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Activities of Alkoxyalkyl Esters of Cidofovir (CDV), Cyclic CDV, and (*S*)-9-(3-Hydroxy-2-Phosphonylmethoxypropyl)Adenine against Orthopoxviruses in Cell Monolayers and in Organotypic Cultures

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The potencies of several alkoxyalkyl esters of acyclic nucleoside phosphonates against vaccinia virus and cowpox virus were evaluated in cell monolayers and three-dimensional epithelial raft cultures. Prodrugs were at least 20-fold more active than their parent compounds. Octadecycloxyethyl-(*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine emerged as the most potent derivative.

Smallpox has been declared eradicated since 1980 (5). However, due to the potential threat of variola virus as a weapon for bioterrorism (4) and the increasing number of infections with monkeypox virus (11), it is essential that adequate antiviral drugs be developed. Cidofovir (CDV) has already proven to be active against orthopoxviruses (9, 10, 14, 16, 18). However, this drug needs to be administered intravenously and is potentially nephrotoxic. Recently, it was shown that alkoxyalkyl and alkyl esters of CDV and cyclic cidofovir (cCDV) were more active against vaccinia virus (VV) and cowpox virus (CPV) in human foreskin fibroblasts than their parent compounds due in part to an increased penetration through the cell membrane (13, 15). In vivo studies indicate that the prodrugs are highly orally bioavailable and are as effective as parenterally administered CDV for the treatment of VV- or CPV-infected mice (6, 7, 17).

The activities of various alkoxyalkyl esters of the acyclic nucleoside phosphonate (ANP) analogues CDV [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; HPMPC; Vistide],

TABLE 1. Activities of alkoxyalkyl esters of CDV, cCDV, and HPMPA against VV and CPV in HEL fibroblast monolayers

Compound	VV		CPV		Cytotoxicity (μg/ml)		SI[e]	
	EC_{50} (μg/ml)[a]	Fold decrease[b]	EC_{50} (μg/ml)[a]	Fold decrease[b]	MCC (μg/ml)[c]	CC_{50} (μg/ml)[d]	VV	CPV
HDP-CDV	0.013 ± 0.006	360	0.035 ± 0.004	390	>5	0.37 ± 0.09	28	11
ODE-CDV	0.0051 ± 0.0043	910	0.014 ± 0.001	900	>5	0.16 ± 0.11	31	11
OLP-CDV	0.019 ± 0.012	260	0.032 ± 0.000	420	>5	1.04 ± 0.51	55	33
ODBG-CDV	0.044 ± 0.017	130	0.069 ± 0.004	230	>5	0.30 ± 0.09	7	4
OLE-CDV	0.0083 ± 0.0029	570	0.018 ± 0.004	720	>5	0.18 ± 0.09	22	10
CDV	2.52 ± 1.45		6.83 ± 0.34		>50	37.9 ± 0.7	15	6
OLE-cCDV	0.0035 ± 0.0034	2,800	0.011 ± 0.005	1,500	>5	0.26 ± 0.18	74	24
cCDV	5.25 ± 1.23		8.97 ± 1.46		>50	42.4 ± 12.2	8	5
HDP-HPMPA	0.00051 ± 0.00044	670	0.0017 ± 0.0022	250	>2	0.044 ± 0.003	86	26
ODE-HPMPA	0.000023 ± 0.000023	15,300	0.000062 ± 0.000035	7,200	>2	0.01 ± 0.00	435	161
HPMPA	0.19 ± 0.10		0.24 ± 0.13		>20	2.80 ± 0.07	15	12

[a] Concentration required to inhibit 50% of virus-induced CPE. The EC_{50} values of each compound represent the mean ± standard deviation of the EC_{50} values of at least two independent experiments.

[b] Fold decrease in EC_{50} relative to that of the parental compound based on molar values, given the following molecular weights: HDP-CDV, 583.68; ODE-CDV, 581.71; OLP-CDV, 609.71; ODBG-CDV, 717.85; OLE-CDV, 595.68; CDV, 315.12; OLE-cCDV, 559.65; cCDV, 297.2; HDP-HPMPA, 585.72; ODE-HPMPA, 599.38; HPMPA, 325.

[c] Minimum cytotoxic concentration required to alter cell morphology.

[d] Concentration required to reduce cell growth by 50%. The CC_{50} values of each compound represent the mean ± standard deviation of the CC_{50} values of at least two independent experiments.

[e] Selectivity index (ratio of CC_{50} to EC_{50}).

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TABLE 2. Activities of alkoxyalkyl esters of CDV, cCDV, and HPMPA against VV and CPV in PHK monolayers

Compound	VV		CPV		Cytotoxicity (μg/ml)		SI[e]	
	EC_{50} (μg/ml)[a]	Fold decrease[b]	EC_{50} (μg/ml)[a]	Fold decrease[b]	MCC (μg/ml)[c]	CC_{50} (μg/ml)[d]	VV	CPV
HDP-CDV	0.48 ± 0.52	20	0.32 ± 0.19	30	20	0.18 ± 0.18	0.4	0.6
ODE-CDV	0.081 ± 0.079	130	0.053 ± 0.037	180	20	0.012 ± 0.011	0.2	0.3
OLP-CDV	0.40 ± 0.44	30	0.33 ± 0.23	30	20	0.034 ± 0.027	0.1	0.1
ODBG-CDV	0.058 ± 0.070	230	0.072 ± 0.071	170	>5	0.012 ± 0.011	0.2	0.2
OLE-CDV	0.065 ± 0.037	170	0.035 ± 0.022	290	>20	0.014 ± 0.013	0.2	0.4
CDV	5.8 ± 4.2		5.3 ± 2.1		>50	7.2 ± 6.7	1.2	1.4
OLE-cCDV	0.31 ± 0.32	110	0.12 ± 0.17	160	>20	0.055 ± 0.080	0.2	0.5
cCDV	18.9 ± 12.9		9.9 ± 5.7		>50	41.7 ± 34.9	2.2	4.2
HDP-HPMPA	0.079 ± 0.059	30	0.022 ± 0.023	50	2	0.021 ± 0.025	0.3	1.0
ODE-HPMPA	0.005 ± 0.000	560	0.0015 ± 0.0008	750	2	0.0078 ± 0.0110	2	5.2
HPMPA	1.5 ± 0.9		0.61 ± 0.28		20	0.53 ± 0.05	0.4	0.9

[a] Concentration required to inhibit 50% of virus-induced CPE. The EC_{50} values of each compound represent the mean ± standard deviation of the EC_{50} values of at least two independent experiments.

[b] Fold decrease in EC_{50} relative to that of the parental compound based on molar values, given the following molecular weights: HDP-CDV, 583.68; ODE-CDV, 581.71; OLP-CDV, 609.71; ODBG-CDV, 717.85; OLE-CDV, 595.68; CDV, 315.12; OLE-cCDV, 559.65; cCDV, 297.2; HDP-HPMPA, 585.72; ODE-HPMPA, 599.38; HPMPA, 325.

[c] Minimum cytotoxic concentration required to alter cell morphology.

[d] Concentration required to reduce cell growth by 50%. The CC_{50} values of each compound represent the mean ± standard deviation of the CC_{50} values of at least two independent experiments.

[e] Selectivity index (ratio of CC_{50} to E_{50}).

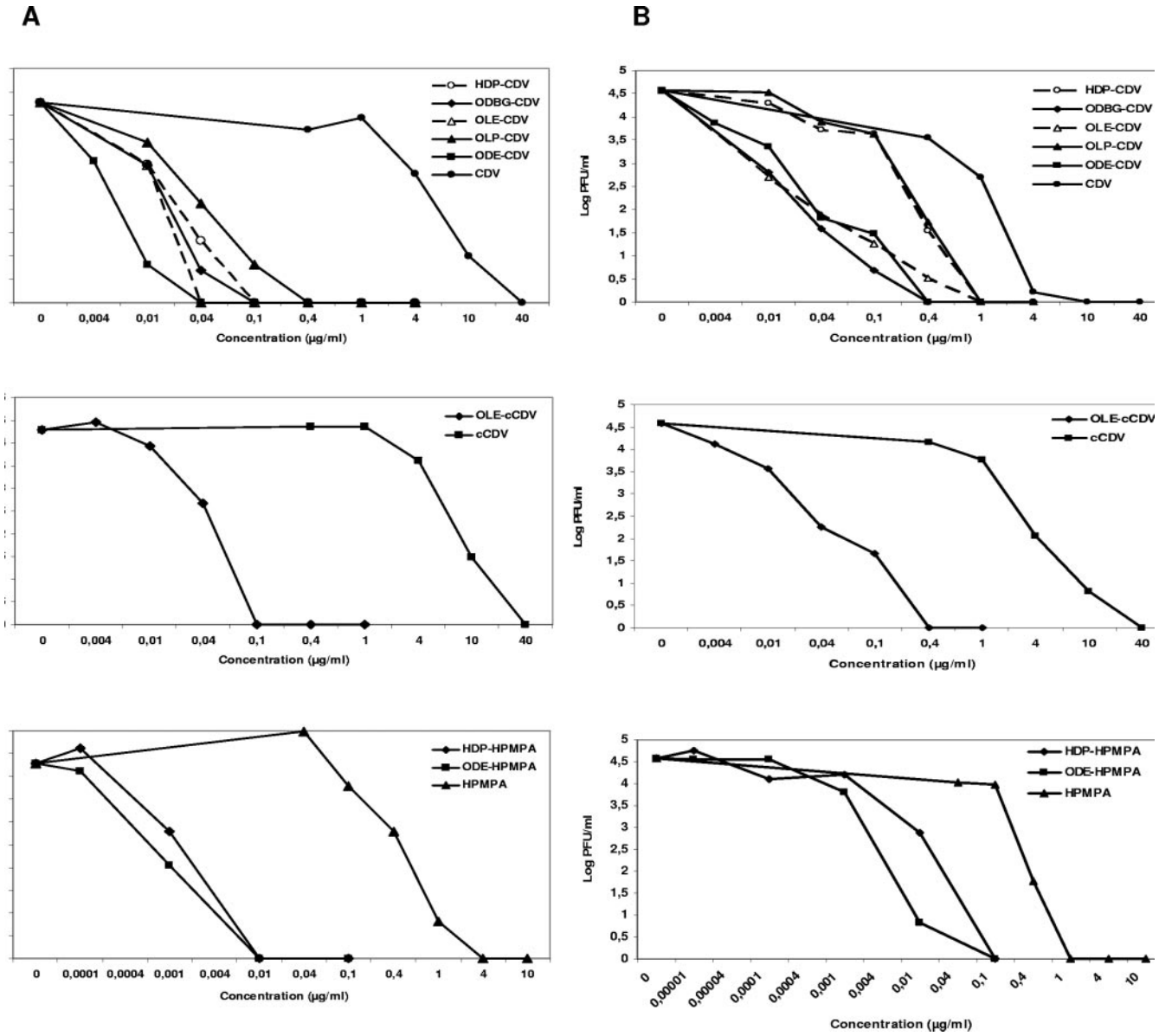

FIG. 1. Effects of alkoxyalkyl esters of CDV, cCDV, and HPMPA on extracellular VV production in HEL fibroblast monolayers (A) and PHK monolayers (B).

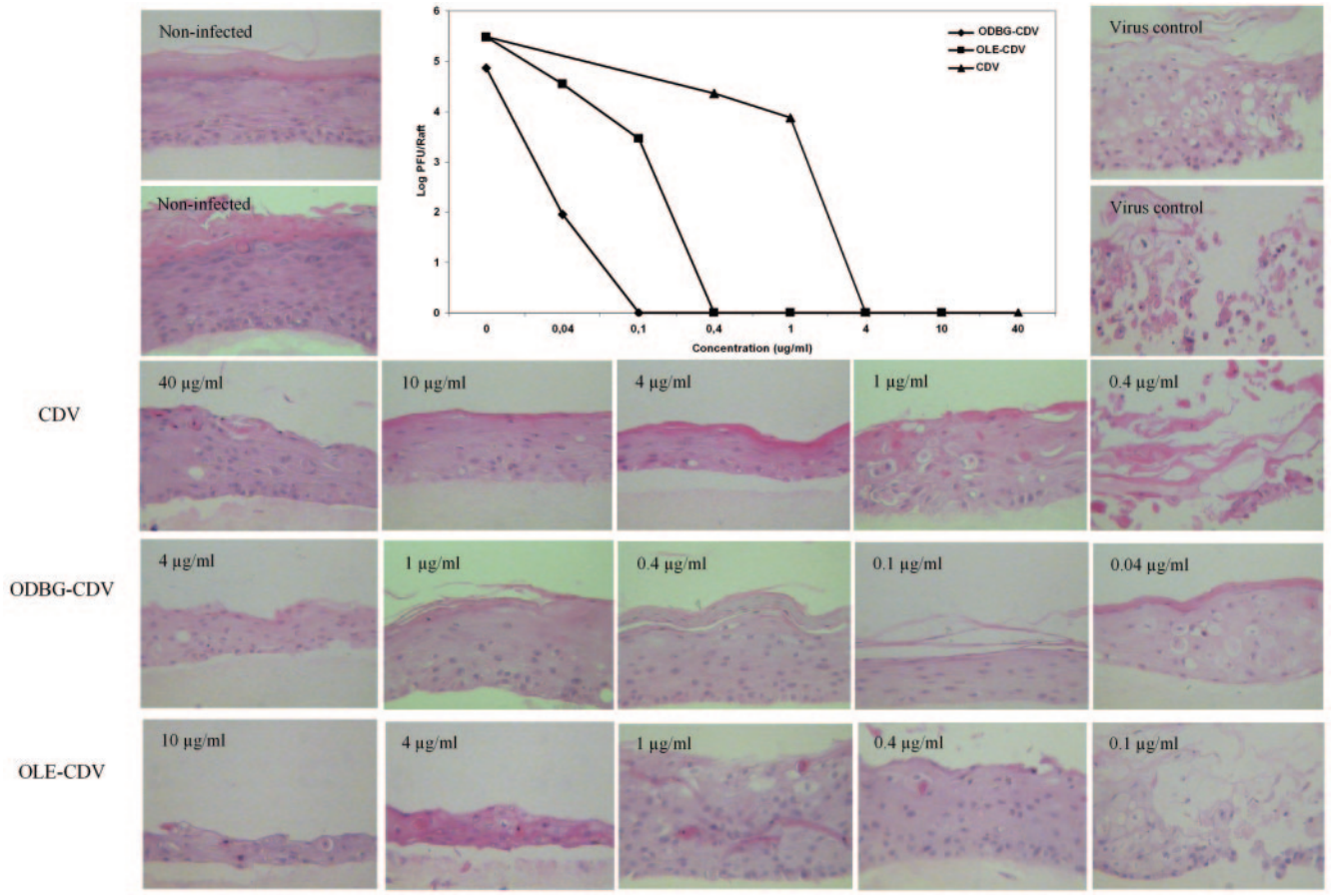

FIG. 2. Activities of CDV, ODBG-CDV, and OLE-CDV against VV in organotypic epithelial raft cultures of PHK cells.

cCDV (cHPMPC), and (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) against VV (strain Lederle-Chorioallentoic; ATCC VR-118) and CPV (strain Brighton; ATCC VR-302) were evaluated. Stock solutions of the parent compounds were made in phosphate-buffered saline (PBS) at a concentration of 2 mg/ml and stored at 4°C. The following alkoxyalkyl esters were used: 1-*O*-octadecyl-2-*O*-benzyl-glyceryl–CDV (ODBG-CDV), oleyloxyethyl-CDV (OLE-CDV), hexadecycloxypropyl-CDV (HDP-CDV), octadecycloxyethyl-CDV (ODE-CDV), oleyloxypropyl-CDV (OLP-CDV), oleyloxyethyl-cCDV (OLE-cCDV), hexadecycloxypropyl-HPMPA (HDP-HPMPA), and octadecycloxyethyl-HPMPA (ODE-HPMPA). The structures and the synthesis of these compounds were described elsewhere (3, 12, 15). Stock solutions in a concentration of 2 to 10 mg/ml were made in PBS/dimethyl sulfoxide (3:1) and stored at −20°C. Antiviral assays with monolayers of both human embryonic lung (HEL) fibroblasts (ATCC CCL-137) and primary human keratinocyte (PHK) cells isolated from neonatal foreskins were carried out as described before (18). The viral cytopathic effect (CPE) was recorded, and the 50% effective concentration (EC_{50}) was defined as the compound concentration required to reduce the viral CPE by 50% compared to that for the untreated control. Cytotoxicity assays were carried out as reported previously (18). The 50% cytotoxic concentration (CC_{50}) was defined as the concentration required to reduce the cell number by 50% compared to that for the untreated controls. The selectivity index (SI) was defined as the ratio of the CC_{50} for cell growth to the EC_{50} for viral CPE. All the prodrugs tested were far more potent than the parent compounds against VV and CPV in HEL fibroblasts (Table 1). ODE-CDV and OLE-CDV emerged as the most active CDV derivatives. ODBG-CDV exhibited the weakest activity but was still at least 130-fold more active than CDV. OLE-cCDV was 2,800-fold more active than its parent compound against VV, while ODE-HPMPA was up to 15,300-fold more active than HPMPA. These strong activities result in high degrees of selectivity of the prodrugs, despite their higher cytotoxicities, in comparison with those of the original compounds. OLP-CDV appeared to be the most selective prodrug of CDV, while ODE-HPMPA showed up to 30-fold more selectivity than HPMPA. The minimal cytotoxic concentration (MCC) reflects the effects of the compounds on the change in the structure of the monolayer due to ballooning and detachment of the cells. The highest concentrations of the ANP derivatives that were used to test the HEL fibroblasts did not alter the morphology of the cells (Table 1). Since poxviruses have an epithelial tropism, the antiviral activities of the ANP derivatives were analyzed by using epithelial cell monolayers (Table 2). As in HEL fibroblasts, all prodrugs were more potent than the parent compounds. The differences in the activities in comparison to those of the parent compounds were, however, not as pronounced as in the HEL fibroblasts. The order of the decreasing activities of the CDV derivatives was different when PHK cells were used. ODBG-CDV, OLE-CDV, and ODE-

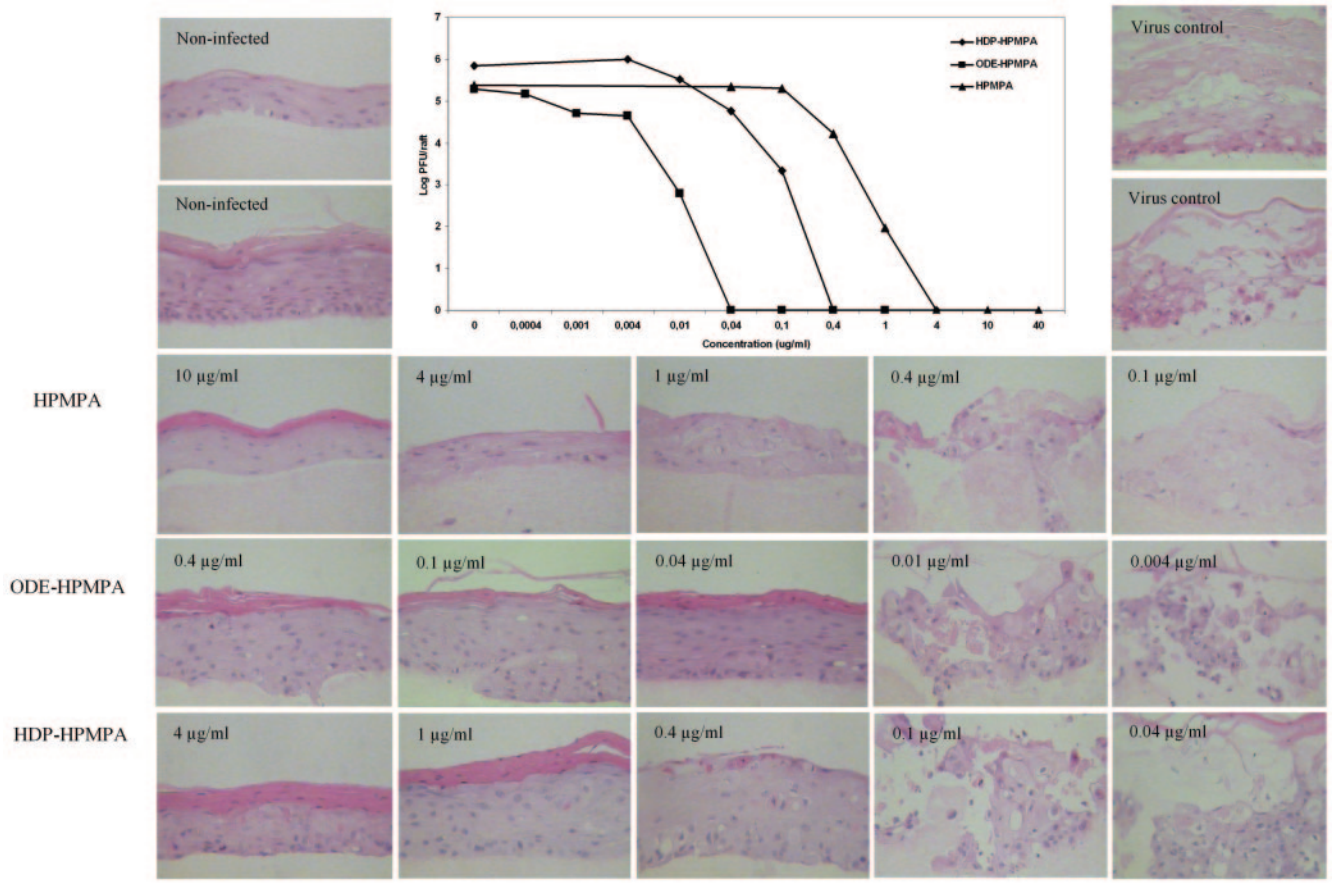

FIG. 3. Activities of HPMPA, ODE-HPMPA, and HDP-HPMPA against VV in organotypic epithelial raft cultures of PHK cells.

CDV were the most active compounds. The decrease in EC_{50} varied from 290-fold for the most active compounds to 20-fold for the least potent compounds. ODE-HPMPA was clearly the most potent prodrug. Its activity was increased up to 750-fold in comparison to that of HPMPA. Due to the high sensitivity of growing PHK cells to the compounds, no selectivity could be seen. Most prodrugs affected the morphology of PHK cells at only the highest concentrations used (Table 2).

The more potent activity of the alkoxyalkyl derivatives against VV was confirmed in a virus yield reduction assay carried out as described before (1). HEL fibroblasts and PHK cells were infected with VV (850 PFU/well), and at 3 days postinfection samples were taken to determine the virus yield. Results showing the extracellular VV production are depicted in Fig. 1. Similar data were obtained for intracellular virus production (data not shown).

Because three-dimensional organotypic epithelial raft cultures more closely mimic the differentiated epithelium, data concerning the activities of the prodrugs against the dermotropic poxviruses in this ex vivo model would be more representative than the results obtained with keratinocyte monolayers. As shown before, the lesions in the raft cultures infected with VV, which can be seen after histological examination, are similar to the ones seen in the clinic (18). These three-dimensional epithelial culture systems have recently been successfully used to determine the activities of ANP derivatives against VV, orf virus, and herpesviruses (2, 8, 18). Organotypic cultures were prepared as described previously (2, 18). The cultures were infected in duplicate with VV at day 7 after the raft cultures were lifted, and the compounds were added to the medium. Every other day, the medium was removed and replenished with growth medium containing the compounds. At day 12 after the raft cultures were lifted, the organotypic cultures were harvested. One series was fixed in 10% buffered formalin for histological examination. The other series of the rafts was frozen in PBS and thawed to release the virus from the infected epithelium. Virus yield was determined by a plaque assay with HEL fibroblasts. The results obtained with CDV, ODBG-CDV, and OLE-CDV are shown in Fig. 2. ODBG-CDV was the most active CDV derivative, showing full protection against VV at a concentration of 0.1 μg/ml, which was confirmed in the virus yield assay and which is reflected by EC_{90} and EC_{99} values of <0.04 μg/ml. The order of increasing activity of the CDV derivatives obtained with the organotypic epithelial cultures was comparable to that obtained with the PHK cell monolayers. OLE-cCDV also proved to be more active than cCDV in the ex vivo model. The EC_{90} and EC_{99} values of OLE-cCDV, 0.10 μg/ml and 0.33 μg/ml, respectively, were 10-fold lower than those of the parent compound. ODE-HPMPA appeared to be the most potent prodrug of HPMPA and inhibited virus production up to a concentration of 0.04 μg/ml (Fig. 3). The EC_{90} of this derivative was 700-fold lower

than that of HPMPA, which was 0.57 μg/ml. Although ODBG-CDV exhibited the weakest activity in HEL fibroblasts, it was one of the CDV prodrugs with the strongest activities in PHK cell monolayers. This strong activity was confirmed on both morphology and virus yield in the ex vivo model. These data prove the importance of using a model that bears the most resemblance to the natural environment of the virus.

It has already been reported that alkoxyalkyl derivatives containing an ethanediol linker (ODE, OLE) show the highest activities against orthopoxvirus replication in human foreskin fibroblasts (13). Similar effects were observed when activity against viral replication in HEL cells was evaluated. In PHK cells, derivatives that contain an ethanediol or glycerol linker (ODBG) showed the highest antiviral activities. These analogues also appeared to be the most toxic. The presence of a double bond had no influence on antiviral activity.

In summary, we have demonstrated the activities of several alkoxyalkyl esters of CDV, cCDV, and HPMPA against orthopox virus replication in vitro on HEL fibroblast and PHK monolayers and ex vivo in differentiated keratinocytes. Due to the epithelial tropism of the poxviruses, these epithelial in vitro and ex vivo systems are appropriate as a prelude for their exploration in an in vivo model. ODE-HPMPA emerged as the most selective molecule against the replication of VV and CPV. Due to the higher levels of activity of these derivatives in comparison to those of their parent compounds and their increased oral bioavailabilities, the results described here are promising for the development of drugs that could eventually be used for treatment in a potential poxvirus outbreak.

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