV. ACV was recovered in the upper phase of the extraction while

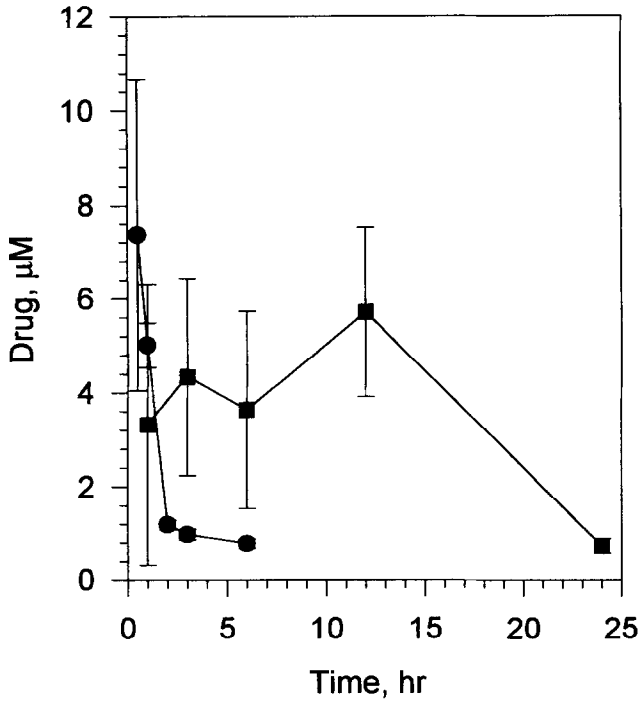


FIG. 4. Plasma drug levels after oral administration of [^{3}H]ACV or ODG-P-[^{3}H]ACV to mice. Key: ACV (●), and ODG-P-ACV (■). Values are means ± SD, N = 3.

ODG-P-ACV was found in the lower phase of the extraction. The lower phases were concentrated and subjected to TLC on silica gel GF plates; only ODG-P-ACV was detected (R_f 0.34) in plasma over the 24-hr experiment (Fig. 4). After oral administration of 88 μmol/kg (20 mg/kg) of ACV, peak levels were observed at 30 min (Fig. 4). Oral ODG-P-ACV administered in a liposomal formulation reached peak plasma levels 12 hr following an oral dose of 55.6 μmol (35 mg/kg). From 1 to 12 hr, plasma levels of ODG-P-ACV were 3.3 to 5.7 μM and 0.73 μM at 24 hr (Fig. 4). Analysis of the plasmas by lipid extraction and TLC of the lipid extract showed that 94–96% of the plasma radioactivity was intact ODG-P-ACV at 1–6 hr, declining to 90% at 12 and 24 hr. The remainder of the plasma radioactivity was recovered as ACV (data not shown). Area under the curve (0 to ∞) for ACV and ODG-P-ACV was determined, and after correction for molar dose, the AUC of ODG-P-ACV was calculated to be 7.8-fold greater than that of ACV.

DISCUSSION

Although ACV-triphosphate is an effective inhibitor of HBV polymerase activity [13], ACV is not highly active in patients infected with HBV [4]. Our studies in HepG2 cells (Table 2) indicate that this is due to poor phosphorylation of ACV. ACV is particularly effective in herpes virus infection because the herpes simplex virus TK phosphorylates ACV readily, and only HSV-infected cells convert ACV to ACV-triphosphate, allowing for a high antiviral selectivity [20, 21]. ACV-triphosphate is 30–100 times more effective as an inhibitor of HSV-1 DNA polymerase than mammalian cell DNA polymerase-α [20, 22]. HBV does not code for a TK, and liver cells do not appear to phosphorylate ACV sufficiently via 5′-nucleotidase or cellular TK to allow for the formation of enough of ACV-triphosphate to inhibit HBV replication.

We have shown previously that lipid analogs of the poorly phosphorylated nucleoside 3′-deoxythymidine (ddT) exhibit 48- to 50-fold increases in activity versus 3′-deoxythymidine in HIV-1-infected cells. ddT diphosphate diacylglycerol was highly effective even in TK-negative CEM cells infected with HIV-1, demonstrating direct conversion of the lipid prodrug to ddT-monophosphate [23]. Furthermore, ACV diphosphate diacylglycerol was fully active in cells infected with TK-negative strains of herpes simplex virus, which are highly resistant to ACV [24]. Thus, nucleoside diphosphate diacylglycerols of poorly phosphorylated nucleosides bypass kinase activation by direct metabolic conversion to the corresponding nucleoside monophosphate. This appears to be catalyzed, at least in part, by a nucleoside diphosphate diglyceride hydrolase present in mitochondria [25]. Welch and coworkers [26] synthesized 1,2-dipalmitoylphosphatidyl-ACV and examined its antiviral activity in HSV-1-infected cells. Phosphatidyl-ACV was substantially less active than ACV (EC_{50} 1.0 vs 0.1 μM). In TK-negative HSV-1, it was similar in activity to ACV (EC_{50} 22 vs 90 μM), suggesting that phosphatidyl-ACV does not bypass TK, giving rise to intracellular ACV-monophosphate. Other workers have employed alternative strategies to achieve kinase bypass utilizing non-lipid types of prodrugs such as "masked" nucleotides, nucleotide phosphate triesters, or nucleoside phosphonates [8, 27, 28].

The present studies show that ODG-P-ACV and a related compound, HDP-P-ACV, have greatly enhanced antiviral activity in cells that constitutively produce HBV. ODG-P-ACV gives rise to much higher intracellular levels of ACV-mono-, ACV-di-, and ACV-triphosphate than observed with free ACV. The exact enzymatic mechanisms of ODG-P-ACV conversion to ACV-monophosphate in HepG2 cells are presently unknown. However, it seems likely that ACV-monophosphate is released by hydrolysis of the phosphoester bond between ACV-monophosphate and the *sn*-3 hydroxyl of glycerol, a phospholipase C-type reaction. Evidence in favor of this includes the fact that we did not observe any glycero-phospho-acyclovir (G-P-ACV) on HPLC analysis of the trichloroacetic acid-soluble metabolites. G-P-ACV would result from oxidation and cleavage of the alkyl-oxy-ether residue of the prodrug. Phospholipase C and lysophospholipase C enzymes have been demonstrated in liver. We reported a lysosomal phospholipase C in liver which has an acid pH optimum and hydrolyzes lysophosphatidylcholine to monoacylglycerol at about 40% of the rate observed with phosphatidylcholine [29]. Lysosomal phospholipase C was not highly specific for the phospholipid polar head group, readily hydrolyzed phospholipids having choline, ethanolamine, inositol, serine, and glycerol as the head group [29], and was present in all rat tissues studied [30]. A phospholipase C capable of hydrolyzing lysophosphatidylcholine at neutral pH was isolated from rabbit liver by Nishihira and Ishibashi [31]. Finally, Okayasu *et al.* [32] observed conversion of 1-O-alkyl-*sn*-glycero-3-phosphocholine to 1-O-alkylglycerol by cultured rat hepatocytes. The two liver phospholipases C studied to date are not highly specific for the polar head group [29, 31] and might act on ODG-P-ACV. Experiments with enzymes from liver cell fractions or purified phospholipases C will be required to identify with certainty the precise enzymatic mechanisms responsible for intracellular generation of ACV-monophosphate from ODG-P-ACV.

Another interesting feature of ODG-P-ACV is its high degree of oral absorption (Table 3) and its long plasma retention (Fig. 4). We hypothesize that this is due, in part, to its structural similarity to lysophosphatidylcholine, a dietary breakdown product of lecithin, much of which is absorbed intact from the small intestine [33, 34]. Preliminary studies in lymph fistula rats with radiolabeled ODG-P-ACV suggest that some absorption occurs via the chylomicrons in intestinal lymph, bypassing the portal venous circulation (Hardison WG and Hostetler KY, unpublished observations, 1996). Octadecylglycerol or propanediol de-

rivatives of other poorly absorbed drugs may provide better oral absorption and favorable pharmacokinetics.

In summary, the octadecylglycerol prodrugs of AZT-monophosphate and ACV-monophosphate exhibit greater *in vitro* antiviral activity than AZT or ACV in 2.2.15 cells, which constitutively replicate HBV. Metabolic studies indicate increased cellular uptake of the ACV-lipid prodrug and suggest direct enzymatic conversion to the nucleoside monophosphates. This strategy may be useful in extending the activity of ACV, AZT, and other nucleosides to viral diseases where viral TK is not produced in the infected cells and cellular enzymes phosphorylate the nucleoside poorly.

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