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Antivaccinia Activities of Acyclic Nucleoside Phosphonate Derivatives in Epithelial Cells and Organotypic Cultures

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Organotypic "raft" cultures of epithelial cells allow the reconstitution of a skin equivalent that is easily infectible with different viruses with cutaneous tropism. Among these, poxvirus and particularly vaccinia virus (VV) are good candidates for use in antiviral tests, giving histological pictures comparable to those observed in humans infected with smallpox. Therefore, we decided to evaluate a series of phosphonate derivatives for their ability to inhibit VV growth in epithelial cell monolayers, and the most powerful derivatives were tested in the organotypic cultures. The most active compound was 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]-adenine [(S)-HPMPA], followed by 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine, cyclic (S)-HPMPA, 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine [(S)-HPMPC; cidofovir, Vistide], and cyclic (S)-HPMPC. Cidofovir, which is on the market for the treatment of human cytomegalovirus retinitis in immunocompromised patients, is potentially a good candidate for the treatment of a poxvirus outbreak, in the absence of any vaccination.

Despite the global eradication of smallpox that led to the discontinuation of vaccination against smallpox about 20 years ago, poxviruses remain a serious health problem. Now that vaccination against smallpox has been discontinued, younger generations are again susceptible to the virus, which is a good candidate, if it is ever released, as an agent for biological warfare or terrorism (3, 5, 14, 15). In addition, other poxviruses, such as monkeypox virus, against which vaccination against smallpox offered protection, are now potential pathogens for humans, as demonstrated by the recent epidemic in the Democratic Republic of Congo (16, 25). Therefore, there is a need for continued study of poxviruses to develop models in order to understand their pathogenicities and to test more powerful drugs, since up to now no antiviral drugs with satisfactory activities against poxviruses have been available.

Here we present the results of a study with a broad range of acyclic nucleoside phosphonates (ANPs) whose activities were tested against vaccinia virus (VV) replication in vitro on epithelial cells both in monolayers and, for the most active ones, in the organotypic "raft" culture system. Organotypic raft cultures use human epithelial cells derived from neonatal foreskins and are able to mimic fully differentiated skin. Previously, organotypic raft cultures have successfully been used for the study of human papillomaviruses (23), and they have recently been applied to the study of herpes simplex virus type 1 infection (18, 33, 34) and adeno-associated virus type 2 (24). Since epithelial cells are the natural host cells for poxviruses, we decided to evaluate VV replication in such a system and use it as a model for evaluation of antiviral compounds. VV gives a histological image in epithelial raft cultures similar to that of cells from patients with clinical poxvirus infections. ANPs have been shown to have antipoxvirus activities (12, 20) both in vitro and in vivo.

Among the different molecules tested in our model, (S)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (HPMPC; cidofovir, Vistide; molecular weight, 315.32) emerged as the most promising molecule.

MATERIALS AND METHODS

Culture of PHKs. Primary human keratinocytes (PHKs) were isolated from neonatal foreskins. Tissue fragments were incubated with trypsin-EDTA for 1 h at 37°C. The epithelial cells were detached and cultured with mitomycin-treated Swiss 3T3 J2 mouse fibroblasts as feeders. The growth medium was a 1/3 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium supplemented with 0.5 μg of hydrocortisone per ml, 10 mg of epidermal growth factor per ml, 10% fetal calf serum, 2 mmol of L-glutamine per liter, 10 mmol of HEPES per liter, 1 mmol of sodium pyruvate per liter, 1×10^{-10} mol of cholera toxin per liter, 5 μg of insulin per ml, 5 μg of human transferrin per ml, and 15×10^{-4} mg of 3,3′,5-triiodo-2-thyronine per ml. PHKs were grown as monolayers to test anti-VV activity, and they were also used to prepare the organotypic raft cultures.

Virus. Stocks of VV (VR-118, strain Lederle-Chorioallentoic; American Type Culture Collection, Manassas, Va.) were prepared in PHKs.

Compounds. The different ANPs were synthesized by A. Holý by procedures described in the literature (17). The compounds tested are listed in Table 1.

Antiviral assays. Confluent PHKs grown in 96-well microtiter plates were infected with VV at 100 $CCID_{50}$s (1 $CCID_{50}$ corresponds to the virus stock dilution that is infective for 50% of the cell cultures). After 2 h of incubation at 37°C, residual virus was removed and the infected cells were further incubated with medium containing serial dilutions of the test compounds (in duplicate). After 24 to 48 h of incubation at 37°C in a 5% CO_2 atmosphere, the viral cytopathic effect (CPE) was recorded and the 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to reduce the viral CPE by 50%. The IC_{50} of each compound represents the mean of the IC_{50}s from two or more experiments.

Cytotoxicity assays. The toxicities of the compounds for the host cells were based on inhibition of cell growth. The cells were seeded at 6×10^3 cells per well in a volume of 0.1 ml into 96-well microtiter plates and allowed to proliferate for 24 h. Then, medium containing serial dilutions (in duplicate) of the test compounds was added (0.1 ml per well). After 3 days of incubation at 37°C in 5% CO_2, the cells were trypsinized and the cell number was determined with a Coulter counter. Cytotoxicity is expressed as the 50% cytotoxic concentration

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TABLE 1. Anti-VV activities of various ANPs, in PHKs

Compound	CC_{50} (μg/ml)	IC_{50} (μg/ml)	SI
9-[2-(Phosphonomethoxy)ethyl]adenine (PMEA)	12.3	>50	<0.2
PMEA di(isooctyl)ester	12.9	9	1.4
9-[2-(Phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP)	31.3	30.5	1.0
9-[2-(Phosphonomethoxy)ethyl]guanine (PMEG)	0.1	1.1 ± 0.2	0.1
Isopropyl 9-[2-(phosphonomethoxy)ethyl]guanine	13.6	>50	<0.3
9-[2-(Phosphonomethoxy)ethyl]-8-azaguanine	6.4 ± 0.7	16.3 ± 11.0	0.4
1-[2-(Phosphonomethoxy)ethyl]-2,4-diaminopyrimidine	17.7	8.0 ± 4.2	2.2
6-[2-(Phosphonomethoxy)ethoxy]-2,4-diaminopyrimidine	21.7	14.0 ± 1.4	1.6
9-[2-(Phosphonomethoxy)ethyl]-2-amino-6-chloropurine hydrobromide	0.9	2.7 ± 0.9	0.3
9-[2-(Phosphonomethoxy)ethyl]-2-amino-6-methylaminopurine	8.9	37.0 ± 4.2	0.2
9-[2-(Phosphonomethoxy)ethyl]-2-amino-6-methylaminopurine	5	≥30.5 ± 27.6	≤0.2
9-[2-(Phosphonomethoxy)ethyl]-2-amino-6-*n*-butylaminopurine	11.3	2.4	4.7
9-[2-(Phosphonomethoxy)ethyl]-2-amino-6-allylamino purine	0.9	7.3 ± 5.2	0.1
9-[(2-Phosphonomethoxy)ethyl]-2-amino-4-propargylaminopurine	2.9	22.8 ± 20.2	0.1
9-[2-(Phosphonomethoxy)ethyl]-2-amino-6-cyclopropylaminopurine	1.3	12.3 ± 5.3	0.1
9-[2-(Diisooctylphosphonylmethoxy)ethyl]-2-amino 6-cyclopropylaminopurine	9.7	10	1.0
9-[2-(Phosphonomethoxy)ethyl]-2-amino-6-cyclopentylaminopurine	7.1	≥45 ± 7	≤0.2
9-[2-(Phosphonomethoxy)ethyl]-2-amino-6-dimethylaminopurine	1.5	5.3 ± 3.3	0.3
9-[2-(Phosphonomethoxy)ethyl]-2-amino-6-(*N*-methyl, *N*-ethyl)aminopurine	2.2 ± 0.2	10.7 ± 3.2	0.2
9-[2-(Phosphonomethoxy)ethyl]-2-amino-N^6-(piperidin-1-yl)purine	>41 ± 13	>50 ± 0	>0.8
9-(R)-[2-(Phosphonomethoxy)propyl]-6-dimethylaminopurine	13.9	>50 ± 0	<0.3
9-(S)-[2-(Phosphonomethoxy)propyl]-2-amino-6-cyclopropylaminopurine	15.9	≥44.0 ± 10.4	≤0.4
9-(S)-[2-(Phosphonomethoxy)propyl]guanine [(S)-PMPG]	5.3	30 ± 0	0.2
1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine [(S)-HPMPC]	≥34 ± 20	4.0 ± 2.6	≥8.5
Cyclic -(S)-HPMPC	≥41 ± 13	8.0 ± 6.0	≥5.1
9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine [(S)-HPMPA]	16.4	0.65 ± 0.33	25.2
Cyclic (S)-HPMPA	17.2 ± 1.3	1.3 ± 0.3	13.2
9-(S)-[(3-hydroxy-2-(phosphonomethoxy)propyl]-8-azaadenine	8.3	7.5 ± 3.5	1.1
9-(S)-[(3-hydroxy-2-(phosphonomethoxy)propyl]-3-deazaadenine [3-deaza-(S)-HPMPA]	7.3 ± 3.8	1.5 ± 1.1	4.9
Cyclic 3-deaza-(S)-HPMPA	12.1 ± 3.8	1.7 ± 0.5	7.1
9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine [(S)-HPMPG]	7.5 ± 7.1	2.1 ± 1.9	3.6
Cyclic (S)-HPMPG	8.2	3.8 ± 1.8	2.2
9-(R)-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine [(R)-HPMPG]	34.4	20 ± 0	1.7
Cyclic (R)-HPMPG	13.6	16.5 ± 4.9	0.8
9-(RS)-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine [(RS)-HPMPG]	6.6	4.6 ± 2.7	1.4
9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine [(S)-HPMPDAP]	>42.5 ± 10.8	2.7 ± 1.2	≥16
9-(RS)-[3-hydroxy-2-(phosphonomethoxy)propyl]-6-hydroxylaminopurine [(RS)-HPMPDAP]	17.3	3.5 ± 2.1	4.9
9-(RS)-[3-azido-2-(phosphonomethoxy)propyl]-2,6-diaminopurine	>50	11.5 ± 4.9	>4.3

(CC_{50}) or the concentration required to reduce cell growth by 50% (relative to the number of cells in the untreated control cell cultures). The CC_{50} of each compound represents the mean of the CC_{50}s from two or more experiments. The selectivity index (SI) is defined as the ratio of the CC_{50} for cell growth to the IC_{50} for viral plaque formation.

Organotypic cultures. For the preparation of dermal equivalents, a collagen matrix solution was made with collagen mixed on ice with Ham's F-12 medium concentrated 10-fold, 10-fold reconstitution buffer, and 3T3 J2 fibroblasts. One milliliter of the collagen matrix solution was poured into 24-well plates. After equilibration of the gel with 1 ml of growth medium overnight at 37°C, 2.5×10^5 normal PHKs were seeded on the tops of the gels and maintained submerged for 24 to 48 h. The collagen rafts were raised and placed onto stainless steel grids at the interface between the air and the liquid culture medium (Fig. 1). The epithelial cells were then allowed to stratify for 10 to 12 days. The cultures were then harvested, fixed in 10% buffered formalin, and embedded in paraffin. Four-micrometer sections were stained with hematoxylin-eosin for histological examination.

Infection of organotypic cultures. At different times after lifting of the rafts, organotypic cultures were infected with 50 μl of the VV stock at the indicated viral inoculum on the top of the in vitro-formed epithelium. At the time of infection, the cultures were incubated in the presence of medium containing different concentrations of the test compounds.

RESULTS

The results for the antiviral activities of the different ANPs on epithelial cell monolayers are summarized in Table 1. Among the ANPs evaluated for their anti-VV activities in PHKs were (S)-HPMPC (cidofovir, Vistide) and its cyclic prodrug cyclic (S)-HPMPC, 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine [(S)-HPMPDAP], 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine [(S)-HPMPA] and its 3-deaza and 8-aza derivatives, as well as selected 9-[2-(phosphonomethoxy)ethyl] derivatives of 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine substituted at the N^6-amino group and selected ANP derivatives of guanine. (S)-HPMPC (cidofovir, Vistide), (S)-HPMPDAP, (S)-HPMPA, cyclic HPMPA, and cyclic 3-deaza-(S)-HPMPA emerged with the highest SIs (SIs = 7.1 to 25.2), followed by 3-deaza-(S)-HPMPA (SI = 4.9) and cyclic (S)-HPMPC (SI = 5.1). Some other HPMP derivatives showed SIs in the range of 3 to 5, i.e., 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine and (RS)-3-azido-HPMPDAP. Except for 9-[2-(phosphonomethoxy)ethyl]-2-amino-6-*n*-butylaminopurine, with an SI of 4.7, none of the phosphonomethoxyethyl derivatives selectively inhibited VV replication in PHKs.

As a rule, the SI was higher when selected drugs were tested in human embryonic lung (HEL) cells (data not shown) than when they were tested in PHKs, with the the difference being particularly marked for the (S)-HPMPA derivatives. The IC_{50}s

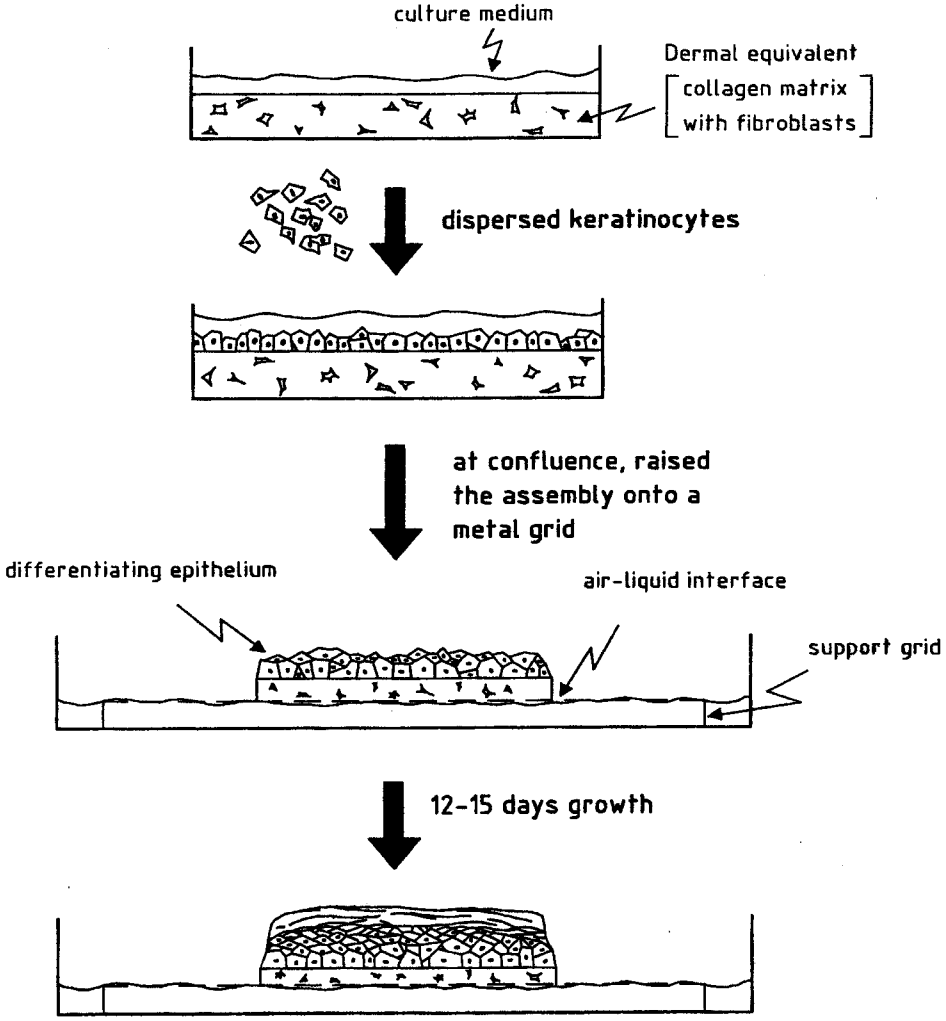

FIG. 1. Scheme showing the different steps used in the production of organotypic epithelial raft cultures.

were generally comparable, but the compounds were systematically more toxic for the PHK cells. The most potent drugs on PHK monolayers were tested in epithelial raft cultures. This system is able to support infection with VV regardless of when the cells are infected at different stages of differentiation. The results obtained are shown in Fig. 2 and compared to those for the uninfected, well-differentiated epithelial raft culture. The cytopathogenic effects obtained and revealed by hematoxylin-eosin staining are characteristic of those seen in clinical lesions induced by poxviruses, i.e., the presence of inclusion bodies.

When the epithelial raft cultures were infected with VV at an input of 1,800 PFU/raft, after 6 days of differentiation, total destruction of the different layers of the epithelia was observed all along the raft (Fig. 3). Treatment of cultures with (S)-HPMPC at 40 μg/ml or (S)-HPMPA at 10 μg/ml resulted in complete protection against VV-induced CPE, while treatment with HPMPC at 10 μg/ml or HPMPA at 4 and 1 μg/ml resulted in partial protection (Fig. 3 and data not shown). Lower concentrations of (S)-HPMPC and (S)-HPMPA were not effective under those culture conditions. If infection with 720 PFU/raft was performed after 8 days of differentiation, only a low number of individual viral plaques were noted in raft cultures treated with (S)-HPMPC at 10 μg/ml, and partial protection was also observed at a concentration of 4 μg/ml (data not shown). Under the same culture conditions, treatment with HPMPA at concentrations of 10 and 4 μg/ml and HPMPDAP at concentrations of 40, 10, and 4 μg/ml resulted in full protection against VV-induced cytopathogenicity, whereas both drugs at 1 μg/ml partially protected the organotypic raft cultures (data not shown).

DISCUSSION

Smallpox, which was eradicated by global vaccination, is the first and only human infectious disease that has disappeared from the surface of the planet. The success of the World Health Organization eradication campaign, which was started in the 18th century by Jenner, has lowered the amount of attention given to the potential pathogenic roles that other poxviruses could play, such as monkeypox virus. In addition, there are strong concerns about the potential release of non-declared smallpox strains as a weapon by bioterrorists (3, 5, 14,

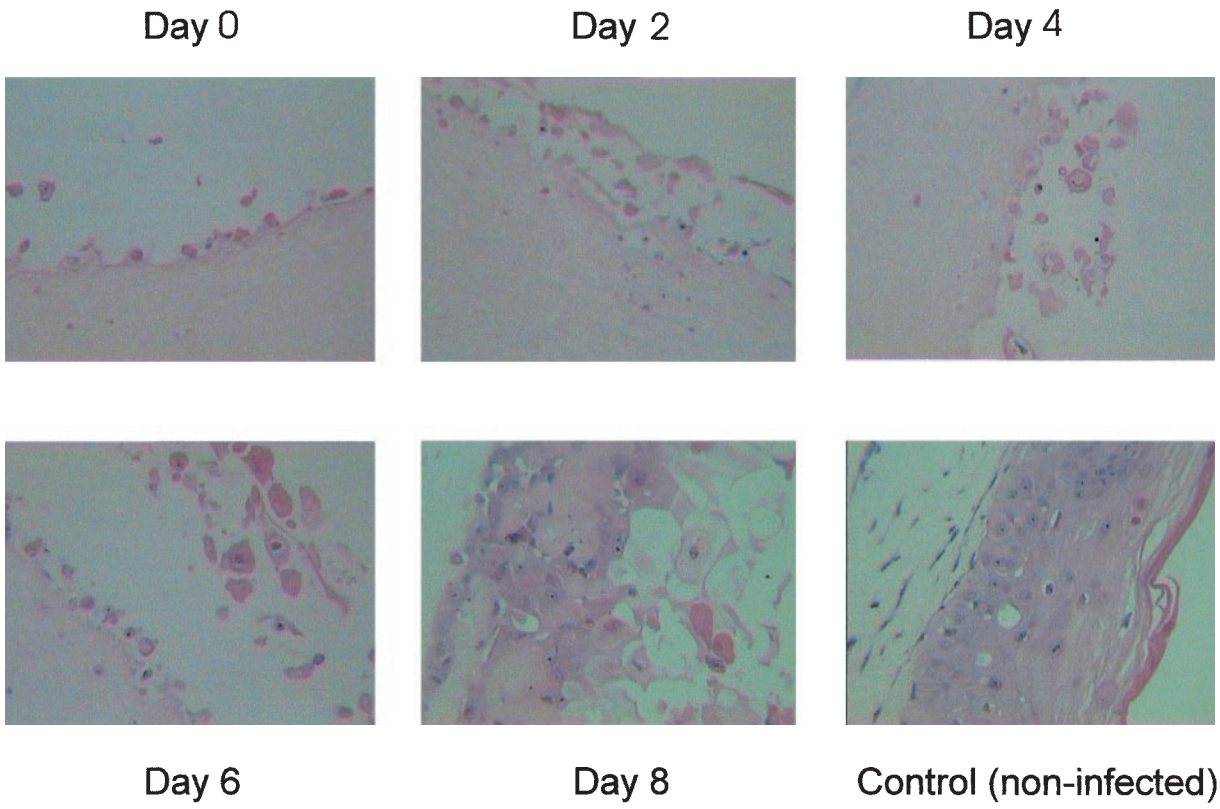

FIG. 2. Epithelial raft cultures infected with VV (720 PFU/raft) at various stages of differentiation. Magnification, ×40.

15). Therefore, the World Health Organization recommended further studies on poxviruses, mostly smallpox virus, in anticipation of complete destruction of the two official stocks in the United States and Russia, that was foreseen in 2002, in order to improve our scientific knowledge based on the use of molecular techniques on the one hand and to establish reliable therapeutic strategies in case of virus release on the other.

In addition to the development of new vaccines and improvements in diagnostic techniques, the development of antiviral drugs with high SIs is also recommended. Several families of compounds have been tested, mostly in the VV model, and most of those that have been identified as anti-VV agents are nucleoside analogues: IMP dehydrogenase inhibitors, *S*-adenosylhomocysteine hydrolase inhibitors, outer membrane protein decarboxylase inhibitors, CTP synthase inhibitors, thymidylate synthase inhibitors, or ANPs (9, 10, 12). (S)-HPMPA can be seen as the lead compound of this last class of compounds. While the structurally related compounds 9-(S)-(2,3-dihydroxypropyl)adenine and phosphonoacetic acid inhibit the replication of VV in primary rabbit kidney cells at IC_{50}s of 20 and 30 μg/ml, respectively, (S)-HPMPA does so at a 100-fold lower concentration, that is, 0.3 μg/ml (12). (S)-HPMPA exhibits a broad-spectrum antiviral activity against DNA viruses, including poxviruses, but also adeno-, herpes-, hepadna-, and iridoviruses (8, 9). (S)-HPMPC (cidofovir, Vistide), the cytosine counterpart of (S)-HPMPA, shows a similar spectrum of activity but proved to be less toxic than the mother compound both in vitro and in vivo (1, 2, 32). Cidofovir has been licensed for the treatment of cytomegalovirus retinitis in AIDS patients, but it also has therapeutic potential by either systemic or topical administration in the treatment of various other herpes-, polyoma-, papilloma-, adeno-, and poxvirus infections (28). Cidofovir confers a pronounced and prolonged inhibition of viral replication that lasts for at least 7 days after treatment with the drug as short as 6 h postinfection. The long-lasting antiviral action is a unique property of cidofovir that allows prophylactic and infrequent dosing of the drug (8, 9, 11, 12).

Poxviruses have an obvious tropism for skin and mucosa, and therefore, we decided to try to infect PHKs with VV. CPEs were easily obtained in a few days, and antiviral assays could be performed, with the assays showing IC_{50}s comparable to those measured by other assays performed initially with HEL fibroblasts. The cytotoxicity measured for PHKs was systematically slightly higher than that measured for HEL cells, making the SI lower for PHKs. Identical CPEs were obtained in infected epithelial raft cultures. The kinetics of infection at different stages of differentiation have shown that the best conditions were obtained when the raft was infected between 6 and 8 days postinfection, with the fixation of the tissue being performed 12 to 14 days after the beginning of differentiation. Different concentrations of the most active phosphonates were added to the medium and showed inhibition of virus growth at concentrations comparable to those observed in the monolayer system. Cidofovir could be used at a concentration as high as 40 μg/ml, while (S)-HPMPA was not toxic at a concentration of about 10 μg/ml. At these concentrations, both drugs were able to afford complete protection against VV-induced CPE in the raft culture system, while both cidofovir and (S)-HPMPA at

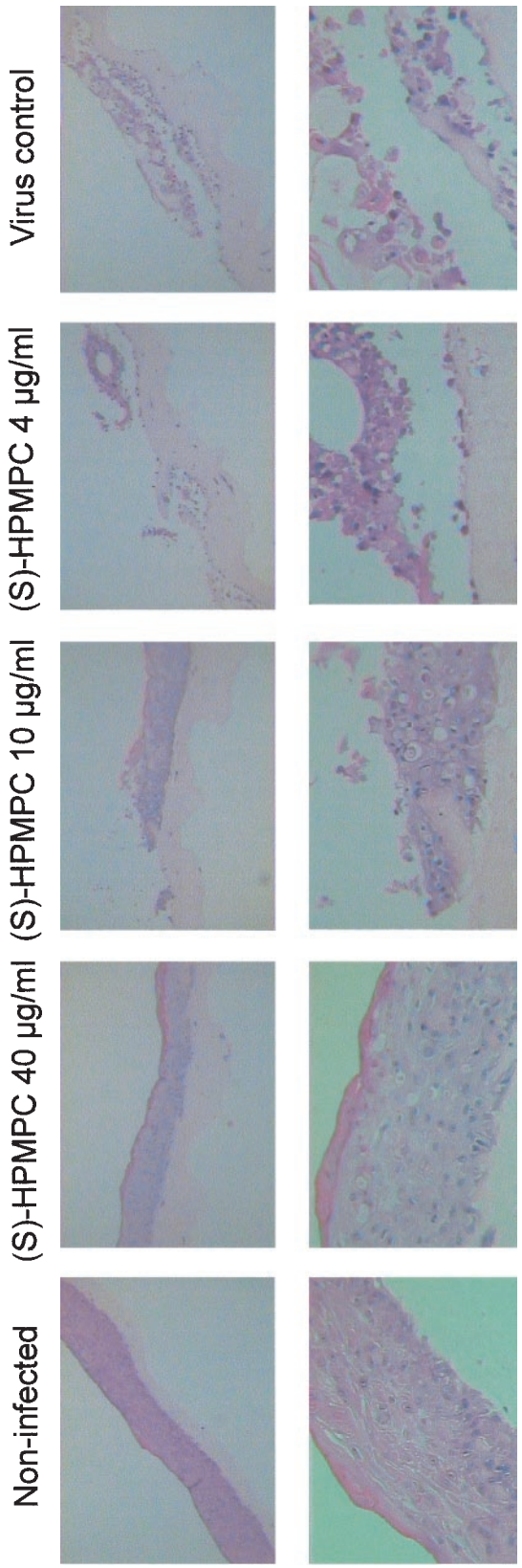

FIG. 3. Epithelial raft cultures infected with VV (1,800 PFU/raft) after 6 days of differentiation and treated with various concentrations of (S)-HPMPC at the time of infection. Magnifications, ×9 (upper panels) and ×36 (lower panels).

concentrations of 10 and 4 μg/ml, respectively, were only partially protective. It should be noted that although it is not possible to calculate IC_{50}s in the raft culture model, the order of potency of the different ANPs observed in the organotypic raft culture system correlated with the IC_{50}s obtained with PHK monolayers.

Recently, several reports have stressed the clinical usefulness of cidofovir for the treatment of poxvirus infections in humans (6, 7, 22). Topical or systemic cidofovir has been shown to be active for the treatment of mollusca contagiosa, which are caused by a poxvirus and which can be responsible for severe disease, particularly in immunocompromised patients. Furthermore, we have recently seen a particularly severe case of orf, also known as ecthyma contagiosum (which is caused by a member of the poxvirus family) that completely regressed under local cidofovir treatment (13). ANPs, particularly cidofovir, are good candidates for the treatment and the prophylaxis of poxvirus infections, based on the efficiency of cidofovir against cowpox virus, monkeypox virus, and VV infections in animal models (4, 10, 19, 21, 27, 30, 31; J. W. Huggins, D. Smee, M. J. Martinez, and M. Bray, Abstr. Eleventh Int. Conf. Antivir. Res., Antivir. Res. **37:**A73, 1998.) and the results of clinical case reports (6, 7, 13, 22). Cidofovir could also be used in combination with other antiviral drugs with different molecular targets in order to synergize the activities and to minimize the emergence of resistant viruses. Recently, treatment of cowpox virus respiratory infection in mice with a combination of cidofovir and ribavirin was reported (29).

Organotypic cultures are powerful tools not only because they provide a histological picture similar to that observed in vivo for the viruses with a tropism for the skin but also because for one given experimental condition, several parameters (cellular and viral) can be studied by different techniques. It is also possible to resuspend the cells in order to quantify some parameters by flow cytometry.

Our results obtained with the organotypic epithelial raft culture model confirmed results previously obtained in vitro (26) and in vivo (4, 10, 19, 26; Huggins et al., Abstr. Eleventh Int. Conf. Antivir. Res.), demonstrating the activity of cidofovir against the poxvirus class of viruses. The monolayer cell system could be used to screen new molecules since these cells represent the natural host for poxviruses. The raft culture will help to confirm, visualize, and analyze antiviral activities. The use of epithelial cells to evaluate antiviral drugs is complementary to the existing models, with none of them giving activities 100% similar to those obtained in a surrogate model when antivariola activity is concerned.

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