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In vitro evaluation of the anti-orf virus activity of alkoxyalkyl esters of CDV, cCDV and (*S*)-HPMPA

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Abstract

Acyclic nucleoside phosphonates (ANPs) and in particular (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (HPMPC, cidofovir, CDV, Vistide®) and its adenine counterpart (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine [(*S*)-HPMPA] are highly active against orf virus infections. This parapoxvirus commonly causes infection in sheep, goats, but also humans. Alkoxyalkyl esters of CDV have an increased oral bioavailability and are more active against orthopoxviruses than the parent compounds. In the present study, the potency of several alkoxyalkyl esters of CDV, cyclic cidofovir (cCDV) and (*S*)-HPMPA was evaluated against different orf virus isolates in two cell types, human embryonic lung (HEL) fibroblast and primary lamb keratinocytes. Each prodrug was at least 10-fold more active than its parent compound in both cell types. Of all the compounds tested, the (*S*)-HPMPA alkoxyalkyl esters showed the highest activity and selectivity against orf virus. Our results support the development of alkoxyalkyl esters of ANPs as antivirals not only for the treatment of complicated human orf lesions, but also in the therapy and prophylaxis of contagious ecthyma in sheep and goats.

Keywords: Orf virus; Contagious ecthyma; Acyclic nucleoside phosphonates; Oral bioavailability; Prodrugs

1. Introduction

Orf virus is the type species of the *Parapoxvirus* genus and causes a skin infection in sheep, goats and humans known as contagious ecthyma or orf. Other members of the *Parapoxvirus* genus, the bovine papular stomatitis virus (BPSV) and the pseudocowpox virus (PCPV), both commonly affecting cattle, also cause human infection (Haig and Mercer, 1998). Human orf is defined as an occupational zoonosis, but infections have also been reported in non-professional individuals as a consequence of direct contact with infected products in religious sacrifices (Gunduz et al., 2005; Uzel et al., 2005). The lesions are localized on the fingers, hands and arms, but they can also occur on the face (Bodnar et al., 1999; Chahidi et al., 1993). Orf virus infection in humans, as well as in sheep and goats, typically progresses through the stages of erythema, papula, pustule and ends with the formation of a scab, with the resolution of the lesions within 6–8 weeks (Groves et al., 1990; Leavell et al., 1968). Occasionally associated bacterial infections, fever, regional lymphoadenopathy, erythema multiforme and bullous pemphigoid have been described; these cases often require medical treatment in a hospital setting (Agger and Webster, 1983; Hansen et al., 1984; Mourtada et al., 2000; Murphy and Ralfs, 1996; Uzel et al., 2004). In burned and immunocompromised patients, tumour-like nodules known as "giant orf" have been described; these lesions, which often relapse after cryotherapy or excision of the mass, may ultimately require amputation (Ballanger et al., 2006; Degraeve et al., 1999; Gurel et al., 2002; Hunskaar, 1986; Pether et al., 1986; Tan et al., 1991). In order to treat these complicated, extensive and relapsing lesions, development of an effective antiviral therapy seems to be essential.

The efficacy of the (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (HPMPC, cidofovir, CDV, Vistide®) has been demonstrated in vitro against orf virus and pseudocowpox virus (Nettleton et al., 2000). Recently, the

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antiviral activity of CDV, or its adenine counterpart (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine [(*S*)-HPMPA] and other acyclic nucleoside phosphonates (ANPs) have been proved against several orf virus strains in vitro and in organotypic ovine raft cultures (Dal Pozzo et al., 2005). In vivo, CDV has been used as a cream in a Beeler base vehicle for the topical treatment of a giant orf lesion in a transplanted patient (Geerinck et al., 2001) and in orf virus lesions in experimentally infected lambs (Scagliarini et al., 2007). Despite the observation that CDV can be nephrotoxic after intravenous administration, no significant systemic side effects have been noted with topical treatment (Geerinck et al., 2001; Scagliarini et al., 2007). Topical cidofovir has been used in the treatment of other infections caused by epitheliotropic viruses, such as herpesviruses, human papillomaviruses and molluscum contagiosum (Koonsaeng et al., 2001; Pontes et al., 2006; Snoeck et al., 1994; Toro et al., 2000). The low oral bioavailability of CDV does not allow effective oral administration but lipid derivatives of CDV and cyclic-CDV (cCDV), have been shown to have substantial oral bioavailability and less kidney accumulation compared to the respective unmodified compounds (Buller et al., 2004; Ciesla et al., 2003; Quenelle et al., 2004). Alkoxyalkyl and alkyl esters of CDV and cCDV demonstrated a higher activity in vitro and in animal models against vaccinia virus (VV) and cowpox virus (CPV) infection (Buller et al., 2004; Keith et al., 2004; Kern et al., 2002; Quenelle et al., 2004). Recently, the activity of alkoxyalkyl esters of CDV, cCDV and (*S*)-HPMPA has been demonstrated against VV and CPV in primary human keratinocytes (PHK) and in organotypic raft cultures (Lebeau et al., 2006). Here, we report the in vitro activity of various prodrugs of CDV, cCDV and (*S*)-HPMPA against orf virus in human embryonic lung cells (HEL) and in primary lamb keratinocyte (PLK) cells.

2. Materials and methods

2.1. Cells and virus

Different Italian orf virus strains were used in this work (IT-01, IT-Mi90, IT-To, IT-C2). IT-01 was isolated from a proliferative form of contagious ecthyma in sheep, while the other Italian isolates were obtained from classical lesions in a lamb (IT-C2) and in chamois (IT-Mi90 and IT-To) (Scagliarini et al., 2006). The New Zealand orf virus NZ2 isolate was kindly provided by A. Mercer (Otago University Dunedin, New Zealand).

Primary lamb keratinocytes were isolated and propagated in cell culture as described elsewhere (Dal Pozzo et al., 2005). Human embryonic lung fibroblasts (HEL-299, ATCC CCL-137) were cultured as previously described (Andrei et al., 2004). Orf virus was propagated and subsequently titrated in PLK and in HEL cells.

2.2. Antiviral molecules

CDV, hexadecycloxypropyl-CDV (HDP-CDV), octadecycloxyethyl-CDV (ODE-CDV), oleyloxypropyl-CDV (OLP-CDV), 1-*O*-octadecyl-2-*O*-benzyl-glyceryl-CDV (ODBG-CDV), oleyloxyethyl-CDV (OLE-CDV), cyclic cidofovir (cCDV, cHPMPC), oleyloxyethyl-cCDV (OLE-cCDV), (*S*)-HPMPA, hexadecycloxypropyl-(*S*)-HPMPA [HDP-(*S*)-HPMPA] and octadecycloxyethyl-(*S*)-HPMPA [ODE-(*S*)-HPMPA] were used in this study. Structures and synthesis of the different alkoxyalkyl esters of CDV, cCDV and (*S*)-HPMPA have been described elsewhere (Beadle et al., 2002; Hartline et al., 2005; Kern et al., 2002).

2.3. Cytotoxicity assay

HEL and PLK cells were seeded in 96-well microtiter plates at a density of 3.5×10^3 and 5×10^3 per well, respectively. After 24 or 48 h of incubation for HEL and PLK, respectively, serial dilutions (in duplicate) of the test compounds were added. After 3–4 days of incubation, cells were trypsinized and the cell number was determined with a Coulter Counter. Cytotoxicity was expressed as the 50% cytotoxic concentration (CC_{50}) or the concentration required to reduce cell growth by 50% (referring to the number of cells in the untreated cell control). The CC_{50} values represent the means from two independent experiments.

2.4. Antiviral assay

The antiviral activity of the alkoxyalkyl esters of CDV, cCDV, (*S*)-HPMPA and of the parental compounds was tested against each of the orf virus strains listed above. Confluent HEL and PLK cells in 96-well microtiter plates were infected with a viral inoculum ranging from 20 to 60 PFU/100 μl/well. After 2 h of incubation at 37 °C and 5% CO_2, the viral inoculum was removed and replaced by medium containing serial dilutions of the test compounds (in duplicate). After 3 days of incubation at 37 °C and 5% CO_2, the viral cytopathic effect (CPE) was recorded and the 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to reduce 50% of the viral CPE. The IC_{50} values of the test compounds against the individual virus strains was calculated as the means of two or more independent experiments. The selectivity index (SI) (defined as the ratio of the CC_{50} for cell growth to the IC_{50} for viral cytopathic effect) for each compound tested was calculated first for each orf virus strain and then expressed as the mean SI.

3. Results

3.1. Cytotoxicity

For both the prodrugs and the parent compounds, the toxicity on growing PLK cells was lower than on growing HEL fibroblast monolayers (Tables 1 and 2). In both cell lines the CC_{50} values of the alkoxyalkyl esters were uniformly lower than the CC_{50} values of the parent compounds. In growing HEL cells, ODE-CDV was the most cytotoxic CDV derivative, while ODE-(*S*)-HPMPA and HDP-(*S*)-HPMPA showed the highest cytotoxicity among all the prodrugs. In PLK cells, the order of cytotoxicity of the test compounds was different among the CDV prodrugs, with OLE-CDV being the most toxic molecule while the (*S*)-HPMPA derivatives again showed the highest cytotoxicity.

Table 1
Activity of alkoxyalkyl esters of CDV, cCDV and (*S*)-HPMPA against different strains of orf virus (ORFV) in HEL fibroblast monolayer

Human embryonic lung fibroblasts														
Compound	Antiviral activity IC_{50} (μg/ml)[a]							Cytotoxicity (μg/ml)	SI[b]					
	ORFV strains					Average	Fold[c]	CC_{50}[d]	ORFV strains					Average
	IT-C2	IT-Mi-90	IT-To	NZ2	IT-01				IT-C2	IT-Mi-90	IT-To	NZ2	IT-01	
HDP-CDV	0.0005 ± 0.0000	0.0004 ± 0.0001	0.0017 ± 0.0016	0.0015 ± 0.0007	0.0004 ± 0.0002	0.0009 ± 0.0006	344	0.37 ± 0.09	740	925	218	247	925	611 ± 354
ODE-CDV	0.0010 ± 0.0006	0.0018 ± 0.0018	0.0018 ± 0.0003	0.0012 ± 0.0002	0.0007 ± 0.0003	0.0013 ± 0.0005	238	0.16 ± 0.11	160	89	89	133	229	140 ± 58
OLP-CDV	0.0017 ± 0.0004	0.0008 ± 0.0004	0.0016 ± 0.0006	0.0029 ± 0.0020	0.0007 ± 0.0003	0.0015 ± 0.0009	207	1.04 ± 0.51	612	1300	650	359	1486	881 ± 485
ODBG-CDV	0.0006 ± 0.0002	0.0005 ± 0.0001	0.0023 ± 0.0020	0.0027 ± 0.0023	0.0009 ± 0.0005	0.0014 ± 0.0010	221	0.30 ± 0.09	500	600	130	111	333	335 ± 218
OLE-CDV	0.0007 ± 0.0002	0.0004 ± 0.0001	0.0026 ± 0.0008	0.0018 ± 0.0007	0.0009 ± 0.0002	0.0013 ± 0.0009	238	0.18 ± 0.09	257	450	69	100	200	215 ± 151
CDV	0.37 ± 0.29	0.17 ± 0.05	0.35 ± 0.13	0.41 ± 0.24	0.24 ± 0.07	0.31 ± 0.10		37.9 ± 0.7	102	223	108	92	158	137 ± 54
OLE-cCDV	0.0020 ± 0.0000	0.0012 ± 0.0011	0.0029 ± 0.0030	0.0008 ± 0.0004	0.0014 ± 0.0001	0.0017 ± 0.0008	512	0.26 ± 0.18	90	150	62	225	129	131 ± 63
cCDV	0.87 ± 0.32	1.00 ± 0.00	0.67 ± 0.29	0.83 ± 0.38	1.00 ± 0.92	0.87 ± 0.14		42.4 ± 12.2	49	42	63	51	42	50 ± 9
HDP-HPMPA	0.000014 ± 0.000011	ND	ND	0.000013 ± 0.00	ND	0.000013 ± 0.000007	5078	0.044 ± 0.003	3143	ND	ND	3438	ND	3291 ± 209
ODE-HPMPA	0.000030 ± 0.000025	ND	ND	0.000021 ± 0.000012	ND	0.000026 ± 0.000017	2538	0.01 ± 0.00	333	ND	ND	478	ND	406 ± 103
HPMPA	0.068 ± 0.030	0.050 ± 0.000	0.057 ± 0.012	0.064 ± 0.019	0.093 ± 0.040	0.066 ± 0.016		2.80 ± 0.07	41	56	49	44	30	44 ± 10

Values represent the means for at least two independent experiments. ND: not determined.

[a] Concentration required to inhibit virus-induced CPE by 50%.

[b] Selectivity index: ratio of CC_{50} to IC_{50}.

[c] Fold decrease in IC_{50} values compared to the corresponding parent compound.

[d] Concentration required to reduce cell growth by 50%.

Table 2
Activity of alkoxyalkyl esters of CDV, cCDV and (*S*)-HPMPA against different strains of orf virus (ORFV) in PLK monolayer

Primary lamb keratinocytes														
Compound	Antiviral activity IC_{50} (μg/ml)[a]							Cytotoxicity (μg/ml)	SI[b]					
	ORFV strains					Average	Fold[c]	CC_{50}[d]	ORFV strains					Average
	IT-C2	IT-Mi-90	IT-To	NZ2	IT-01				IT-C2	IT-Mi-90	IT-To	NZ2	IT-01	
HDP-CDV	0.037 ± 0.023	0.08 ± 0.085	0.018 ± 0.009	0.18 ± 0.13	0.12 ± 0.02	0.087 ± 0.065	28	>5	>179	>63	>278	>28	>43	118 ± 107
ODE-CDV	0.0032 ± 0	0.023 ± 0.025	0.0095 ± 0.0064	0.03 ± 0.01	0.03 ± 0.02	0.019 ± 0.012	127	3.21 ± 1.95	465	140	338	107	107	229 ± 159
OLP-CDV	0.067 ± 0.032	0.19 ± 0.25	0.031 ± 0.028	0.32 ± 0.14	0.4 ± 0.17	0.20 ± 0.16	12	>5	>76	>26	>161	>16	>13	58 ± 63
ODBG-CDV	0.017 ± 0.014	0.15 ± 0.18	0.0055 ± 0.0021	0.11 ± 0.07	0.093 ± 0.04	0.075 ± 0.061	32	3.93 ± 0.49	218	26	715	36	42	207 ± 295
OLE-CDV	0.0038 ± 0.018	0.047 ± 0.047	0.014 ± 0.009	0.03 ± 0.021	0.06 ± 0.036	0.031 ± 0.023	78	2.73 ± 1.99	144	58	195	91	46	107 ± 62
CDV	0.24 ± 0.07	2.6 ± 0.85	0.5 ± 0	1.26 ± 0.7	7.5 ± 3.54	2.42 ± 2.98		>50	>53	>19	>100	>40	>9	44 ± 36
OLE-cCDV	0.021 ± 0.016	0.13 ± 0.11	0.0095 ± 0.0064	0.13 ± 0.13	0.15 ± 0.05	0.088 ± 0.067	35	4.53 ± 0.81	105	35	477	35	30	136 ± 193
CCDV	0.24 ± 0	3.2 ± 2.55	1.25 ± 1.06	5.33 ± 3.51	5.63 ± 3.79	3.13 ± 2.40		>50	>27	>16	>40	>9	>9	20 ± 13
HDP-HPMPA	0.011 ± 0.001	ND	ND	0.036 ± 0.006	ND	0.023 ± 0.018	31	1.60 ± 0.57	145	ND	ND	44	ND	94 ± 71
ODE-HPMPA	0.0026 ± 0.0013	ND	ND	0.006 ± 0.001	ND	0.0043 ± 0.0024	169	1.15 ± 1.20	442	ND	ND	192	ND	317 ± 177
HPMPA	0.18 ± 0.09	0.83 ± 0.24	0.41 ± 0.01	0.61 ± 0.36	1.65 ± 0.92	0.74 ± 0.56		22.18 ± 14.19	72	27	54	36	13	40 ± 23

Values represent the means for at least two independent experiments. ND: not determined.

[a] Concentration required to inhibit virus-induced CPE by 50%.

[b] Selectivity index: ratio of CC_{50} to IC_{50}.

[c] Fold decrease in IC_{50} values compared to the corresponding parent compound.

[d] Concentration required to reduce cell growth by 50%.

3.2. Antiviral activity

In HEL as well as in PLK cell monolayers, no significant variation was observed in the potency of the test compounds against the different orf virus strains (Tables 1 and 2).

In HEL cell monolayers, all the prodrugs proved more active against orf virus than their parent compounds (Table 1). HDP-CDV was the most active prodrug of CDV, with an average IC_{50} value of 0.0009 ± 0.0006 μg/ml compared to 0.31 ± 0.1 μg/ml for its parent compound. The activity increased 200-fold (OLP-CDV) to 340-fold (HDP-CDV) in comparison to CDV. OLE-cCDV was 500-fold more active than cCDV. The two (*S*)-HPMPA prodrugs showed the highest activity of all prodrugs, with IC_{50} values that were 2500-fold [ODE-(*S*)-HPMPA] or 5000-fold [HDP-(*S*)-HPMPA] lower than the parent compound.

In PLK monolayer cultures, all the alkoxyalkyl esters showed stronger antiviral activity than the parent compounds (Table 2), although the difference was less pronounced than in HEL cells. In PLK cells, the order of activity of the CDV derivatives against orf virus was different from their ranking in HEL cells. In PLK cells, ODE-CDV was the most active molecule among CDV derivatives, followed by OLE-CDV and ODBG-CDV, with IC_{50} values 130-, 80- and 30-fold lower than for CDV. OLP-CDV and HDP-CDV were the least active prodrugs with IC_{50} values of 0.2 ± 0.16 and 0.087 ± 0.06 μg/ml, respectively. In PLK cells, ODE-(*S*)-HPMPA clearly showed the highest activity among all the prodrugs, with a 170-fold reduced IC_{50} value.

3.3. Selectivity

Despite their higher toxicity in both cell lines, the prodrugs revealed an increased selectivity in comparison to their parent compounds due to their greater potency (Tables 1 and 2). By comparing the average SI values in HEL cells, OLP-CDV proved to be the most selective CDV prodrug while HDP-(*S*)-HPMPA emerged as the most selective of all prodrugs tested against orf virus replication.

In PLK cells, the selectivity of the prodrugs towards orf virus replication was reduced compared to HEL cells, but still remarkably high. OLP-CDV was the least selective compound among all the alkoxyalkyl esters, whereas ODE-CDV and ODE-(*S*)-HPMPA revealed the highest SI, with values of 230 and 320, respectively.

4. Discussion

Human orf is known as a secondary zoonosis because of the low incidence of the infection and of the usual self-limiting characteristic of the disease in hosts with a normal immune system. The exposure to orf virus occurs after contact with infected animals or fomites and is mostly confined to rural areas. With classical and localized lesions, symptomatic treatment and antibiotics to prevent secondary bacterial infections are sufficient to reduce further complications. In immunocompromised and transplant patients, the occurrence of opportunistic bacterial, mycotic and viral infections is often observed. Some cases of giant orf have been described in this group of patients and several therapies have been used including topical treatment with idoxuridine 40% in dimethylsulphoxide (Hunskaar, 1984, 1986), injections with α-interferon (Tan et al., 1991), cryotherapy (Degraeve et al., 1999), radiation therapy and surgical excision (Ballanger et al., 2006; Tan et al., 1991). The use of a steroid cream during an orf infection complicated by generalized atopic eczema, has led to further propagation of the orf lesion (Dupré et al., 1981). In the past, amputation has been required to halt the spread of a giant orf lesion in a patient with lymphoma (Savage and Black, 1972). The first use of an antiviral, with a specific activity towards orf virus, occurred recently with the treatment based on cidofovir cream of a giant orf lesion on a finger of a renal transplant recipient (Geerinck et al., 2001). Although CDV can be nephrotoxic when given systemically, topical application did not alter the renal function in this patient who already had diminished renal function. Topical CDV has been recently used also in the therapy of contagious ecthyma in experimentally infected lambs. In this case, a reduction in the severity and the duration of the lesions was observed, together with a decrease in viral viability in the scabs, which represent the most important vehicle of environmental contamination (Scagliarini et al., 2007).

Alkoxyalkyl prodrugs of CDV have shown enhanced oral bioavailability and lower kidney accumulation compared to the parent compound; these characteristics would make possible their oral administration in patients with compromised general conditions. Oral administration of an antiviral in a flock would be easier than the use of a topical formulation.

In this study, human embryonic lung fibroblasts and primary lamb keratinocytes were used to assess the antiviral activity of the alkoxyalkyl esters of CDV, cCDV and (*S*)-HPMPA against orf virus replication in vitro. Several orf virus strains have been used in order to evaluate the antiviral activity of the prodrugs and their parent compounds against clinical isolates obtained from classical (IT-C2, IT-Mi90, IT-To) and proliferative forms of the disease (IT-01), as well as from more (NZ2, IT-To) and less cell-adapted strains (IT-01, IT-Mi90 and IT-C2) (Scagliarini et al., 2006). As previously reported when testing the ANPs against orf virus (Dal Pozzo et al., 2005), no significant variation was observed in the activity of the prodrugs against the different orf virus strains. Consequently, the results were presented as IC_{50} values for each individual strain as well as an average IC_{50}s value for all the strains.

Although the selectivity of the parent compounds was already high in PLK cells, the SI increased up to 10-fold when the prodrugs were tested. Among all the alkoxyalkyl esters, the (*S*)-HPMPA prodrugs showed the highest activity and selectivity against orf virus. In particular, HDP-(*S*)-HPMPA and ODE-(*S*)-HPMPA were the most potent compounds in HEL and in PLK cells, respectively. Similarly, ODE-(*S*)-HPMPA exhibited the strongest activity against both vaccinia virus and cowpox virus (Lebeau et al., 2006). The antiviral activity of the alkoxyalkyl esters of CDV containing an ethanediol linker (ODE, OLE) was previously reported first in human foreskin fibroblasts against orthopoxvirus replication (Keith et al., 2004) and human cytomegalovirus (Beadle et al., 2002) and later in HEL and PHK

cells infected with VV and CPV (Lebeau et al., 2006). In our study, it was confirmed that in PLK cell monolayers, ODE-CDV and OLE-CDV were the most potent CDV prodrugs against a parapoxvirus. ODBG-CDV was previously found to have high activity in PHK cells infected with VV and CPV (Lebeau et al., 2006), and this compound also showed high activity against orf virus-infected PLK monolayers. Our results obtained testing the alkoxyalkyl esters of CDV, cCDV and (*S*)-HPMPA against a member of the *Parapoxvirus* genus are consistent with the ones obtained with members of the *Orthopoxvirus* genus, underlying the possibility of using orf virus as a model for the study of antiviral strategies against poxviruses.

The high activity of lipid derivatives of CDV, cCDV and (*S*)-HPMPA encourages the development of these compounds for use in normal and immunocompromised patients suffering from proliferative and recurrent orf virus infections, and also for the prevention and treatment of poxvirus infections in case of a poxvirus outbreak. Orally, bioavailable derivatives of CDV would also represent promising antivirals for the treatment of contagious ecthyma in animals. In large-scale treatment of a flock, oral administration of an effective antiviral would be a great advantage in the therapy or prophylaxis of orf virus infection.

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