

Increased Antiviral Activity of 1-O-Hexadecyloxypropyl-[2-¹⁴C]cidofovir in MRC-5 Human Lung Fibroblasts Is Explained by Unique Cellular Uptake and Metabolism

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ABSTRACT

Recently, there has been renewed interest in finding orally active drugs against smallpox. Cidofovir (CDV) given by parenteral injection has been shown to protect against lethal poxvirus infection. We have been interested in the synthesis and evaluation of orally active derivatives of CDV. Previous studies showed that the CDV and cyclic cidofovir (cCDV) analogs 1-O-hexadecyloxypropyl-CDV (HDP-CDV) and 1-O-hexadecyloxypropyl-cCDV (HDP-cCDV), show >100-fold increases in antiviral activity versus the unmodified nucleosides against cells infected with orthopoxviruses, cowpox, and vaccinia virus. In contrast to CDV, HDP-CDV is orally bioavailable and has been reported to be orally active in lethal cowpox virus infection in mice. To assess the metabolic basis for the increased antiviral activity of HDP-CDV in vitro, we studied the cellular uptake

and anabolic metabolism of ¹⁴C-labeled CDV, cCDV, and their alkoxyalkanol esters HDP-CDV and HDP-cCDV. HDP-CDV and HDP-cCDV were taken up rapidly by MRC-5 human lung fibroblasts in vitro, but uptake of CDV and cCDV was much slower. Analysis of cellular metabolites showed that levels of cidofovir diphosphate (CDV-DP), the active antiviral compound, were >100 times greater with HDP-CDV than levels observed with CDV. When cells were exposed to HDP-CDV, the intracellular half-life of CDV-DP was 10 days versus 2.7 days reported when cells are exposed to CDV. HDP-CDV seems to circumvent poor cellular uptake by rapid association with cellular membrane phospholipids, whereas CDV uptake proceeds via the slow process of fluid endocytosis.

Cidofovir (1-[(S)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine; CDV) is an acyclic phosphonate analog of cytosine that has been shown to have activity against all double-stranded DNA viruses studied to date, including herpes group viruses, orthopoxviruses, parapoxviruses, adenoviruses, and papovaviruses (De Clercq et al., 1987; Snoeck et al., 1988; De Clercq, 1997). CDV (Vistide; Gilead Sciences, Foster City, CA) is approved as an intravenous treatment for cytomegalovirus retinitis in AIDS patients but has dose-limiting renal side effects (Lea and Bryson, 1996; Plosker and Noble, 1999). CDV given intravenously protects mice against lethal vaccinia or cowpox virus infection (Neyts and De Clercq, 1993; Bray et al., 2000; Smee et al., 2000). Topical cidofovir has also been reported to have activity against mollusca contagiosum (Zabawski, 2000). Intralesional CDV

has been used to treat laryngeal papillomatosis (Snoeck et al., 1998; Stragier et al., 2002).

It would be useful to have highly active antiviral analogs of CDV that are less toxic and orally bioavailable. Our laboratory has developed a strategy to enhance absorption of poorly absorbed nucleotides, such as acyclovir monophosphate and ganciclovir monophosphate, by attaching certain ether lipid residues, such as 1-O-hexadecylpropanediol (Hostetler et al., 1997, 2000, 2001; Beadle et al., 2000). As part of this program, we synthesized 1-O-hexadecyloxypropyl-CDV (HDP-CDV) and tested it against MRC-5 human lung fibroblasts infected with cytomegaloviruses and herpes simplex viruses, type 1 and type 2. HDP-CDV exhibited multiple log increases in antiviral activity in vitro against CMV and HSV-1 compared with CDV (Beadle et al., 2002) (Table 1). HDP-CDV was also active against various ganciclovir-resistant CMV isolates. Multiple log enhancement of antiviral activity was also noted against various strains of cowpox and vaccinia virus infected cells in vitro (Kern et al., 2002) (Table 1) and against variola virus-infected cells in vitro (J. W. Huggins, personal communication).

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ABBREVIATIONS: CDV, cidofovir; HDP, 1-O-hexadecyloxypropyl; HSV, herpes simplex virus; CMV, cytomegalovirus; cCDV, cyclic cidofovir; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; CDV-MP, cidofovir monophosphate; CDV-DP, cidofovir diphosphate; HCMV, human cytomegalovirus.

In this article, we have compared the cellular uptake and intracellular metabolism of HDP-[2-¹⁴C]CDV and [2-¹⁴C]CDV to assess the mechanisms leading to the remarkable increase in antiviral activity observed in our prior studies.

Materials and Methods

Cells and Media. MRC-5 human lung fibroblasts were obtained from American Type Culture Collection (Manassas, VA) at an early pass number. Cells were grown in minimal essential medium with Earle's salts containing 2% fetal bovine serum. Fetal bovine serum was obtained from Invitrogen (Carlsbad, CA).

Chemicals and Radiochemicals. Cidofovir and cyclic cidofovir were provided by Gilead Sciences. [2-¹⁴C]CDV (specific activity, 53 mCi/mmol), cyclic[2-¹⁴C]CDV (specific activity, 56 mCi/mmol), and HDP-cyclic[2-¹⁴C]CDV (specific activity, 50 mCi/mmol) were prepared by custom synthesis by Moravek Biochemicals (Brea, CA). HDP-cyclic[2-¹⁴C]CDV was treated with dilute NaOH to open the ring, and HDP-[2-¹⁴C]CDV was isolated as the monosodium salt. Cidofovir monophosphate and cidofovir diphosphate were prepared by custom synthesis by TriLink BioTechnologies (San Diego, CA).

Cell Uptake Studies. Radiolabeled CDV, cCDV, HDP-CDV or HDP-cCDV at concentrations of 1, 3, or 10 μ M (specific activity, 50 to 56 μ Ci/ μ mol) were added to 24-well plates containing subconfluent monolayers of MRC-5 human lung fibroblast cells and incubated at 37°C for the times indicated. The medium was then removed and the cell monolayers were washed with cold phosphate-buffered saline (PBS), lysed with (0.5 N) sodium hydroxide and transferred to scintillation vials for counting.

Metabolism Experiments. [2-¹⁴C]CDV or HDP-[2-¹⁴C]CDV (10 μ M; specific activity, 50 and 56 mCi/mmol, respectively) was added to 25-cm² flasks of nearly confluent MRC-5 cells and incubated for 6, 24, or 48 h. The cell monolayers were treated as follows: the media was removed and the cell monolayer was washed twice with cold PBS. Then 0.6 ml of distilled water was added and the flasks were frozen and thawed twice, followed by sonication in a cold bath sonicator for 5 min. The cells were removed by scraping and transferred to a glass tube. Cold trichloroacetic acid was added to a final concentration of 8%, and the contents were vortexed and centrifuged for 10 min at 4°C. The supernatant was removed, counted, and immediately analyzed by HPLC. HPLC was done by applying the sample to a 4.6- × 15-cm Partisil 10 SAX column with a SAX guard column. The column was eluted at a flow rate of 1 ml/min using a potassium phosphate buffer gradient of 20 to 700 mM, pH 5.8, beginning at 9 min for a duration of 20 min and a terminal hold of 5 min. Fractions (1 min/ml) were collected, FloScint IV scintillation fluid was added, and the samples were analyzed by liquid scintillation counting.

For the drug washout experiments, cells were incubated for 24 h with HDP-[¹⁴C]CDV. The media was removed and the cell monolayer

was washed twice with cold PBS. Drug-free complete medium was added, and the cells were incubated and harvested at 0, 2, 4, 6, 8, and 10 days and analyzed by Partisil SAX HPLC as noted above. The retention times of CDV, CDV-MP, and CDV-DP were identical to pure reference standards of chemically synthesized CDV, CDV-MP, and CDV-DP.

Results

HDP-CDV was roughly 400-, 200-, and 50-fold more active than CDV against HCMV, HSV-1/HSV-2, and poxviruses, respectively (Table 1). Similar trends were noted with HDP-cCDV against HCMV and HSV, but the increase in antiviral activity against poxviruses was lower (~10-fold versus CDV). Studies were done to assess the reasons for the marked increases in antiviral activity.

To assess drug uptake, MRC-5 cells were exposed to 10 μ M CDV or HDP-CDV for times ranging from 1 to 24 h and drug uptake was assessed. Cellular uptake of CDV was maximal at 1 to 4 h but remained stable or declined slightly by 24 h. In contrast, the cellular drug content of HDP-CDV increased nearly linearly for 6 h and progressively to 24 h (Fig. 1). Similar results were observed with HDP-cCDV, except that cellular drug levels stopped rising after 4 h and declined slowly thereafter. The uptake of HDP-cCDV was about twice that observed with HDP-CDV. Cyclic CDV uptake was generally similar to that of CDV (Fig. 1).

To assess the effect of concentration on drug uptake, we evaluated the cellular uptake of 1, 3, and 10 μ M CDV, cCDV, HDP-cCDV, and HDP-CDV at 4 h during the linear phase of cellular drug uptake. At 4 h, the cellular drug content observed with 1 μ M CDV was 1.2 pmol/well versus 28 pmol/well with 1.0 μ M HDP-CDV. At concentrations of 3 and 10 μ M, drug uptake of HDP-CDV was 77 and 245 pmol/well, an increase of 11- to 23-fold versus CDV (Fig. 2). At 3 and 10 μ M, the uptake of HDP-cCDV was approximately twice that of HDP-CDV and 32-fold greater than observed with cCDV (Fig. 2).

We next exposed cells to 10 μ M drug for various times and evaluated the intracellular levels of CDV, CDV monophosphate (CDV-MP), and CDV diphosphate (CDV-DP). HPLC analysis of extracts of cells exposed to 10 μ M HDP-CDV revealed readily detectable peaks at the same retention times as authentic standards of CDV, CDV-MP, and CDV-DP, as well as a peak identified as (S)-1-[3-hydroxy-2-(phosphonyl-methoxy)propyl]uridine (Table 2). However, cellular levels of CDV-MP and CDV-DP were much lower in cells exposed to 10 μ M CDV. After 24 h, CDV-MP level was 1.0 picomole/flask with CDV versus 63 pmol/flask with HDP-CDV. CDV-DP, the active antiviral, was 1.3 with CDV versus 133 with HDP-CDV, an increase of 102-fold. After 48 h, cellular CDV-DP was 1.8 pmol/flask with CDV versus 184 with HDP-CDV, and increase of 102-fold (Table 2). Interestingly, we did not observe a radioactive peak eluting between CDV and CDV-MP, reported previously as CDV diphosphocholine by others (Ho et al., 1992; Aduma et al., 1995). However, small amounts of a radioactive compound eluting before CDV were noted and seemed to correspond to (S)-1-[3-hydroxy-2-(phosphonyl-methoxy)propyl]uridine, the deamination product of CDV [(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine].

To assess the intracellular levels of the metabolites of HDP-[2-¹⁴C]CDV, we exposed cells to 7.5 μ M HDP-[2-

TABLE 1

Activity of cidofovir and 1-O-hexadecyloxypropyl cidofovir against herpes group viruses and poxviruses in vitro

Numbers in parentheses are the -fold increases in antiviral activity versus CDV. Data were abstracted from Kern et al. (2002) and Beadle et al. (2002) and used with permission.

Virus	EC ₅₀ by Plaque Reduction		
	CDV	HDP-CDV	HDP-cCDV
	μ M		
HCMV-AD169	0.38	0.0009 (422)	0.001 (380)
HCMV-Towne	0.40	0.0009 (444)	0.001 (400)
HSV-1	15.2	0.06 (253)	0.06 (253)
HSV-2	10.5	0.08 (131)	0.23 (46)
Cowpox-Brighton	44.7	0.60 (74)	2.10 (21)
Vaccinia-Copenhagen	46.2	0.80 (58)	3.80 (12)
Vaccinia-WR	45.8	1.10 (42)	5.60 (8)
Vaccinia-Elstree	41.6	1.20 (35)	3.80 (11)

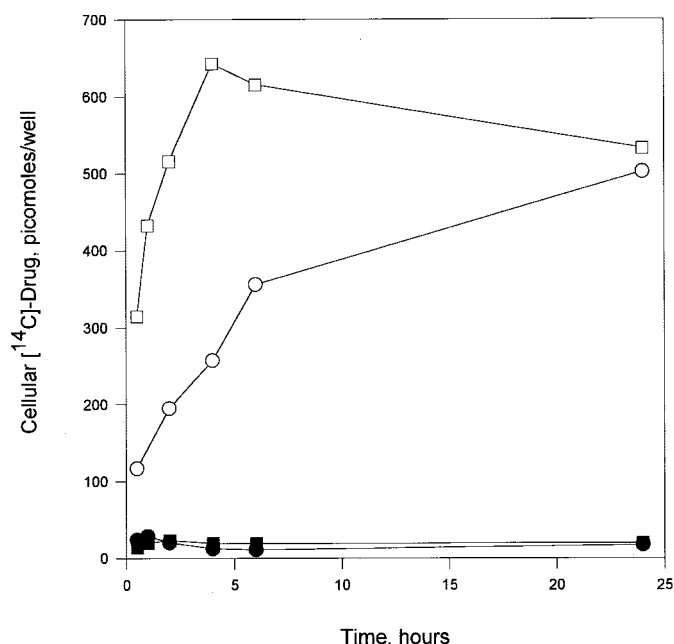


Fig. 1. Uptake of ^{14}C -labeled drugs by MRC-5 human lung fibroblasts. Data are the average of two determinations. ●, CDV; ■, cyclic CDV; ○, HDP-CDV; □, HDP-cyclic CDV.

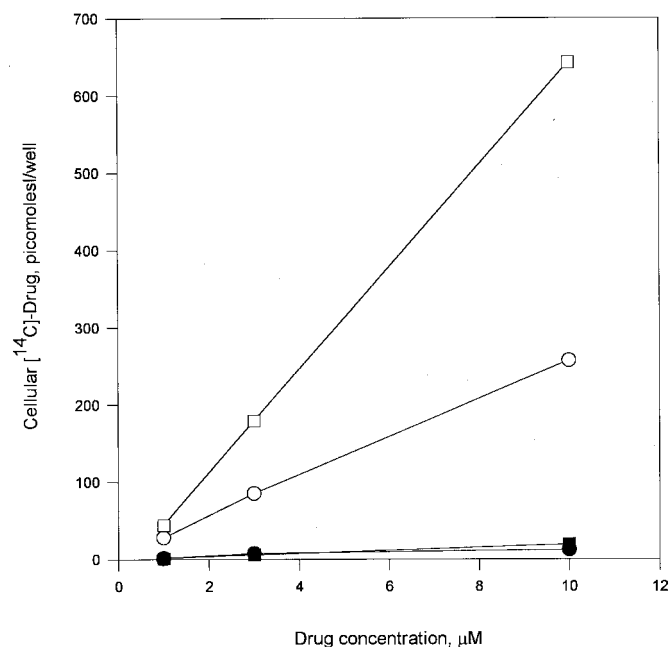


Fig. 2. Effect of concentration of cellular uptake of ^{14}C -labeled drugs. Cells were exposed to drugs for 4 h and analyzed for drug content. ●, CDV; ■, cyclic CDV; ○, HDP-CDV; □, HDP-cyclic CDV.

TABLE 2

Comparison of metabolite levels found in MRC-5 cells after exposure to $[2\text{-}^{14}\text{C}]\text{CDV}$ ($10\ \mu\text{M}$) or $\text{HDP-}[2\text{-}^{14}\text{C}]\text{CDV}$ ($10\ \mu\text{M}$)

Times are the time of exposure to radioactive CDV or HDP-CDV. Each data point represents an analysis of the cells from a single T-75 flask.

Metabolite	CDV			HDP-CDV		
	6 h	24 h	48 h	6 h	24 h	48 h
	<i>pmol/flask</i>					
HPMPU	4.8	6.9	4.2	8.8	27.3	36.0
CDV	146.4	273.8	129.3	166.7	697.4	702.0
CDV-MP	3.9	1.0	1.2	11.8	63.2	71.4
CDV-DP	6.3	1.3	1.8	11.2	132.6	184.4

HPMPU, (S)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]uridine.

$^{14}\text{C}]\text{CDV}$ for 24 h. Then the radioactive drug was washed away with PBS and the medium was replaced with drug-free growth medium and incubation continued for 2 to 10 days. Cell metabolites were analyzed by HPLC at 0, 2, 4, 6, 8, and 10 days after removal of the drug. Cell extracts were prepared by freezing and thawing in water, and the membrane fraction was isolated by centrifugation. The membrane fraction contained unmetabolized HDP- $[2\text{-}^{14}\text{C}]\text{CDV}$, which represented 2084 pmol/flask at time 0. The water-soluble metabolites consisted of 460 pmol/flask CDV, 45 pmol/flask CDV-MP, and 83 pmol/flask CDV-DP at zero time (Fig. 3). Two days after the washout of HDP- $[2\text{-}^{14}\text{C}]\text{CDV}$ from the flask, membrane levels of HDP- $[2\text{-}^{14}\text{C}]\text{CDV}$ declined by 52%, whereas the water-soluble metabolites $[2\text{-}^{14}\text{C}]\text{CDV}$, $[2\text{-}^{14}\text{C}]\text{CDV-MP}$, and $[2\text{-}^{14}\text{C}]\text{CDV-DP}$ increased by 58, 102, and 64%, reaching peak levels of 722, 74, and 166 pmol/flask, respectively. Thereafter, CDV, CDV-MP, and CDV-DP declined gradually to 267, 24, and 83 pmol/flask at 10 days. The $T_{1/2}$ values for HDP-CDV, CDV, CDV-MP, and CDV-DP were estimated to be 2, 8, 7, and 10 days, respectively (Fig. 3).

Discussion

Cellular uptake of CDV is slow and has been shown to occur by fluid phase endocytosis (Connelly et al., 1993). Covalent addition of the 1-*O*-hexadecyloxypropyl ester to the phosphonate of CDV results in remarkable increases in the antiviral activity of HDP-CDV versus CDV against HCMV and HSV (Beadle et al., 2002) and against vaccinia virus and cowpox viruses (Kern et al., 2002) (Table 1). The present study indicates that this is caused, at least in part, by increased cell penetration of HDP-CDV relative to CDV. Furthermore, the intracellular levels of the active antiviral metabolite, CDV-DP, formed after intracellular cleavage of HDP-CDV by phospholipase C-like enzymes and phosphorylation by cellular kinases, is more than two logs greater than the levels observed with equimolar concentrations of CDV. The intracellular half-life of CDV-DP is approximately 10 days in cells exposed to HDP-CDV versus a reported half-life in cells exposed to CDV of 17 h (Ho et al., 1992) or 1 to 2.7 days in Vero cells, where a biphasic decay of CDV-DP was observed (Aduma et al., 1995). The ratios of CDV to CDV-MP and CDV-DP observed when cells were exposed to HDP-CDV were generally similar to that seen with CDV alone, as reported by Ho and coworkers (1992) and Aduma et al. (1995) (i.e., $\text{CDV} \gg \text{CDV-DP} > \text{CDV-MP}$). Surprisingly, we did not observe conversion of CDV to CDV diphosphate choline in these experiments, in contrast to prior reports (Ho et al., 1992; Aduma et al., 1995). An important cause of the 10-day $T_{1/2}$ value observed for CDV-DP after exposure of cells to

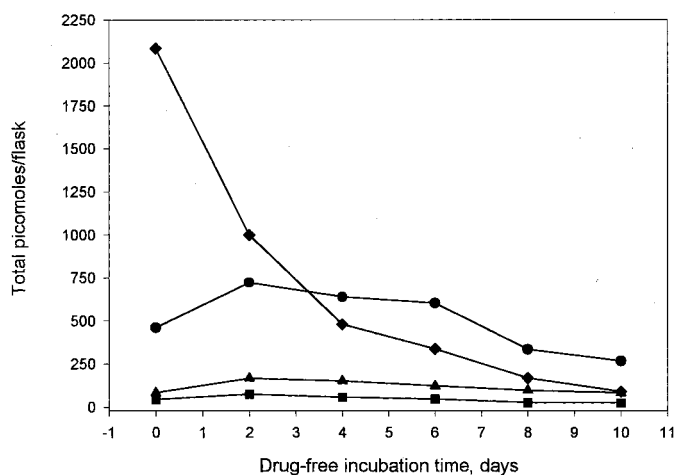


Fig. 3. Persistence of cellular metabolites of HDP- ^{14}C CDV in MRC-5 cells flasks of near-confluent MRC-5 cells were exposed to drug for 24 h. Media was removed, the cell monolayer was washed, drug-free complete medium was replaced, and incubation was continued. At the indicated times, the metabolites were analyzed by HPLC (cell extract) or by lipid extraction (cell pellet). ●, CDV; ▲, CDV-diphosphate; ■, CDV-monophosphate; ◆, HDP-CDV

HDP-CDV in this study is the presence in MRC-5 cellular membranes of a large pool of HDP-CDV that is metabolized by cellular phospholipase C and phosphodiesterases to release intracellular CDV, which may be anabolized in turn to CDV-DP by cellular kinases.

In conclusion, the present study shows that the cellular uptake of HDP-CDV is 11- to 23-fold greater than that of CDV in MRC-5 cells *in vitro*. With 10 μM HDP-CDV, the intracellular level of the active antiviral, CDV-DP, was 102 times greater than that observed with CDV at both 24 and 48 h. This seems to explain, at least in part, the multiple log increases in antiviral activity observed with HDP-CDV in cells infected with CMV, HSV-1, cowpox, and vaccinia virus *in vitro*. Finally, the intracellular half-life of CDV-DP was 10 days in MRC-5 human lung fibroblasts exposed to HDP-CDV, suggesting that long-lasting antiviral activity may be provided by relatively infrequent exposures of cells to drug. Because HDP-CDV and other compounds of this class are also orally bioavailable, at least in rodents, the compounds are worthy of further investigation as possible oral therapies for viral disease caused by susceptible viruses, including HCMV, HSV, and orthopoxviruses [including smallpox (variola major) and vaccinia]. Preliminary studies by Huggins et al. (2002) show that HDP-CDV is orally active in a lethal cowpox virus infection model in mice.

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