



Alkoxyalkyl prodrugs of acyclic nucleoside phosphonates enhance oral antiviral activity and reduce toxicity: Current state of the art

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ABSTRACT

Although the acyclic nucleoside phosphonates cidofovir, adefovir and tenofovir are approved for treating human cytomegalovirus, hepatitis B and HIV infections, respectively, their utility is limited by low oral bioavailability, renal toxicity and poor cell penetration. Research over the past decade has shown that these undesirable features can be eliminated by esterifying the compounds with an alkoxyalkyl group, in effect disguising them as lysophospholipids. In this modified form, the drugs are readily taken up in the gastrointestinal tract and have a prolonged circulation time in plasma. The active metabolite also has a long half life within cells, permitting infrequent dosing. Because these modified drugs are not recognized by the transport mechanisms that cause the accumulation of acyclic nucleoside phosphonates in renal tubular cells, they lack nephrotoxicity. Alkoxyalkyl esterification also markedly increases the *in vitro* antiviral activity of acyclic nucleoside phosphonates by improving their delivery into cells. For example, an alkoxyalkyl ester of cyclic-cidofovir, a less soluble compound, retains anti-CMV activity for 3 months following a single intravitreal injection. Two of these novel compounds, hexadecyloxypropyl-cidofovir (CMX001) and hexadecyloxypropyl-tenofovir (CMX157) are now in clinical development. This article focuses on the hexadecyloxypropyl and octadecyloxyethyl esters of cidofovir and (S)-HPMPA, describing their synthesis and the evaluation of their *in vitro* and *in vivo* activity against a range of orthopoxviruses, herpesviruses, adenoviruses and other double-stranded DNA viruses. The extension to other nucleoside phosphonate antivirals is highlighted, demonstrating that this novel approach can markedly improve the medicinal properties of these drugs.

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Abbreviations: Ad5, adenovirus type 5; ALT, alanine aminotransferase; AUC, area under curve; CDV, cidofovir; CDVp, cidofovir monophosphate; CDVpp, cidofovir diphosphate; CMV, cytomegalovirus; cPr-DAP, N⁶-cyclopropyl-2,6-diaminopurine; CPX, cowpox; dATP, deoxyadenosine triphosphate; dCTP, deoxycytosine triphosphate; ECTV, ectromelia virus; GCV, ganciclovir; HBV, hepatitis B virus; HCMV, human cytomegalovirus; HD, hexadecyl; HDP, hexadecyloxypropyl; HDP-CDV, hexadecyloxypropyl-cidofovir; HDP-PMEDAP, hexadecyloxypropyl-9-(2-phosphono-methoxyethyl)-2,6-diaminopurine; HDP-PMMPA, hexadecyloxypropyl-(R)-9-(2-phosphono-methoxypropyl)adenine; HHV, human herpes virus; HIV, human immunodeficiency virus; HPMP, 3-hydroxy-2-(phosphonomethoxy)propyl; HPMPA, (S)-9-(3-hydroxy-2-phosphono-methoxypropyl)adenine; HPMPAp, HPMPA monophosphate; HPMPApp, HPMPA diphosphate; HPPMP, 3-hydroxy-2-(phosphonomethoxy)propyl; PMPA, (R)-9-(2-phosphonomethoxypropyl)adenine; PPen, 5-phosphono-pent-2-en-1-yl; PPM, phosphonopropoxymethyl; PPMMP, phosphonopropoxymethyl-2,6-diaminopurine; RPV, rabbitpox virus; SCID, severe combined immunodeficiency; TCID, tissue culture infectious dose; VV, vaccinia virus; VV-LED, vaccinia virus-Lederle; VV-WR, vaccinia virus Western Reserve; VZV, varicella zoster virus; Wt, wild type.

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1. Introduction

Acyclic nucleoside phosphonates were first synthesized and their antiviral activity evaluated by the groups of Holy and De Clercq (De Clercq et al., 1987; Snoeck et al., 1988). There are currently three antivirals of this class approved for human use against cytomegalovirus (cidofovir, CDV, HPMPC), hepatitis B (adefovir, PMEA) and HIV (tenofovir, PMPA)(reviewed in De Clercq and Holy, 2005 and De Clercq, 2007). These compounds are analogs of deoxycytosine monophosphate (CDV) and deoxyadenosine monophosphate (PMEA, PMPA), in which the deoxyribose has been replaced with an acyclic moiety (Fig. 1). The intrinsic phosphonate of the acyclic nucleoside phosphonates is beneficial because it allows for bypass of the first phosphorylation required for activation

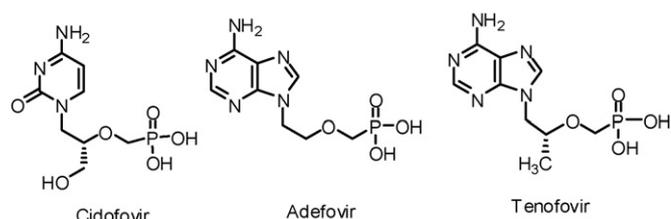


Fig. 1. Acyclic nucleoside phosphonate antivirals in clinical use.

of nucleosides during anabolic phosphorylation to the triphosphates. Therefore, compounds like cidofovir are fully active against thymidine kinase or UL-97 mutant viruses which are resistant to acyclovir or ganciclovir due to poor phosphorylation.

The principal drawbacks of acyclic nucleoside phosphonates are their poor oral bioavailability, low cellular uptake and their tendency to concentrate in the kidney proximal tubule, resulting in nephrotoxicity (Cundy, 1999). To achieve oral bioavailability, Gilead Sciences masked the dual negative charges on adefovir with dipivoxil (Noble and Goa, 1999) and on tenofovir with disoproxil fumarate (Kearney et al., 2004) leading to better oral absorption, but releasing adefovir or tenofovir in plasma where kidney proximal tubule organic anion transporters are prone to concentrate them.

This review will focus on an alternative method which improves the pharmacokinetic and antiviral performance of acyclic nucleoside phosphonates. The strategy relies on the disguise of acyclic nucleoside phosphonates as partially metabolized phospholipids (Painter and Hostetler, 2004). The rationale for these chemical modifications will be presented and the biochemistry, pharmacokinetics, *in vitro* and *in vivo* antiviral efficacy will be reviewed. The focus will be on the new alkoxyalkyl analogs of CDV (HPMPC) and (S)-HPMPA, but the generality of the approach as applied to many different types of acyclic nucleoside phosphonates will also be highlighted. The status of clinical development of two agents of

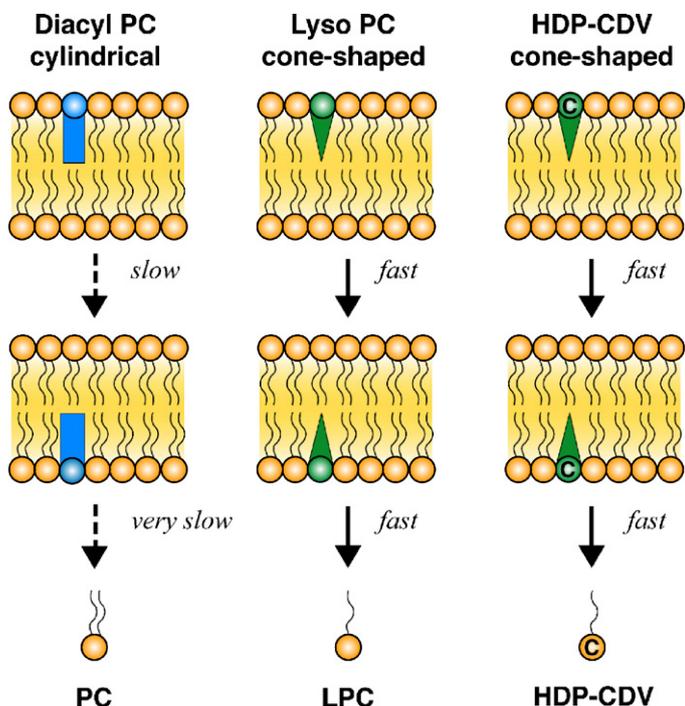


Fig. 2. Interactions of phosphatidylcholine, lysophosphatidylcholine and HDP-CDV with phospholipid bilayer membranes.

this class, HDP-CDV (CMX001) and HDP-tenofovir (CMX157), will be reviewed.

2. Lysophospholipid prodrug strategy for acyclic nucleoside phosphonates

2.1. Phospholipid membrane interactions

In the fluid mosaic model, biological membranes are fluid phospholipid bilayers with embedded proteins as described by Singer and Nicolson (1972). Membrane phospholipids are comprised primarily of diacylglycerophosphocholine (phosphatidylcholine, PC) and other diacyl species such as phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol. Membrane phospholipids have two acyl esters and assume a cylindrical shape. They do not readily translocate from the outer to the inner leaflet of phospholipid bilayer membranes (Fig. 2). In cellular membranes, the distribution of phospholipids and sphingomyelin between inner and outer membrane leaflets of plasma membranes is asymmetric implying mechanisms controlling transverse distribution (Van Deenen, 1981; Zachowski, 1993). Membrane proteins have been identified (aminophospholipid translocases,

flippases) which facilitate transbilayer movement of diacylphospholipids (Zachowski, 1993; Raggars et al., 2002; Kol et al., 2004). These proteins may be ATP-dependent or ATP-independent (Raggars et al., 2002). Intracellular or plasma phospholipid transfer proteins are also known which have the ability to transfer lipids between plasma lipoproteins or intracellularly between membranes (Wirtz, 2006). Finally, lipid rafts (detergent resistant microdomains) enriched in sphingomyelin, cholesterol and certain proteins, are another important membrane feature involved with cellular traffic of membranes, lipids and proteins (Simons and Vaz, 2004; Helms and Zurzolo, 2004; Hanzal-Bayer and Hancock, 2007).

In contrast to cylinder-shaped diacylphospholipids, lysophospholipids lack the acyl ester at the *sn*-2 hydroxyl of glycerol, are cone-shaped and may disturb the bilayer structure which results in spontaneous transbilayer movement (Fig. 2). Because lysophospholipids have a single acyl chain, their off-rate from phospholipid bilayer membranes is higher than that of diacylphospholipids which allows for rapid rates of diffusion of lysophospholipids inside the cell (Raggars et al., 2002). In principle, lysophospholipids might also undergo protein-mediated transbilayer movement but this has not been extensively studied.

Acyclic nucleoside phosphonates have a double negative charge at physiologic pH and have been found to enter cells very slowly by endocytosis (Connelly et al., 1993). In designing lipid analogs of acyclic nucleoside phosphonates to increase oral absorption in the small intestine and to facilitate cellular uptake and metabolism, we chose to focus on lysophospholipid analogs because of the ease with which they cross cellular membranes and desorb into the cytoplasm where they undergo metabolism and conversion to their diphosphates.

2.2. Design paradigm for acyclic nucleoside phosphonate analogs

Previous studies of lysophosphatidylcholine (LPC) absorption in rats or from inverted intestinal sacs indicated that about 40% of lysophosphatidylcholine is absorbed intact without further enzymatic cleavage (Scow et al., 1967; Nilsson and Borgstrom, 1967). In the small intestinal enterocyte, LPC is reacylated to PC and incorporated into chylomicrons which are secreted into intestinal lymphatics. To enhance absorption, we chose to disguise acyclic nucleoside phosphonate antivirals as LPC analogs, making some key changes in the molecule to optimize stability and oral bioavailability. The structure of LPC is shown in Fig. 3 (left panel) and the arrows indicate the enzymatic cleavage points of phospholipases A₁, C, D and lysophospholipase (Van den Bosch, 1982). There are several key features of the structure of HDP-CDV (right panel, Fig. 3) that make it more stable and more prone to good absorption from the intestine. First, the acyl ester bond at the *sn*-1 position of LPC has been replaced with an ether linkage, preventing hydrolysis of the acyl group by lysophospholipase during absorption. The alkyl ether also increases compound stability. Second, the hydroxyl

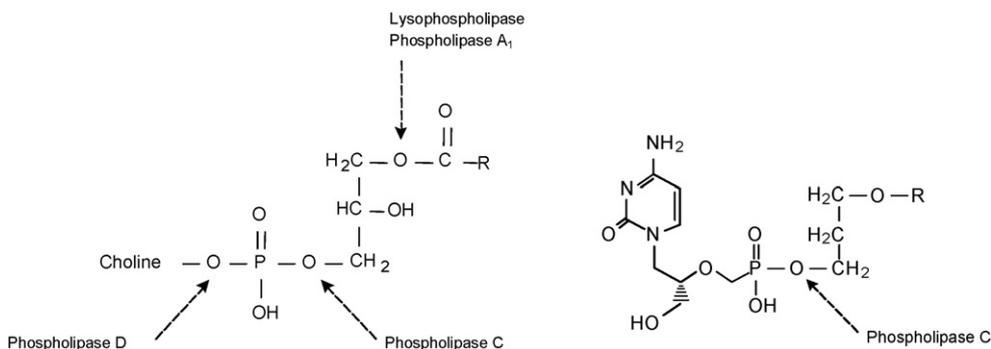


Fig. 3. Predicted major metabolic cleavage sites for lysophosphatidylcholine and HDP-CDV.

at the *sn*-2 position of glycerol in LPC has been replaced with a hydrogen atom to prevent reacylation by lysophosphatidylcholine acyltransferases present in small intestinal enterocytes and other tissues. Finally, it should be noted that compounds like CDV and (*S*)-HPMPA have a phosphonate ($-P-CH_2-$) linkage to the acyclic nucleoside which is not subject to cleavage by a phospholipase D or a phosphodiesterase. This means that the only metabolic cleavage which can readily occur is that catalyzed by phospholipase C (Fig. 3), an enzyme which we first demonstrated in mammalian tissues in 1980 (Matsuzawa and Hostetler, 1980; Hostetler and Hall, 1980). Phospholipase C is not present in plasma or pancreatic secretions, providing stability for HDP-CDV and other compounds of this type during oral absorption and transport in plasma to tissues. This general approach is applicable to all nucleoside phosphonates.

3. Antiviral evaluation of hexadecyloxypropyl and octadecyloxyethyl esters of CDV and (*S*)-HPMPA

3.1. Antiviral effects *in vitro*

Cidofovir (CDV) and (*S*)-HPMPA were converted to their hexadecyloxypropyl and octadecyloxyethyl esters (Fig. 4) and compared their antiviral activity with CDV and (*S*)-HPMPA (Beadle et al., 2002, 2006; Beadle, 2007).

3.1.1. CDV analogs

HDP-CDV and ODE-CDV showed large increases in antiviral activity against four poxviruses compared with CDV (Table 1). Unmodified CDV has EC_{50} values ranging from 12 to 46 μ M while HDP-CDV and ODE-CDV had EC_{50} s ranging from 0.003 to 0.9 (Kern et al., 2002; Buller et al., 2004). Variola appeared to be more sensitive to inhibition by HDP-CDV and ODE-CDV than other poxviruses with EC_{50} values of 0.10 and 0.03 μ M, respectively (Huggins et al., 2002). Against herpesviruses, HDP and ODE esters of CDV showed remarkable activity in the low nanomolar EC_{50} range (0.9 nM for HCMV and 8–60 nM for HSV-1,-2 and HHV-8), versus unmodified CDV which ranged from 0.38 (HCMV) to 65 μ M (EBV) (Williams-Aziz et al., 2005). Increases in antiviral activity of several logs were routinely noted versus unmodified CDV. HDP and ODE esters were generally similar in activity against herpesviruses. Against five strains of adenovirus, the order of activity was ODE-CDV > HDP-CDV \gg CDV, and increases of activity of 2–3 logs were commonly seen versus unmodified CDV (Hartline et al., 2005). HDP-CDV and ODE-CDV also showed increased activity against BK virus (Randhawa et al., 2006) and orf viruses (Dal Pozzo et al., 2007).

Table 1

Antiviral effects of CDV, HDP-CDV and ODE-CDV on replication of poxviruses, herpesviruses, adenoviruses and polyoma viruses, *in vitro*.

	EC_{50} (μ M)		
	CDV	HDP-CDV	ODE-CDV
Poxviruses			
Cowpox, Brighton	44.7 ^a	0.9 ^a	0.3 ^a
Vaccinia, Copenhagen	46.2 ^a	0.8 ^a	0.2 ^a
Variola, Bangladesh	27.3 ^b	0.10 ^b	0.03 ^b
Ectromelia virus	12.0 ^c	0.50 ^c	0.20 ^c
Rabbitpox virus	39.0 ^d	0.50 ^d	–
Herpes viruses			
HCMV	0.38 ^e	0.0009 ^e	0.0009 ^e
HSV-1	5.5 ^f	0.06 ^f	0.02 ^f
HSV-2	5.1 ^f	0.01 ^f	0.008 ^f
HHV-8	2.6 ^f	0.02 ^f	0.03 ^f
EBV	65.6 ^f	0.03 ^f	0.10 ^f
VZV	0.5 ^f	0.0004 ^f	0.0001 ^f
Adenoviruses			
AdV-3	2.0 ^g	0.01 ^g	<0.006 ^g
AdV-5	0.5 ^g	<0.009 ^g	<0.008 ^g
AdV-7	1.3 ^g	0.02 ^g	<0.009 ^g
AdV-8	1.0 ^g	0.03 ^g	<0.008 ^g
AdV-31	1.4 ^g	0.28 ^g	0.090 ^g
Polyoma virus			
BK virus	115 ^h	0.13 ^h	0.083 ^h
Orf virus			
IT-C2	0.24 ⁱ	0.037 ⁱ	0.0032 ⁱ
NZ2	1.26 ⁱ	0.18 ⁱ	0.03 ⁱ

^a Kern et al. (2002).

^b Huggins et al. (2002).

^c Buller et al. (2004).

^d Prichard (personal communication, 2008).

^e Beadle et al. (2002).

^f Williams-Aziz et al. (2005).

^g Hartline et al. (2005).

^h Randhawa et al. (2006).

ⁱ Dal Pozzo et al. (2007).

3.1.2. (*S*)-HPMPA analogs

(*S*)-HPMPA is the adenine analog of CDV (HPMPC) and is known to be more active against poxviruses than CDV. HDP and ODE esters of (*S*)-HPMPA were tested against cowpox, vaccinia, variola and ectromelia viruses *in vitro* (Beadle et al., 2006; Huggins, personal communication, 2006). (*S*)-HPMPA itself was 3–10 times more active against poxvirus replication than CDV. Esterification to HDP-(*S*)-HPMPA or ODE-(*S*)-HPMPA resulted in large increases

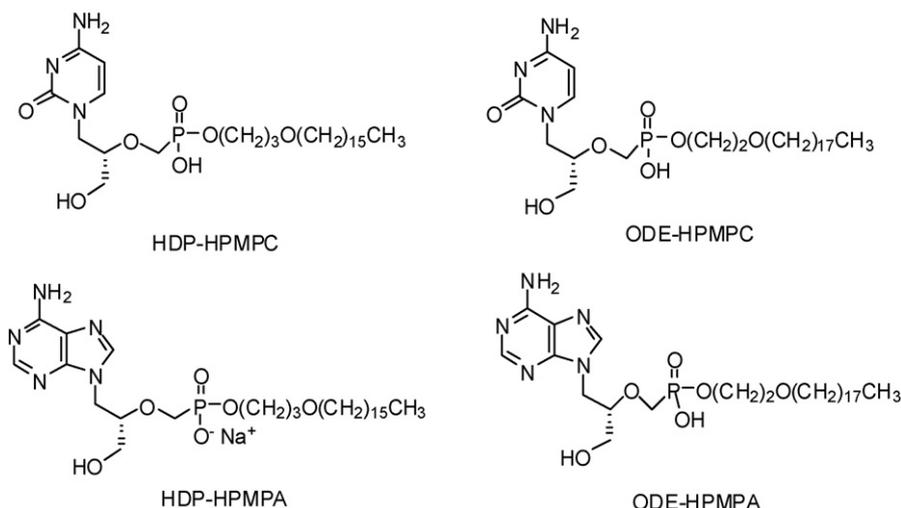


Fig. 4. Structure of key alkoxyalkyl esters of CDV and (*S*)-HPMPA.

in antipoxvirus activity ranging from 160 to 270-fold (Beadle et al., 2006). In HCMV infected cells, the antiviral activity of HDP-(S)-HPMPA and ODE-(S)-HPMPA was increased by 270-fold versus (S)-HPMPA (Beadle et al., 2006) (Table 2). Studies with other herpesviruses have not yet been reported. In adenovirus infected cells, ODE-(S)-HPMPA was the most active compound with EC₅₀ values of 0.04–0.16 μM compared with 0.19–1.1 μM with HDP-(S)-HPMPA (Hartline et al., 2005). (S)-HPMPA was previously shown to be inactive against HIV-1 (Balzarini et al., 1993). However, when HDP and ODE esters were prepared and tested against HIV-1, EC₅₀ values in the 0.4–7 nM range were found (Hostetler et al., 2006). HDP-(S)-HPMPA and ODE-(S)-HPMPA are also active in the low nanomolar range against BK virus (Randhawa et al., 2006; Randhawa et al., 2008) and orf viruses (Dal Pozzo et al., 2007).

Esterification of CDV and (S)-HPMPA with HDP and ODE was designed to increase oral bioavailability based on the resemblance to lysophosphatidylcholine (Fig. 3). However, the large increases in antiviral activity *in vitro* were unexpected. Both CDV and (S)-HPMPA have been reported to inhibit replication of all double-stranded DNA viruses and the increased antiviral activity observed after alkoxyalkyl esterification does not appear to be specific for a particular type of virus (Tables 1 and 2). Although (S)-HPMPA was reported to be inactive against HIV (Balzarini et al., 1993), HDP-(S)-HPMPA and ODE-(S)-HPMPA are highly active with low nanomolar EC₅₀ values *in vitro* (Hostetler et al., 2006), suggesting that the alkoxyalkyl ester strategy can extend a compound's range of antiviral activity in some cases (Table 2).

Table 2

Antiviral effects of (S)-HPMPA, HDP-(S)-HPMPA and ODE-(S)-HPMPA on replication of poxviruses, herpesviruses, adenoviruses, HIV, Polyoma, orf and hepatitis B viruses, *in vitro*.

	EC ₅₀ (μM)		
	(S)-HPMPA	HDP-(S)-HPMPA	ODE-(S)-HPMPA
Poxviruses			
Cowpox	4.0 ^a	0.02 ^a	0.02 ^a
Vaccinia	2.7 ^a	0.01 ^a	0.01 ^a
Variola	7.9 ^b	<0.05 ^b	<0.05 ^b
Ectromelia	0.24 ^c	0.049 ^c	0.032 ^c
Herpes viruses			
HCMV	0.82 ^a	0.003 ^a	0.003 ^a
Adenoviruses			
AdV-3	1.9 ^d	0.43 ^d	0.14 ^d
AdV-5	0.50 ^d	0.19 ^d	0.04 ^d
AdV-7	2.2 ^d	1.0 ^d	0.06 ^d
AdV-8	1.1 ^d	1.1 ^d	0.07 ^d
AdV-31	2.5 ^d	0.29 ^d	0.16 ^d
HIV-1			
HIV-1 _{lai}	78 ^e	0.007 ^e	0.0004 ^e
Polyoma virus			
BK virus	n.d.	0.02 ^f	0.03 ^f
Orf virus			
IT-C2	0.18 ^g	0.011 ^g	0.0026 ^g
NZ2	0.61 ^g	0.036 ^g	0.006 ^g
Hepatitis B virus			
HBV wt	0.99 ^h	0.25 ^h	0.11 ^h

^a Beadle et al. (2006).

^b Huggins (personal communication 2006).

^c Buller and Hostetler (unpublished, 2008).

^d Hartline et al. (2005).

^e Hostetler et al. (2006).

^f Randhawa et al. (2008).

^g Dal Pozzo et al. (2007).

^h Hostetler et al. (2005).

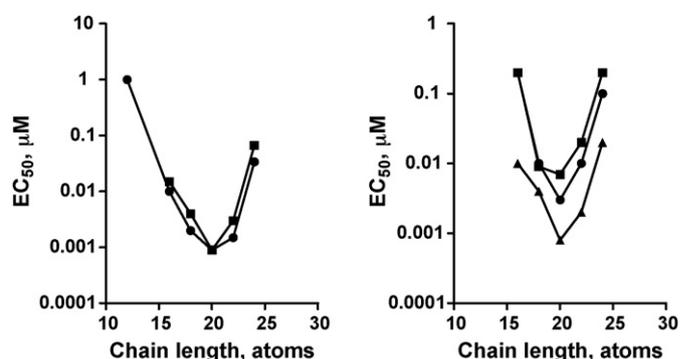


Fig. 5. Effect of alkoxypropyl-cidofovir chain length on antiviral activity against HCMV, MCMV and drug-resistant HCMV isolates. Left panel: Wild type CMVs; HCMV (AD169), circles; MCMV, squares; right panel: Drug resistant CMV isolates: VR4760 (GCV/PFA resistant, UL97/DNA pol mutations), triangles; C8914-6 (GCV resistant, UL97 mutation), circles; 759 D100 (GCV/CDV resistant, DNA pol mutation), squares. Adapted from Wan et al. (2005) and Williams-Aziz et al. (2005).

3.2. Effect of alkyl chain length and linker moiety

To determine the optimal length for the alkoxyalkyl chain, a series of analogs of CDV were synthesized with alkoxyalkyl chains ranging from 12 to 24 atoms. Their activity against HCMV and ganciclovir-resistant HCMV isolates is shown in Fig. 5. The optimal chain length is 20 atoms, representing a 16 carbon alkyl group (hexadecyl) and an oxypropyl linker. HDP-CDV represents the optimal structure for both wild type HCMV (left panel) and three drug resistant isolates of HCMV (right panel) having UL97 and DNA polymerase mutations (Wan et al., 2005; Williams-Aziz et al., 2005). Alkoxyalkyl esters with shorter chains (12–16 atoms) or long chains (24 atoms) exhibit less activity. This is probably due to increased water solubility of the short chains, while the 24 atom compound has a lower fluidity and may be hindered in its ability to traverse membranes or by slow hydrolysis by phospholipase C. HDP-CDV retains substantial antiviral activity against the CMV strains resistant to ganciclovir, foscarnet and CDV, even those having DNA polymerase mutations or double mutations in UL97 and DNA polymerase retaining excellent EC₅₀ values from 0.8 to 7 nM (Fig. 5). CDV compounds lacking a linker are generally less active and those having the oxyethyl linker are usually the most active at any given chain length compared with the oxypropyl moiety (Wan et al., 2005; Williams-Aziz et al., 2005).

4. Mechanism of increased antiviral activity of HDP-CDV and HDP-(S)-HPMPA

4.1. Cell uptake and anabolic phosphorylation

Antiviral studies with CDV, HDP-CDV and (S)-HPMPA and HDP-(S)-HPMPA showed dramatic increases in antiviral activity against many dsDNA viruses as discussed in the prior section. In some cases, compounds previously reported to be inactive against HIV-1 ((S)-HPMPA) were shown to have low nanomolar EC₅₀ values when converted to HDP-(S)-HPMPA or ODE-(S)-HPMPA (Hostetler et al., 2006). To evaluate the mechanisms responsible for the increased antiviral activity, ¹⁴C-labeled CDV, HDP-CDV, (S)-HPMPA, and HDP-(S)-HPMPA were incubated with MRC-5 human lung fibroblasts and cell uptake and anabolic phosphorylation of the compounds to the active metabolites (CDV and (S)-HPMPA diphosphates) was determined (Aldern et al., 2003; Magee et al., 2008). CDV and (S)-HPMPA uptake increased very slowly over 24 h reaching levels of 3.6 and 2.8 pmol/well (Fig. 6), consistent with their reported uptake by

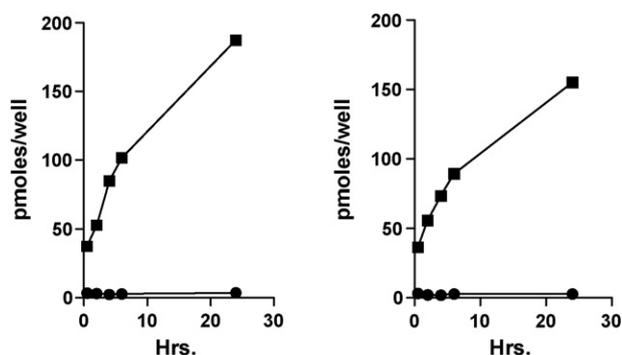


Fig. 6. Cellular uptake of CDV, (S)-HPMPA and their HDP esters in MRC-5 cells. Left panel: CDV, circles; HDP-CDV, squares; right panel: (S)-HPMPA, circles; HDP-(S)-HPMPA, squares. Adapted from Aldern et al. (2003) and Magee et al. (2008).

fluid phase endocytosis (Connelly et al., 1993; Palu et al., 1991). In contrast, HDP-CDV and HDP-(S)-HPMPA uptake increased rapidly over 6 h and progressively to 24 h achieving cellular levels of 187 and 155 pmol/well, respectively (Fig. 6), representing an increase of 52–55-fold versus unmodified CDV and (S)-HPMPA. The area under curve for HDP-CDV and HDP-(S)-HPMPA was 41 and 42-fold greater than that of CDV and (S)-HPMPA. Interestingly, the uptake profile for the two HDP esters in MRC-5 cells is very similar, suggesting that the hexadecyloxypropyl lipid moiety (not the nucleoside polar head group) is driving cellular uptake because of the propensity of these compounds to insert spontaneously into the phospholipid bilayer membrane of cells (Fig. 2).

Increased entry of HDP-CDV and HDP-(S)-HPMPA into the cell is not sufficient to explain the marked increases in antiviral activity noted above. To have antiviral activity, HDP-CDV and HDP-(S)-HPMPA must be hydrolyzed to CDV and (S)-HPMPA by a cellular phospholipase C followed by anabolic phosphorylation to their diphosphates (CDVpp and (S)-HPMPApp) (Hostetler, 2007). In the MRC-5 uptake studies, we examined the metabolites in the cells and measured levels of CDV, CDVp and CDVpp and (S)-HPMPA, (S)-HPMPAp and (S)-HPMPApp by Partisil SAX HPLC (Fig. 7). With exposure to 10 μ M CDV, cell levels of CDVpp at 24 h were 1.3 pmol/well versus 132 pmol/well with HDP-CDV, an increase of 100-fold (Aldern et al., 2003). When exposed to 10 μ M (S)-HPMPA, (S)-HPMPApp levels at 24 h were 21 pmol/well compared with 451 pmol/well with HDP-(S)-HPMPA, and increase of 21-fold (Magee et al., 2008). A timed study showed that HDP-(S)-HPMPA conversion to (S)-HPMPApp is much more rapid than of

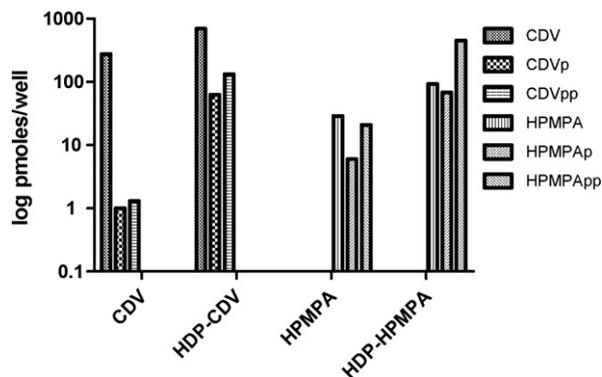


Fig. 7. Metabolism and anabolic phosphorylation of CDV, (S)-HPMPA and their HDP esters in MRC-5 cells *in vitro*. 10 μ M 14 C-labeled CDV, (S)-HPMPA, HDP-CDV and HDP-(S)-HPMPA were applied to wells containing confluent MRC-5 cells and incubated for 24 h. Anabolic phosphorylation to their monophosphates (CDVp and (S)-HPMPAp) and diphosphates (CDVpp and (S)-HPMPApp) was measured by Partisil SAX HPLC. Adapted from Aldern et al. (2003) and Magee et al. (2008).

HDP-CDV (Aldern et al., 2006). When the cells were exposed to HDP-CDV or HDP-(S)-HPMPA for 24 h followed by washout of the compounds, the disappearance of the respective diphosphates in the cell was slow with a $T_{1/2}$ of about 7 days which suggests that less frequent dosing might be possible *in vivo* (Aldern et al., 2003, 2006).

The cellular metabolism studies comparing CDV with HDP-CDV and (S)-HPMPA with HDP-(S)-HPMPA indicate that the cell uptake and conversion of the HDP analogs to their diphosphates is 0.5–1 log greater than the unmodified compounds. This, in turn, probably accounts for the greater antiviral activity of the alkoxyalkyl esters of the acyclic nucleoside phosphonates. Slow uptake of CDV and (S)-HPMPA is due to poor penetration of the cell membranes because of the dual negative charge at physiologic pH and the slow nature of fluid phase endocytosis, their common mechanism of uptake (Connelly et al., 1993; Palu et al., 1991). In contrast, HDP-CDV and HDP-(S)-HPMPA uptake is very rapid because these compounds spontaneously insert into cell membranes, translocate to the inner membrane leaflet and desorb readily to be metabolized by phospholipase C and anabolic phosphorylation to the antiviral diphosphates (Figs. 2, 6 and 7).

4.2. Mechanism of action of cidofovir and (S)-HPMPA diphosphates on the vaccinia E9L DNA polymerase

4.2.1. Cidofovir inhibition of the CMV and vaccinia polymerases

CDV undergoes anabolic phosphorylation in the cell to CDV diphosphate (CDVpp), the active antiviral. Prior studies with the DNA polymerase of CMV showed that CDVpp, an analog of dCTP, inhibits the polymerase by incorporation into the growing DNA chain opposite template Gs. Incorporation of a single CDV causes a 31% decrease in the rate of elongation but when two successive CDVs are added, chain termination results and 3'-to-5' exonuclease activity is blocked (Xiong et al., 1997). However, with the E9L DNA polymerase of vaccinia virus, the mechanism of inhibition is somewhat different. CDVpp is a poor substrate for DNA synthesis relative to dCTP but when incorporated into the growing DNA strand, CDV terminated primers are good substrates for the next dNTP addition. However, after the addition of CDV + 1 base by the E9L polymerase, the rate of primer extension is greatly slowed and the 3'-to-5' exonuclease activity is completely blocked (Magee et al., 2005). Recently, an additional mechanism of action was discovered; it was shown that templates containing a CDV residue cannot be extended beyond the CDV base by the E9L polymerase, effectively blocking further rounds of replication (Table 3) (Magee et al., 2008).

4.2.2. (S)-HPMPA inhibition of the vaccinia DNA polymerase

(S)-HPMPA was found to block adenovirus DNA polymerase at the level of chain elongation although the detailed mechanism is not clear (Mul et al., 1989). Against vaccinia virus replication, (S)-HPMPA is 17 times more active than CDV (Tables 1 and 2). Surprisingly, unlike CDVpp, (S)-HPMPApp is an excellent substrate for the E9L polymerase with a K_m and V_{max} similar to that of dATP. It is readily incorporated into the growing DNA strand and, unlike CDVpp, it does not slow chain extension but blocks 3'-to-5' exonuclease activity when in the penultimate position. At the primer terminus HPMPA can still be excised. Why then is (S)-HPMPA 17 times more inhibitory to vaccinia replication than CDV? Because (S)-HPMPApp is readily incorporated into DNA and does not slow chain extension, many (S)-HPMPApp residues may be incorporated into the template strand and templates containing (S)-HPMPA cannot be extended, blocking further rounds of replication leading to template strand inhibition (Magee et al., 2008).

Table 3
Mechanism of action of CDV and (S)-HPMPA diphosphates versus CMV and vaccinia DNA polymerases.

Viral polymerase	Compound	Action	Result	3'-to-5' exo-nuclease	Template strand extension	Refs.
CMV DNA polymerase	CDVpp	Incorporation of 1 CDV base + any base Incorporation of 2 consecutive CDV bases	Slows chain extension Chain termination	Inhibited	Not determined	Xiong et al. (1997)
Vaccinia E9L DNA polymerase	CDVpp	Incorporation of CDV + any base	Primer extension greatly slowed	Inhibited	Blocked	Magee et al. (2005, 2008)
Vaccinia E9L DNA polymerase	(S)-HPMPApp	Incorporation of one or more (S)-HPMPAs	No effect on primer extension	Inhibited	Blocked	Magee et al. (2008)

5. Pharmacokinetics and tissue distribution of HDP-CDV and HDP-(S)-HPMPA in mice

5.1. Oral pharmacokinetics of HDP-CDV

HDP-CDV was designed to resemble lysophosphatidylcholine, a natural phospholipid metabolite which is substantially absorbed without further hydrolysis (Scow et al., 1967). We replaced the phosphocholine moiety of LPC with CDV, eliminated the *sn*-2 hydroxyl of glycerol to prevent reacylation by acylCoA:1-acyl-*sn*-glycero-3-phosphocholine-*O*-acyltransferase [EC 2.3.1.62]. The *sn*-1 acyl ester bond was changed to an ether linkage to prevent cleavage by pancreatic or cellular lysophospholipases. To see if this approach would increase oral bioavailability, radioactive CDV and HDP-CDV were prepared. Oral administration of equimolar doses (17.8 $\mu\text{mol/kg}$) of [2- ^{14}C]-CDV or HDP-[2- ^{14}C]-CDV to mice produced very low plasma levels with CDV and substantially greater levels with HDP-CDV (Fig. 8). The C_{max} for CDV was 0.8 μM versus

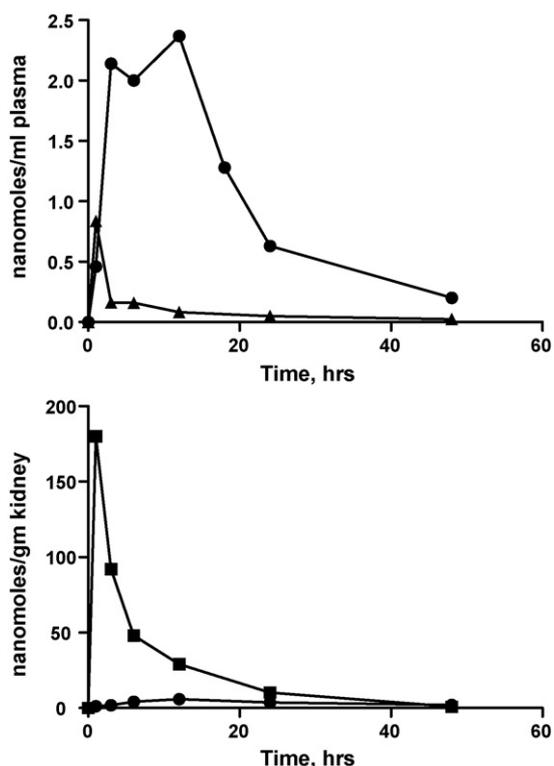


Fig. 8. Key pharmacokinetic features of [2- ^{14}C]-CDV and HDP-[2- ^{14}C]-CDV after administration to mice: plasma and kidney levels of drug and metabolites. Upper panel: Plasma levels of [^{14}C]-CDV (triangles) or HDP-[^{14}C]-CDV (circles) after oral administration to mice at 17.8 $\mu\text{mol/kg}$. Lower panel: Kidney levels of [2- ^{14}C]-CDV (squares) after intraperitoneal administration (17.8 $\mu\text{mol/kg}$) or HDP-[2- ^{14}C]-CDV (circles) after oral administration (17.8 $\mu\text{mol/kg}$) to mice. Adapted from Ciesla et al. (2003).

2.3 μM for HDP-CDV and the area under curve for HDP-CDV was 12-fold greater than that of CDV. The relative oral bioavailability was estimated to be 88% for HDP-CDV and the $T_{1/2}$ for HDP-CDV was 14.9 h (Ciesla et al., 2003). Analysis of the plasma indicated that about 50% of the radioactivity was intact HDP-CDV, the remainder being CDV and a metabolite. Similar results were obtained with HDP-[8- ^{14}C]-HPMPA which was estimated to have a C_{max} of 1.1 μM , a relative oral bioavailability of 73% and a $T_{1/2}$ of 11 h (Hostetler et al., 2005; Magee et al., 2008).

Nephrotoxicity is the dose limiting toxicity of intravenous CDV in humans because the compound is concentrated by the organic anion transporter in renal proximal tubule cells (Izzedine et al., 2005). With HDP-CDV, another key pharmacokinetic finding was that 17.8 $\mu\text{mol/kg}$ of HDP-CDV given orally results in very low kidney uptake, C_{max} 5.9 nmol/g. However, in mice, when an equimolar dose of CDV was given intraperitoneally, the C_{max} in kidney was 180 nmol/g, a 30-fold increase (Fig. 8). The area under curve in kidney was 170 nmol h/g for HDP-CDV versus 1170 nmol h/g for CDV, an increase in AUC of 6.9-fold (Ciesla et al., 2003). Thus, masking of one of the negative charges on the phosphonate of CDV by esterification with HDP greatly reduces kidney uptake, the major site of dose-limiting toxicity with CDV.

A scheme showing the proposed pathways of intestinal absorption of HDP-CDV is shown in Fig. 9. Like lysophosphatidylcholine (LPC), HDP-CDV is thought to be absorbed at the apical brush border in mixed micelles of bile salts, monoglycerides and lysophospholipids. Following absorption, HDP-CDV enters the portal vein directly leading to high levels of drug and metabolites in liver (Ciesla et al., 2003). LPC is reacylated in the enterocyte endoplasmic reticulum to phosphatidylcholine (PC) and PC and HDP-CDV (red symbols) are incorporated in to lipid-rich chylomicrons. Assembled chylomicrons contain triglyceride and cholesterol esters in the core while the surface coat consists of apolipoprotein B48, PC and HDP-CDV (Fig. 9). Chylomicrons are secreted into the small intestinal lymphatic system which leads successively to the thoracic duct, left subclavian vein, innominate vein and ultimately to the superior vena cava. After returning to the right side of the heart, chylomicrons pass through the lung and then, via the left side of the heart, into the arterial circulation. Interestingly, it appears to be possible to manipulate the fraction of the acyclic nucleoside phosphonate lipid prodrug passing through the chylomicron pathway (lung) versus the portal vein (liver) by making the lipid ester more hydrophobic. For example, 1-*O*-octadecyl-2-*O*-benzyl-*sn*-glycero-CDV, a more hydrophobic prodrug than HDP-CDV because of the 2-*O*-benzyl group (instead of hydrogen), has been shown to give rise to higher lung levels than HDP-CDV after oral administration due to greater partitioning into the chylomicron pathway (Hostetler et al., 2007).

5.2. Oral pharmacokinetics of HDP-(S)-HPMPA

Oral administration of HDP-(S)-[8- ^{14}C]-HPMPA to mice as a single dose of 10 mg/kg gives a C_{max} for HDP-(S)-HPMPA

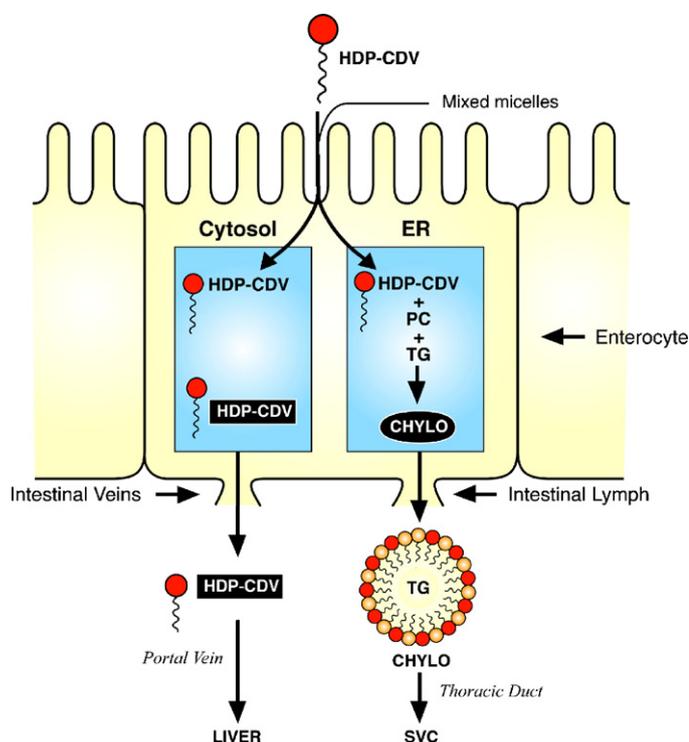


Fig. 9. Proposed pathways of intestinal absorption of HDP-CDV. HDP-CDV (dark symbols) in mixed micelles of bile salts, monoglycerides, and lysophospholipids is absorbed at the brush border of the enterocyte. HDP-CDV enters the portal vein directly. Chylomicrons are formed in the endoplasmic reticulum with a core consisting of triglycerides (TG) and cholesterol esters and a surface coat consisting of apoprotein B-48 (not shown in the diagram), phosphatidylcholine (yellow symbols) and HDP-CDV (red symbols) and secreted into the small intestinal lymphatics.

and metabolites of $1.1 \mu\text{M}$ at 1 h. The plasma $\text{AUC}_{0-24\text{h}}$ was 12.1 nmol h/ml versus 16.4 nmol h/ml for intraperitoneal administration indicating a relative oral bioavailability of 74% compared with 24% for (S)-HPMPA (Quenelle et al., 2007a). The dose-limiting toxicity of HDP-(S)-HPMPA in rodents was gastrointestinal and no evidence of nephrotoxicity was apparent (Hostetler, unpublished data, 2007). Thus, the esterification of CDV and HPMPA with HDP leads to marked increases in oral bioavailability and is probably a general finding with compounds of this class.

In summary, conversion of CDV and (S)-HPMPA to HDP-CDV and HDP-(S)-HPMPA results in the following:

1. Increased cellular uptake and conversion to CDVpp and (S)-HPMPApp, the active antiviral metabolites.
2. Multiple log increases antiviral activity against double-stranded DNA viruses compared with the unmodified nucleobases.
3. Bypass of the initial phosphorylation required with conventional nucleoside analogs.
4. Oral bioavailability
5. Full activity against ganciclovir and acyclovir resistant herpes group viruses
6. Elimination of nephrotoxicity
7. Oral activity in animal models of vaccinia, cowpox, mousepox, rabbitpox, human CMV, murine CMV, adenovirus infections and viral retinitis.

6. Efficacy of HDP-CDV and related compounds in animal models of disease

6.1. Orthopoxvirus infections

Treatment of cowpox and vaccinia infection: HDP-CDV was originally designed to address the need for oral prophylaxis or treatment of smallpox because of concerns about possible bioterrorism with this agent. HDP-CDV was first tested orally in lethal models of cowpox (CPX) and vaccinia virus (VV) infection in mice. Both HDP-CDV and ODE-CDV were effective in reducing mortality when given orally prior to or 1, 2 or 3 days following intranasal infection with CPX or VV in BALB/c mice (Quenelle et al., 2004). Smee et al. (2004) treated mice 24 h after infection with VV-IHD strain with a single oral dose of 100, 50 or 25 mg/kg of HDP-CDV and found highly significant protection with zero to 20% mortality versus 100% mortality in untreated mice.

6.1.1. Treatment of ectromelia virus infection

In lethal aerosol challenge model with ectromelia virus (ECTV) infection, HDP-CDV and ODE-CDV given orally at 5 and 10 mg/kg prevented mortality from a high dose challenge in A/Ncr mice. Viral titers in liver and spleen were reduced below the limit of detection at 7 days while lung titers were reduced only slightly at 10 mg/kg (Buller et al., 2004).

In more detailed studies, high dose ($10,000 \times \text{LD}_{50}$) ECTV infection by the aerosol or intranasal route was treated with HDP-CDV (CMX001) 10 mg/kg on the first day followed by 2.5 mg/kg on day 3. Untreated A/Ncr mice had 100% mortality by day 12 versus 25% mortality in HDP-CDV treated animals by day 20 (Parker et al., 2008). With high dose intranasal infection, animals treated with 10 mg/kg on day 1 and 2.5 mg/kg of HDP-CDV on day 3 were completely protected versus 100% mortality by day 7 in untreated mice. The dose of HDP-CDV required to produce 100% survival varies with the infectious dose of ECTV in the intranasal model. At high viral input doses (500–5000 pfu), 8 mg/kg HDP-CDV provided complete protection while 4 mg/kg \times 5 days gave 62–75% protection. With 50 pfu of ECTV, 2 mg/kg of HDP-CDV for 5 days provided 87% protection. However, with low dose infections with less than 5 pfu, 2 mg/kg of HDP-CDV for 5 days provided 100% protection (Parker et al., 2008). Since the upper limit of natural infections with variola may approach 50 pfu, attempts were made to determine the dose of HDP-CDV required providing full protection. Infected A/Ncr mice were treated with doses of HDP-CDV ranging from 0.3 to 5 mg/kg daily for 14 days. Daily treatment with doses of 1.25 mg/kg or greater for 14 days provided complete protection from mortality but 2.5 mg/kg or greater was required to minimize infection-related weight loss in this model (Parker et al., 2008).

6.1.2. Synergistic efficacy of HDP-CDV and ST-246 treatment of cowpox infection

ST-246 is an orally bioavailable drug which inhibits poxvirus infection by interfering with intracellular maturation of the virus (Yang et al., 2005), while HDP-CDV inhibits the viral DNA polymerase by inhibiting chain elongation (Magee et al., 2005) and produces template strand inhibition when the templates contain CDV residues (Magee et al., 2008). Quenelle et al. (2007a) found that in mice infected with CPX where treatment was delayed until 6 days post-infection, various doses of ST-246 (1–10 mg/kg) or HDP-CDV (0.3–3 mg/kg) alone did not separately provide survival benefits. However, when several ineffective doses of ST-246 and HDP-CDV were used in combination starting 6 days after infection and continuing for 5 days, nearly full protection could be achieved (Quenelle et al., 2007b).

6.1.3. Targeting CDV analogs to lung in ectromelia virus infection

HDP-CDV and ODE-CDV protect from poxvirus mortality and lower viral titers in liver and spleen but do not greatly reduce viral titers in lung (Quenelle et al., 2004; Buller et al., 2004). By making the lipid ester more hydrophobic by adding an *O*-benzyl group (1-*O*-octadecyl-2-*O*-benzyl-*sn*-glycero-3-CDV, ODBG-CDV) (Hostetler et al., 2007), it is possible to increase the incorporation of the compound into the chylomicron surface coat during assembly in the enterocyte (see Fig. 9). This leads to a higher proportion of the absorbed compound passing through the intestinal lymphatics to the superior vena cava and eventually to the right side of the heart followed by passage through the lungs (instead of passing to the liver first via the portal vein). Although the relative oral bioavailability of ODBG-[2-¹⁴C]-CDV was 32% compared with 88% for HDP-[2-¹⁴C]-CDV, the peak level in lung for ODBG-CDV was 100 nmol/g compared with 1 nmol/mg for HDP-CDV. Oral administration of 2 and 8 mg/kg of HDP-CDV and ODBG-CDV to animals given a high dose intranasal challenge with ECTV resulted in 80% survival at 2 mg/kg and 100% survival at 8 mg/kg versus 100% mortality in untreated A/Ncr mice (Hostetler et al., 2007). While this approach does not appear to confer any survival benefits in poxvirus infections, it may represent a useful way to design other drugs to target lung infections and neoplasia.

6.1.4. HDP-CDV treatment of rabbitpox virus infection

Infection of rabbits with rabbitpox virus (RPV) has been reported to exhibit all aspects of human infection with variola virus (Adams et al., 2007). This is important because approval of an anti-smallpox drugs by the FDA requires demonstration of activity in two or more poxvirus infection models having a well defined pathogenesis resembling that of human variola virus infection (Roberts et al., 2008). Rabbits injected intradermally with RPV develop a systemic disease with fever, viremia, secondary skin and mucocutaneous lesions, severe respiratory disease, natural aerosol transmission between animals and death in a high percentage of infected animals by nine days. In this system, HDP-CDV was given at doses of either 1 mg/kg or 5 mg/kg twice a day for 5 days beginning 24 h before challenge with 500 pfu of RPV. Untreated animals exhibited typical symptoms of RPV infection including fever, weight loss, skin and mucocutaneous lesions and 100% mortality by day 8. The group treated with 1 mg/kg of HDP-CDV exhibited signs of disease and 50% mortality at day 8 while animals receiving 5 mg/kg showed little sign of disease except for a transient fever and <5 secondary skin lesions. The authors concluded that RPV infection of rabbits reprises most of the typical features of human smallpox, providing an excellent model for testing of drugs and vaccines (Adams et al., 2007).

6.1.5. Treatment of CDV-resistant vaccinia virus infection

Serial passage of vaccinia virus (VV) in the presence of increasing concentrations of CDV gives rise to CDV-resistant virus (Smee et al., 2002; Andrei et al., 2006; Kornbluth et al., 2006). The CDV-resistant VV-WR was found to have five mutations, four in the exonuclease domain (H296Y, A314V, H319W, S338F) of the vaccinia E9L polymerase and one in the polymerase domain (R604S). The CDV resistant VV-WR had an EC₅₀ value of 29 μM representing 11–14-fold resistance (Table 4). The CDV-resistant VV-WR was found to be 10–30 times less virulent in mice. However, a single dose of HDP-CDV of 100 or 50 mg/kg given once 24 h after exposure provided 80–90% survival versus 20% in untreated animals. CDV was also effective; a single intraperitoneal dose of CDV 50 or 100 mg/kg gave 60–80% survival (Table 4) (Kornbluth et al., 2006). Andrei et al. (2006) did similar studies with vaccinia Lederle (VV-LED) and found two mutations, A314T and A684V, which led to 9-fold resistance to CDV *in vitro* (Table 4). The CDV resistance mutations, A314T and A684V in a vaccinia WR background, were nonlethal in mice with

doses of 4000 pfu/mouse. Nevertheless, it was shown that 10 or 50 mg/kg of subcutaneous CDV daily for 5 days reduced weight loss in mice infected with a high dose of CDV resistant VV-WR (Andrei et al., 2006). Investigators agree that CDV resistant vaccinia is less virulent and in spite of the 9–14 fold *in vitro* resistance, the disease in mice can be treated effectively with parenteral CDV (Smee et al., 2005; Andrei et al., 2006) or oral HDP-CDV (Kornbluth et al., 2006).

HDP-(S)-HPMPA and ODE-(S)-HPMPA treatment of cowpox and vaccinia infections: The *in vitro* antiviral activity of HDP- and ODE-(S)-HPMPA EC₅₀s against vaccinia Copenhagen are 0.01 μM versus 0.8 μM for HDP-CDV, representing an 80-fold increase in activity (Beadle et al., 2006). These two compounds are somewhat less active against vaccinia WR *in vitro* with EC₅₀s ranging from 0.1 to 0.3 μM versus 1.1 μM for HDP-CDV (Quenelle et al., 2007a). When BALB/c mice were infected intranasally with lethal doses of vaccinia WR and oral treatment delayed for 24, 48 or 72 h, 10 mg/kg and 30 mg/kg HDP-(S)-HPMPA and ODE-(S)-HPMPA daily for 5 days both provided significant protection (87–100% survival). Similar results were observed in animals infected with cowpox Brighton. When treatment was delayed for 72 h, 30 mg/kg treatment with both compounds provided lesser, but statistically significant protection (Quenelle et al., 2007a). In spite of their greater *in vitro* antiviral activity, HDP-(S)-HPMPA and ODE-(S)-HPMPA do not appear to offer significant benefits over HDP-CDV (CMX001).

6.2. HCMV infection

HDP-CDV and ODE-CDV are highly active *in vitro* against human cytomegalovirus (HCMV) with EC₅₀s of 1 nM (Beadle et al., 2002; Wan et al., 2005). The antiviral properties of oral HDP-CDV and ODE-CDV were tested in SCID mice having either human fetal retinal tissue or human fetal thymus/liver implants which were later infected with HCMV. Implants were infected with HCMV and 24 h later oral treatment was started with HDP-CDV or ODE-CDV at 5 and 10 mg/kg daily for 28 days. In fetal retinal implants at 28 days, HDP-CDV at 5 and 10 mg/kg reduced HCMV infection in the implants with 7% positive for HCMV versus 71% positive in untreated implants. In thy/liv implants, 5 mg/kg HDP-CDV and ODE-CDV both eliminated HCMV infection in 100% of implants at 28 days (Bidanset et al., 2004). In SCID implanted thy/liv mice, HDP-(S)-HPMPA and ODE-(S)-HPMPA at 10 mg/kg for 28 days were also effective reducing the percentage of HCMV infected implants from 45% in untreated to 9% and 11%, respectively. Viral load in the treated implants remaining infected was reduced by 1.4–2.7 logs (Quenelle et al., 2008). While effective, HDP-(S)-HPMPA and ODE-(S)-HPMPA do not appear to offer any advantages over HDP-CDV (CMX001) in the animal models of HCMV infection.

6.3. Murine CMV infection

The Smith strain of murine CMV produces lethal model of disseminated disease after intraperitoneal injection and has been used to evaluate potential antiviral therapies (Kern, 1991). HDP-CDV and ODE-CDV have *in vitro* EC₅₀s of 1 nM against MCMV *in vitro* (Kern et al., 2004). When treatment was delayed for 24 or 48 h after intraperitoneal injection of MCMV, both HDP-CDV and ODE-CDV given daily for 5 days produced statistically significant survival benefits, 67–100%, compared with no survival in untreated animals. Surprisingly, with a treatment delay of 24 h, a single oral dose of 3, 10 or 30 mg/kg HDP-CDV or ODE-CDV resulted in 87–100% survival compared with 7–10% survival in untreated animals. Survival benefits were also noted at 48 h treatment delays (Kern et al., 2004). HDP-(S)-HPMPA was also active in the lethal MCMV model at 10 and 30 mg/kg following a 24 h treatment delay. However, the compound does not show activity

Table 4
Properties of CDV-resistant vaccinia virus.

Virus	EC ₅₀ (μM)		Fold Res.	Mutations: E9L DNA polymerase		CDV-R virulence	Treatment of CDV-R vaccinia infections in mice	
	Wt	CDV-R		Exonuclease domain	Polymerase domain			
Vaccinia WR ^a	2.1	29	13.8	H296Y A314V H319W S338F		R604S	Decreased 10–30-fold	Single oral dose of 50 or 100 mg/kg HDP-CDV 10–20% mortality versus 100% mortality in untreated
Vaccinia Lederle ^b	2	18	9	A314T		A684V	Nonlethal in vaccinia-WR background	10 or 50 mg/kg CDV s.c. for 5 days reduced weight loss compared with the untreated controls. All animals survived

Abbreviations: Wt, wild type; WR, Western Reserve, CDV-R, CDV-resistant.

^a Kornbluth et al. (2006).

^b Andrei et al. (2006).

superior to HDP-CDV in the lethal MCMV model (Quenelle et al., 2008).

6.4. Adenovirus infection

HDP-CDV is highly active *in vitro* against adenovirus serotypes 3, 5, 7, 8 and 31 (Table 1 and Hartline et al., 2005). Toth et al. (2008) recently found that HDP-CDV (CMX001) given orally at 2.5 mg/kg 1 day prior to infection with adenovirus type 5 (Ad5) and daily for 18 days thereafter prevented Ad5-induced mortality in immunosuppressed Syrian hamsters. Untreated animals had 50% mortality by day 6 post-infection. Treated animals also had significantly lower levels of serum ALT (a liver enzyme) and lower serum viral titers. In animals treated with CMX001, serum and liver viral titers were undetectable at 15 days post-infection versus serum and liver viral titers of 10⁴ TCID₅₀/ml and 10⁶ TCID₅₀/g in surviving untreated animals. CMX001 also reduced Ad5 titers in salivary gland and pancreas and prevented extensive liver damage, a major target of adenovirus infection (Toth et al., 2008).

CMX001 was also effective when given 6 h to 2 days after infection with Ad5. Treatment with 2.5 mg/kg of CMX001 given 6 h or 1 day after infection provided complete protection while one animal of eight in the 2 day post-infection group died versus three of eight in the untreated group. Animals with a 2-day treatment delay had a lower virus burden in the liver at day 7 and no infectious virus was found in liver at day 16. Animals with a 6 h or 1 day treatment delay had liver enzyme and viral titer parameters indistinguishable from animals treated prophylactically (Toth et al., 2008).

6.5. Prevention of viral retinitis by pretreatment with intraocular HDP-cyclic-CDV

The animal studies discussed above utilized the HDP or ODE esters of CDV or (S)-HPMPA because of their tendency to insert into biological membranes and their oral bioavailability. However, less soluble forms of these compounds can be prepared by utilizing HDP esters of cyclic-CDV. Because both negative charges of CDV are masked, HDP-cyclic-CDV is sparingly soluble and has poor oral pharmacokinetics (Hostetler, unpublished data). To obtain long acting antiviral preparations for intraocular use, HDP-cyclic-CDV and HDP-cyclic-HPMPA have been evaluated (Cheng et al., 2004; Tammewar et al., 2007).

Intraocular CDV is effective in treating human CMV retinitis but causes a decrease in intraocular pressure, an unacceptable side effect. In fact a large number of acyclic nucleoside phosphonates, including (S)-HPMPA, had this property in animal testing (Banker et al., 1998). However, HDP-cyclic-CDV, a poorly soluble crystalline analog of CDV, given by intravitreal injection in rabbits was nontoxic

and doses of 100 μg/eye did not cause decreased intraocular pressure. The depot of HDP-cyclic-CDV cleared slowly from the vitreous with a half life of 6.3 days. Control and pretreated rabbits were then challenged with intraretinal HSV-1 at varying times after pretreatment. A single intravitreal dose of HDP-cyclic-CDV prevented HSV retinitis for 68 days versus pretreatment with vehicle or CDV, and statistically significant reductions in HSV retinitis were observed 100 days (Cheng et al., 2004). HDP-cyclic-CDV is expected to be even more active against HCMV retinitis because its EC₅₀ value is 42 nM, lower than the EC₅₀ for HSV-1100–600 nM (Wan et al., 2005; Williams-Aziz et al., 2005).

HDP-cyclic-(S)-HPMPA is also highly active against HCMV with an EC₅₀ of 20 nM (Tammewar et al., 2007). Intravitreal injection of the highest nontoxic dose of the sparingly soluble compound in the rabbit eye (55 mg/eye) gives an estimated intravitreal “concentration” of 65 μM, more than 3000 times the EC₅₀ against HCMV. HDP-cyclic-(S)-HPMPA did not cause decreased intraocular pressure in the guinea pig eye and has a residence time in vitreous of over 4 months (Tammewar et al., 2007). Both HDP-cyclic-CDV and HDP-cyclic-(S)-HPMPA are worthy of further evaluation as possible long acting intraocular treatments for CMV retinitis.

7. Application of the same strategy to other acyclic nucleoside phosphonates

In the foregoing sections, the effect of esterification of HPMPA (CDV) and HPMPA with alkoxyalkyl groups was reviewed including their effects to enhance antiviral activity and oral bioavailability and to reduce nephrotoxicity. In this section, the effect of alkoxyalkyl esterification of compounds with other acyclic nucleoside phosphonate side chains and other nucleobases will be discussed and the most interesting results highlighted.

There are numerous alternative acyclic structures. Structures of the side chains of the ones in current clinical use, such as those of adefovir (PME) and tenofovir (PMP), are shown in Fig. 10. In this figure, B denotes a nucleobase (A, C, T, G or substituted nucleobases) and R represents an alkoxyalkyl group such as hexadecyloxypropyl or octadecyloxyethyl. The antiviral activity of alkoxyalkyl esterified acyclic nucleoside phosphates of interest is shown grouped by the respective acyclic structures and compared with the unmodified compounds (Table 5).

7.1. HPMP nucleosides

Cidofovir (HPMPA, Vistide[®]) is a representative of his class, having the phosphonomethoxypropyl acyclic side chain (Fig. 10). Cidofovir is in clinical use for cytomegalovirus retinitis and was the first to be synthesized and evaluated as its hexadecyloxypropyl

Table 5
Antiviral activity of alkoxyalkyl esters of various types of acyclic nucleoside phosphonates.

Compound	EC ₅₀ (μM)		
	Vaccinia	HCMV	HIV-1
HPMP nucleosides			
(S)-HPMPA	2.7 ^a	0.82 ^a	77.5 ^b
HDP-(S)-HPMPA	0.01	0.003	0.007
(S)-HPMPC (Cidofovir, CDV)	46.2 ^c	0.38 ^d	–
HDP-CDV (CMX001)	0.8		0.009
cHPMP-5-aza-C	2.12 ^e		0.070 ^e
HDE-cHPMP-5-aza-C	0.037	0.00038	>0.80
	Vaccinia	HCMV	HIV-1
PME nucleosides			
PMEA (Adefovir)	–	–	1.1 ^f
HDP-PMEA	–	–	0.000015
PMEDAP	>10 ^g	13 ^g	0.08 ^f
HDP-PMEDAP	0.6	0.5	0.000016
PME-N ⁶ -cyPr-DAP	4.0 ^g	0.4 ^g	0.07 ^f
HDP-PME-N ⁶ -cyPr-DAP	0.10	0.02	<0.00001
	HIV-1	HBV (2.2.15)	HBV (AD38)
PPen nucleosides			
PPen A, C, G, T	>100 ^h	>100 ^h	>30 ^h
HDP-PPen-A	>30	15.7	1.5
HDP-PPen-C	>20	2.9	3.9
HDP-PPen-G	7.5	1.8	2.4
HDP-PPen-T	>20	0.66	2.0
	HIV-1	HBV (2.2.15)	HBV (AD38)
PMP nucleosides			
PMPA (Tenofovir)	3.20 ⁱ	7.2 ⁱ	2.25 ⁱ
HDP-PMPA (CMX157)	0.012	1.6	0.49
		HCMV	
PPM nucleosides			
PPMG (ACV methylene phosphonate)		5.4 ^j	
HDP-PPMG		0.24	
PPMDAP		>100 ^j	
HDP-PPMDAP		2.85	
PPM-N ⁶ -cyPr-DAP		>90 ^j	
HDP-PPM-N ⁶ -cyPr-DAP		1.6	
	Vaccinia	HCMV	HSV-1
HPPMP nucleoside			
HPMPC (CDV)	31	1.2	3.3
HDP-P-HPMPC	3.2 ^k	0.26 ^k	0.06 ^k

Abbreviations: HPMPA, 9-(S)-(3-hydroxy-2-phosphonomethoxy-propyl)adenine; PMEA, 9-(2-phosphonomethoxyethyl)adenine; PMEDAP, 9-2-(phosphonomethoxyethyl)-2,6-diaminopurine; PME-N⁶-cyPr-DAP, 9-(2-phosphonomethoxyethyl)-2-amino-6-cyclopropylaminopurine; PPen-A, 9-(5-phosphono-pent-2-en-1-yl)adenine; PMPA, 9-(R)-(2-phosphonomethoxypropyl)adenine; PPMG, 9-(phosphono-propoxymethyl)-guanine; HDP-P-HPMPC, hexadecyloxypropyl-phospho-cidofovir; HDP, hexadecyloxypropyl; HDE, hexadecyloxyethyl.

^a Beadle et al. (2006).

^b Hostetler et al. (2006).

^c Kern et al. (2002).

^d Beadle et al. (2002).

^e Krečmerová et al. (2007).

^f Valiaeva et al. (2006).

^g Prichard et al. (2008).

^h Choo et al. (2007b).

ⁱ Painter et al. (2007).

^j Ruiz et al. (2006).

^k Ruiz et al. (2007).

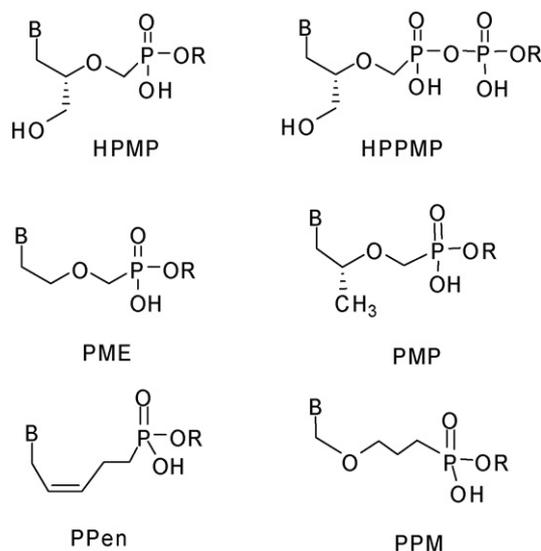


Fig. 10. Structure of alkoxyalkyl esters of various antiviral acyclic nucleoside phosphonates. **Abbreviations:** B, nucleobase; R, alkoxyalkyl; HPMP, 3-hydroxy-2-(phosphono-methoxy)propyl; PME, 2-(phosphonomethoxy)ethyl; PPen, 5-phosphono-pent-2-en-1-yl; HPPMP, 3-hydroxy-2-(phosphonomethoxy)propyl; PMP, 3-hydroxy-2-(phosphono-methoxy)propyl; PPM, phosphono-propoxymethyl.

ester (HDP-CDV, CMX001). HDP esters of both (S)-HPMPA and CDV show multiple log increases in antiviral activity when tested *in vitro* against vaccinia (50–270×) or HCMV (40–270×) (Table 5). (S)-HPMPA had been reported previously to be inactive against HIV (Balzarini et al., 1993) but the HDP ester was found to have an EC₅₀ value of 7 nM (Table 5; Hostetler et al., 2006). This appears to be due to extraordinarily poor cellular lymphocyte uptake and conversion of (S)-HPMPA to HPMPA diphosphate versus that observed with HDP-(S)-HPMPA (Aldern and Hostetler, unpublished 2007). Esterification of HPMP-cyclic-5-aza-cytosine with hexadecyloxyethyl results in a multiple log increase in the activity of this compound against vaccinia and HCMV (Krečmerová et al., 2007).

7.2. PME nucleosides

Adefovir (PMEA, Hepsara[®]) is a drug used for treating hepatitis B (as the dipivoxil ester). It is also active against HIV infection (Kahn et al., 1999). The acyclic side chain of PMEA has the phosphonomethoxyethyl structure (Fig. 10). In contrast to HPMPA and CDV, PMEA lacks a secondary hydroxylmethyl group in its acyclic side chain and is an obligatory chain terminator. Like CDV, nephrotoxicity is the most significant toxicity in humans chronically treated with adefovir (Kahn et al., 1999). HDP-PMEA is strikingly active against HIV-1 with an EC₅₀ of 15 pM (Table 5) and selectivity index of 4000 (Valiaeva et al., 2006). The increased activity against HIV-1 of HDP-PMEA versus PMEA is the largest increase in activity noted to date with HDP esters of acyclic nucleoside phosphonates. The PME analogs of diaminopurine (PMEDAP) and N⁶-cyclopropyl-diaminopurine (PME-N⁶-cPr-DAP) also show dramatic increases in antiviral activity against HIV-1 when converted to HDP-PMEDAP and HDP-PME-N⁶-cPr-DAP with EC₅₀ values of 16 and <10 pM. Increased activity is also noted for both compounds against vaccinia and HCMV (Prichard et al., 2008). It is worth noting that HDP-PME-N⁶-cPr-DAP has an excellent EC₅₀ against HCMV of 20 and 100 nM against vaccinia virus suggesting possible preclinical evaluation for use against poxvirus and herpesvirus diseases (Table 5).

7.3. PMP nucleosides

The PMP nucleosides have a side chain with the structure phosphonomethoxypropyl (Fig. 10). The acyclic side chain has a methyl group in place of the hydroxymethyl group in the HPMP nucleoside phosphonates making PMPA an obligatory chain terminator. PMPA is an example of a drug in clinical use for treatment of HIV infection as tenofovir disoproxil fumarate (Viread®). Esterification of PMPA with HDP increases antiviral activity from EC₅₀ values of 3.2 μM for PMPA to 0.012 μM for HDP-PMPA in HIV-1 infected PBMCs (Painter et al., 2007), representing 260-fold increase (Table 5). HDP-PMPA also exhibits increased activity versus PMPA in HBV although the increased activity is less, 4–5-fold (Table 5). HDP-PMPA (CMX157) is in preclinical development for HIV infection and will be discussed in more detail in Section 8.

7.4. PPen nucleosides

A new class of acyclic nucleoside phosphonate was recently reported having a 5-phosphono-pent-2-en-1-yl acyclic side chain loosely based on the structure of d4T (Choo et al., 2007a). Unmodified acyclic nucleoside phosphonates of this class are inactive against HSV-1. However, when esterified with HDP, the HDP-PPen-T compound exhibits antiviral activity against HSV-1 *in vitro* (Choo et al., 2007a). None of the unmodified PPen nucleosides are active against HIV or HBV (Choo et al., 2007b). However, when converted to their HDP esters (Table 5), HDP-PPen-G is active against HIV-1 and HBV with EC₅₀ values of 7.5 μM (HIV) and 1.8–2.4 μM (HBV). Against HBV, HDP-PPen-T was the most active compound against HBV with an EC₅₀ of 0.66 μM. All HDP-PPen nucleosides (A, C, G, and T) were active against HBV, but only HDP-PPen-G had significant activity against HIV (Table 5). It is presumed that the lack of activity of unmodified PPen nucleosides is due to poor cellular uptake as reported previously for CDV (Aldern et al., 2003) and (S)-HPMPA (Magee et al., 2008).

7.5. PPM nucleosides

PPM nucleosides have a phosphonopropoxymethyl acyclic side chain (Fig. 10); an example is phosphonopropoxymethylguanidine (PPMG) which is also known as acyclovir methylene phosphonate. PPMG is a weak inhibitor of HCMV with an EC₅₀ of 5.4 μM. HDP-PPMG has an EC₅₀ value of 0.24 μM, an increase of 22-fold (Ruiz et al., 2006). PPM-DAP and PPM-N⁶-cyPr-DAP are essentially inactive against HCMV *in vitro*, but their HDP esters have EC₅₀s of 2.8 and 1.6 μM, respectively (Table 5).

7.6. HPPMP nucleoside

When HPMPA (CDV) is esterified with HDP-phosphate, by forming a pyrophosphate link between the phosphonate and the phosphate, one obtains HDP-P-CDV (Ruiz et al., 2007). This compound shows increased antiviral activity against vaccinia, HCMV and HSV-1 compared with unmodified CDV (Table 5).

7.7. Summary: alkoxyalkyl prodrugs of other nucleoside phosphonates

From the results summarized in this section and in Table 5, it is clear that the enhancement of antiviral activity resulting from esterification of an acyclic nucleoside phosphonate with an alkoxyalkyl residue like hexadecyloxypropyl is not confined to one particular type of acyclic phosphonate side chain or to one nucleoside base. All types of acyclic nucleoside phosphonates show enhancement of antiviral activity, presumably due to increased cellular penetration of the alkoxyalkyl esterified compounds and increased

metabolic conversion to their diphosphates (triphosphate equivalents) (Aldern et al., 2003, Magee et al., 2008). In a number of instances, acyclic nucleoside phosphonates which were reported to be inactive showed antiviral activity as their HDP esters (Hostetler et al., 2006; Choo et al., 2007a,b; Prichard et al., 2008). This emphasizes the probability that earlier *in vitro* antiviral screening with unmodified acyclic nucleoside phosphonates in virus-infected cells has systematically underestimated the antiviral activity of most members of this class.

8. Status of clinical development of HDP-CDV (CMX001) and HDP-tenofovir (CMX157)

HDP-CDV was originally synthesized and evaluated as an oral treatment for smallpox under biodefense initiatives of the National Institutes of Allergy and Infectious Disease (NIAID) and the Department of Defense. However, as the antiviral evaluations proceeded, it became apparent that HDP-CDV was a broad spectrum antiviral with potent activity against orthopoxviruses (including variola), cytomegalovirus (wild type and drug resistant), herpes simplex virus, adenovirus, Epstein Barr virus, VZV, HHV-6, HHV-8, BK virus, human papilloma virus and orf virus. Under an NIAID grant to Chimerix Inc. of Durham, NC, HDP-CDV (CMX001) is currently in Phase I/II human clinical trials (www.ClinicalTrials.gov, NCT00780182 and NCT00793598).

8.1. HDP-CDV (CMX001)

8.1.1. Preclinical studies

HDP-CDV (CMX001) is being developed for prevention and treatment of smallpox and for various double-stranded DNA virus infections. HDP-CDV antiviral activity against orthopoxviruses, various herpes viruses, adenoviruses, BK virus and orf virus has been described (Table 1). CMX001 is active against HCMV strains and drug resistant HCMV (Fig. 3), all herpes group viruses (Williams-Aziz et al., 2005) and human papilloma virus (HPV 18) (Painter et al., 2008a). CMX001 is orally bioavailable and results in substantial systemic exposure in rabbits, mice and monkeys. Doses of 4 and 10 mg/kg were effective in models of orthopoxvirus, CMV and adenovirus infections as discussed above. Safety studies in mice, rats and monkeys showed dose-dependent enteritis which was reversible after discontinuance of the drug. It is expected that reversible gastrointestinal toxicity will be the dose limiting toxicity of CMX001 (Painter et al., 2008a). Animals maintained normal clinical pathology values and, in contrast to CDV, there was no evidence of nephrotoxicity. CMX001 undergoes omega oxidation to a short chain carboxylic acid metabolite (CMX064) due to small intestine and liver cytochrome P450 enzymes. CMX064 is devoid of antiviral activity. Formation of CMX064 is greatest in the monkey and much lower in humans and mice (Painter et al., 2008a).

8.1.2. Clinical studies

A placebo-controlled, dose-escalating Phase I safety pharmacokinetic study in healthy volunteers showed dose-proportional C_{max} and AUC with single doses ranging from 0.1 to 1 mg/kg (Painter et al., 2008a,b). Oxidative metabolism of CMX001 to CMX064 when matched by AUC was much lower in humans than in monkeys, rabbits or mice and multiple dose studies in man are currently in process (NCT00780182). It was concluded that plasma concentrations of CMX001 in normal human volunteers are as much as 20-fold greater than those observed in animals at comparable doses per kg of body weight (Painter et al., 2008b). BK virus infection is an important cause of renal allograft rejection (Hirsch et al., 2002) and there is no approved treatment. A Phase Ib/IIa study is currently in

progress for a post-organ transplant study of CMX001 in patients exhibiting viruria with BK virus (NCT00793598).

8.2. HDP-tenofovir (CMX157)

8.2.1. Preclinical studies

The HDP ester of PMPA (tenofovir) was synthesized and evaluated *in vitro* against HIV-1 in MT-2 cells where it was found to have an EC₅₀ of <0.00001 μM versus 0.65 μM for tenofovir. In PBMCs the EC₅₀ was 0.012 μM compared with 3.20 μM for tenofovir (Painter et al., 2007). Selectivity indexes for CMX157 in MT-2 cells and PBMCs were >5 × 10⁶ and 12,000, respectively. Oral administration of single or multiple doses of 10, 30 or 100 mg/kg to rats showed dose proportional plasma levels of CMX157. On day one the T_{max} was noted at 0.5–2 h and with doses of 10, 30 and 100 mg/kg, the C_{max} and AUC_{0–24h} values were 85, 379 and 905 ng/ml and 442, 1830 and 5410 ng h/ml. Similar results were noted at day 7. Tenofovir was the other significant metabolite in plasma. Toxicity studies in rats given 10, 30 and 100 mg/kg for 7 days showed no signs of toxicity, no alterations in body weight, no changes in clinical chemistry parameters and no mortality. On necropsy, there were no changes in organ weights or abnormalities of histopathology. CMX157 was tested against a panel of thirty NRTI resistant HIV variants and found to be substantially more active (295–472-fold) than tenofovir with low nanomolar EC₅₀ values. This appears to be due in part to higher levels of tenofovir diphosphate (TFV-PP) in cells exposed to CMX157 compared to the levels observed with tenofovir exposure; in PBMCs incubated with 1 μM CMX157, TFV-PP was 33 fold higher than in cells incubated with the same amount of tenofovir (Lanier et al., 2008). The increased activity may also be attributed, in part, to CMX157 binding directly to HIV virions and subsequently inhibiting viral replication in “untreated” cells. Neither direct binding to HIV nor subsequent inhibition of viral replication was observed with tenofovir (Lanier et al., 2009). CMX157 is currently in preclinical development for treatment of HIV infection.

9. Conclusions

HDP-CDV and HDP-(S)-HPMPA are alkoxyalkyl prodrugs of CDV and (S)-HPMPA which exhibit a several log increase in antiviral activity against orthopoxviruses and herpes group viruses because of their ability to traverse biological membranes more rapidly than CDV and (S)-HPMPA, resulting in much higher levels of their diphosphates in the cells. Alkoxyalkyl esters with 18–22 atoms are optimally active. These compounds are orally bioavailable and are highly efficacious in animal models of orthopoxvirus, cytomegalovirus and adenovirus infections. Esterification of acyclic nucleoside phosphonates with alkoxyalkyl groups produces increases their antiviral activity and oral bioavailability, and is not specific for any particular nucleobase or acyclic phosphonate side chain. Alkoxyalkyl ester prodrugs of a wide variety of acyclic nucleoside phosphonates exhibit multiple-log increases in antiviral activity. Certain acyclic nucleoside phosphonates which were reported to be inactive *in vitro*, were found to have substantial antiviral activity after conversion to their hexadecyloxypropyl or octadecyloxyethyl esters. Preclinical and clinical studies of HDP-CDV reveal no nephrotoxicity, in contrast to CDV and adefovir, where nephrotoxicity is dose-limiting.

HDP-CDV (CMX001) is in clinical development for orthopoxvirus and dsDNA virus infections, and studies to date have revealed no problematic adverse effects. Another compound of this class, HDP-tenofovir (CMX157), is also orally bioavailable, highly active against NRTI resistant variants of HIV-1 and has the unique attribute of inserting directly into the phospholipid bilayer of the virus and inhibiting subsequent replication of HIV virions. Animal safety

studies have been benign. CMX157 is now in preclinical development for treatment of HIV infection. The alkoxyalkyl ester strategy for acyclic nucleoside phosphonates reviewed here is a general approach which provides many benefits for developing new orally available drugs for a range of viral infections.

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