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# Activities of Acyclic Nucleoside Phosphonates against Orf Virus in Human and Ovine Cell Monolayers and Organotypic Ovine Raft Cultures

F. Dal Pozzo,[1,4] G. Andrei,[1] A. Holý,[2] J. Van Den Oord,[3] A. Scagliarini,[4] E. De Clercq,[1] and R. Snoeck[4]*

*Rega Institute for Medical Research, K.U. Leuven, Belgium[1]; Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Praque, Czech Republic[2]; Pathology Department, U.Z. Leuven, Belgium[3]; and Department of Veterinary Public Health and Animal Pathology, Alma Mater Studiorum, Bologna, Italy[4]*

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**Orf virus, a member of the *Parapoxvirus* genus, causes a contagious pustular dermatitis in sheep, goats, and humans. Previous studies have demonstrated the activity of (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]-cytosine (HPMPC; cidofovir; Vistide) against orf virus in cell culture and humans. We have evaluated a broad range of acyclic nucleoside phosphonates (ANPs) against several orf virus strains in primary lamb keratinocytes (PLKs) and human embryonic lung (HEL) monolayers. HPMPC, (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPDAP), and (*R*)-9-[3-hydroxy-2-(phosphonomethoxy)propoxy]-2,4-diaminopyrimidine (HPMPO-DAPy) were three of the most active compounds that were subsequently tested in a virus yield assay with PLK and HEL cells by virus titration and DNA quantification. HPMPC, HPMPDAP, and HPMPO-DAPy were evaluated for their activities against orf virus replication in organotypic epithelial raft cultures from differentiated PLK cells. At the highest concentrations (50 and 20 μg/ml), full protection was provided by the three drugs, while at 5 μg/ml, only HPMPDAP and HPMPC offered partial protection. The activities of the three compounds in the raft culture system were confirmed by quantification of infectious virus and viral DNA. These findings provide a rationale for the use of HPMPC and other ANPs in the treatment of orf (contagious ecthyma) in humans and animals.**

Orf virus is the prototype of the *Parapoxvirus* genus, and it is the causative agent of a worldwide contagious skin infection of sheep and goats known as contagious ecthyma, contagious pustular dermatitis, or scabby mouth (9). The disease causes economic loss due to its impact on the agricultural sector as well as on public health, since orf represents a typical occupational zoonosis that affects workers who come into direct contact with infected animals or their products (2).

Orf virus is an epitheliotropic virus that infects damaged skin and replicates in regenerating epidermal keratinocytes (20). The lesions appear after an incubation time of 3 to 10 days and progress through the stages of erythema, papule, pustule, and scab formation. It is commonly a self-limiting disease that resolves within 6 to 8 weeks; but complications can include bacterial infections, regional lymphadenopathy, lymphangitis, erythema multiforme, and bullous pemphigoid (11, 23, 24). In animals and humans, especially in burned and immunocompromised subjects, extensive and recurrent lesions have been described; these lesions result in the development of giant orf or tumor-like lesions (15, 16, 28, 31). While no treatment except for antibiotic therapy to prevent secondary bacterial infections is required for the self-limiting forms of the disease, in the complicated forms, cryotherapy, excision of the mass, and in the worse cases, amputation may be necessary (6).

(*S*)-1-[3-Hydroxy-2-(phosphonomethoxy)propyl]cytosine (HPMPC; cidofovir; Vistide) belongs to the nucleotide analogues (i.e., acyclic nucleoside phosphonates [ANPs]) and has a broad spectrum of antiviral activity, including activities against virtually all DNA viruses, such as polyoma-, papilloma-, adeno-, herpes-, and poxviruses. After the ANPs have been taken up by the cells and converted to their diphosphorylated active metabolites (the deoxynucleoside triphosphate analogues), they can interact with their target enzyme, the viral DNA polymerase (4, 30). The first ANPs accredited with antiviral activity against vaccinia virus (5) were HPMPC; (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine (HPMPA); the cyclic derivative of HPMPA, cHPMPA; and the corresponding guanine and 2,6-diaminopurine derivatives (termed HPMPG and HPMPDAP, respectively). The antiviral activities of cidofovir and other ANPs against vaccinia virus have been confirmed and subsequently extended to other poxviruses, including cowpox, camelpox, monkeypox, and molluscum contagiosum viruses (3, 29, 34). Recently, the activity of cidofovir has been demonstrated against orf virus in cell culture (25) and by topical treatment of cases of giant orf in humans (7; unpublished data).

The purpose of this study was to investigate the antiviral activities of cidofovir and other ANPs against members of the *Parapoxvirus* genus and, in particular, orf virus. In this way we could further examine the potencies of the ANPs against members of the poxvirus family, since, despite the global eradication of smallpox, poxviruses remain a serious health threat.

* Corresponding author. Mailing address: Rega Institute for Medical Research, Katholieke Universtiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium. Phone: 32 16 337395. Fax: 32 16 337340. E-mail: Robert.Snoeck@rega.kuleuven.ac.be.

TABLE 1. Compounds tested against orf virus

| Compound no. | Compound |
|---|---|
| 1a | (*S*)-1-[3-Hydroxy-2-(phosphonomethoxy)propyl]cytosine (HPMPC) |
| 1b | (*S*)-9-[3-Hydroxy-2-(phosphonomethoxy)propyl]adenine (HPMPA) |
| 1c | (*S*)-9-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPDAP) |
| 1d | (*S*)-9-[3-Hydroxy-2-(phosphonomethoxy)propyl]-3-deazaadenine (3-deaza-HPMPA) |
| 2a | Cyclic-(*S*)-HPMPC (cHPMPC) |
| 2b | Cyclic-(*S*)-HPMPA (cHPMPA) |
| 3a | 9-[2-(Phosphonomethoxy)ethyl]adenine (PMEA) |
| 3b | 9-[2-(Phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP) |
| 3c | 9-[2-(Phosphonomethoxy)ethyl]-2-amino-6-chloropurine |
| 3d | 9-[2-(Phosphonomethoxy)ethyl]guanine (PMEG) |
| 3e | 9-[2-(Phosphonomethoxy)ethyl]-6-dimethylaminopurine |
| 3f | 9-(2-Phosphonomethoxyethyl)-2-amino-6-methylpurine |
| 3g | 9-(2-Phosphonomethoxyethyl)-2-amino-6-cyclopropylaminopurine |
| 4 | *N*-(3-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}amino)propanoic acid |
| 5 | *N*-(4-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}amino)butanoic acid |
| 6 | *N*-(*RS*)-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}iminodiacetic acid |
| 7 | *N*-(*RS*)-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}serine |
| 8 | *N*-(*RS*)-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}aspartic acid |
| 9 | *N*-(*RS*)-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}phenylalanine |
| 10 | *N*-(*RS*)-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}leucine |
| 11a | *N*-(*RS*)-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}threonine |
| 11b | *N*-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}-(2R,3S)-threonine (L enantiomer) |
| 11c | *N*-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}-(2S,3R)-threonine (D enantiomer) |
| 12 | *N*-(*RS*)-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}aminomethylsulfonic acid |
| 13 | *N*-(*RS*)-2-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}aminoethylsulfonic acid |
| 14 | *N*-(*RS*)-2-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}aminoethylphosphonic acid |
| 15 | *N*-(*RS*)-2-{2-Amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}aminoethoxymethylphosphonic acid |
| 16a | 2,4-Diamino-6-[2-(phosphonomethoxy)ethoxy]pyrimidine (PMEO-DAPy) |
| 16b | 5-Bromo-2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]pyrimidine |
| 17 | (*R*)-{2,4-diamino-3-hydroxy-6-[2-(phosphonomethoxy)propoxy]}pyrimidine (HPMPO-DAPy) |
| 18 | Cyclic-(*R*)-{2,4-diamino-3-hydroxy-6-[2-(phosphonomethoxy)propoxy]}pyrimidine |
| 19a | 9-(*S*)-2-(Phosphonomethoxypropyl)adenine (*S*)-PMPA |
| 19b | 9-(*S*)-(3-Fluoro-2-phosphonomethoxypropyl)adenine (*S*)-FPMPA |
| 20a | 9-(*R*)-2-(Phosphonomethoxypropyl)adenine (*R*)-PMPA |
| 20b | 9-(*R*)-(3-Fluoro-2-phosphonomethoxypropyl)adenine (*R*)-FPMPA |

We tested a broad range of ANPs against orf virus replication in vitro (on cell monolayers) and, for the most active ones, in an ex vivo organotypic raft culture system. Organotypic human skin equivalent has already successfully been used for the study of different epitheliotropic viruses, like human papillomaviruses (21), herpes simplex virus type 1 (32), adenovirus type 2 (22), and vaccinia virus (29). In order to investigate the antiviral activities of some ANPs against orf virus, we developed an ovine raft culture system from differentiated lamb keratinocytes which could reproduce the morphology of the in vivo ovine skin.

## MATERIALS AND METHODS

**Cells.** Primary lamb keratinocytes (PLKs) were isolated from the foreskin tissue of 3- to 12-month-old lambs. Thin sheets of foreskin tissue were cut into small pieces and then incubated with trypsin-EDTA (Gibco, Invitrogen Corporation, United Kingdom) for 30 min at 37°C. Trypsinized cells were filtered with 70-μm-pore-size filters and then centrifuged at 1,200 rpm for 10 min. The cell pellet was resuspended in the growth medium, a 1/3 mixture of Ham's F12 (Gibco, Invitrogen Corporation) and Dulbecco's modified Eagle's medium (Gibco, Invitrogen Corporation) supplemented with 10% of fetal calf serum (FCS), 2 mmol of L-glutamine per liter, 1 mmol of sodium pyruvate per liter, 0.5 μg/ml of hydrocortisone, 2 ng/ml of epidermal growth factor, 5 μg/ml of transferrin, 5 μg/ml of insulin, 0.1 nmol of cholera toxin per liter, and $1.5 \times 10^{-3}$ μg/ml of 3,3′,5-triiodo-2-thyronine. This growth medium was also used in the raft cultures. PLKs were cultured at 37°C and in a 5% $CO_2$ atmosphere, and when they reached confluence, they were applied in the antiviral and the cytotoxicity assays, as well as for the preparation of the organotypic raft cultures.

Human embryonic lung fibroblasts (HEL-299; ATCC CCL-137) were grown in minimal essential medium supplemented with 10% FCS, 2 mmol of L-glutamine per liter, and 7.5% sodium bicarbonate.

3T3 J2 murine fibroblasts, added in the collagen matrix as feeder cells for the keratinocytes in the raft culture system, were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS.

**Virus.** Several orf virus strains (strains IT-Mi90, IT-To, IT-C2, IT-01, and NZ2) were propagated in PLKs and used to test the activities of the ANPs. IT-Mi90 and IT-To isolated from chamois and IT-C2 isolated from sheep were adapted to grow in cell culture; IT-01 is a recently isolated strain from a proliferative form of contagious ecthyma in sheep. The reference NZ2 strain (27) was kindly provided by A. Mercer (Otago University Dunedin, New Zealand).

**Compounds.** A list of the compounds whose activities were tested against orf virus is presented in Table 1.

ANPs derived from 9-[2-(phosphonomethoxy)ethyl]-2-aminopurine with substitutions at position 6 of the amino group of amino acids (compounds 4 to 11), aminoalkylsulfonic acids (compounds 12 and 13), or aminoalkylphosphonic acids (compounds 14 and 15) were prepared by the treatment of diisopropyl 2-amino-6-chloro-9-[2-(phosphonomethoxy)ethyl]purine with the tetrabutylammonium salt of the corresponding acid in dioxane, followed by transsilylation and hydrolysis (14). All other ANP derivatives were synthesized as described in the literature (12, 13) (Fig. 1).

**Antiviral assay.** The activities of the ANPs against each of the orf virus strains listed above were evaluated in PLK and HEL cells. Both cell lines were cultured in 96-well microtiter plates, and the confluent monolayers were infected with a viral inoculum with a titer ranging from 20 to 60 PFU/100 μl/well. After 2 h of incubation at 37°C with 5% $CO_2$, residual virus was removed and the infected cells were further incubated with medium containing serial dilutions of the compounds (in duplicate). After 3 days of incubation at 37°C and 5% $CO_2$, the viral cytopathic effect was recorded, and the 50% inhibitory concentration ($IC_{50}$) was defined as the compound concentration required to reduce 50% of the viral

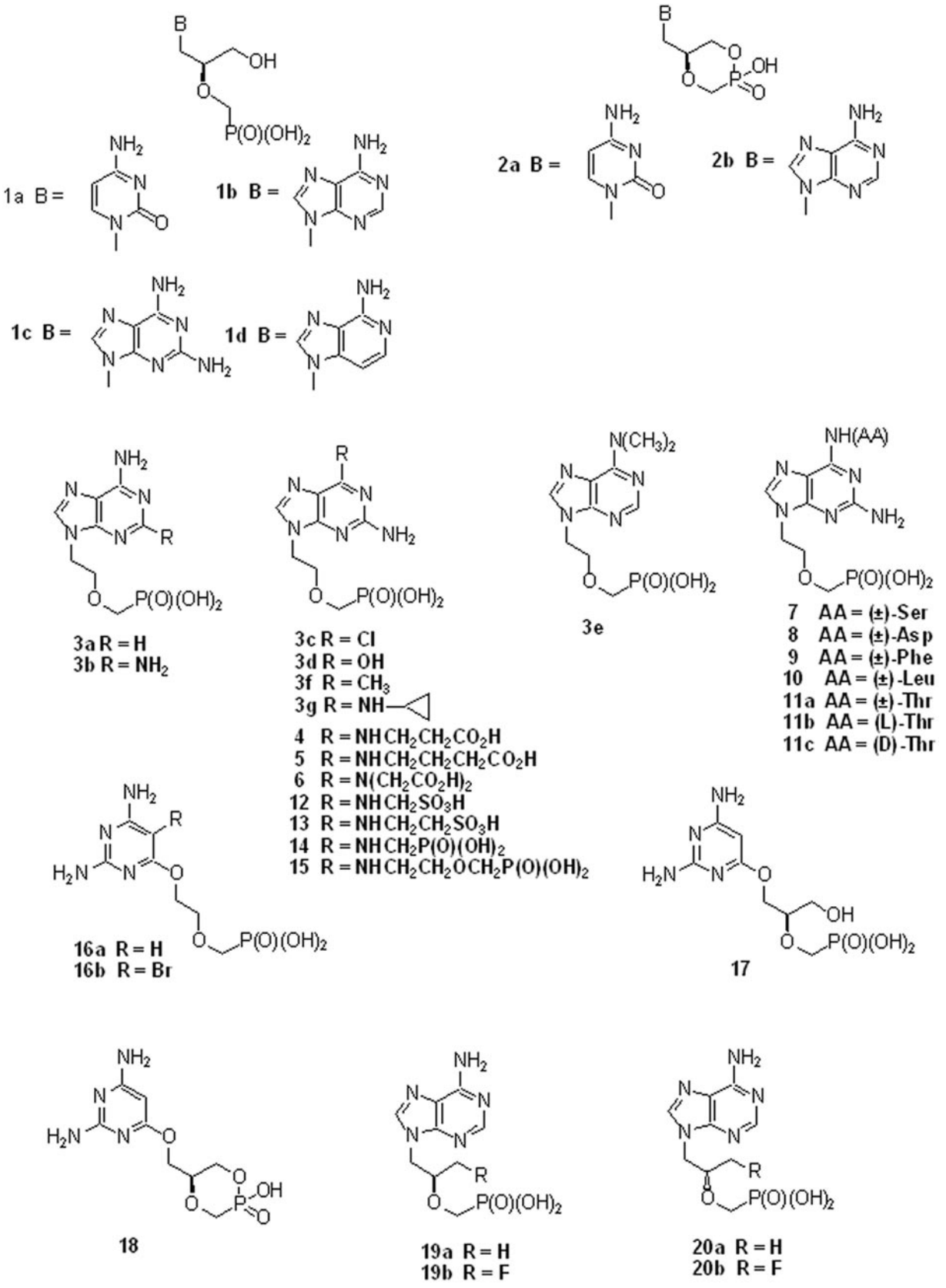


FIG. 1. Structures of the compounds tested.

cytopathic effect. The $IC_{50}$s of the compounds tested against each strain were calculated as the means of two or more independent experiments.

**Cytotoxicity assay.** The cell toxicities of the compounds were evaluated based upon the inhibition of cell growth. The cells were seeded into 96-well microtiter plates at $3.5 \times 10^3$ and $5 \times 10^3$ per well for HEL and PLK cells, respectively, in a volume of 100 μl. After 24 and 48 h of incubation for HEL and PLK cells, respectively, 100 μl of medium containing serial dilutions (in duplicate) of the tested compounds were added. After 4 days of incubation, the cells were trypsinized and the cell number per well 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% (in reference to the number of cells in the untreated control cell). The $CC_{50}$ of each compound resulted from the mean $CC_{50}$ of two independent experiments. The selectivity index (SI), defined as the ratio of the $CC_{50}$ for cell growth to the $IC_{50}$ for viral plaque formation, was calculated for each compound.

**Virus yield assays.** The effects of several dilutions (50, 20, 5, 2, and 0.5 μg/ml) of HPMPC, (*R*)-9-[3-hydroxy-2-(phosphonomethoxy)propoxy]-2,4-diaminopyrimidine (HPMPO-DAPy), and HPMPDAP were evaluated with the orf virus yields from the HEL and PLK cells. Both cell lines were grown in six-well microtiter plates, and the confluent monolayers were infected with orf virus NZ2 at a multiplicity of infection of approximately 0.5. After 2 h of incubation at 37°C in a 5% $CO_2$ atmosphere, residual virus was removed and was replaced by medium containing different concentrations of the test compounds. A virus and a cell control were included at each time point.

The supernatants and the infected cell monolayers were recovered separately in order to investigate the reduction of the production of extracellular and cell-associated virions. At 24, 72, and 96 h postinfection, the supernatants were harvested and frozen at −80°C; the cell monolayers were also frozen at −80°C after the addition of 3 ml of phosphate-buffered PBS to each well.

In order to titrate the extracellular virus, the supernatants were thawed and the virus yield was evaluated by virus titration in 96-well microtiter plates. After the cell cultures were thawed, the disrupted cells contained in 3 ml PBS were collected from the bottom of the microplates and then centrifuged at 1,200 rpm for 10 min. The supernatants with the intracellular virus were used for virus titration. The titration was performed with serial 10-fold dilutions of the samples, and the viral titer was expressed in PFU per ml. The $IC_{90}$ and the $IC_{99}$ were defined and calculated at each time point as the compound concentrations required to reduce 90% and 99% of the viral growth, respectively.

**Real-time PCR.** Real-time PCR was applied to the quantification of the viral DNA in the supernatant and the cell monolayers. The samples were prepared as described above, and the viral DNA was purified with a QIAamp DNA Blood Mini kit (QIAGEN, Hilden, Germany) and then stored at −20°C until use. To quantify orf virus DNA, a real-time PCR assay was developed based on the TaqMan technology. Primers and probe were designed by using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA), and a highly conserved region of the orf virus genome, delimited by the primer pair PPP-1 and PPP-4 (17). The nucleotide sequences of the forward and the reverse primers were 5′-CAGCAGAGCCGCGTCAA-3′ and 5′-CATGAACCGCTACAACAC CTTCT-3′, respectively. An MGB probe of 15 nucleotides (5′-CACCTTCGG CTCCAC-3′) was designed, and it was labeled with the 6-carboxyfluorescein reporter dye at the 5′ end. The TaqMan PCR assay was performed, and the products were evaluated on an ABI PRISM 7000 sequencer (Applied Biosystems, Foster City, CA). The PCR was performed in a final volume of 25 μl, which contained 12.5 μl of 2× TaqMan Universal PCR master mix, No AmpErase UNG (Applied Biosystems, Branchburg, NJ), 900 nmol of each primer, 200 nmol of the probe, and 5 μl of the DNA template. A plasmid was constructed to be used as an internal standard during the reaction. After amplification of the viral target region with the primer pair PPP-1 and PPP-4, the PCR product was purified and cloned into the pCR 4-TOPO vector (TOPO TA Cloning kit; Invitrogen, Groningen, The Netherlands). The plasmid was purified, and the sequence of the cloned insert was verified. Immediately before each PCR was set up, the plasmid was diluted in nine serial dilutions (from $10^{-2}$ to $10^{-10}$). The standard curve for the TaqMan PCR was constructed by plotting serial dilutions of the plasmid against the corresponding cycle threshold ($C_T$) values. The amplification steps were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles consisting of 95°C for 15 s and 60°C for 1 min. Each sample was retested in triplicate during the same reaction and the DNA concentration was calculated as the mean of the three measurements. The reproducibility of the technique was evaluated by repeating the experiments twice with large and small amounts of orf virus DNA. The specificity of the assay was tested by including negative controls, like DNA extracted from uninfected cells and supernatants. The reduction of the viral DNA was expressed as a percentage of the amount of DNA in the untreated control.

**Organotypic cultures.** For the preparation of the raft cultures, a collagen matrix solution was made to mimic the dermal function and the physiology of the skin in vivo. The "collagen beds" were prepared on ice by mixing Ham's F-12 medium (Gibco, Invitrogen Corporation) concentrated 10-fold, reconstitution buffer (a solution of 22% $NaHCO_3$, 2% NaOH, and 47.6% HEPES) concentrated 10-fold, collagen type I (Becton Dickinson, Pharmingen, San Diego, CA), and 3T3 J2 fibroblasts. One milliliter of the collagen matrix solution was poured into 24-well plates and was solidified at 37°C for 1 h. After equilibration of the gel with 1 ml of growth medium overnight at 37°C, $2 \times 10^5$ PLKs were seeded on top of the collagen beds and maintained by submersion in the growth medium 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. The epithelial cells were then allowed to stratify for 6 days, and at that time, the cultures were infected with 100 μl of orf virus strain NZ2 (360 PFU/100 μl), which was placed on the tops of the rafts. To test the effects of HPMPC, HPMPO-DAPy, and HPMPDAP on the replication of orf virus ex vivo, at the time of infection, medium containing different concentrations of these compounds (50, 20, 5, 2 and 0.5 μg/ml) was added. In the same assay, uninfected as well as infected untreated rafts were included as controls for the normal differentiated epithelium and viral replication, respectively. The three compounds were tested in two independent experiments. Furthermore, in order to correlate the histopathological features of the raft cultures with the reduction of viral replication, each condition was tested in duplicate in the same experiment. At 5 days postinfection, the first set of raft cultures was fixed in 10% buffered formalin and embedded in paraffin. Micrometer sections were stained with hematoxylin-eosin for histological examination. The second set of the rafts was immerged in 5 ml of sterile PBS and frozen at −20°C. After the rafts were thawed, the PBS was collected and centrifuged at 1,800 rpm for 10 min, and the supernatants containing the released virus were used for quantification of infectious virus and viral DNA. Titration of infectious virus was performed with 10-fold serial dilutions of the samples, and the viral titer was expressed as the number of PFU per raft. Viral DNA was quantified by real-time PCR.

## RESULTS

**Antiviral and cytotoxicity assays.** The results of the antiviral activities of the ANPs in cell culture (HEL and PLK cells) are presented in Table 2. No variation in the activities of the ANPs against the different orf virus strains was observed. Therefore, the results are presented as the means and the standard deviation of the $IC_{50}$s obtained for all the strains. Furthermore, the mean for each strain was the result of at least two independent experiments.

When the drugs were tested with human fibroblasts, the $IC_{50}$s were consistently lower than those when the drugs were tested with lamb keratinocytes. HPMPC (compound 1a), HPMPA (compound 1b), 3-deaza-HPMPA (compound 1d), and HPMPDAP (compound 1c) were the most active among the compounds tested and showed the highest SIs, ranging from 50.5 (compound 1d) to 99.7 (compound 1a) in HEL cells and from 12 (compound 1b) to ≥19.9 (compound 1c) in PLKs. Interestingly, both cyclic forms of ANPs (cHPMPC [compound 2a] and cHPMPA [compound 2b]), which had decreased electronegative charges, were several times less active and more toxic than their parent compounds, compounds 1a and 1b, respectively. Among the new subclass of "open-ring" ANPs, neither 2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]pyrimidine (compound 16a) nor its 5-bromo derivative (compound 16b) was active against orf virus, with $IC_{50}$s of 25.4 μg/ml (compound 16a) and 14.2 μg/ml (compound 16b) in HEL cells and >50 μg/ml in PLK cells. On the contrary, HPMPO-DAPy (17) had an $IC_{50}$ of 0.51 ± 0.15 μg/ml in HEL cells and 5.45 ± 1.85 μg/ml in PLKs, with comparatively high SIs in both cell lines (39.2 in HEL cells and 6.4 in PLKs). Also in this case, the cyclic form (compound 18) was less active, although it was approximately equally toxic for the cells.

TABLE 2. Activities and cytotoxicities of the compounds tested against orf virus

| Compound | Activities of ANPs in HEL cells | | | Activities of ANPs in PLK cells | | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ (μg/ml)[a] | $CC_{50}$ (μg/ml)[b] | SI[c] | $IC_{50}$ (μg/ml) | $CC_{50}$ (μg/ml) | SI |
| 1a, HPMPC | 0.33 ± 0.12 | 32.9 | 99.7 | 2.18 ± 1.82 | ≥38.6 | ≥17.7 |
| 1b, HPMPA | 0.08 ± 0.03 | 3.6 | 45 | 0.86 ± 0.43 | 10.4 | 12 |
| 1c, HPMPDAP | 0.43 ± 0.16 | 23.6 | 54.9 | 1.8 ± 0.85 | ≥35.9 | ≥19.9 |
| 1d, 3-deaza-HPMPA | 0.22 ± 0.04 | 11.1 | 50.5 | 1.49 ± 0.38 | ≥22.2 | ≥14.9 |
| 2a, cHPMPC | 1.04 ± 0.27 | 89.7 | 86.3 | 3.61 ± 1.88 | >50 | >13.8 |
| 2b, cHPMPA | 0.43 ± 0.15 | 6.3 | 14.7 | 1.07 ± 0.3 | ≥8.6 | ≥8 |
| 3a, PMEA | >50 | 41.9 | <0.8 | >50 | 13.05 | <0.3 |
| 3b, PMEDAP | >50 | 27.1 | <0.5 | >50 | 6.15 | <0.12 |
| 3c | 1.05 ± 0.7 | 1 | 0.9 | 1.72 ± 0.53 | 0.15 | 0.09 |
| 3d, PMEG | 2.62 ± 0.88 | 7.25 | 2.8 | 5.98 ± 1.83 | 0.72 | 0.12 |
| 3e | 1.82 ± 0.72 | 3.9 | 2.1 | 1.41 ± 0.2 | 0.44 | 0.3 |
| 3f | 32 | 34.7 | 1.08 | ≥50 | >50 | 1 |
| 3g | 0.6 ± 0.14 | <0.5 | <0.83 | 1.7 ± 0.8 | 6.3 | 3.7 |
| 4 | >50 | >50 | 1 | >50 | >50 | 1 |
| 5 | >50 | >50 | 1 | >50 | >50 | 1 |
| 6 | >50 | >50 | 1 | >50 | >50 | 1 |
| 7 | 1.5 ± 0.77 | 2.2 | 1.5 | 10.12 ± 6.7 | 0.67 | 0.07 |
| 8 | 2.04 ± 1 | 2.75 | 1.3 | 16.94 ± 6.63 | 0.95 | 0.06 |
| 9 | 8.5 ± 4.09 | >50 | >5.9 | 28.8 ± 10.7 | 23 | 0.79 |
| 10 | 2.16 ± 0.65 | 2.8 | 1.3 | 10.37 ± 7.11 | 1.19 | 0.11 |
| 11a | 0.87 ± 0.26 | 1.8 | 2 | 5.71 ± 2.68 | 0.35 | 0.06 |
| 11b | 0.46 ± 0.04 | 3.6 | 7.9 | 4.9 ± 1.2 | 0.2 | 0.04 |
| 11c | 3.08 ± 0.68 | 9.2 | 2.9 | 15.1 ± 5.8 | 0.2 | 0.01 |
| 12 | 0.12 ± 0.06 | 0.17 | 1.4 | 0.43 ± 0.17 | 0.035 | 0.08 |
| 13 | >50 | 170.8 | <3.4 | >50 | 109.2 | <2.2 |
| 14 | 8.38 ± 5.77 | >50 | >5.96 | 21.4 ± 0.7 | 5 | 0.23 |
| 15 | >50 | >50 | 1 | >50 | >50 | 1 |
| 16a, PMEO-DAPy | 25.4 ± 4.3 | 18.3 | 0.7 | >50 | 37.35 | <0.7 |
| 16b | 14.17 ± 2.2 | 14 | 0.9 | >50 | 3.35 | <0.07 |
| 17, HPMPO-DAPy | 0.51 ± 0.15 | 20 | 39.2 | 5.41 ± 1.85 | 34.4 | 6.4 |
| 18 | 3.13 ± 1.41 | 26.1 | 8.3 | 16 ± 2.1 | >50 | >3.1 |
| 19a, (*S*)-PMPA | >50 | >50 | 1 | >50 | >50 | 1 |
| 19b, (*S*)-FPMPA | >50 | >50 | 1 | >50 | >50 | 1 |
| 20a, (*R*)-PMPA | >50 | >50 | 1 | >50 | >50 | 1 |

[a] The values are expressed as the means ± standard deviations of the $IC_{50}$s calculated for the five orf virus strains, with each of them being tested in at least two independent experiments.
[b] The values are the means of two cytotoxicity assays.
[c] SI = $CC_{50}/IC_{50}$.

As expected, 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA; compound 3a) and 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (compound 3b) did not reveal any anti-orf virus activity either in HEL or in PLK cells, with $IC_{50}$s >50 μg/ml. However, the N-6-dimethyl derivative of PMEA (compound 3e) showed moderate activity in both cell lines.

Among the new subclass of N-6-substituted 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine derivatives, the molecules with a sulfonic acid and/or a phosphonic acid residue were not particularly active, although the aminomethanesulfonic acid derivative (compound 12) showed the maximum toxicity among this group of derivatives tested. As expected, the toxicities of the guanine derivative 9-[2-(phosphonomethoxy)ethyl]quanine (compound 3d) and its 6-chloropurine precursor (compound 3c) were also very high in both cell lines and particularly in PLK cells, where the SIs were ranged from 0.12 to 0.09.

Among the group of compounds derived from amino acids linked to the 6 position of the purine residue by their amino function, we first examined the racemic compounds. While compounds bearing the linear (nonbranched chain) ω-aminoalkanoic acids β-alanine (compound 4), *N*-(4-{2-amino-9-[2-(phosphonomethoxy)ethyl]purin-6-yl}amino)butanoic acid (compound 5), or iminodiacetic acid (compound 6) were essentially inactive, all the other compounds of this subgroup derived from "natural" amino acids exhibited activities against orf virus in vitro, with $IC_{50}$s in the range of 0.87 to 2.16 μg/ml, without regard to the acid (Asp) or neutral (Ser, Leu, Thr) character of the amino acid involved. Only the phenylalanine derivative (compound 9) was quite inactive. The best activity against orf virus was observed for the racemic threonine derivative (compound 11a). Therefore, the individual enantiomers were synthesized from L- and D-threonine and subjected to the assay. The data obtained for compounds 11b and 11c showed clearly that the biological effect was enantiomer specific, with the compound containing the L-threonine residue (compound 11b) being more active (6.7 times in HEL cells and 3.3 times in PLK cells) than the compound containing the D enantiomer (compound 11c). Nonetheless, very low SIs (between 1 and 2 in HEL cells and 2 orders of magnitude lower for PLKs) clearly indicated that the suppression of multiplication of orf virus was due to the interference of the compounds with the host cell metabolism rather than to a specific antiviral activity.

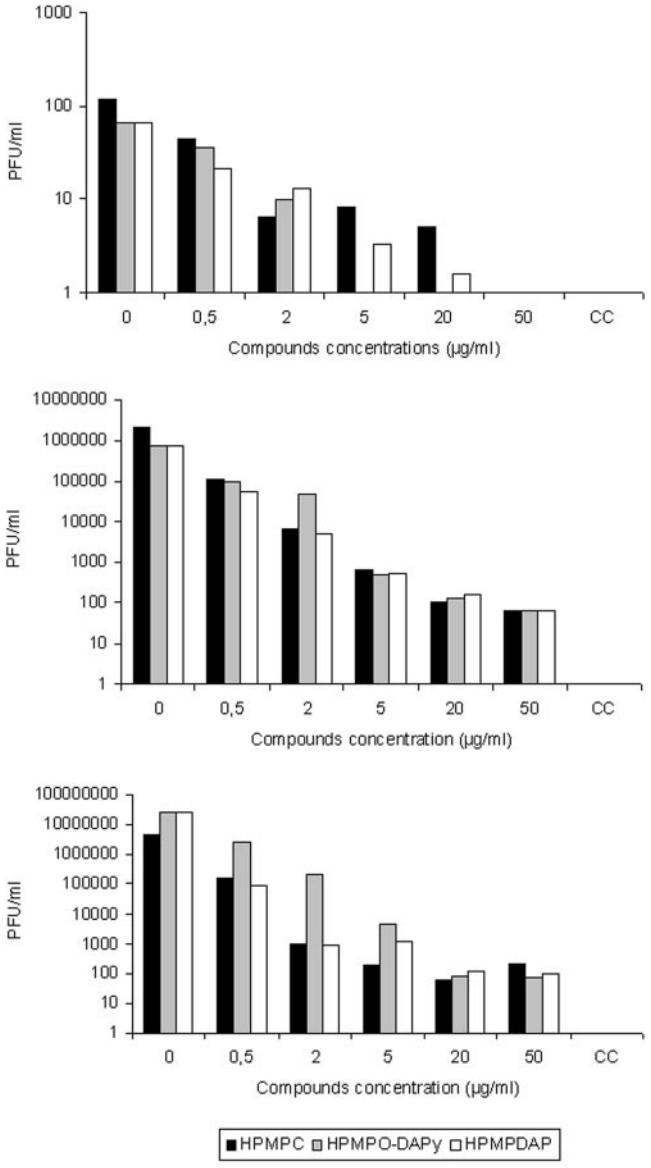


FIG. 2. Virus yield assay with PLK cells. The effects of several concentrations (0.5, 2, 5, 20, and 50 μg/ml) of HPMPC, HPMPDAP, and HPMPO-DAPy on cell-associated orf virus were determined. The viral growth was measured by virus titration and is expressed as the number of PFU/ml on a log scale. The virus yield was evaluated at 24 h (top panel), 72 h (middle panel), and 96 h (bottom panel) postinfection. At each time point, a virus control (0 μg/ml) and a cell control (CC) were also included.

**Virus yield assay.** In PLK and HEL cells the activities of HPMPC, HPMPDAP, and HPMPO-DAPy were confirmed by inhibition of extra- and intracellular virus yields at 24, 72, and 96 h postinfection. The virus titer was expressed as the $\log_{10}$ number of PFU per ml. At each time point in the treated samples as well as in the virus controls, the intracellular virus yield was 0.5 to 2 $\log_{10}$ units higher than the level of extracellular virus production (data not shown). Figure 2 presents the time- and concentration-dependent decreases in the intracellular virus yield in PLK cells treated with the different concentrations of the three compounds. At 24 h postinfection (Fig. 2, top panel), virus replication was undetectable at concentrations of 50, 20, and 5 μg/ml of HPMPO-DAPy and at 50 μg/ml of HPMPC and HPMPDAP. At 72 h postinfection (Fig. 2, middle panel), the three compounds afforded similar reductions of virus growth. However, at 96 h postinfection (Fig. 2, bottom panel), HPMPC and HPMPDAP proved to be the most active compounds. Thus, at a concentration of 2 μg/ml, HPMPC and HPMPDAP inhibited virus growth by 4 to 5 $\log_{10}$ units, while at the same concentration, HPMPO-DAPy reduced the virus yield by 1 $\log_{10}$ unit.

The $IC_{90}$s and $IC_{99}$s were $<0.5$ and 1.3 μg/ml, respectively, for HPMPC; $<0.5$ and $<0.5$ μg/ml, respectively, for HPMPDAP; and 0.5 and 1.96 μg/ml, respectively, for HPMPO-DAPy.

Similar results were obtained for the reduction of the orf virus yield in HEL cells (data not shown).

**Real-time PCR.** The real-time PCR technique demonstrated a high degree of interassay reproducibility when samples with large and small amounts of orf virus DNA were tested in independent experiments. The coefficient of variation of the $C_T$ value was 0.3% to 0.1%. The quantitative PCR was performed to evaluate the inhibition of viral DNA production in samples collected during the virus yield assay. Figure 3 shows the time- and concentration-dependent reductions of viral DNA in the supernatants of PLK cells. Similar results were obtained for the reduction of the cell-associated virus in the PLKs (data not shown). At each concentration, the viral DNA content is presented as the mean with the standard deviation of three measurements and is expressed as a percentage of the amount of the virus control. At the highest concentrations (50 and 20 μg/ml) of the three compounds tested, the viral DNA was undetectable in the supernatants (Fig. 3) as well as in the cell monolayers (data not shown). For the lowest concentrations of the three drugs tested, the inhibition of viral DNA production was higher with HPMPDAP than with HPMPC and HPMPO-DAPy.

**Organotypic raft cultures.** The inhibition of orf virus replication offered by HPMPC, HPMPDAP, and HPMPO-DAPy was evaluated in ovine organotypic raft cultures in two independent experiments. The histological features of the rafts in the presence of different concentrations of HPMPC are shown in Fig. 4. In the uninfected and untreated raft, after hematoxylin-eosin staining, the ability of the lamb keratinocytes to generate a fully differentiated epithelium with all the epidermal layers was constantly clearly observed (Fig. 4). The infected but untreated raft allowed orf virus replication with evidence of the typical cytopathic effect, characterized by balloon degeneration and the presence of cytoplasmatic inclusion bodies. At 6 days postinfection, complete destruction of the differentiated epithelium was observed in the infected and untreated raft (Fig. 4). Treatment of the cultures with HPMPC at the highest concentrations tested (50 and 20 μg/ml) resulted in complete protection of the epithelium. Similar results were obtained with the same concentrations of HPMPDAP and HPMPO-DAPy (data not shown), while at 5 μg/ml, only HPMPC and HPMPDAP offered partial protection (Fig. 4 and data not shown). The lowest concentrations (2 and 0.5 μg/ml) of each of the compounds tested were not effective, with the histological features of the epithelium being similar to those of the virus-treated control epithelium (Fig. 4 and data not shown).

Histological examination of cultures from two indepen-

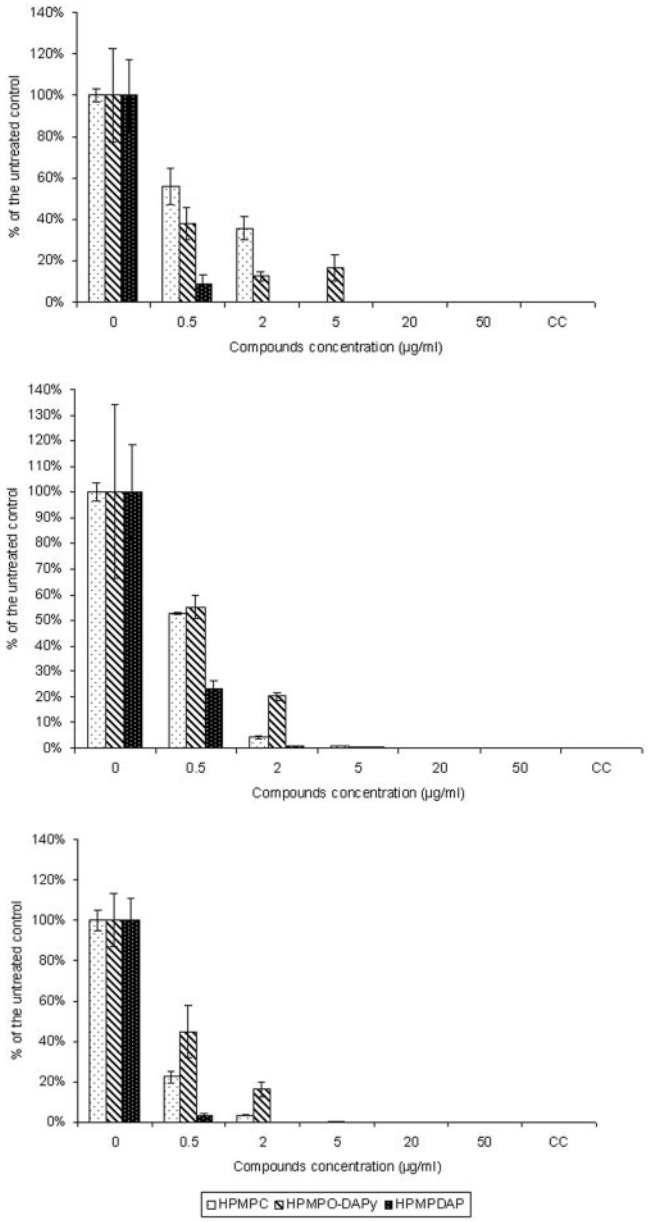


FIG. 3. Effects of several concentrations of HPMPC, HPMPDAP, and HPMPO-DAPy on viral DNA concentration measured in the supernatants of PLK infected cells after 24 h (top panel), 72 h (middle panel), and 96 h (bottom panel). Orf virus DNA was quantified by real-time PCR, and the concentration was calculated in reference to those in serial 10-fold dilutions of an internal standard. The DNA concentration was calculated as the mean of three measurements, and the reduction of the viral DNA was expressed as a percentage of the amount of DNA for the untreated control (0 μg/ml). Error bars represent the standard deviations of three measurements for each sample. CC, cell control.

dent experiments showed similar results, demonstrating the reproducibility of the technique.

The histopathological features of the raft cultures were correlated with the reduction of orf virus replication in the rafts.

The level of orf virus production in the rafts in the presence of the highest doses (50 and 20 μg/ml) of the three compounds was 4 $\log_{10}$ units lower than that of the virus control, while the DNA concentration was undetectable (Fig. 5). For the raft treated with HPMPO-DAPy at a concentration of 5 μg/ml, the virus yield increased compared to the virus yield achieved with the other two compounds tested at the same concentration. A similar profile was obtained when viral DNA was analyzed (Fig. 5). For the lowest concentrations, a similar trend was observed for the different molecules, with HPMPO-DAPy clearly being the less active.

## DISCUSSION

Contagious ecthyma is an infectious disease found throughout the world, and its spread is closely linked to the presence and size of animal herds. Although sheep and goats are the natural hosts for orf virus, the infection can also arise in humans. Orf virus infection occurs frequently in rural areas and among individuals within occupational categories at risk (farmers, sheep shearers, and veterinarians). Orf virus infection is the main occupational zoonosis reported to the Public Health Laboratory in the United Kingdom (10), and thousands of cases are registered annually in Australia and New Zealand (2).

No vaccine is able to prevent the disease in humans, and it has been observed that preexisting immunity to vaccinia virus (after smallpox vaccination) does not provide any protection against orf virus (33). In animals, vaccination can limit the severity of the disease, but it does not prevent the infection; in addition, vaccine strains have been the source of outbreaks of ecthyma (8).

Orf virus infection is usually a self-limiting disease without systemic spread, but the rising numbers of patients treated with immunosuppressants following transplantation and affected by immunodeficiency diseases contribute to the steadily increasing occurrence of atypical and relapsing forms of the infection. In these cases, cryotherapy and surgery are the most common treatments, but there is a need for a specific therapy.

The interest in the anti-orf virus activities of the ANPs is further prompted by the assumption that orf virus is a surrogate model in the study of the potencies of molecules of this class against the poxvirus family. The ANP derivatives are among the most potent drugs available against poxviruses (3). Studies on the spectra of activity of the ANPs are of relevance due to the increasing evidence that smallpox and monkeypox viruses could be weaponized by bioterrorists.

We have evaluated the antiviral activities of the compounds on HEL fibroblasts and epithelial PLK cells, which are both permissive for orf virus growth. Compounds that were active and nonactive against orf virus were distinguished in both cell lines.

The most active molecules in both HEL and PLKs were HPMPA (compound 1b), 3-deaza-HPMPA (compound 1d), HPMPC (compound 1a), and HPMPDAP (compound 1c); and the last two compounds also showed the highest selectivity. Interestingly, among the new generation of ANPs, HPMPO-DAPy (compound 17) demonstrated marked anti-orf virus activity, but it had a higher $IC_{50}$ and a lower selectivity index than HPMPC in both cell lines (1). While the HPMP derivative (compound 1b) is highly active against the model poxviruses, the related molecules containing a methyl group [9-(*S*)-2-(phosphonomethoxypropyl)adenine and 9-(*R*)-2-(phosphonomethoxypropyl)adenine; compounds 19a and 20a, respectively] or a fluoromethyl group [9-(*S*)-(3-fluoro-2-phosphonomethoxypropy-

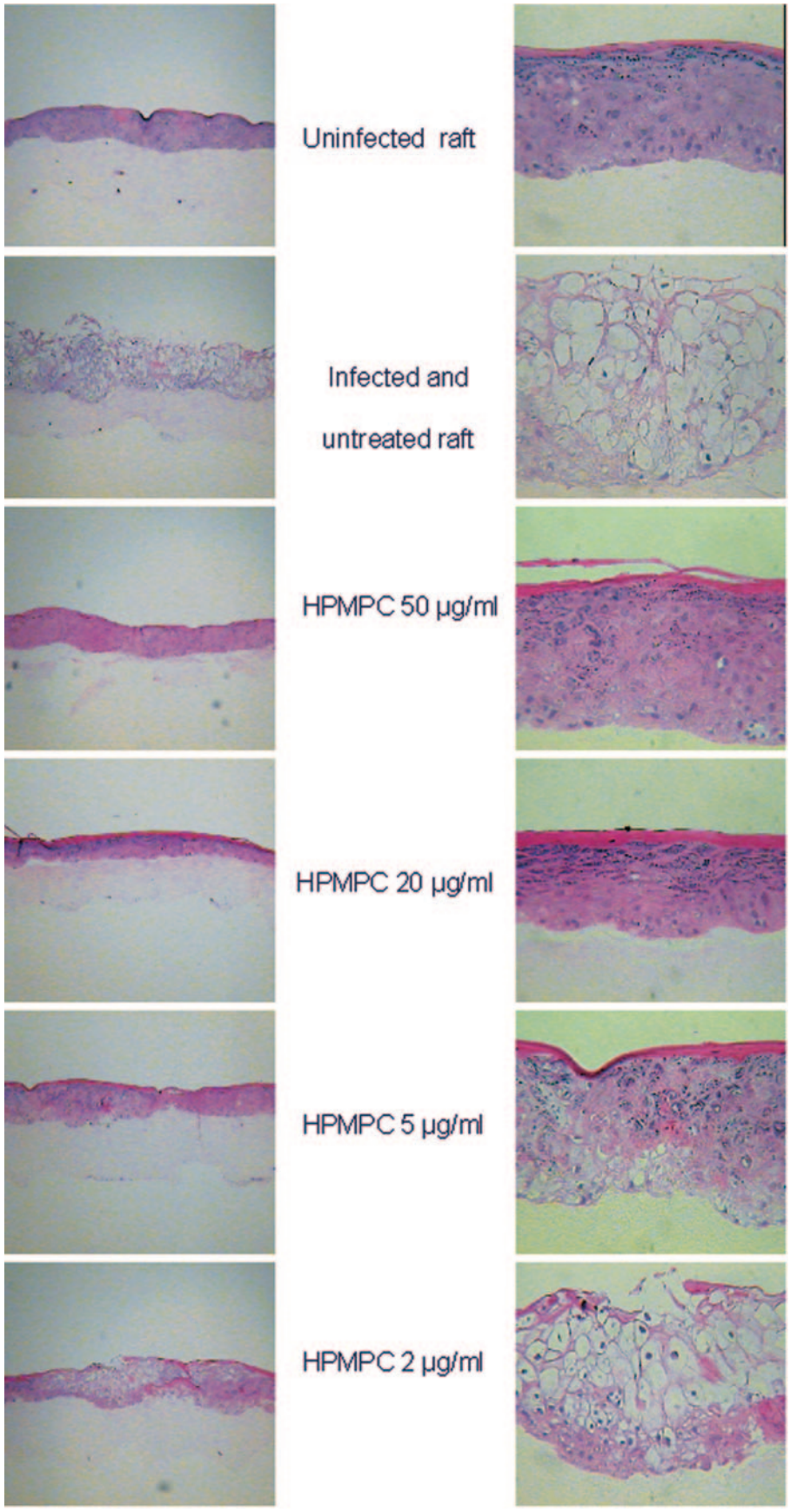


FIG. 4. Ovine raft cultures. The rafts were infected with orf virus after 6 days of differentiation and treated with various concentrations (50, 20, 5, and 2 μg/ml) of HPMPC. At 11 days of differentiation, the rafts were fixed and stained with hematoxylin-eosin. Magnifications, ×9 and ×36 in the right and left panels, respectively.

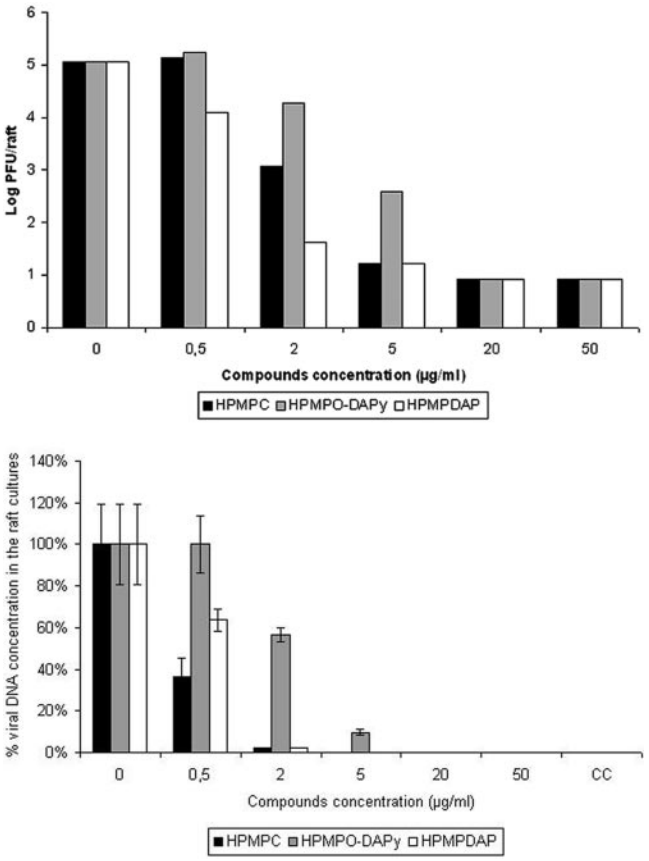


FIG. 5. (Top panel) Orf virus yield from the organotypic cultures is presented on a logarithmic scale; (bottom panel) quantification of orf virus DNA from the rafts, reported as a percentage of the amount of DNA in the infected but untreated raft. Error bars represent the standard deviations of three measurements for each sample. CC, cell control.

l)adenine and 9-(*R*)-(3-fluoro-2-phosphonomethoxypropyl)adenine; compounds 19b and 20b, respectively] instead of the hydroxymethyl group are devoid of any other activity except antiretroviral activity.

The anti-orf virus activity of HPMPO-DAPy was compared with those of HPMPC and HPMPDAP in a virus yield assay, which confirmed the order of decreasing activity measured in the antiviral assay (HPMPDAP > HPMPC > HPMPO-DAPy). The differential activities of the three compounds were confirmed by quantification of the viral DNA in the supernatants and in the cells.

Animal models commonly used for in vivo studies (mice, rats, *nu*/*nu* mice, guinea pigs, rabbits) do not allow orf virus replication (18, 19); consequently, sheep are the animals most commonly used. Nevertheless, the increasing concern about the use of animals in scientific work represents a further reason to apply alternative protocols. In this study we generated a three-dimensional model to reproduce the morphological and physiological characteristics of the ovine skin. The differentiation of the keratinocytes into a pluristratified epithelium was obtained after 11 to 12 days of growth from either young or adult foreskin tissue. The raft system has previously given successful results in the study of epitheliotropic viruses, such as papilloma-, herpes-, and poxviruses (20, 21, 28, 31). Orf virus has a high epitheliotropism, and it develops a local epidermal infection by replicating in the germinal layer of the epithelium. Orf virus could replicate in the ovine raft cultures, giving a histological picture similar to that in infected skin in vivo; in this system a correlation between the histological features in the untreated and treated rafts and viral replication was demonstrated. Furthermore, this system has proved to be a reliable model for the study of the antiviral activities of several compounds. In particular, we applied the ovine raft cultures to the study of the inhibitory effects of HPMPC, HPMPDAP, and HPMPO-DAPy. After 5 days of treatment at the highest concentrations tested (50 and 20 μg/ml), none of the three compounds caused toxic effects on the epithelium, resulting in full protection from orf virus growth.

Our in vitro and ex vivo study results demonstrated the activities of several ANP derivatives against orf virus. In particular, we further corroborated the anti-orf virus activity of HPMPC, as has already been shown in previous studies in vitro (25). Clinical applications of cidofovir cream for the topical treatment of human orf demonstrated the therapeutic activity of the compound (7; our unpublished data). The protocol consisted of once-daily application for several courses of 5 days on and 5 days off therapy, and it resulted in the complete resolution of the lesions. This therapeutic approach was chosen by consideration of the long-lasting activity of HPMPC, which confers prolonged inhibition of viral DNA synthesis and virus replication that lasts for at least 7 days after a single exposure (26). This feature allows infrequent dosing of the drug and limits its nephrotoxicity, which has been observed mainly after intravenous administration but not upon topical or intralesional administration (34).

Among the list of ANPs active against orf virus, cidofovir can be considered the main candidate for the therapy of natural infections in humans and animals not only because of its well-documented activity but also because since 1996 it has been formally approved for the intravenous treatment of cytomegalovirus retinitis in AIDS patients.

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