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Antiviral evaluation of octadecyloxyethyl esters of (*S*)-3-hydroxy-2-(phosphonomethoxy)propyl nucleosides against herpesviruses and orthopoxviruses☆

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ABSTRACT

Our previous studies showed that esterification of 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl] adenine (HPMPA) or 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)-propyl]cytosine (HPMPC) with alkoxyalkyl groups such as hexadecyloxypropyl (HDP) or octadecyloxyethyl (ODE) resulted in large increases in antiviral activity and oral bioavailability. The HDP and ODE esters of HPMPA were shown to be active in cells infected with human immunodeficiency virus, type 1 (HIV-1), while HPMPA itself was virtually inactive. To explore this approach in greater detail, we synthesized four new compounds in this series, the ODE esters of 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)-propyl]guanine (HPMPG), 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]thymine (HPMPT), 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPDAP) and 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-2-amino-6-cyclopropylaminopurine (HPMP-cPrDAP) and evaluated their antiviral activity against herpes simplex virus, type 1 (HSV-1), human cytomegalovirus (HCMV), and vaccinia, cowpox and ectromelia. Against HSV-1, subnanomolar EC_{50} values were observed with ODE–HPMPA and ODE–HPMPC while ODE–HPMPG had intermediate antiviral activity with an EC_{50} of 40 nM. In HFF cells infected with HCMV, the lowest EC_{50} values were observed with ODE–HPMPC, 0.9 nM. ODE–HPMPA was highly active with an EC_{50} of 3 nM, while ODE–HPMPG and ODE–HPMPDAP were also highly active with EC_{50}s of 22 and 77 nM, respectively. Against vaccinia and cowpox viruses, ODE–HPMPG and ODE–HPMPDAP were the most active and selective compounds with EC_{50} values of 20–60 nM and selectivity index values of 600–3500. ODE–HPMPG was also active against ectromelia virus with an EC_{50} value of 410 nM and a selectivity index value of 166. ODE–HPMPG and ODE–HPMPDAP are proposed for further preclinical evaluation as possible candidates for treatment of HSV, HCMV or orthopoxvirus diseases.

1. Introduction

Phosphonate nucleoside analogs are an important class of antiviral agents including cidofovir, adefovir and tenofovir which are approved for treatment of cytomegalovirus, hepatitis B and HIV infections, respectively (De Clercq and Holý, 2005; Holý, 2003). However, their cellular uptake is limited by their double negative charge which leads to lower antiviral activity (Aldern et al., 2003; Magee et al., 2008). For example, HPMPA was previously reported to be inactive in HIV-infected cells (Balzarini et al., 1993; De Clercq, 1991). However, we recently found that esterification of HPMPA with alkoxyalkyl groups such as hexadecyloxypropyl (HDP) or octadecyloxyethyl (ODE) results in a marked increase in cell uptake and antiviral activity (Hostetler et al., 2006). We also synthesized a series of 5-phosphono-pent-2-en-1-yl (PPen) nucleosides and found that the unmodified PPen nucleosides lacked antiviral activity in vitro (Choo et al., 2007). However, the PPen compounds were active antivirals when esterified with HDP groups (Choo et al., 2007). Thus, it appears likely that the antiviral activity of phosphonate nucleoside analogs has

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been systematically underestimated because of poor cell penetration.

To evaluate this in more detail, we compared the ODE esters of HPMPA (Beadle et al., 2006) and HPMPC (Beadle et al., 2002) with four newly synthesized compounds, the ODE esters of HPMPG, HPMPT, HPMPDAP and HPMP-cPrDAP. The antiviral activity of these compounds was determined in cells infected with HSV-1, human cytomegalovirus (HCMV), murine cytomegalovirus (MCMV), vaccinia virus, cowpox virus and ectromelia virus. The ODE esters were substantially more active against HSV-1 than published data for the unmodified acyclic nucleoside phosphonates. The ODE esters were also highly active against HCMV and MCMV and were several orders of magnitude more active than ganciclovir. The most active compound against orthopoxviruses was ODE–HPMPA, but it was also the most cytotoxic of the series. The most active and selective compounds against orthopoxviruses were ODE–HPMPG and ODE–HPMPDAP.

2. Materials and methods

2.1. General chemistry methods

^{1}H and ^{31}P nuclear magnetic resonance (NMR) spectra were recorded on a Varian HG-300 spectrometer at 300 MHz for ^{1}H NMR and are reported in units of ppm relative to internal tetramethylsilane at 0.00 ppm. Mass spectra were recorded on either a Finnigan LCQDECA mass spectrometer or ThermoFinnigan MAT900XL high-resolution mass spectrometer at the small molecule facility in Department of Chemistry at University of California, San Diego. In addition to the spectral data provided, purity, of the target compounds (>98%) was confirmed by analytical thin layer chromatography (TLC) using Analtech Uniplate™ silica gel-GF (250 μm) plates. The plates were developed using the solvent system $CHCl_3$/MeOH/con NH_4OH/H_2O (70:30:3:3, v/v) and visualized with UV light, phospray (Supelco; Bellefonte, PA, USA) and charring at 400 °C. Flash chromatography was performed with silica gel (Merck silica gel 60, 230–400 mesh).

2.2. General method for synthesis of 3-trityloxy-2-hydroxypropyl nucleosides (**5**–**8**) (Scheme 1)

A suspension of the corresponding heterocyclic base **1**–**4** (1 mmol) and cesium carbonate (0.1 mmol) or sodium hydride (0.1 mmol) in dry DMF (25 ml) was stirred at room temperature for 1 h. (*S*)-Trityl glycidyl ether (DAISO Co., Ltd.) (0.9 mmol) was added in one portion. The mixture was stirred at 80 °C overnight. DMF was evaporated, the residue was purified by column chromatography on silica gel (eluent: dichloromethane–methanol, 0–20%) to give product.

2.2.1. 1-(S)-(3-Trityloxy-2-hydroxypropyl)-4-methoxy-5-methyl-2-pyrimidone (**5**)

It was synthesized from 4-methoxy-5-methyl-2-pyrimidone and S-trityl glycidyl ether (cesium carbonate base) as described by Wong and Fuchs (1970). Yield 87%. ^{1}H NMR ($CDCl_3$): δ 8.00 (s, 1H), 7.21–7.47 (m, 15H), 4.04–4.14 (m, 2H), 3.65–3.74 (m, 1H), 3.30 (s, 3H), 3.06–3.22 (m, 2H), 1.85 (s, 3H). MS-ESI (*m*/*z*) 479.18 $(M+Na)^+$.

2.2.2. 9-(S)-(3-Trityloxy-2-hydroxypropyl)-6-O-benzylguanine (**6**)

It was synthesized from 6-O-benzylguanine and S-trityl glycidyl ether (sodium hydride base) as reported by Liu et al. (2003). Yield 57%. ^{1}H NMR ($CDCl_3$): δ 8.00 (s, 1H), 7.19–7.50 (m, 20H), 5.49 (s, 2H), 5.20 (br.s, 1H), 4.20–4.30 (m, 1H), 4.10–4.20 (m, 2H), 2.98–3.22 (m, 2H). MS-ESI (*m*/*z*) 558.24 $(M+H)^+$.

2.2.3. 9-(S)-(3-Trityloxy-2-hydroxypropyl)-2,6-diaminopurine (**7**)

It was synthesized from 2,6-diaminopurine activated by sodium hydride and S-trityl glycidyl ether. Yield 75%. ^{1}H NMR ($CDCl_3$): δ 7.35–7.50 (m, 7H), 7.15–7.35 (m, 9H), 5.83 (br.s, 2H), 4.91 (br.s, 2H), 4.05–4.25 (m, 3H), 3.22–3.33 (m, 1H), 2.93–3.05 (m, 1H). MS-ESI (*m*/*z*) 467.21 $(M+H)^+$.

2.2.4. 9-(S)-(3-Trityloxy-2-hydroxypropyl)-2-amino-6-cyclopropylaminopurine (**8**)

It was synthesized from 2-amino-6-cyclopropylaminopurine activated by sodium hydride and S-trityl glycidyl ether. Yield 12%. ^{1}H NMR ($CDCl_3$): δ 8.00 (s, 1H), 7.21–7.45 (m, 15H), 5.91 (br.s, 2H), 4.05–4.30 (m, 2H), 3.55–3.75 (m, 1H), 3.15–3.35 (m, 2H), 0.45–0.70 (m, 2H), 0.70–95 (m, 2H). MS-ESI (*m*/*z*) 507.21 $(M+H)^+$.

2.3. General method for synthesis of 2-(octadecyloxy)ethyl (S)-3-trityloxy-2-(phosphonomethoxy)propyl nucleosides **9**–**12**

2-(Octadecyloxy)ethyl p-toluene-sulfonyloxymethyl-phosphonate (1.5 mmol) prepared as described by Beadle et al. (2006) was added to a mixture of the corresponding (*S*)-(3-trityloxy-2-hydroxypropyl)-nucleoside **5**–**8** (1.0 mmol) and sodium tert-butoxide (2.0 mmol) in dry triethylamine (50 ml). The mixture was stirred at 70 °C for 24 h. The solvent was evaporated; the residue was purified by column chromatography to give the product.

2.3.1. 2-(Octadecyloxy)ethyl 1-(S)-[3-trityloxy-2-(phosphonomethoxy)propyl] 4-methoxy-5-methyl-2-pyrimidone (**9**)

Yield 23%. ^{1}H NMR ($CDCl_3$ + methanol-d_4): δ 7.43–7.7.48 (m, 8H), 7.24–7.33 (m, 7H), 7.12 (s, 1H), 3.91–4.09 (m, 4H), 3.80–3.91 (m, 1H), 3.50–3.61 (m, 4H), 3.39–3.43 (m, 2H), 3.36 (s, 3H), 3.25–3.32 (m, 2H), 1.74 (s, 3H), 1.48–1.60 (m, 2H), 1.18–1.38 (m, 30H); 0.88 (t, J = 7 Hz, 3H). MS-ESI (*m*/*z*) 845.49 $(M-H)^-$.

2.3.2. 2-(Octadecyloxy)ethyl 9-(S)-[3-trityloxy-2-(phosphonomethoxy)propyl] 6-O-benzylguanine (**10**)

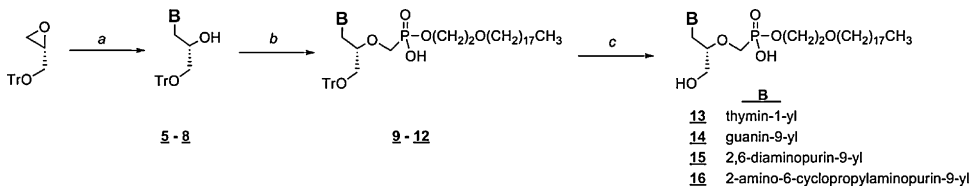
Yield 23%. ^{1}H NMR ($CDCl_3$ + methanol-d_4): δ 7.90 (s, 1H), 7.18–7.33 (m, 20H), 5.50 (s, 2H), 3.88–4.03 (m, 2H), 3.60–3.80 (m, 2H), 3.39–3.50 (m, 3H), 3.22–3.39 (m, 4H), 3.08–3.20 (m, 2H), 1.45–1.62 (m, 2H), 1.00–1.40 (m, 30H); 0.88 (t, J = 7 Hz, 3H). MS-ESI (*m*/*z*) 948.15 $(M+H)^+$.

2.3.3. 2-(Octadecyloxy)ethyl 9-(S)-[3-trityloxy-2-(phosphonomethoxy)propyl] 2,6-diaminopurine (**11**)

Yield 31%. ^{1}H NMR ($CDCl_3$ + methanol-d_4): δ 7.56 (s, 1H), 7.20–7.48 (m, 15H), 4.00–4.15 (m, 2H), 3.55–3.68 (m, 4H), 3.40–3.55 (m, 3H), 3.20–3.40 (m, 4H), 1.42–1.62 (m, 2H), 1.15–1.40 (m, 30H); 0.89 (t, J = 7 Hz, 3H). MS-ESI (*m*/*z*) 615.41 $(M+H)^+$, 637.31 $(M+Na)^+$, 653.32 $(M+K)^+$, 613.36 $(M-H)^-$.

2.3.4. 2-(Octadecyloxy)ethyl 9-(S)-[3-trityloxy-2-(phosphonomethoxy)propyl] 2-amino-6-cyclopropylaminopurine (**12**)

Yield 49%. ^{1}H NMR ($CDCl_3$ + methanol-d_4): δ 7.96 (s, 1H), 7.26–7.62 (m, 15H), 4.15–4.40 (m, 2H), 3.60–3.72 (m, 4H), 3.40–3.58 (m, 3H), 3.25–3.48 (m, 4H), 1.42–1.62 (m, 2H), 1.15–1.40 (m, 31H); 0.89 (t, J = 7 Hz, 3H), 0.50–0.78 (m, 4H). MS-ESI (*m*/*z*) 895.53 $(M-H)^-$.



Scheme 1. Synthesis of octadecyloxyethyl esters of 3-hydroxy-2-(phosphonomethoxy)-propyl nucleosides. Reagents: (a) protected nucleobases **1**, 4-methoxy-5-methyl-2-pyrimidone, **2**, 6-O-benzylguanine, **3**, 2,6-diaminopurine, **4**, 2-amino-6-cyclopropylaminopurine, Cs_2CO_3 or NaH, N, N-DMF, 80 °C; (b) octadecyloxyethyl p-toluene-sulfonyloxymethyl-phosphonate, NaH, DMF, 70 °C; (c) 80% AcOH.

2.4. General method for synthesis of 2-(octadecyloxy)ethyl (S)-3-hydroxy-2-(phosphonomethoxy)propyl nucleosides (**13**, **15**, **16**)

A mixture of the corresponding 2-(octadecyloxy)ethyl (S)-3-trityloxy-2-phosphonomethoxypropyl nucleoside (**9**, **11**, or **12**) (0.1 mmol) and 80% AcOH in water (10 ml) was stirred at room temperature overnight. The solvents were evaporated, then co-evaporated with water. The residue was purified by column chromatography on silica gel (eluent: chloroform–methanol–ammonium hydroxide–water 70:58:8:8) to give the product.

2.4.1. 2-(Octadecyloxy)ethyl 1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl] thymine (**13**)

Yield 71%. ^{1}H NMR ($CDCl_3$ + methanol-d_4): δ 7.72 (s, 1H), 3.93–4.09 (m, 2H), 3.73–3.88 (m, 2H), 3.65–3.72 (m, 1H), 3.54–3.65 (m, 2H), 3.20–3.54 (m, 2H), 3.24–3.38 (m, 4H), 1.90 (s, 3H), 1.48–1.60 (m, 2H), 1.18–1.38 (m, 30H); 0.89 (t, J = 7 Hz, 3H). ^{31}P NMR ($CDCl_3$ + methanol-d_4): δ 15.30. MS-ESI (m/z) 591.30 $(M+H)^+$, 613.35 $(M+Na)^+$, 629.32 $(M+K)^+$.

2.4.2. 2-(Octadecyloxy)ethyl 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl] 2,6-diaminopurine (**15**)

Yield 86%. ^{1}H NMR ($CDCl_3$ + methanol-d_4): δ 7.80 (s, 1H), 4.00–4.05 (m, 2H), 3.78–3.82 (m, 2H), 3.55–3.65 (m, 2H), 3.42–3.50 (m, 2H), 3.23–3.98 (m, 3H), 1.50–1.62 (m, 2H), 1.15–1.33 (m, 30H), 0.89 (t, J = 7 Hz, 3H). ^{31}P NMR ($CDCl_3$ + methanol-d_4): δ 16.76. MS-ESI (m/z) 613.39 $(M-H)^-$.

2.4.3. 2-(Octadecyloxy)ethyl 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl] 2-amino-6-cyclopropylaminopurine (**16**)

Yield 46%. ^{1}H NMR ($CDCl_3$ + methanol-d_4): δ 7.75 (s, 1H), 4.22–4.58 (m, 2H), 4.00–4.10 (m, 2H), 3.62–3.90 (m, 2H), 3.58–3.62 (m, 1H), 3.40–3.58 (m, 3H), 3.24–3.40 (m, 3H), 1.42–1.62 (m, 2H), 1.15–1.40 (m, 31H), 0.80–1.00 (m, 4H), 0.72 (t, J = 7 Hz, 3H). ^{31}P NMR ($CDCl_3$ + methanol-d_4): δ 16.89. MS-ESI (m/z) 655.52 $(M+H)^+$, 693.50 $(M+Na)^+$.

2.4.4. 2-(Octadecyloxy)ethyl 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl] guanine (**14**)

A mixture of compound **10** (0.1 mmol) and 10% TFA in dichloromethane (20 ml) was stirred at room temperature for 2 days. The solvents were evaporated, then co-evaporated with water. The residue was purified by column chromatography on silica gel (eluent: chloroform–methanol–ammonium hydroxide–water 70:58:8:8) to give the product. Yield 68%. ^{1}H NMR ($CDCl_3$ + methanol-d_4): δ 7.98 (s, 1H), 3.95–4.06 (m, 2H), 3.55–3.78 (m, 4H), 3.40–3.55 (m, 3H), 3.30–3.40 (m, 4H), 1.50–1.62 (m, 2H), 1.18–1.38 (m, 30H); 0.89 (t, J = 7 Hz, 3H). ^{31}P NMR ($CDCl_3$ + methanol-d_4): δ 16.64. MS-ESI (m/z) 616.37 $(M+H)^+$, 638.30 $(M+Na)^+$, 654.43 $(M+K)^+$, 614.38 $(M-H)^-$.

2.5. In vitro evaluation of antiviral activity

2.5.1. HSV-1 assays

HSV-1 was measured by ELVIRA yield reduction assay as previously described by Stránská et al. (2004). Briefly, subconfluent MRC-5 cells in 24 well culture plates were inoculated by removing the medium and adding HSV-1 at a dilution that causes 3–4+ cytopathic effect in a non-drug well in 24 h. The HSV-1 virus, strain F (American Type Culture Collection, Manassas, VA), was absorbed for 1 h at 37 °C, aspirated and replaced with various concentrations of drugs as indicated in Eagles MEM containing 2% FBS, followed by incubation at 37 °C for 24 h. The supernatant was then aspirated and 1 ml of a suspension of reporter ELVIRA cells (Diagnostic Hybrids Inc., Athens, OH) was added to each well according to the manufacturers instructions. After incubation for 24 h, all wells were aspirated, 0.3 ml of ELVIRA lysis solution was added followed by addition of ELVIRA detection buffer 5 min later. After 15–90 min of color development, the optical density (570 nm) of each well was read spectrophoto-metrically. The susceptibility of HSV-1 to each compound was determined graphically and is expressed as EC_{50}, the concentration required to inhibit viral replication by 50%.

2.5.2. HSV-1 cytotoxicity assays

MRC-5 cells were seeded into 96-well tissue culture plates at 2.5×10^4 cells/well. After 24 h incubation, media was replaced with MEM containing 2% FBS and drug was added to the first row, then diluted serially 5-fold from 100 to 0.03 μM. The plates were incubated for 7 days and cells stained with neutral red and incubated for 1 h. Plates were shaken on a plate shaker for 15 min and neutral red was solubilized with 1% glacial acetic acid/50% ethanol. The optical density was read at 540 nm. The concentration of drug that reduced cell viability by 50% (CC_{50}) was calculated.

2.5.3. HCMV and MCMV assays

Plaque reduction assays were used to assess antiviral activity against both HCMV and MCMV by methods reported previously (Prichard et al., 2008). For HCMV, human foreskin fibroblast (HFF) cells cultivated in minimum essential media (MEM) with Earle's salts supplemented with 10% fetal bovine serum (FBS) and standard concentrations of penicillin and gentamicin. Monolayers were infected with the AD169 strain of HCMV (American Type Culture Collection, Manassas, VA) to yield approximately 20–30 plaques per well. Drug dilutions were prepared in growth media and were added to duplicate wells in six well plates. The infected plates were incubated for 8 days and monolayers were stained with a 1.5% solution of neutral red. Similar methods were used to evaluate the efficacy against the Smith strain of MCMV (American Type Culture Collection, Manassas, VA) but were performed in monolayers of primary mouse embryo fibroblast cells in 12-well plates and cell monolayers were stained after 7 days of infection. For both assays, plaques were enumerated and drug concentrations sufficient to reduce plaque number 50% (EC_{50}) values were interpolated from the experimental data.

Table 1
Antiviral activity and selectivity of octadecyloxyethyl esters of 3-hydroxy-2-(phosphonomethoxy)propyl nucleosides against HSV-1, strain F.

Compounds	Method	EC_{50}, μM	CC_{50}, μM	Selectivity
ODE-(*S*)-HPMPA	ELV	<0.00001 (7)	4.9 ± 6.3 (4)	>650,000
ODE-(*S*)-HPMPC	ELV	0.00047 ± 0.0011 (5)	1.8 ± 2.9 (3)	3,800
ODE-(*S*)-HPMPT **13**	ELV	0.34 ± 0.3 (3)	35 ± 12 (3)	100
ODE-(*S*)-HPMPG **14**	ELV	0.04 ± 0.3 (3)	1.2 ± 1.2 (4)	30
ODE-(*S*)-HPMPDAP **15**	ELV	0.43 ± 0.3 (3)	7.5 ± 1.2 (3)	17
ODE-(*S*)-HPMP-cPrDAP **16**	ELV	0.78 ± 0.04 (3)	17 ± 3.7 (4)	22
Unmodified HPMP nucleosides (reference)				
(*S*)-HPMPA (a,b)	CPE	23.1, 6.1[a]	330	14
(*S*)-HPMPC (a,b)	CPE	7.2, 14.3[a]	358	50
(*S*)-HPMPC (c)	DNAr	3.3	1000	300
(*S*)-HPMPC (c)	PRA	15.2	1000	66
(*S*)-HPMPT (a,b)	CPE	535, 250[a]	1360	2.5
(*R*,*S*)-HPMPG (a,b)	CPE	23.1, 23.1[a]	1250	54.
(*R*,*S*)-HPMPDAP (a,b)	CPE	23.1, 31.4[a]	628	27

For the ELVIRA HSV assay results in the upper section of the table, the numbers in parentheses are the number of replicate determinations. Cytotoxicity measurements were made by the neutral red method in MRC-5 cells. References: (a) Holý et al. (1987); (b) De Clercq et al. (1987); (c) Beadle et al. (2002). In the cases where there are two numbers for EC_{50}s in column 3, the first number is from reference (a), the second number from reference (b). All data are for the HSV-1 strain F unless otherwise indicated. Methods abbreviations: DNAr, DNA reduction; ELV, ELVIRA; CPE, cytopathic effect assay, PRA, plaque reduction assay.

[a] The average of the data from 3 HSV strains: G, 196, and Lyons.

2.5.4. Vaccinia and cowpox antiviral assays

Vaccinia virus, strain Copenhagen, and cowpox virus, strain Brighton, were obtained from Dr. John Huggins of the U.S. Army Medical Research Institute for Infectious Diseases, Frederick, MD. Monolayers of HFF cells were infected with either vaccinia virus or cowpox virus would yield 20–30 plaques per well. Following a 1 h adsorption, the medium was aspirated from the wells, drug dilutions were added in an agarose overlay, and plates were incubated for 3 days. Infected monolayers were stained with a 0.02% solution of neutral red in PBS, plaques were enumerated, and EC_{50} values were calculated as described above.

2.5.5. Neutral red cytotoxicity assays

HFF cells were seeded into 96-well plates containing 2.5×10^4 cells/well and incubated for 24 h. Growth media was replaced with MEM containing 2% FBS and dilutions of the test compounds. The plates were incubated for 7 days, the media aspirated and the cells stained with 0.01% neutral red in PBS. The optical density was measured at 540 nm to determine the number of viable cells. Cytotoxicity is expressed as the concentration of drug that reduced cell viability by 50% (CC_{50}), and was interpolated from the experimental data.

2.5.6. Ectromelia virus and cells

BS-C-1 cells (ATCC CCL 26) were grown in Eagle's minimum essential medium (MEM) containing 10% fetal clone III (Hyclone, Logan, UT), 2 mM L-glutamine (GIBCO, Grand Island, NY), 100 U/ml penicillin (GIBCO, Grand Island, NY), and 100 μg/ml streptomycin (GIBCO, Grand Island, NY). A plaque-purified isolate of the MOS strain of ECTV (ATCC VR-1374) designated Mos-3-P2, was propagated in an African green monkey kidney cell line, BS-C-1 (Chen et al., 1992). Virus was purified through a sucrose cushion as described elsewhere (Moss and Earl, 1998). Virus infectivity was estimated as described previously (Wallace and Buller, 1985).

2.5.7. Ectromelia plaque reduction assay

CV-1 cells were plated in wells of a 24 well cluster plate. Each monolayer was infected with ~75 plaque forming units (PFU) of indicator virus in 0.1 ml of DMEM +5% fetal clone III for 60 min at 37 °C. Media was removed by aspiration and standard virus overlay medium, Dubecco's modified Eagle's Medium (Biowhittaker), 5% fetal clone II (Hyclone) and −1% carboxymethylcellulose (Sigma), containing no drug or the test drug at concentrations ranging from 0.05 to 50 μM was added. The plates were incubated at 37 °C for 3–4 days for ECTV and 2 days for VACV, monolayers were stained and plaques counted using a stereomicroscope. The EC_{50} concentration for each drug was calculated.

2.5.8. MTS cytotoxicity assay

Serial dilutions of the compound were made in DMEM supplemented with 2% fetal clone II (Hyclone). The compound dilutions were mixed with BSC-1 cells at 5000 cells per reaction in 100 μl total volume for 72 h. At the given time point, 20 μl of MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) was added to each reaction and incubated 2–4 h. The OD_{490} was read and the % cell viability for each reaction was calculated based on untreated cells. The compound concentration was plotted versus the % cell viability to determine the concentration at which the cell viability was 50%.

3. Results

3.1. Synthetic chemistry

The synthesis of ODE–HPMPC and ODE–HPMPA has been reported previously (Beadle et al., 2002, 2006). Scheme 1 illustrates the synthetic route utilized for the ODE esters of HPMPT, HPMPG and HPMPDAP and HPMP-N^6-cyclopropyl-DAP. Protected bases **1**–**4** reacted with (*S*)-trityl glycidyl ether to give the corresponding 3-trityloxy-2-hydroxypropyl nucleosides **5**–**8** which were then alkylated by octadecyloxyethyl p-toluenesulfonyloxymethyl-phosphonate to give **9**–**12**. Acidic deprotection (80% aqueous CH_3COOH) gave the desired octadecyloxyethyl esters of 3-hydroxy-2-(phosphonomethoxy)propyl nucleosides **13**–**16**.

3.2. Antiviral activity in HSV-1 infected cells

ODE esters of the HPMP nucleosides were added to MRC-5 cells previously infected with HSV-1 and the effect on viral replication was assessed using the ELVIRA assay (Table 1). The most active compound, ODE–HPMPA, exhibited an EC_{50} < 10 pM and a selectivity index >6×10^5. ODE–HPMPC was the next most active antiviral with an EC_{50} of 0.47 nM and a selectivity index of 3800. ODE-(*S*)-HPMPG was intermediate in activity with an EC_{50} value of 40 nM and a selectivity index of 30. The ODE esters of HPMPT, HPMPDAP and HPMP-cPrDAP were less active with EC_{50} values of 0.34, 0.43 and 0.78 μM, respectively. The order of activity of the ODE esters

Table 2
Antiviral activity and selectivity of octadecyloxyethyl esters of 3-hydroxy-2-(phosphonomethoxy)propyl nucleosides against HCMV and MCMV.

Compound	HCMV EC_{50}[a]	HCMV CC_{50}[b]	SI[c]	MCMV EC_{50}[d]	MCMV CC_{50}[d]
GCV	2.7 ± 1.2	>392	>145	4.7	>100
ODE–HPMPC	0.0009 ± 0.0001	2.5 ± 0.1	2700	0.0006	>1
ODE-(S)-HPMPA	0.003 ± 0.001	0.5 ± 0.1	170	0.009	1.2
ODE-(S)-HPMPG	0.022 ± 0.02	8.7 ± 0.6	400	0.10	8.0
ODE-(S)-HPMPDAP	0.077 ± 0.03	37 ± 4	480	0.23	>10
ODE-(S)-HPMPT	0.35 ± 0.24	39 ± 6	110	0.27	>10

[a] Concentration (μM) sufficient to reduce plaque formation by 50% (EC_{50}) expressed as the mean of two or more replicates with standard deviation values shown.
[b] Concentration (μM) that reduced cell viability by 50% (CC_{50}) in a neutral red uptake assay. Values shown represent the mean of two or more replicates with standard deviation values.
[c] Selectivity index (SI) is calculated as the CC_{50} value divided by the EC_{50} value.
[d] Values shown represent a single determination of EC_{50} and CC_{50} values.

Table 3
Antiviral activity of octadecyloxyethyl esters of 3-hydroxy-2-(phosphonomethoxy)propyl nucleosides against vaccinia and cowpox viruses in HFF cells in vitro.

Compound	HFF cells CC_{50}, μM[a]	Vaccinia EC_{50}, μM[b]	Vaccinia SI[c]	Cowpox EC_{50}, μM[b]	Cowpox SI[c]
ODE–HPMPC	25 ± 19	0.3 ± 0.3	83	0.09 ± 0	278
ODE-(S)-HPMPA	1.4 ± 0.6	0.003 ± 0.007	466	0.008 ± 0.001	175
ODE-(S)-HPMPG	36 ± 28	0.02 ± 0	1800	0.06 ± 0.007	600
ODE-(S)-HPMPT	66 ± 16	2.1 ± 1.1	31	5.9 ± 2.8	11
ODE-(S)-HPMPDAP	70 ± 14	0.02 ± 0.004	3500	0.03 ± 0.02	2330

[a] Concentration (μM) that reduced cell viability by 50% (CC_{50}) in a CellTiter Glo assay. Values shown represent the mean of three or more replicates with standard deviation values.
[b] Concentration (μM) sufficient to reduce plaque formation by 50% (EC_{50}) expressed as the mean of three or more replicates with standard deviation values shown.
[c] Selectivity index (SI) is calculated as the CC_{50} value divided by the EC_{50} value.

is HPMPA ≫ HPMPC ≫ HPMPG > HPMPT = HPMPDAP > HPMPcPrDAP.

3.3. Antiviral activity in HCMV and MCMV infected cells

The efficacy of the ODE esters was also assessed against HCMV and the HPMPC analog was the most active with an EC_{50} of less than 1 nM with a selectivity index of 3970 (Table 2). ODE–HPMPA was also highly effective with an EC_{50} value of 3 nM, but it was the most cytotoxic of this series with a CC_{50} value of 0.5 μM. All the compounds had selectivity index values of ≥500, with the exception of HPMPT. Similar results were obtained in a single experiment with MCMV, and the order of efficacy was the same as with HCMV. The overall order of efficacy of the analogs against HCMV was ODE–HPMPC > ODE–HPMPA > ODE–HPMPG = ODE–HPMPDAP ≫ ODE–HPMPT. The most selective compounds were ODE–HPMPC > ODE–HPMPDAP > ODE–HPMPG > ODE–HPMP ≫ ODE–HPMPT.

3.4. Antiviral activity against orthopoxviruses

We tested the antiviral activity of the ODE ester series against vaccinia virus and cowpox virus (Table 3). ODE–HPMPA was the most active compound with an EC_{50} of 3 nM, followed by ODE–HPMPG and ODE–HPMPDAP with EC_{50} values of 20 nM. ODE esters of HPMPC and HPMPT were less active. Generally similar results were noted with cowpox virus (Table 3). The most selective compounds of this series against vaccinia and cowpox were ODE–HPMPG and ODE–HPMPDAP with selective index values of 600 and 2330, respectively. We also tested ODE analogs of HPMPG, HPMPT and HPMPDAP against ectromelia virus replication in vitro (Table 4). ODE–HPMPG was the most active and selective of these compounds with an EC_{50} of 0.41 μM and a selectivity index of 166.

4. Discussion

Previous authors have reported the antiviral activity of the unmodified HPMP nucleoside phosphonates (Holý et al., 1987; De Clercq et al., 1987; Beadle et al., 2002) as shown in Table 1. It is readily apparent from an examination of Table 1 that the ODE–HPMP nucleosides are several orders of magnitude more active in HSV-1 infected cells than the corresponding unmodified nucleoside phosphonates. The EC_{50} values for the unmodified nucleoside phosphonates from the literature were as follows: (*S*)-HPMPA 6.6–23.1 μM, (*S*)-HPMPC 3.3–21.9 μM, (*R,S*)-HPMPG 23.1 μM, (*R,S*)-HPMPDAP 23.1–31.4 μM and (*S*)-HPMPT 250–535 μM (Holý et al., 1987; De Clercq et al., 1987; Beadle et al., 2002). However, these comparisons should not be considered as definitive because the work was done by different groups using different methods, different strains of HSV and different cell types.

In HCMV infected cells, ODE–HPMPC is clearly the most active and selective compound. A close analog of this compound, CMX001 (hexadecyloxypropyl-cidofovir, HDP-CDV), is currently entering Phase II clinical trials for diseases caused by BK virus and HCMV (ClinicalTrials.gov identifier: NCT00793598 and NCT00942305). Our current results indicate that ODE–HPMPG and ODE–HPMPDAP are less cytotoxic then ODE–HPMPC and have good selectivity indexes of 400 and 480. These compounds are suitable for further investigation with regard to their oral pharmacokinetics and toxicology. It would also be of interest to know if either ODE–HPMPG or ODE–HPMPDAP is synergistic with CMX001 in vitro.

In orthopoxvirus infected cells (Tables 3 and 4), the situation is quite different with the ODE esters of HPMPA, HPMPG and HPMPDAP being the most active compounds. Of these, the most interesting compounds for further evaluation are ODE–HPMPG

Table 4
Antiviral activity and selectivity of some octadecyloxyethyl esters of 3-hydroxy-2-(phosphonomethoxy)propyl nucleosides against ectromelia virus.

Compound	CC_{50}[a], μM	EC_{50}, μM	SI
ODE-(S)-HPMPG	68 ± 12	0.41 ± 0.003	166
ODE-(S)-HPMPDAP	119 ± 4.5	1.15 ± 0.11	104
ODE-(S)-HPMPT	37 ± 5.8	21.2 ± 0.93	1.75

[a] MTS cytotoxicity in BSC-1 cells; data expressed as mean ± SD of three or more replicates; SI, selective index as defined in Table 2.

and ODE–HPMPDAP which are less cytotoxic and have the highest selectivity index values of 1800 and 3500, respectively. Again, these two compounds should be studied further to evaluate their oral pharmacokinetics and toxicology and possible synergy with CMX001 against orthopoxviruses.

The reason for the increased antiviral activity of ODE esters of the HPMP nucleosides has not been studied. However, our prior studies with the related esters, hexadecyloxypropyl-(*S*)-HPMPA and hexadecyloxypropyl-(*S*)-HPMPC, have shown that the ether lipid modified compounds exhibit 50–60 times greater cell uptake and 20–100-fold greater cellular conversion to HPMPC diphosphate and HPMPA diphosphate than the unmodified nucleoside phosphonates (Aldern et al., 2003; Magee et al., 2008). This was also true for ODE–HPMPC (Aldern et al., 2003) but uptake and metabolism of the other ODE–HPMP nucleosides has not yet been studied. Nevertheless, it is likely that the increased antiviral activity observed in this study is due to the same mechanism.

HPMPA and HPMPC are reported to inhibit the replication of all double stranded DNA viruses (Holý, 2003; De Clercq and Holý, 2005). In the case of HCMV, this occurs after incorporation of two consecutive HPMPC residues opposite template Gs resulting in chain termination (Xiong et al., 1997). However, HPMPCpp inhibits vaccinia virus replication by substantially slowing viral DNA chain extension after incorporation of an HPMPC residue followed by incorporation of any additional base (Magee et al., 2005). Interestingly, the mechanism of action of HPMPA differs from HPMPC in vaccinia infections. In this case, HPMPApp is readily incorporated into vaccinia viral DNA and chain extension is not slowed by the incorporation of one or more HPMPA bases. Template strand inhibition occurs because the vaccinia DNA polymerase is unable to bridge HPMPA bases in the new viral template, resulting in chain termination (Magee et al., 2008). Template strand inhibition also occurs if an HPMPC residue is present in the template (Magee et al., 2008). The effects of HPMPG and HPMPDAP diphosphates on the DNA polymerases of HSV-1, HCMV or vaccinia virus have not yet been examined; this represents an important area for future research.

In conclusion, the 3-hydroxy-2-(phosphonomethoxy)propyl (HPMP) nucleosides were previously identified as rather weak inhibitors of HSV and other double stranded DNA viruses (De Clercq and Holý, 2005; Holý, 2003). Esterification of one of the two phosphonate oxygens with an octadecyloxyethyl ester moiety blocks one of the negative charges and facilitates cellular uptake (Aldern et al., 2003; Magee et al., 2008). We have shown that the anti-HSV activity of 6 different HPMP nucleosides is increased by several orders of magnitude when they are esterified with an octadecyloxyethyl moiety. The two most active compounds against HSV-1 were ODE–HPMPA and ODE–HPMPC with subnanomolar EC_{50} values. The antiviral activity of five 3-hydroxy-2-(phosphonomethoxy)propyl nucleosides was also tested against HCMV, MCMV, vaccinia, cowpox and ectromelia viruses. The ODE esters of HPMPC and HPMPA were the most active against HCMV and MCMV in vitro. ODE–HPMPC was the most selective of the five compounds tested. Against the orthopoxviruses, the most active compound was ODE–HPMPA, but it was also the most cytotoxic compound. For the orthopoxviruses, the most promising analogs were ODE–HPMPG and ODE–HPMPDAP because of their excellent antiviral activity and low cytotoxicity. Future research should evaluate their oral pharmacokinetics, toxicology and possible synergy with HDP-CDV (CMX001), a compound in clinical development for BK and HCMV viruses.

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