Short communication

Synthesis and antiviral evaluation of alkoxyalkyl-phosphate conjugates of cidofovir and adefovir[☆]

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Abstract

Esterification of cidofovir (CDV), an antiviral nucleoside phosphonate, with alkyl or alkoxyalkyl groups increases antiviral activity by enhancing cell uptake and conversion to CDV diphosphate. Hexadecyloxypropyl-CDV (HDP-CDV) has been shown to be 40–100 times more active than CDV in vitro in cells infected with herpes group viruses, variola, cowpox, vaccinia or ectromelia viruses. Since the first phosphorylation of CDV may be rate limiting, we synthesized the hexadecyloxypropyl-phosphate (HDP-P-) and octadecyloxyethyl-phosphate (ODE-P-) conjugates of CDV and phosphonomethoxy-ethyl-adenine (PMEA, adefovir). We tested the CDV analogs in cells infected with human cytomegalovirus, herpes simplex virus, cowpox virus and vaccinia virus; the analogs of PMEA were tested in cells infected with the human immunodeficiency virus, type 1. In general, the alkoxyalkyl-phosphate conjugates of CDV were substantially more active than CDV. HDP-P-CDV and ODE-P-CDV were 4.6–40 times more active against HCMV and 7–30 times more active against cowpox and vaccinia in vitro. Although the compounds of this type were more cytotoxic than the unmodified bases, their selectivity for virally infected cells was generally greater than the parent nucleotides except that HDP-P-PMEA showed little or no selectivity in HIV-1 infected MT-2 cells. Although the new compounds with an interposed phosphate were generally less active than the corresponding alkoxyalkyl esters of CDV and PMEA, the present approach provides a possible alternative method for enhancing the antiviral activity of drugs of this class.
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Acyclic nucleoside phosphonates are well known and are in clinical use as antiviral agents for cytomegalovirus (cidofovir, CDV), HIV (tenofovir) and hepatitis B (adefovir, PMEA) (Holy, 2003). The limitations of acyclic nucleoside phosphonates relate to their poor oral bioavailability and nephrotoxicity (Cundy, 1999). In general, the uptake of nucleoside phosphonates such as CDV into target cells is poor because of the dual negative charges on the phosphonate moiety and the slow uptake by fluid phase endocytosis (Connelly et al., 1993; Aldern et al., 2003). Once in the cell, they require two subsequent anabolic phosphorylations to achieve activity as their diphosphates (Ho et al., 1992).

We showed previously that the antiviral activity of CDV could be markedly increased by conversion to HDP-CDV (Beadle et al., 2002; Kern et al., 2002). Furthermore, HDP-CDV is orally active in mice against lethal infections with ectromelia virus (Buller et al., 2004), vaccinia and cowpox virus infections (Quenelle et al., 2004), lethal murine cytomegalovirus (CMV) infection (Kern et al., 2004) as well as in human CMV (HCMV) infection in SCID-hu mice (Bidanset et al., 2004). Cellular uptake studies of [2-^{14}C]CDV and HDP-[2-^{14}C]CDV in MRC-5 cells indicated that cellular drug and metabolite levels were many fold higher with HDP-CDV. In cells exposed to HDP-CDV for 48 h, cellular levels of the metabolites for CDV, CDVp and CDVpp were 702, 71 and 184 pmol/flask, respectively (Aldern et al., 2003). Similar results were reported in cellular metabolism studies of CDV in MRC-5 cells (Ho et al.,

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i) DCC, morpholine, t-butyl alcohol, reflux.
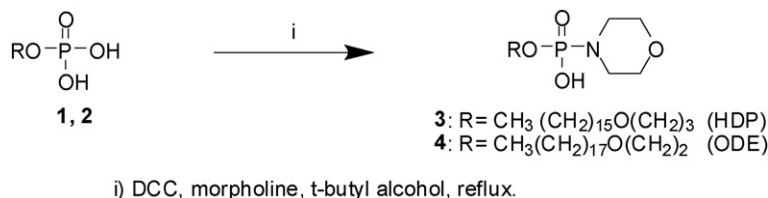
Scheme 1. Synthesis of alkoxyalkyl-phosphomorpholidates.

1992). Thus, it appears that the first phosphorylation may be the rate-limiting step in activation of CDV to CDVpp. Bypassing the first phosphorylation step from CDV to CDVp could increase the activity of antiviral nucleoside phosphonate drugs. Therefore, it would be of interest to see if alkoxyalkyl phosphate conjugates of CDV also exhibit greater antiviral activity due to enhanced cell uptake and favorable cellular metabolism.

To address this question, we synthesized alkoxyalkyl-phosphate adducts of (S)-1-[3-hydroxy-2-(phosphonome-thoxy)-propyl]cytosine (cidofovir, CDV) and phosphono-methoxyethyl-adenine (PMEA) and tested the former for antiviral activity in cells infected with HCMV, HSV-1, vaccinia and cowpox and the latter in cells infected with HIV-1.

The nucleoside phosphonate–phosphate conjugates were prepared as depicted in Schemes 1 and 2. Scheme 1 outlines the synthesis of alkoxyalkyl phosphomorpholidates **3** and **4**. The alkoxyalkyl phosphomorpholidates were coupled to the nucleoside phosphonates as shown in Scheme 2. (S)-1-(3-hydroxy-2-phosphono-methoxypropyl)cytosine (CDV) was treated with dimethoxytritylchloride in DMSO by the method of Otmar et al. (1999) to give intermediate **5** that was condensed with HDP-phosphate morpholidate **3** and **4** in pyridine, tributylamine and catalytic acetic acid at room temperature. Finally, hydrolysis with TFA in CHCl$_3$ gave compounds **6**, hexadecyloxypropyl-P-cidofovir (HDP-P-CDV), and **7**, octadecyloxyethyl-phospho-cidofovir (ODE-P-CDV). Compound **9** was prepared from the condensation of com-

pound **8**, 9-(2-phosphonomethoxyethyl)adenine (PMEA), and compound **3** in pyridine and acetic acid as catalyst. Detailed synthetic methods are as follows.

2-(Octadecyloxy)ethyl dihydrogen phosphate (**2**): To a cold solution of phosphorus oxychloride, 3 ml (32 mmol) in THF was added dropwise a solution of 2-octadecyloxy-1-ethanol (5 g,16 mmol) and triethylamine (4.4 ml, 32 mmol) in THF, while the temperature was maintained below 20 °C. The mixture was kept an additional hour, water was added and the stirring continued overnight followed by extraction with ethyl ether. The crude solid from the ether layer was recrystallized from hexane to give **2** as a white solid in 72% yield.

2-(Octadecyloxy)ethyl hydrogen morpholinophosphate (**4**): To a solution of 3 g (7.6 mmol) of 2-(octadecyloxy)ethyl phosphate in *tert*-butyl alcohol was added 2 g (24 mmol) of mor-pholine and 5.8 g (30 mmol) of DCC, added in four portions and refluxed over 48 h. Ethyl ether was added to the mixture, then it was filtered, and the filtrate concentrated to give **4** as an oil. Mass spectrum: (ESI) *m/z* 462 (M–H)$^-$

3-(Hexadecyloxy)propyl dihydrogen phosphate (**1**): Compound **1** was prepared following the same procedure described above except that 3-(hexadecyloxy)-1-propanol was substituted for 2-octadecyloxy-1-ethanol. The yield was 71%.

3-(Hexadecyloxy)propylhydrogenmopholinophosphonate (**3**): HDP-phosphonomorpholidate was prepared using the procedure described in example 1 using 1.92 g (15 mmol) of 3-(hexadecyloxypropyl) dihydrogen phosphate, 1.3 g (15 mmol)
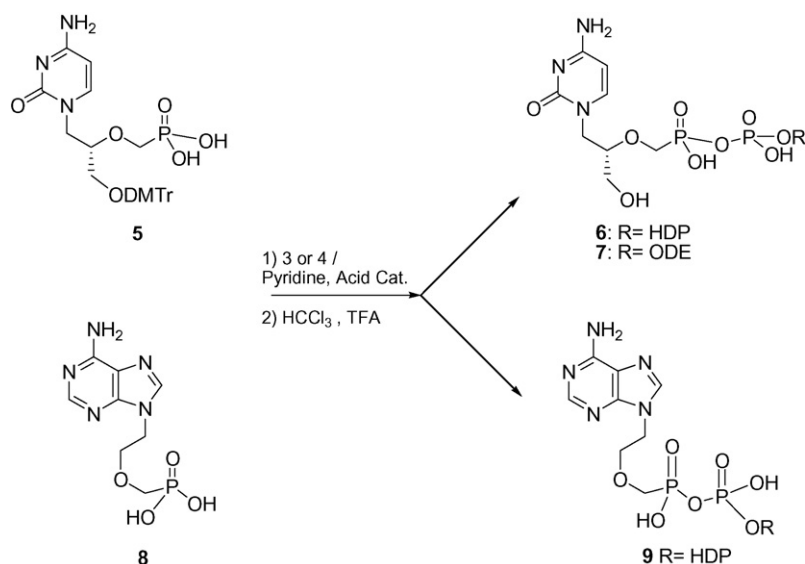
Scheme 2. Synthesis of alkyoxyalkyl-phosphate esters of acyclic nucleoside phosphonates.

of morpholine and 35 mmol of DCC. **3** was obtained as an oil. Mass spectrum: (ESI) *m/z* 449 (M–H)$^-$.

(S)-1-(3-(4,4′-Dimethoxytrityloxy)-2-phosphonomethoxy-propyl)cytosine (**5**): (S)-1-(3-hydroxy-2-phosphonomethoxy-propyl)cytosine dihydrate (free acid, 0.25 g, 0.75 mmol) was mixed with methanol and 3 ml of tributylamine. After solution was achieved, solvents were removed and the solid residue dissolved in DMSO; 0.9 g of tributylamine and 0.7 g of dimethoxytritylchloride was added and the mixture was stirred overnight at room temperature. Solvents were removed and the solid residue was recrystallized from ethyl acetate and dried to obtain 0.35 g (91%) of the product **5** as a white solid. MS (ESI) *m/z* 943 (M–H)$^-$.

(1-(4-Aminooxopyrimidin-1(2*H*)-yl)-3-hydroxypropan-2-yloxy)methyl-phosphonic(3-hexadecyloxy)propylphosphoric) anhydride (HDP-P-CDV, **6**): To a suspension of **5**, 0.1 g (0.17 mmol) in pyridine was added 0.1 ml of tributylamine. After solution was achieved, 1 g of **3** in pyridine and 0.1 ml of acetic acid were added and the solution was stirred for 48 h. Solvents were removed and the residue was mixed with a solution of $CHCl_3$ and TFA (5:0.5) and stirred at room temperature. Solvents were removed and the crude mixture was purified by flash chromatography using silica gel and eluting with 70:30:3:3 ($CHCl_3$:methanol:ammonium hydroxide:water) to obtain 0.04 g (40%) of **6** as a white solid. MS (ESI) *m/z* 642 (M–H)$^-$. ^{1}H NMR 300 MHz (DMSO) δ ppm: 7.47 (d, 1H, 6.9 Hz), 5.66 (d, 1H, 7.2 Hz), 3.8–3.1 (mm, 16H), 1.7 (m, 2H), 1.4 (m, 2H), 1.2 (s, 26H), 0.8 (t, 3H). ^{31}P NMR (DMSO-d$_6$) 12.5 (s, P1), 0.4 (s, P2).

(1-(4-Aminooxopyrimidin-1(2*H*)-yl)-3-hydroxypropan-2-yloxy)methyl-phosphonic-((3-octadecyloxy)ethyl phosphoric) anhydride (ODE-P-CDV, **7**): To a suspension of **5** (0.14 g, 0.17 mmol) in pyridine was added 0.1 ml of tributylamine followed by a solution of 1 g of **4** in pyridine and 0.1 ml of acetic acid were added and stirred for 48 h. Solvents were removed and the residue was mixed with a solution of $CHCl_3$:TFA in (5:0.5) and stirred at room temperature. After complete deprotection, the mixture was purified by column chromatography using silica gel and eluting with 70:30:3:3 ($CHCl_3$:methanol:amonia:water) to obtain 0.03 g (20%) of **7** as a white solid. MS (ESI) *m/z* 654 (M–H)$^-$. ^{1}H NMR 300 MHz ($CDCl_3/CD_3OD$) δ ppm:

6 (d, 1H, *J* = 8Hz), 5.8 (d, 1H, *J* = 7.2 Hz), 4.2–3.4 (mm, 16H), 1.7 (m, 2H), 1.58 (m, 2H), 1.27 (s, 28H), 0.89 (t, 3H). ^{31}P NMR ($CDCl_3/CD_3OD$), 12.38 (m, P1), 5.6 (d, P2, *J* = 25.5 Hz).

((2-(6-Amino-9H-purin-9-yl)ethoxy)methylphosphonic (3-hexadecyloxy)-propylphos-phonic)anhydride (HDP-P-PMEA, **9**): 9-(2-phosphonylmethoxyethyl)-adenine (PMEA) 0.5 g was dissolved in pyridine and a solution of 1 g of **3** in pyridine was added along with 1 ml of acetic acid. The reaction mixture was heated at 40 °C overnight. Solvents were removed and the oily residue was washed with 10% methanol in ethyl ether and filtered. The filtrate was concentrated and purified by column chromatography using silica gel and eluting with 25% methanol in dichloromethane to obtain 0.19 g of **9** (17%) as a white solid. Mass spectrum: (ESI) *m/z* 635 (M–H)$^-$, 636 (M + H)$^+$. ^{1}H NMR 300 MHz (DMSO) δ 8.35 (s, 1H), 8.09 (s, 1H), 7.86 (bs, 2H), 7.12 (bs, 2h), 0.83 (s, 3H). ^{31}P NMR (DMSO) δ 1.4 (d, P1, J_{PP} = 24.32 Hz), −12 (d, P2, J_{PP} = 24.32 Hz).

The effect of the various compounds on HIV replication was measured by p24 reduction as previously described (Hostetler et al., 1996; Hammond et al., 2001). Drug effects on HCMV replication were assessed by plaque reduction assay in HFF cells as described previously (Beadle et al., 2002; Wan et al., 2005). Antiviral activity against HSV-1 was assessed in MRC-5 cells as previously reported using a DNA reduction assay (Beadle et al., 2002). Drug activity against cowpox and vaccinia virus infected human foreskin fibroblast cells (HFF) was determined by plaque reduction assay (Keith et al., 2004) Cytotoxicity was assessed in HFF or MRC-5 cells by neutral red uptake and the CC_{50}s determined as reported previously (Beadle et al., 2002; Keith et al., 2004; Wan et al., 2005). The antiviral activity of the various compounds is expressed as the EC_{50}, the concentration required to inhibit p24, plaques or viral DNA production by 50%.

Both HDP-P-CDV (**6**) and ODE-P-CDV (**7**) were more active than CDV with EC_{50} values of 0.26 and 0.03 μM versus 1.2 μM for CDV in vitro against HCMV; this represents an increase of 5–40-fold (Table 1). Similar results were noted against HSV-1 with EC_{50} values for HDP-P-CDV and ODE-P-CDV of 0.06 and 0.00002 μM versus 3.3 μM for CDV. Although the cyto-

Table 1

Antiviral activity of cidofovir, alkoxyalkyl and alkoxyalkyl phosphate conjugates of cidofovir against herpesviruses and orthopoxviruses, in vitro

Compound	EC_{50} (μM)				CC_{50} (μM)
	HCMV[1]	HSV-1[2]	Cowpox[3]	Vaccinia[4]	
CDV	1.2 ± 0.43[a,c]	3.3 ± 3.7[a]	42 ± 5.4[b]	31 ± 5.4[b]	>317
HDP-CDV	0.0009 ± 0.0001[a]	0.0001 ± 0.0001[a]	0.5 ± 0.3[b]	0.6 ± 0.4[b]	31 ± 4
ODE-CDV	0.0009 ± 0.0001[a]	0.001 ± 0.002[a]	0.2 ± 0.2[b]	0.2 ± 0.1[b]	18 ± 3
HDP-P-CDV	0.26 ± 0.20[d]	0.06 ± 0.02	6.2 ± 3.3	3.2 ± 0.5	77 ± 4.4
ODE-P-CDV	0.03 ± 0.04[d]	0.00002 ± 0.00002	0.9 ± 0.5	1.1 ± 1.1	9.5 ± 4.3

Results expressed as EC_{50} and CC_{50} in μM and are the mean ± S.D. of three or more determinations unless otherwise indicated. *Abbreviations*: EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration; HDP-CDV, hexadecyloxypropyl-cidofovir; ODE-CDV, octadecyloxyethyl-cidofovir, HDP-P-CDV, 3-(hexadecyloxy)propyl-phospho-cidofovir (**6**), ODE-P-CDV, 2-(octadecyloxy)ethyl-phospho-cidofovir. Antiviral assays: [1]HCMV (AD-169) plaque reduction assay in HFF cells; [2]HSV-1 DNA reduction assay in MRC-5 Cells, [3]Cowpox Brighton, plaque reduction assay in HFF cells; [4]Vaccinia Copenhagen plaque reduction assay in HFF cells; [a]data from Beadle et al. (2002); [b]data from Kern et al. (2002); [c]data from Wan et al. (2005); [d]mean ± S.D. of two determinations.

Table 2
Antiviral activity of alkoxyalkyl and alkoxyalkyl phosphate conjugates of phosphonomethoxyethyladenine against HIV-1, in vitro

Compound	HIV-1 EC_{50}	MT-2 CC_{50}	S.I.
PMEA	1.30 ± 0.70 (7)	157 ± 54 (3)	121
HDP-PMEA	0.00002 ± 0.00003 (4)	0.06 ± 0.09 (3)	3000
HDP-P-PMEA	0.003 ± 0.005 (3)	0.018 ± 0.013 (3)	6

Results expressed as EC_{50} and CC_{50} in μM and are the mean $\pm$ S.D. of three or more determinations. *Abbreviations*: PMEA, phosphonomethoxyethyadenine; HDP-PMEA, hexadecyloxypropyl-phosphonomethoxyethyadenine; HDP-P-PMEA, 3-(hexadecyloxy)propyl-phospho-phosphonomethoxy-ethyadenine; assays: HIV-1$_{lai}$ p24 reduction assay in MT-2 cells. CC_{50} is the micromolar concentration which reduces viable cell number by 50% in a propidium iodide staining assay.

toxicity of HDP-P-CDV and ODE-P-CDV was higher than that of CDV, the selectivity index for the former two compounds against HCMV and HSV-1 ranged from 770 to >400,000. However, when compared against the antiviral activity of the HDP- and ODE-esters of CDV (Beadle et al., 2002), HDP-P-CDV and ODE-P-CDV are less active with the exception of ODE-P-CDV versus HSV-1 (Table 1). In cells infected with cowpox and vaccinia generally similar results were noted. Again, the antiviral activity of HDP-P-CDV and ODE-P-CDV was substantially greater than CDV against cowpox and vaccinia, but less than noted previously with HDP-CDV and ODE-CDV versus CDV (Kern et al., 2002; Keith et al., 2004).

We prepared HDP-P-PMEA (HDP-P-adefovir) and assessed its antiviral activity in MT-2 cells infected with HIV-1$_{lai}$ by p24 reduction (Table 2). HDP-P-PMEA had an EC_{50} of 3 nM against HIV-1 but its cytotoxicity in MT-2 cells was much greater than that of PMEA itself, $0.018\,\mu M$ versus $157\,\mu M$. The selectivity index of HDP-P-PMEA was only 6 versus 121 for PMEA. However, HDP-PMEA, the hexadecyloxy-propyl ester of PMEA, had an EC_{50} of 0.2 nM and a CC_{50} of 60 nM. The selectivity index for HDP-PMEA was 3000, twenty-five times greater than that of PMEA (Table 2).

In summary, our data suggest HDP-P-CDV and ODE-P-CDV are able to enter the cell more rapidly than CDV and are able to be metabolized to CDVpp. A phospholipase C type of enzyme might generate CDVp directly. However, HDP-P-CDV and ODE-P-CDV are less active than HDP-CDV and ODE-CDV indicating that their uptake and conversion to CDVpp is less extensive. Similar conclusions can be drawn with respect to PMEA, HDP-PMEA and HDP-P-PMEA against HIV-1 (Table 2). Thus, interposition of an additional phosphate residue between the alkoxyalkyl and CDV or PMEA leads to greater in vitro activity against herpes viruses and poxviruses than the activity observed with the unmodified nucleotide. However, the alkoxyalkyl-phosphate esters of CDV are generally less active than the corresponding alkoxyalkyl esters.

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