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In vitro activity of *cyclo*Sal-nucleoside monophosphates and polyhydroxycarboxylates against orthopoxviruses

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Abstract

Because variola virus might be used as a pathogen in biological attacks, there is an urgent need to provide effective antiviral drugs for the treatment of orthopoxvirus infections. Thus, the aim of the present study was to test the antiviral activity of 3 pro-nucleotides of the acyclic nucleoside analogues aciclovir (ACV), 3 of penciclovir (PCV) and 38 of the cyclic nucleoside analogue brivudin (BVDU), on the basis of *cyclo*Saligenyl-nucleoside monophosphate approach against vaccinia virus and cowpox virus in vitro. In further experiments, 13 synthetic humic acid-like polymers, so-called polyhydroxycarboxylates, were examined. Antiviral screening was performed by means of the plaque reduction assay and for quantification of the cytotoxicity of the test compounds the XTT-based tetrazolium reduction assay EZ4U was used. As result, three *cyclo*Sal-monophosphate derivatives of ACV proved to be potent inhibitors of both vaccinia virus and cowpox virus replication in vitro. Among the tested monophosphate derivatives of *cyclo*Sal-PCV and *cyclo*Sal-BVDU, selected substances showed a promising antiviral activity against vaccinia virus and cowpox virus. For the polyanionic compounds, no relevant antiviral activity was detected. In conclusion, by the delivery of nucleoside monophosphates from neutral, membrane-permeable prodrugs on the basis of the *cyclo*Saligenyl-nucleotide concept, different ACV, PCV and BVDU derivatives can act as potent and selective inhibitors of orthopoxvirus replication. However, most of the *cyclo*Sal-monophosphate derivatives of BVDU had a higher cytotoxicity than their parent nucleosides.

Keywords: Vaccinia virus; Cowpox virus; Antiviral activity; Pro-nucleotides; Polyanionic compounds

1. Introduction

Smallpox belongs to one of the oldest recorded infectious diseases of mankind. During many centuries, it has been a serious endemic disease all over the world. The last case of naturally occurring smallpox was observed in 1977 in Somalia. Because of successful preventive measures by immunization, the World Health Organization (WHO) declared global eradication of the disease in 1980 (World Health Organization, 1980). In the following years, the vaccination programs were discontinued in all countries. Since then, the causative agent of smallpox, variola virus, is retained in two central repositories, the Centers for Disease Control and Prevention (CDC) in Atlanta (USA) and the State Research Center of Virology and Biotechnology (VECTOR labs) in Novosibirsk (Russia). Since the events of September 11, 2001, in New York City and the subsequent deaths related to anthrax (Atlas, 2002), it seems to be possible that variola virus might be used as a pathogen in biological attacks (Henderson et al., 1999). Virtually, all children and a considerable part of the adult population are now fully susceptible to smallpox. Additionally, there is only a limited stock of efficient and safe vaccine available. Therefore, the CDC has listed variola virus in the category A of the most dangerous biological agents which may be used in biowarfare and bioterrorism (CDC; http://www.bt.cdc.gov/Agent/Agentlist.asp). In January 2002, the WHO Smallpox Advisory Group recommended that the existing variola virus stocks should be retained until satisfactory antiviral drugs and a safer vaccine are developed.

The search for antiviral agents to be effective against pox viruses started over 50 years ago when the thiosemicarbazones, which were introduced as tuberculostatic agents,

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were found to be active against vaccinia virus (Domagk et al., 1946; Bauer, 1955). To date, several substances have been reported to display antiviral activity against orthopoxviruses, but none of them have been approved for the therapy of pox virus infections in humans. Most of the effective compounds fall within the group of nucleoside analogues (De Clercq, 2001a). In particular, the acyclic nucleoside phosphonate (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [(*S*)-HPMPC, cidofovir] has been proved to be effective against variola virus, monkeypox virus, cowpox virus and vaccinia virus in vitro (Safrin et al., 1997) and in vivo in several animal models (Neyts and De Clercq, 1993; Bray et al., 2002; Huggins et al., 2002; Smee et al., 2002). After two consecutive phosphorylation steps, the diphosphate of cidofovir serves as a competitive inhibitor or alternative substrate in the viral DNA polymerase reaction (De Clercq, 2002). However, because of its nephrotoxicity an intravenous medication of cidofovir is limited in practice (Bray and Roy, 2004).

Among the first generation of nucleoside analogues which have been reported as inhibitors of herpes simplex virus (HSV) replication, there are also several potent compounds against pox viruses. These substances, targeting at the viral DNA synthesis, include, in particular, adenine arabinoside (Ara-A) (Walton et al., 1969) and 5-iodo-2′-deoxyuridine (5-iodo-dUrd) (Neyts and De Clercq, 2002). The second generation of nucleoside analogues is represented by 9-(2-hydroxyethoxymethyl)guanine (aciclovir, ACV) and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (brivudin, BVDU) which have been licensed for antiviral treatment of HSV and varicella-zoster virus (VZV) infections in humans since several years (De Clercq, 2001b). These agents are selectively activated by the herpesvirus thymidine kinase and, therefore, do not significantly inhibit the replication of pox viruses. However, this handicap might be changed by the use of lipophilic prodrugs of their monophosphates (Meier et al., 1998, 1999; Meier, 2004). The so-called *cyclo*Saligenyl-nucleotide concept was designed to release the nucleotides selectively by controlled, chemical induced hydrolysis involving a successive coupled cleavage of the phenyl- and benzylester of the *cyclo*Sal-phosphotriester (tandem reaction). Using this concept, the synthesis of pro-nucleotide derivatives of the acyclic nucleoside analogues ACV, penciclovir (PCV) and the cyclic nucleoside analogue BVDU has been described by Meier et al. (1998, 2001). To date, several compounds have been demonstrated to be potent inhibitors of HSV and Epstein-Barr virus (EBV) replication (Meerbach et al., 2000; Meier et al., 2002, 2004).

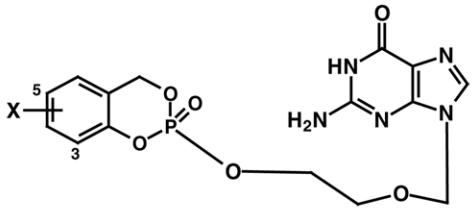
The aim of the present study was to test pro-nucleotides of acyclic and cyclic nucleoside analogues, based on the *cyclo*Sal-nucleoside monophosphate approach, against vaccinia virus and cowpox virus in vitro. Thus, different *cyclo*Sal-monophosphate (MP) derivatives of ACV, PCV and BVDU were used in comparison with the reference substances cidofovir and 5-iodo-dUrd. In further experiments, synthetic humic acid-like polymers, the so-called polyhydroxycarboxylates, which have been shown to inhibit replication of HSV (Helbig et al., 1997), were also tested.

2. Materials and methods

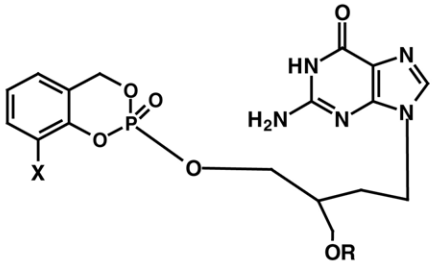
2.1. Chemicals

The synthesis of the new pro-nucleotides on the basis of *cyclo*Sal-phosphotriesters of the acyclic nucleoside analogues ACV, PCV and the cyclic nucleoside analogue BVDU was described in detail by Meier et al. (1998, 2001). In the present study, 3 different *cyclo*Sal-MP derivatives of ACV, 3 of PCV and 38 of BVDU were used as test compounds (Table 1; Fig. 1). In control experiments, the unphosphorylated parent nucleosides ACV, PCV and BVDU as well as the reference substances cidofovir and 5-iodo-dUrd were tested.

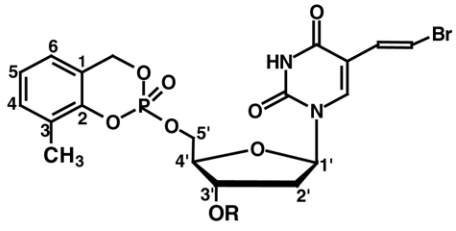
The different polyhydroxycarboxylates used in this study were produced by enzymatic or non-enzymatic oxidation of *ortho*- and *para*-di- as well as triphenolic substances



***cyclo*Sal-aciclovir monophosphate (*cyclo*Sal-ACVMP)**



***cyclo*Sal-penciclovir monophosphate (*cyclo*Sal-PCVMP)**



***cyclo*Sal-5-bromovinyl-2'-deoxyuridine monophosphate (*cyclo*Sal-BVDUMP)**

Fig. 1. Structure of *cyclo*Sal test compounds.

Table 1
Test compounds

Test group	Substances
*Cyclo*Sal-monophosphates (*cyclo*Sal-MP) from aciclovir (ACV)	3-Methyl-*cyclo*Sal-ACVMP 5-Methyl-*cyclo*Sal-ACVMP 5-H-*cyclo*Sal-ACVMP
*Cyclo*Sal-MP from penciclovir (PCV)	3-Methyl-*cyclo*Sal-PCVMP 3-Methyl-*cyclo*Sal-O-acetyl-PCVMP 5-H-*cyclo*Sal-PCVMP
*Cyclo*Sal-MP from brivudin (BVDU)	3-Methyl-*cyclo*Sal-3′-OH-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-acetyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-pivaloyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-methyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-propionyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-hexanoyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-decanoyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-*i*butyryl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-levulinyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-glycinyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-L-alaninyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-D-alaninyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-L-valinyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-D-valinyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-L-leucinyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-D-leucinyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-L-isoleucinyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-D-isoleucinyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-L-prolinyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-D-prolinyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-L-phenylalanyl-BVDUMP 3-Methyl-*cyclo*Sal-3′-O-D-phenylalanyl-BVDUMP 5-H-*cyclo*Sal-3′-O-acetyl-BVDUMP 5-H-*cyclo*Sal-3′-O-*i*butyryl-BVDUMP 5-H-*cyclo*Sal-3′-O-levulinyl-BVDUMP 5-H-*cyclo*Sal-3′-O-L-alaninyl-BVDUMP 5-Chloro-*cyclo*Sal-3′-OH-BVDUMP 5-O-Methyl-*cyclo*Sal-3′-OH-BVDUMP 3,5-Dimethyl-*cyclo*Sal-3′-OH-BVDUMP 3-*t*-Butyl-*cyclo*Sal-3′-OH-BVDUMP 6-Chloro-7-methyl-*cyclo*Sal-3′-OH-BVDUMP 6-Chloro-*cyclo*Sal-3′-OH-BVDUMP 3-Phenyl-*cyclo*Sal-3′-OH-BVDUMP 5-Phenyl-*cyclo*Sal-3′-OH-BVDUMP Benzo[*b*]-*cyclo*Sal-3′-OH-BVDUMP Benzo[*c*]-*cyclo*Sal-3′-OH-BVDUMP 6-Chloro-7-ECM-*cyclo*Sal-3′-OH-BVDUMP 6-Chloro-7-*n*-butyl-*cyclo*Sal-3′-OH-BVDUMP
Phenolic polymers	KOP/caffeic acid GALOP/gallic acid GENOP/gentisinic acid HYKOP/hydrocaffeic acid PYROP/pyrogallol CHOP/chlorogenic acid POP/protocatechuic acid 2,5-DHPOP/2,5-dihydroxy-phenylacetic acid 3,4-DHPOP/3,4-dihydroxy-phenylacetic acid KHYKOP/caffeic acid plus hydrocaffeic acid (1:1) KGALOP/caffeic acid plus gallic acid (1:1) GALEEOP/gallic acid ethylester GALMEOP/gallic acid methylester

according to the methods described previously (Meerbach et al., 2002). Table 1 lists the phenolic polymers tested as well as their starting compounds. The mean molecular weight (MW) and the number of carboxylic groups correspond to the data reported previously (Helbig et al., 1997; Klöcking et al., 2002; Meerbach et al., 2002) with the exception of GALOP (starting compound: gallic acid, MW: 7300, carboxylic groups: 18.2 equivalents per mol) and PYROP (starting compound: pyrogallol, MW: 7300, carboxylic groups: 12.5 equivalents per mol). Dextran sulfate was included as reference substance of the polyanionic compounds.

2.2. Viruses and cell cultures

The following viruses belonging to the genus *orthopoxvirus* were included in this study:

(i) vaccinia virus, strain Elstree, received from Prof. Herzberg, Greifswald, Germany;
(ii) cowpox virus, strain Marina (Meyer et al., 1999), isolated from an elephant in 1961.

The viruses were grown and titrated using monolayers of the permanent African green monkey kidney cells Vero76 (ATCC, CRL 1587). The cells were cultured in Eagle's minimum essential medium (EMEM) with Hanks' salts (Cambrex, Verviers, Belgiun) supplemented with 2 mM L-glutamine (Cambrex), 5% fetal calf serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Cambrex) and 100 μg/ml streptomycin sulfate (Cambrex). Titers of viral stocks were $10^{7.4}$ tissue culture infectious dose 50%

($TCID_{50}$) per ml for vaccinia virus and $10^{7.9}$ $TCID_{50}$/ml for cowpox virus.

2.3. Testing of cytotoxicity

The cytotoxicity induced by the test compounds was quantified in microtiter plates by means of the XTT-based tetrazolium reduction assay EZ4U (Biozol, Eching, Germany) described previously (Klöcking et al., 1995). Cytotoxicity tests were run in confluent monolayers of Vero76 cells. After measuring extinction from three wells, substance concentrations at the half-maximum cytotoxicity (50% cytotoxic concentration, CC_{50}) were calculated as arithmetic means.

2.4. Antiviral assay

Antiviral screening was performed in 24-well flat-bottomed microtiter plates by means of the plaque reduction assay which is used as a standard method for antiviral testing against pox viruses in vitro. Each well of the microtiter plates was seeded with 10^5 7-day-old Vero76 cells in 1 ml of growth medium. Cells were cultivated for 2–3 days at 37 °C in a humid atmosphere containing 5% CO_2. After confluence of cells, the medium was removed and the cell monolayers were washed with phosphate-buffered saline (PBS). Each well was inoculated with 1 ml virus which was diluted in growth medium. Vaccinia virus was used at a 1:5000 dilution, which corresponds to $10^{3.7}$ tissue culture infective dose 50% ($TCID_{50}$) per ml and 0.05 multiplicity of infection (m.o.i.). The cowpox virus strain was diluted 1:10,000 corresponding to $10^{3.9}$ $TCID_{50}$/ml and 0.08 m.o.i. After adsorption of virus for 1 h at 37 °C, the inocula were removed and the wells were overlaid with 1 ml of a 1:1 mixture consisting of purified agar (Difco Laboratories, Detroit, USA) at a final concentration of 0.5% and the antiviral substances at a final half log dilution over a range between 0.125 and 512 μg/ml. The agar was suspended in distilled water and the antiviral compounds were diluted with EMEM without phenol red (Cambrex). Both components were added to the cell monolayers at a temperature of 40 °C. After incubation for 72 h at 37 °C and 5% CO_2, the cells were fixed and stained with 500 μl 0.3% crystal violet in 3% formalin per well for at least 2 h. Subsequently, the overlay was washed off using tap-water and the underlying cells were washed with distilled water. Finally, plaques were counted with the aid of a dissecting microscope (Carl Zeiss, Jena, Germany) with ×100 magnification and an estimate of the 50% inhibitory concentration (IC_{50}) was made.

Each experiment was performed in triplicate and data points of IC_{50} were presented as the arithmetic means ± standard errors of the mean (S.E.M.).

2.5. Statistical analysis

CC_{50} and IC_{50} values were calculated from dose–response curves by regression analysis using the Sigma Statistical Analysis System, Version 1.01 as software. Selectivity index (SI) was expressed as quotient of CC_{50} and IC_{50}. To verify variations in sensitivity of vaccinia virus strain Elstree and cowpox virus strain Marina, IC_{50} values were compared using the double-sided Student's *t*-test. *P*-values were subject to a significance level of 5%.

3. Results

The highest antiviral activity of the test substances was demonstrated for the three *cyclo*Sal-monophosphate derivatives of ACV (Table 2). With IC_{50} values of 6–10 μg/ml, 3-methyl-*cyclo*Sal-ACVMP (entry 1, Table 2) had a much greater antiviral activity than the parent compound against vaccinia virus and cowpox virus and for the 5-methyl-substituded *cyclo*Sal-ACVMP (entry 2, Table 2) an IC_{50} of 17–37 μg/ml could be calculated. With IC_{50} values between 18 and 33 μg/ml, the unsubstituted compound 5-H-*cyclo*Sal-ACVMP (entry 3, Table 2) was found to be effective as well. The selectivity indices of the *cyclo*Sal-ACVMP derivatives tested in this study varied between 8 and 23. Thus, in comparison with the unphosphorylated parent ACV, which was entirely inactive (entry 4, Table 2), *cyclo*Sal-MP derivatives of ACV proved to be potent inhibitors of both vaccinia virus and cowpox virus infection in vitro. The dose–response curve for 3-methyl-*cyclo*Sal-ACVMP, the best compound of this group, is presented in Fig. 2. There were no statistical differences in sensitivity between vaccinia virus strain Elstree and cowpox virus strain Marina to the *cyclo*Sal-MP derivatives.

Among the three tested monophosphate derivatives of *cyclo*Sal-PCV, 3-methyl-*cyclo*Sal-PCVMP (entry 1, Table 3)

Table 2
Antiviral activity of *cyclo*Sal-monophosphates (*cyclo*Sal-MP) from aciclovir (ACV) against vaccinia virus (VV) strain Elstree and cowpox virus (CPV) strain Marina

No.	Compound	CC_{50} (μg/ml)	VV, IC_{50} (μg/ml)[a]	VV, IC_{90} (μg/ml)[a]	CPV, IC_{50} (μg/ml)[a]	CPV, IC_{90} (μg/ml)[a]	SI, VV/CPV
1	3-Methyl-*cyclo*Sal-ACVMP	135	5.9 ± 0.6	12.7 ± 1.5	9.9 ± 2.7	20.4 ± 4.8	22.9/13.6
2	5-Methyl-*cyclo*Sal-ACVMP	>128	16.7 ± 4.3	39.3 ± 9.0	36.8 (n = 1)	64.0	>7.7/>3.5
3	5-H-*cyclo*Sal-ACVMP	283	17.9 ± 3.9	55.1 ± 14.6	33.2 ± 2.8	88.2 ± 4.1	15.8/7.5
4	ACV	>512	>256	>256	>256	>256	–
5	Cidofovir	>512	4.6 ± 0.7	7.8 ± 1.1	4.0 ± 0.6	10.5 ± 3.0	>111/>128
6	5-Iodo-dUrd	>1024	0.4 ± 0.1	1.3 ± 0.3	0.3 ± 0.1	1.0 ± 0.2	>2560/>3413

CC_{50}, 50% cytotoxic concentration; IC_{50}/IC_{90}, 50%/90% inhibitory concentration; SI, selectivity index.
[a] Data are the mean ± standard deviation for three independent experiments.

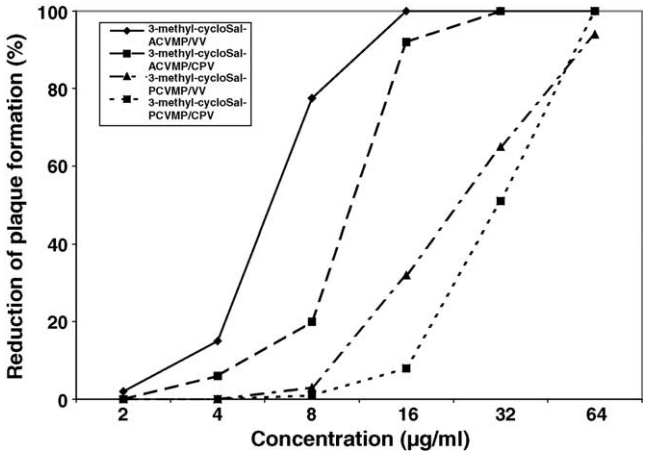


Fig. 2. Dose–response curve for antiviral activity of 3-methyl-*cyclo*Sal-aciclovir-monophosphate (3-methyl-*cyclo*Sal-ACVMP) and 3-methyl-*cyclo*Sal penciclovir-monophosphate (3-methyl-*cyclo*Sal-PCVMP) against vaccinia virus strain Elstree (VV) and cowpox virus strain Marina (CPV).

had a moderate antiviral activity which was comparable between both orthopoxviruses used. The IC_{50} values were calculated as 24 μg/ml against vaccinia virus and about 27 μg/ml against cowpox virus. The dose–response curve for this compound is presented in Fig. 2. 3-Methyl-*cyclo*Sal-O-acetyl-PCVMP (entry 2, Table 3) and the unsubstituted compound 5-H-*cyclo*Sal-PCVMP (entry 3, Table 3) were as ineffective as their unphosphorylated parent compound PCV (entry 4, Table 3).

For the most effective *cyclo*Sal-MP derivatives of BVDU, IC_{50} values of 10–14 μg/ml were estimated (Table 4). This was the case for 3-methyl-*cyclo*Sal-3′-BVDUMP (entry 1, Table 4) and 6-chloro-7-*n*-butyl-*cyclo*Sal-3′-BVDUMP (entry 3, Table 4) against vaccinia virus and for 3-*t*-butyl-*cyclo*Sal-3′-BVDUMP (entry 2, Table 4) against both vaccinia virus and cowpox virus. For other compounds, such as 3-methyl-*cyclo*Sal-3′-O-propionyl-BVDUMP, 6-chloro-*cyclo*Sal-3′-BVDUMP and 6-chloro-7-ECM-*cyclo*Sal-3′-BVDUMP, a moderate antiviral activity with IC_{50} values between 18 and 28 μg/ml could be demonstrated. However, most of these test substances had a relatively marked cytotoxicity. Thus, the selectivity indices were relatively low, except for 3-methyl-*cyclo*Sal-3′-BVDUMP (entry 1, Table 4) and 6-chloro-7-*n*-butyl-*cyclo*Sal-3′-BVDUMP (entry 3, Table 4). Taking all results into account, selected *cyclo*Sal-BVDUMP derivatives showed a promising antiviral activity against vaccinia virus and cowpox virus. However, most of the agents tested in this study were ineffective at therapeutically relevant concentrations or had little selectivity and were, therefore, not listed in Table 4. Most results were comparable with that of the unphosphorylated BVDU (entry 4, Table 4). Vaccinia virus was significantly more sensitive than cowpox virus to 3-methyl-*cyclo*Sal-3′-BVDUMP (entry 1, Table 4). By contrast, 3-methyl-*cyclo*Sal-3′-O-pivaloyl-BVDUMP revealed a higher activity against cowpox virus (IC_{50} 31.2 ± 1.6 μg/ml) than vaccinia virus (IC_{50} 53.8 ± 2.4 μg/ml).

Among the selected synthetic humic acid-like polymers tested in this study, a low antiviral activity against pox viruses was detectable for KOP, the oxidation product of caffeic acid. IC_{50} values varied between 62 μg/ml for the cowpox virus strain Marina and 83 μg/ml for the vaccinia virus strain Elstree. No therapeutically relevant antiviral activity was detectable for the remaining polyanionic compounds including the reference substance dextran sulfate. The oxidation product of the caffeic acid plus hydrocaffeic acid (1:1) had a significantly better antiviral activity against vac-

Table 3
Antiviral activity of *cyclo*Sal-monophosphates (*cyclo*Sal-MP) from penciclovir (PCV) against vaccinia virus (VV) strain Elstree and cowpox virus (CPV) strain Marina

No.	Compound	CC_{50} (μg/ml)	VV, IC_{50} (μg/ml)[a]	VV, IC_{90} (μg/ml)[a]	CPV, IC_{50} (μg/ml)[a]	CPV, IC_{90} (μg/ml)[a]	SI, VV/CPV
1	3-Methyl-*cyclo*Sal-PCVMP	>128	24.0 ± 5.4	57.6 ± 9.5	26.9 ± 3.9	62.8 ± 6.1	>5.3/4.8
2	3-Methyl-*cyclo*Sal-O-acetyl-PCVMP	187	>128	>128	>128	>128	–
3	5-H-*cyclo*Sal-PCVMP	>128	>64.0 (*n* = 2)	>64.0 (*n* = 2)	>64.0	>64.0	–
4	PCV	>512	>256	>256	>256	>256	–

CC_{50}, 50% cytotoxic concentration; IC_{50}/IC_{90}, 50%/90% inhibitory concentration; SI, selectivity index.
[a] Data are the mean ± standard deviation for three independent experiments.

Table 4
Antiviral activity of *cyclo*Sal-monophosphates (*cyclo*Sal-MP) from brivudin (BVDU) against vaccinia virus (VV) strain Elstree and cowpox virus (CPV) strain Marina

No.	Compound	CC_{50} (μg/ml)	VV, IC_{50} (μg/ml)[a]	VV, IC_{90} (μg/ml)[a]	CPV, IC_{50} (μg/ml)[a]	CPV, IC_{90} (μg/ml)[a]	SI, VV/CPV
1	3-Methyl-*cyclo*Sal-3′-OH-BVDUMP	>128	10.8 ± 2.0	31.8 ± 6.5	48.2 ± 0.8 (*n* = 2)	121.3 ± 3.9 (*n* = 2)	>11.9/>2.7
2	3-*t*-Butyl-*cyclo*Sal-3′-OH-BVDUMP	36.2	10.3 ± 2.8	21.0 ± 6.7	13.5 ± 2.1	41.2 ± 5.9	3.5/2.7
3	6-Chloro-7-*n*-butyl-*cyclo*Sal-3′-OH-BVDUMP	143	14.1 ± 2.2	28.0 ± 5.4	>32.0 (*n* = 1)	>32.0 (*n* = 1)	10.1/–
4	BVDU	>512	>128	>128	>128	>128	–

CC_{50}, 50% cytotoxic concentration; IC_{50}/IC_{90}, 50%/90% inhibitory concentration; SI, selectivity index.
[a] Data are the mean ± standard deviation for three independent experiments.

cinia virus (IC_{50} 219 ± 59.7 μg/ml) than cowpox virus (IC_{50} 384 ± 9.3 μg/ml).

None of the test compounds had an in vitro antiviral activity against orthopoxviruses that was comparable to that of the reference substances cidofovir and 5-iodo-dUrd. IC_{50} values of cidofovir against vaccinia virus and cowpox virus were found to be 4–5 μg/ml and the selectivity indices were >111 (entry 5, Table 2). For 5-iodo-dUrd, IC_{50} values of 0.3–0.4 μg/ml were found and selectivity indices were >2500 (entry 6, Table 2).

4. Discussion

The highly selective antiviral drugs ACV, PCV and brivudin possess a well-known antiviral activity against members of the subfamily of viruses called *Alpha-herpesvirinae* (Wutzler, 1997). Their mode of action is based on the presence of viral thymidine kinase which phosphorylates them to the monophosphate and brivudin to the mono- and diphosphate. The mono- and diphosphates are subsequently converted by cellular kinases to the triphosphate forms which inhibit viral DNA polymerase and terminate the growing DNA chain, respectively. Although a highly conserved thymidine kinase gene was identified in the genome of several orthopoxviruses (Schnitzlein and Tripathy, 1991; Hansen et al., 1999), vaccinia virus has been reported to be unsensitive to ACV in normal cells (Darby et al., 1980). However, Darby et al. (1980) could show that vaccinia virus is ACV-sensitive in HSV-specific thymidine kinase-transformed cells in which the thymidine kinase is able to phosphorylate the drug. These results demonstrate the possibility that orthopoxviruses may be sensitive to phosphorylated acyclic nucleoside analogous compounds.

The need of phosphorylation of acyclic nucleoside analogues in virus-infected cells can be avoided by delivery of corresponding nucleotides from neutral, membrane-permeable prodrugs. Such a pro-nucleotide approach was designed by Meier et al. (1999), based on the *cyclo*Saligenyl-nucleotide concept. The basic idea is to achieve nucleotide delivery into cells, bypassing limitations with intracellular formation of nucleotides from their nucleoside precursors. The *cyclo*Saligenyl-nucleotide concept is one of several pro-nucleotide systems reported so far, but it is the only approach in which a pro-nucleotide is cleaved successfully by a simple but selective chemical hydrolysis (Meier et al., 2004). Using the *cyclo*Saligenyl-nucleotide concept, several series of pro-nucleotide derivatives of the acyclic nucleoside analogues ACV, PCV and the cyclic nucleoside analogue BVDU were synthesized (Meier et al., 1998, 2002). Remarkable is the retention of activity of *cyclo*Sal-ACVMP in cells which were infected with thymidine kinase-negative HSV-1 (Meerbach et al., 2000). In addition, various *cyclo*Sal-phosphotriesters derivatives of ACV and BVDU exhibit a pronounced activity against EBV (Meier et al., 2002).

The present study was carried out to evaluate *cyclo*Sal-MP derivatives of ACV, PCV and BVDU against orthopoxviruses, in particular vaccinia virus and cowpox virus. As demonstrated, *cyclo*Sal-phosphotriesters of ACV turned out to be potent inhibitors of vaccinia virus and cowpox virus replication. Most promising are the in vitro data of 3-methyl-*cyclo*Sal-ACVMP which exhibited IC_{50} values of 6–10 μg/ml. The two other *cyclo*Sal-phosphotriesters of ACV, 5-methyl-*cyclo*Sal-ACVMP and 5-H-*cyclo*Sal-ACVMP, also had a good antiviral activity. These findings confirm the assumption of Darby et al. (1980), who postulated a wider antiviral activity for the phosphorylated drug ACV. In addition, our results provide further evidence for the so-called "thymidine kinase bypass", i.e., the intracellular delivery of the monophosphate from the pro-nucleotide (Meier et al., 1998).

Among the PCV-containing *cyclo*Sal-phosphotriesters, only 3-methyl-*cyclo*Sal-PCVMP had a moderate activity against vaccinia virus and cowpox virus. In keeping with these results, *cyclo*Sal-PCVMP derivatives were also found to be less active against HSV-1 in recent studies (Meerbach et al., 2000). As a reason for this failure, the formation of cyclic PCV monophosphate instead of PCV monophosphate during the hydrolysis of the corresponding *cyclo*Sal-triester was suggested (Meier et al., 1998).

Selected *cyclo*Sal-phosphotriesters of BVDU also proved to be active against pox viruses. Compounds with the highest inhibitory effect were 3-methyl-*cyclo*Sal-3′-BVDUMP, 6-chloro-7-butyl-*cyclo*Sal-3′-BVDUMP and 3-*t*-butyl-*cyclo*Sal-3′-BVDUMP. Other compounds, such as 3-methyl-*cyclo*Sal-3′-O-propionyl-BVDUMP, 6-chloro-*cyclo*Sal-3′-BVDUMP and 6-chloro-7-ECM-*cyclo*Sal-3′-BVDUMP, had a good or moderate antiviral activity. Although most of these compounds had a relatively high cytotoxicity, the data show that BVDU monophosphate delivery is sufficient to convert BVDU into an active anti-pox virus drug. However, it cannot be concluded from these data, whether BVDU monophosphate itself is responsible for the antiviral effect or whether further metabolism of BVDU monophosphate to its triphosphate is necessary.

No relevant antiviral activity against vaccinia virus or cowpox virus could be detected for the polyhydroxycarboxylates tested. When present during virus adsorption, several of these polymers exhibit potent activity against HSV, human cytomegalovirus (HCMV) and human immunodeficiency virus (HIV) (Helbig et al., 1997; Klöcking et al., 2002; Meerbach et al., 2002). The "broad spectrum antiviral activity against various enveloped RNA and DNA viruses" of these polymers (Lüscher-Mattli, 2000) apparently did not extend to orthopoxviruses.

Since vaccinia virus is mostly used in antiviral drug screening, variations in sensitivity compared with other members of the genus *orthopoxvirus* are of importance. Our data suggest a similar sensitivity of vaccinia virus and cowpox virus against antiviral drugs targeting the viral DNA polymerase. This observation has also been made by Baker et al.

(2003). Thus, any of the less virulent viruses could serve as surrogates for screening new members of this class of compounds. Of interest is a greater sensitivity of vaccinia virus to antiviral agents as found in two isolated cases of our study. Under consideration of different enzyme targets, drug sensitivity data generated for over 800 compounds have indicated that cowpox virus is the virus that has sensitivities most similar to those of variola virus (Baker et al., 2003). However, it has been concluded that neither cowpox nor monkeypox or vaccinia virus can qualify as surrogate for variola virus. Nevertheless, representative data for the genus *orthopoxvirus* can be expected by inclusion of vaccinia virus as well as cowpox virus in antiviral drug screening as shown in our study. The variola virus itself will be required for confirmatory testing.

In conclusion, pro-nucleotides based on *cyclo*Sal-MP derivatives of ACV, PCV and BVDU can act as potent and selective inhibitors of vaccinia virus and cowpox virus replication in contrast to their parent compounds. Further studies, including in vivo testing of selected compounds which are effective in vitro, would be warranted.

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References

Atlas, R.M., 2002. Bioterrorism: from threat to reality. Annu. Rev. Microbiol. 56, 167–185.

Baker, R.O., Bray, M., Huggins, J.W., 2003. Potential antiviral therapeutics for smallpox, monkeypox and other orthopoxvirus infections. Antiviral Res. 57, 13–23.

Bauer, D.J., 1955. The antiviral and synergistic actions of isathin thiosemicarbazone and certain phenoxypyrimidines in vaccinia infection in mice. Br. J. Exp. Pathol. 36, 105–114.

Bray, M., Martinez, M., Kefauver, D., West, M., Roy, C., 2002. Treatment of aerosolized cowpox virus infection in mice with aerosolized cidofovir. Antiviral Res. 54, 129–142.

Bray, M., Roy, C.J., 2004. Antiviral prophylaxis of smallpox. J. Antimicrob. Chemother. 54, 1–5.

Darby, G., Larder, B.A., Bastow, K.F., Field, H.J., 1980. Sensitivity of viruses to phosphorylated 9-(2-hydroxyethoxymethyl)guanine revealed in TK-transformed cells. J. Gen. Virol. 48, 451–454.

De Clercq, E., 2001a. Vaccinia virus inhibitors as a paradigm for the chemotherapy of poxvirus infections. Clin. Microbiol. Rev. 14, 382–397.

De Clercq, E., 2001b. Antiviral drugs: current state of the art. J. Clin. Virol. 22, 73–89.

De Clercq, E., 2002. Cidofovir in the treatment of poxvirus infections. Antiviral Res. 55, 1–13.

Domagk, G., Behnisch, R., Mietzch, F., Schmidt, H., 1946. Über eine neue, gegen Tuberkelbazillen in vitro wirksame Verbindungsklasse. Naturwissenschaften 10, 315.

Hansen, H., Sandvik, T., Tryland, M., Olsvik, O., Traavik, T., 1999. Comparison of thymidine kinase and A-type inclusion protein gene sequences from Norwegian and Swedish cowpox virus isolates. APMIS 107, 667–675.

Helbig, B., Klöcking, R., Wutzler, P., 1997. Anti-herpes simplex virus type 1 activity of humic acid-like polymers and their *o*-diphenolic starting compounds. Antiviral Chem. Chemother. 8, 265–273.

Henderson, D.A., Inglesby, T.V., Barlett, J.G., Ascher, M.S., Eitzen, E., Jahrling, P.B., Hauer, J., Layton, M., McDade, J., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T., Russell, P.K., Tonat, K., Working Group on Civilian Biodefense, 1999. Smallpox as a biological weapon: medical and public health management. JAMA 281, 2127–2137.

Huggins, J.W., Baker, R.O., Beadle, J.R., Hostetler, K.Y., 2002. Orally active ether lipid prodrugs of cidofovir for the treatment of smallpox. Antiviral Res. 53, A66.

Klöcking, R., Schacke, M., Wutzler, P., 1995. Primärscreening antiherpetischer Verbindungen mit EZ4U. Chemother. J. 4, 141–147.

Klöcking, R., Helbig, B., Schötz, G., Schacke, M., Wutzler, P., 2002. Anti-HSV-1 activity of synthetic humic acid-like polymers derived from *p*-diphenolic starting compounds. Antiviral Chem. Chemother. 13, 241–249.

Lüscher-Mattli, M., 2000. Polyanions—a lost chance in the fight against HIV and other virus diseases? Antiviral Chem. Chemother. 11, 249–259.

Meerbach, A., Klöcking, R., Meier, C., Lomp, A., Helbig, B., Wutzler, P., 2000. Inhibitory effect of *cyclo*Saligenyl-nucleoside monophosphates (*cyclo*Sal-NMP) of acyclic nucleoside analogues on HSV-1 and EBV. Antiviral Res. 45, 69–77.

Meerbach, A., Neyts, J., Balzarini, J., Helbig, B., De Clercq, E., Wutzler, P., 2002. In vitro activity of polyhydroxycarboxylates against herpesviruses and HIV. Antiviral Chem. Chemother. 12, 337–345.

Meier, C., 2004. *Cyclo*Sal-pronucleotides—design of the concept, chemistry and antiviral activity. In: De Clercq, E. (Ed.), Advances in Antiviral Drug Design. Elsevier, Amsterdam, pp. 147–213.

Meier, C., Habel, L., Haller-Meier, F., Lomp, A., Herderich, M., Klöcking, R., Meerbach, A., Wutzler, P., 1998. Chemistry and anti-HSV-1 evaluation of *cyclo*Sal-nucleotides of acyclic nucleoside analogues. Antiviral Chem. Chemother. 9, 389–402.

Meier, C., Knispel, T., De Clercq, E., Balzarini, J., 1999. *Cyclo*Sal-pronucleotides of 2′,3′-dideoxyadenosine and 2′,3′-dideoxy-2′,3′-didehydroadenosine: synthesis and antiviral evaluation of a highly efficient nucleotide delivery system. J. Med. Chem. 42, 1604–1614.

Meier, C., Lomp, A., Meerbach, A., Wutzler, P., 2001. Synthesis, hydrolysis and anti-EBV activity of a series of 3′-modified *cyclo*Sal-BVDUMP pronucleotides. Nucleosides Nucleotides Nucleic Acids 20, 307–314.

Meier, C., Lomp, A., Meerbach, A., Wutzler, P., 2002. *Cyclo*Sal-BVDUMP pronucleotides: how to convert an antiviral-inactive nucleoside analogue into a bioactive compound against EBV. J. Med. Chem. 45, 5157–5172.

Meier, C., Meerbach, A., Balzarini, J., 2004. *Cyclo*Sal-pronucleotides—development of first and second generation chemical Trojan horses for antiviral chemotherapy. Front. Biosci. 9, 873–890.

Meyer, H., Schay, C., Mahnel, H., Pfeffer, M., 1999. Characterization of orthopoxviruses isolated from man and animals in Germany. Arch. Virol. 144, 491–501.

Neyts, J., De Clercq, E., 1993. Efficacy of (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine for the treatment of lethal vaccinia virus infections in severe combined immune deficiency (SCID) mice. J. Med. Virol. 41, 242–246.

Neyts, J., De Clercq, E., 2002. Effect of 5-iodo-2′-deoxyuridine on vaccinia virus (orthopoxvirus) infections in mice. Antimicrob. Agents Chemother. 46, 2842–2847.

Safrin, S., Cherrington, J., Jaffe, H.S., 1997. Clinical uses of cidofovir. Rev. Med. Virol. 7, 145–156.

Schnitzlein, W.M., Tripathy, D.N., 1991. Identification and nucleotide sequence of the thymidine kinase gene of swinepox virus. Virology 181, 727–732.

Smee, D.F., Sidwell, R.W., Kefauver, D., Bray, M., Huggins, J.W., 2002. Inhibition of wild-type and cidofovir-resistant strains of camelpox,

cowpox, monkeypox, and vaccinia viruses by selected antiviral agents. Antiviral Res. 53, A66.

Walton, E., Jenkins, S.R., Nutt, R.F., Holly, F.W., Nemes, M., 1969. Branched-chain sugar nucleosides. V. Synthesis and antiviral properties of several branched-chain sugar nucleosides. J. Med. Chem. 12, 306–309.

World Health Organization, 1980. The global eradication of smallpox: final report of the global commission for the certification of smallpox eradication. World Health Organization, Geneva, Switzerland.

Wutzler, P., 1997. Antiviral therapy of herpes simplex and varicella-zoster virus infections. Intervirology 40, 343–356.