ELSEVIER

Antiviral Research 45 (2000) 69–77

Antiviral Research

www.elsevier.com/locate/antiviral

# Inhibitory effect of *cyclo*Saligenyl-nucleoside monophosphates (*cyclo*Sal-NMP) of acyclic nucleoside analogues on HSV-1 and EBV

Astrid Meerbach [a,*], Renate Klöcking [a], Chris Meier [b], Andreas Lomp [b], Björn Helbig [a], Peter Wutzler [a]

[a] *Institute for Antiviral Chemotherapy, Friedrich-Schiller-University Jena, Nordhäuser Str. 78, D-99089 Erfurt, Germany*
[b] *Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany*

Received 2 April 1999; accepted 20 November 1999

**Abstract**

The in vitro antiviral activity of a new series of *cyclo*Sal-pro-nucleotides derived from the acyclic nucleoside analogues aciclovir and penciclovir against herpes simplex virus type 1 (HSV-1), thymidine kinase deficient ($TK^-$) HSV-1, and Epstein–Barr virus (EBV) was evaluated. Using the XTT-based tetrazolium reduction assay EZ4U, the *cyclo*Sal derivatives were examined for their antiviral and cytotoxic effects in HSV-1 as well as HSV-1-$TK^-$-infected Vero cells. The anti-EBV activity was assessed by means of an EBV DNA hybridization assay using a digoxigenin-labeled probe specific for the Bam H1-W-fragment of the EBV genome and by measuring viral capsid antigen (VCA) expression in P3HR-1 cells by indirect immunofluorescence. Among the new *cyclo*Sal-phosphotriesters the three aciclovir monophosphates proved to be potent and selective inhibitors of HSV-1 replication, EBV DNA synthesis and EB-VCA expression. Of interest is the retention of activity of the aciclovir monophosphates in HSV-1-$TK^-$-infected cells. Particularly 3-methyl-*cyclo*Sal-aciclovir monophosphate retained the same effectiveness, as compared to the wild type virus strain. In contrast to the aciclovir pro-nucleotides the penciclovir *cyclo*Sal-phosphotriesters exhibited at best only a marginal antiviral effect on HSV and EBV replication. 

*Keywords:* Pro-nucleotides; Anti-HSV-1; Anti-EBV

## 1. Introduction

Diseases caused by herpesviruses play an important role in viral infections of humans. Especially the incidence of infections by herpes simplex virus (HSV) is high throughout the world. Clinical manifestations of diseases with HSV range from mucosal ulcerations with HSV-1 (herpes labialis)

* Corresponding author. Tel.: +49-361-7411314; fax: +49-361-7411114.
*E-mail address:* meerbach@zmkh.ef.uni-jena.de (A. Meerbach)

0166-3542/00/$ - see front matter 
PII: S0166-3542(99)00076-5

to ulcerating vesicular lesions with HSV-2 (herpes genitalis). More rarely, HSV infections can result in keratoconjunctivitis, eczema herpeticatum, herpes encephalitis, generalized disseminated diseases in neonates and immunosuppressed patients (Rawls, 1973).

Aciclovir has been the 'gold standard' for the therapy and suppression of HSV infections for more then 16 years (Wagstaff et al., 1994). In contrast to former antiviral drugs such as vidarabine (Pavan-Langston, 1977) or iododeoxyuridine (Francois, 1963), aciclovir was the prototype of the first generation of selective antiviral agents (Elion et al., 1977). It is selectively phosphorylated in virus-infected cells to its triphosphate, which inhibits viral DNA polymerase. Now, one has entered already the second generation of antiviral drugs for herpesvirus infections (for a review, see Alrabiah and Sacks, 1996). Members of this generation are penciclovir, famciclovir, valaciclovir, brivudin and cidofovir. They appear particularly promising for the treatment of herpesvirus infections for instance because of high antiviral potency (i.e. brivudin against varicella-zoster virus), prolonged intracellular half-life of the triphosphate (penciclovir), increased oral bioavailability, as compared to the parent compound (i.e. famciclovir, valaciclovir) or long-lasting and broad-spectrum antiviral activity (i.e. cidofovir) (De Clercq, 1995).

Another emerging area of concern are Epstein–Barr virus (EBV) infections and the role they play particularly in post-transplant lymphoproliferative disorders. EBV is the causative agent of infectious mononucleosis, and is also associated with the development of several human malignancies (for review, see Anagnostopoulos and Hummel, 1996). Tumours classically linked with EBV are Burkitt's lymphoma (Burkitt, 1958; Epstein et al., 1964) and nasopharyngeal carcinoma (zur Hausen et al., 1970). More recently, an association of EBV and B-cell lymphomas in immunosuppressed including AIDS patients (Shibata et al., 1993), certain rare T-cell lymphomas (Su et al., 1991; Meijer et al., 1996) and lymphoproliferative syndromes (Savage and Waxman, 1997; Cao et al., 1998), cases of Hodgkin's lymphomas (Niedobitek, 1996; Glaser et al., 1997) and gastric carcinomas (Leoncini et al., 1993; Osato and Imai, 1996) were reported. EBV has also been linked to other diseases such as chronic mononucleosis (Okano et al., 1991) as well as oral hairy leukoplakia in patients with AIDS (Brandwein et al., 1996). Despite of an observed regression of oral hairy leukoplakia in AIDS patients after aciclovir/desciclovir treatment (Greenspan et al., 1990) until now there has been no clear evidence for clinical benefit of aciclovir treatment in EBV-associated lymphoproliferative disorders (Pagano et al., 1983; Andersson et al., 1986; Van der Horst et al., 1991).

As mentioned above, intracellular conversion of the nucleoside analogues into their mono-, di- or triphosphate forms is necessary after cell penetration to act as inhibitors of viral enzymes or to lead to DNA chain termination. Due to altered or deficient enzymes necessary for phosphorylation to the monophosphate as well as structural differences with the natural nucleosides, this metabolization is often inefficient, and, thus, the therapeutic activity can be limited. One attempt to improve the therapeutic potential is the use of neutral lipophilic prodrugs of their monophosphates (Meier et al., 1997; Meier, 1998). This so-called *cyclo*Saligenyl-nucleotide concept was designed to release the nucleotides selectively by controlled, chemically induced hydrolysis involving a successive coupled cleavage of the phenyl- and benzylester of the *cyclo*Sal-phosphotriester (tandem reaction). Using this concept a new series of pro-nucleotide derivatives of the acyclic nucleoside analogues aciclovir and penciclovir was synthesized by Meier et al. (1998). The present study was aimed at evaluating the in vitro sensitivity of these novel derivatives against HSV and EBV.

## 2. Material and methods

### 2.1. Chemicals

The synthesis of new pro-nucleotides on the basis of *cyclo*Sal-phosphotriesters of the acyclic nucleoside analogue aciclovir (ACV) and penciclovir (PCV) was described in detail by Meier et

al. (1998). The following compounds were used in the present study (Fig. 1): 3-methyl-*cyclo*Sal-aciclovir monophosphate (3-methyl-*cyclo*Sal-ACVMP), 5-methyl-*cyclo*Sal-aciclovir monophosphate (5-methyl-*cyclo*Sal-ACVMP), the unsubstituted *cyclo*Sal-aciclovir monophosphate (5-H-*cyclo*Sal-ACVMP), 3-methyl-*cyclo*Sal-penciclovir monophosphate (3-methyl-*cyclo*Sal-PCVMP), 3-methyl-*cyclo*Sal-*O*-acetyl-penciclovir monophosphate (3-methyl-*cyclo*Sal-*O*-acetyl-PCVMP), and the unsubstituted *cyclo*Sal-penciclovir monophosphate (5-H-*cyclo*Sal-PCVMP). ACV, PCV, and Cidofovir (HPMPC) served as reference compounds.

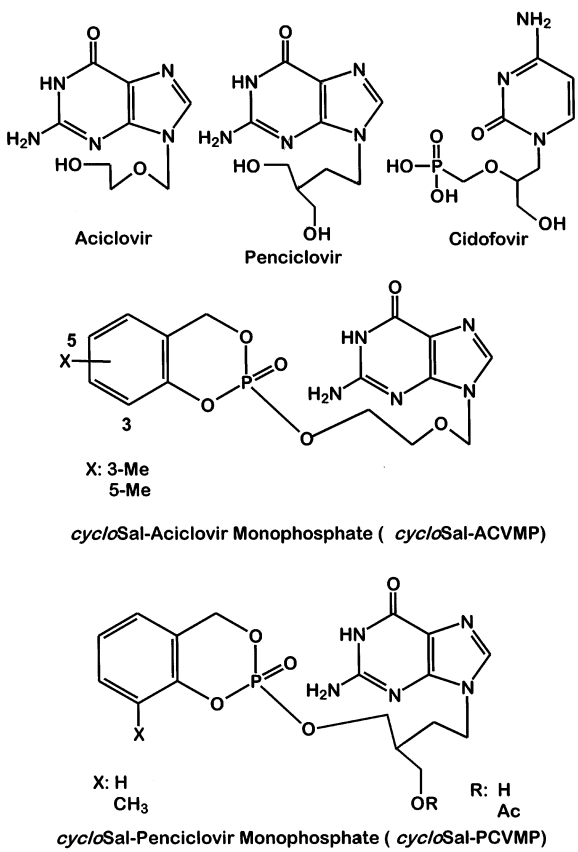


Fig. 1. Structure of test compounds.

## 2.2. Detection of anti-HSV-1 activity

### 2.2.1. Cells and virus strains

Vero cells were cultivated in MEM with L-glutamine supplemented with 10% heat-inactivated FBS and antibiotics. HSV-1 strain Kupka isolated by Benda in 1962 from a patient with herpes labialis (Benda et al., 1972) was propagated in these cells. The TK-negative strain HSV-1/TK$^-$ (reference number B2006) was obtained from E. De Clercq, Leuven, Belgium, and was originally described by Dubbs and Kit (1964). It was propagated in rabbit testis primary cells. All experiments concerning antiviral activity and cytotoxicity were carried out in Vero cells.

### 2.2.2. Antiviral screening assay

Antiviral screening was performed in 96-well flat-bottomed microtiter plates by means of an XTT-based tetrazolium reduction assay (EZ4U, Biomedica GmbH, Vienna, Austria). The method described by Klöcking et al. (1995) allows to determine both the inhibition of viral cytopathogenicity and substance-induced cytotoxic effects at the same time. Briefly, 120 h after infection of the cells with $10^5$ $TCID_{50}$/ml of HSV-1 and addition of test substances, the EZ4U test was carried out. Optical density (OD) was measured at 492 and 620 nm (reference wave length) in a microplate reader (Ceres 900C, Bio-Tek, Winooski VT). Percentages of antiviral and cytotoxic activities of the test compound were calculated from the measured $OD_{492}$-$OD_{620}$ values according to Pauwels et al. (1988). Each experiment was performed in triplicate. The mean values are representative for three independent experiments. Substance concentrations at half-maximum virus inhibition ($EC_{50}$) and at half-maximum cytotoxic effectiveness ($CC_{50}$), respectively, were calculated from dose-response curves by regression analysis.

## 2.3. Detection of anti-EBV activity

### 2.3.1. Cell cultures

The EBV producer cell line P3HR-1, the EBV genome carrying cell lines Raji and Namalwa as well as the EBV negative cell line Ramos were grown in RPMI 1640 medium supplemented with

10% heat-inactivated FBS, L-glutamine and antibiotics at 36.5°C in a humified 5% $CO_2$ containing atmosphere.

*2.3.2. Exposure of P3HR-1 cells to drugs*

Exponentially growing P3HR-1 cells were centrifuged, resuspended in fresh medium and seeded at a density of $10^6$ cells/ml in 25 $cm^2$ cell culture flasks. The tumour promotor 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma) was added at a concentration of 30 ng/ml to induce virus production (Lin et al., 1979). Cell cultures were incubated with compounds at different concentrations for 7 days.

*2.3.3. DNA isolation*

Control and drug treated cells were pelleted, washed with PBS twice and resuspended in 200 μl PBS. DNA was extracted using a DNA extraction kit (QIAamp Blood Kit, Qiagen). DNA concentration was determined by UV spectrometry.

*2.3.4. Slot blot hybridization*

Ten micrograms of total cellular DNA of drug treated P3HR-1 cells and control cells were used to determine the EBV DNA content. The slot blot hybridization assay was done as described previously (Meerbach et al., 1998) using 30 ng/ml of a digoxigenin-11-dUTP-labeled probe specific for the Bam H1-W-fragment of the EBV genome. After hybridization chemiluminescence detection was carried out followed by 2 h exposition to a Kodak film. The amount of EBV DNA was measured using a densitometer (MWG Biotech). Then, the EBV DNA concentration was compared between drug-treated and non-treated P3HR-1 cells and the 50% effective concentration ($EC_{50}$) for inhibition of EBV replication was calculated by regression analysis.

*2.3.5. Preparation of the probe*

The probe was labeled in a PCR reaction according to the method described by Emanuel (1991). The primer sequence 5′-3′: CCAGAGGTAAGTGGACTT and GACCGGTGCCTTCTTAGG delimit a 124 bp sequence within the internal repeat 1 (Bam W region). The PCR reaction mixture consisted of PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl at pH 8.3), 200 μM dNTP (PCR DIG Labeling Mix, Boehringer Mannheim), 1 μM of each primer, 2.5 units Amplitaq DNA polymerase, and 50 ng (per 50 μl reaction mixture) of EBV-Bam HI-W-plasmid DNA. After an initial denaturation step for 5 min at 94°C, 30 cycles were carried out each including 1 min at 95°C for denaturation, 1 min at 60°C for annealing, and 1.5 min at 72°C for chain elongation, followed by an additional 7 min at 72°C.

*2.3.6. Determination of $CC_{50}$ for cell growth*

P3HR-1 cells were grown for 7 days in the presence of the test compounds at different concentrations from an initial density of $2 \times 10^5$ cells/ml in 96-well plates. Cell numbers were determined using a Neubauer counting chamber and 50% inhibitory concentrations for cell growth ($CC_{50}$) were calculated.

*2.3.7. Inhibition of EBV antigen expression*

For detection of virus capsid antigen (VCA) exponentially growing P3HR-1 cells were seeded at a density of $10^6$ cells/ml with addition of TPA and treated with different concentrations of the test compounds for 7 days. Cells were pelleted and washed with PBS. Drops of cell suspension were placed on slides, air dried and fixed in acetone at −20°C for 1 h. VCA was detected by indirect immunofluorescence using human sera with high titres to VCA ($\geq$ 1:2500) and a monoclonal antibody against VCA-gp125 (Chemicon International, Temecula, CA), respectively. The VCA-positive cells were counted under a fluorescence microscope and $EC_{50}$ values were calculated.

## 3. Results

### *3.1. Anti-HSV-1 activity*

The most active ACVMP-triester against HSV-1 Kupka was 3-methyl-*cyclo*Sal-ACVMP. The $EC_{50}$ value of 0.20 μg/ml equals to that of ACV. 5-methyl-*cyclo*Sal-ACVMP and the unsubstituted (5-H) compound were active as well ($EC_{50}$: 0.38

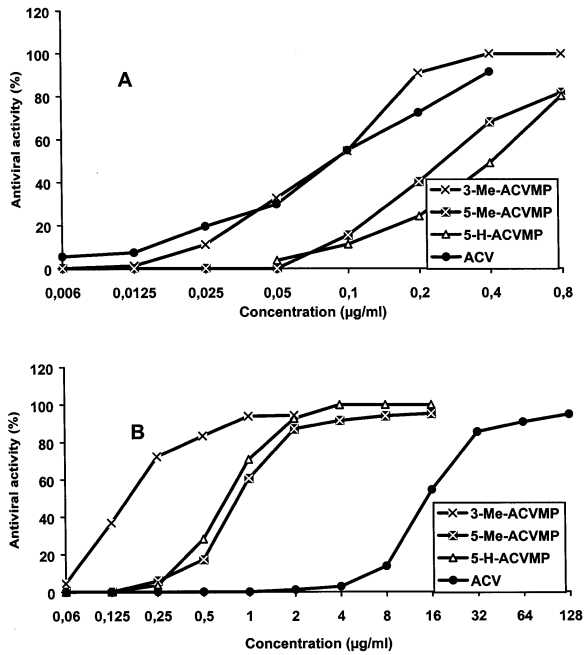


Fig. 2. Dose-response curves of *cyclo*Saligenyl nucleoside monophosphates (*cyclo*Sal-NMP) of acyclic nucleoside analogues against HSV-1 Kupka (A) and HSV-1 TK$^-$ B2006 (B) in Vero cells.

and 0.41 μg/ml, respectively), but they did not achieve the effectiveness of the 3-methyl compound (Fig. 2A). In contrast to aciclovir, the three *cyclo*Sal-ACVMP derivatives showed remarkable antiviral activity also against the HSV-1 TK$^-$ strain B2006. The 3-methyl-*cyclo*Sal-ACVMP derivative proved to be 62-fold more active than ACV. The unsubstituted 5-H-*cyclo*Sal-ACVMP and 5-methyl-*cyclo*Sal-ACVMP surpassed the $EC_{50}$ value of ACV 20- and 19-fold, respectively (Fig. 2B).

The newly synthesized *cyclo*Sal-compounds derived from penciclovir showed only weak antiviral activity. In comparison to penciclovir they were about 32–95-fold less active against HSV-1 Kupka. Only marginal antiviral effects were observed in the anti-HSV-1 TK$^-$ assay.

### 3.2. Anti-EBV activity

Table 1 shows the 50% effective concentrations ($EC_{50}$) for inhibition of EBV DNA synthesis, the $EC_{50}$ for inhibition of VCA expression, the 50% cytostatic concentration ($CC_{50}$), and the ratios of $EC_{50}$ for inhibition of viral DNA synthesis to the 50% cytostatic concentration (SI, selectivity indices). As reference compounds for the anti-EBV activity in vitro, aciclovir, penciclovir, and cidofovir were included (Colby et al., 1980; Boyd et al., 1987; Lin et al., 1991; Bacon and Boyd, 1995). In the present study, selectivity indices of 58, $>31$, and 129, respectively, were obtained for these substances. Among the six tested *cyclo*Sal derivatives the three *cyclo*Sal-ACVMP-phosphotriesters proved to be selective inhibitors of the EBV replicative cycle (Fig. 3 and the EBV structural protein production). They yielded $EC_{50}$ values for inhibition of EBV DNA replication of 2.38 (3-methyl-*cyclo*Sal-ACVMP), 1.85 (5-methyl-*cyclo*Sal-ACVMP), and 4.07 μg/ml (5-H-*cyclo*Sal-ACVMP), and selectivity indices of $>42$, $>54$, and 12, respectively. Of the *cyclo*Sal substances of penciclovir only 3-methyl-*cyclo*Sal-PCVMP and 5-H-*cyclo*Sal-PCVMP showed a marginal anti-EBV activity ($EC_{50} = 21.7$ and 25.2 μg/ml, respectively). 3-methyl-*cyclo*Sal-*O*-acetyl-PCVMP had no influence on EBV replication.

In parallel with the EBV DNA inhibition assay, the indirect immunofluorescence antibody technique was performed to determine the inhibitory effect of the test compounds on the expression of the structural protein VCA. As can be concluded from Table 1, all compounds which reduced EBV DNA synthesis were also effective in reducing VCA expression.

## 4. Discussion

The mode of action of antiviral drugs with significant anti-HSV-activity such as aciclovir, valaciclovir, penciclovir and famciclovir (Wutzler, 1997) bases on the presence of viral thymidine kinase for phosphorylation of the drug into the monophosphate. The monophosphate is subsequently converted by cellular kinases to the di- and triphosphate forms, which inhibit viral DNA polymerase. Problems arise in thymidine kinase deficient strains which occur rarely in immunocompetent patients (Kost et al., 1993; Nyquist et

al., 1994). However, in immunosuppressed patients, aciclovir-resistant HSV strains are observed up to 5–6%. (Englund et al., 1990; Chatis and Crumpacker, 1992). Reyes et al. (1998) reported a prevalence of ACV-resistant isolates of 5.6% among HIV-positive patients. Phosphorylation to the monophosphate can be avoided by delivery of the corresponding nucleotide from neutral, membrane-permeable prodrugs. Such a pro-nucleotide approach was designed and synthesized by Meier et al. (1997), based on the *cyclo*Saligenyl-nucleotide concept. Using this concept, a new series of pro-nucleotide derivatives of the acyclic nucleoside analogues aciclovir and penciclovir were synthesized (Meier et al., 1998).

The present study was carried out for in vitro evaluation of this new group of potentially antiherpetic prodrugs against TK-positive HSV-1, TK-deficient HSV-1, and EBV. The results demonstrate that the test compounds on the basis of *cyclo*Sal-phosphotriesters of aciclovir are potent inhibitors of HSV-1 replication. The most active compound against HSV-1 Kupka was 3-methyl-*cyclo*Sal-ACVMP ($EC_{50}$: 0.20 μg/ml) which reached nearly the same $EC_{50}$ value as aciclovir. The two other ACVMP-phosphotriesters still showed a good anti-HSV-1 effectiveness. Most promising are the results of inhibition the $TK^-$-HSV-1 strain: here, in contrast to ACV, the *cyclo*Sal-ACVMP-phosphotriesters demonstrated a remarkable antiviral effect. The most active compound 3-methyl-*cyclo*Sal-ACVMP elicited a 62-fold higher activity compared to ACV, whereas the unsubstituted 5-H-*cyclo*Sal-ACVMP and the 5-methyl-*cyclo*Sal-ACVMP reached a 20- and 19-fold higher activity than ACV. The complete retention of antiviral activity against $TK^-$-strains provides evidence for the intracellular delivery of the monophosphate from the pro-nucleotide [so-called 'thymidine kinase bypass' (Meier, 1998)].

The PCV-containing *cyclo*Sal-phosphotriester were markedly less active against HSV-1 and $TK^-$-HSV-1. A reason for the failure of PCV derivatives could be due to the formation of cyclic PCVMP instead of PCVMP during the hydrolysis of the corresponding *cyclo*Sal-triester (Meier et al., 1998).

Some nucleoside analogues like aciclovir and penciclovir have proved to be active against EBV in vitro (Colby et al., 1980; Bacon and Boyd, 1995). In clinical trials aciclovir has been used in patients with infectious mononucleosis. Thus, aciclovir infusions were shown to reduce oropharyn-

Table 1
Anti-EBV activity of *cyclo*Sal-phosphotriesters of ACV and PCV

| Compound | $EC_{50}$[b] (μg/ml)[a] | | $CC_{50}$[c] (μg/ml)[a] | SI[d] |
|---|---|---|---|---|
| | DNA-synthesis | VCA-expression | P3HR-1 cells | |
| *cyclo*Sal-ACVMPs | | | | |
| 3-Methyl-*cyclo*Sal -ACVMP | $2.38 \pm 1.19$ | $9.17 \pm 2.52$ | $>100$ | $>42$ |
| 5-Methyl-*cyclo*Sal-ACVMP | $1.85 \pm 0.17$ | $10.0 \pm 2.50$ | $>100$ | $>54$ |
| 5-H-*cyclo*Sal-ACVMP | $4.07 \pm 0.80$ | $9.10 \pm 4.80$ | $48.0 \pm 8.69$ | 12 |
| *cyclo*Sal-PCVMPs | | | | |
| 3-Methyl-*cyclo*Sal-PCVMP | $21.7 \pm 1.13$ | $35.0 \pm 7.55$ | $67.5 \pm 17.7$ | 3 |
| 3-Methyl-*cyclo*Sal-*O*-acetyl-PCVMP | $>50$ | $>50$ | $>100$ | — |
| 5-H-*cyclo*Sal-PCVMP | $25.2 \pm 10.7$ | $33.7 \pm 6.81$ | $87.5 \pm 10.6$ | 4 |
| Reference compounds | | | | |
| ACV | $1.52 \pm 0.59$ | $5.42 \pm 2.35$ | $88.3 \pm 5.77$ | 58 |
| PCV | $3.20 \pm 1.99$ | $6.00 \pm 1.80$ | $>100$ | $>31$ |
| HPMPC | $0.22 \pm 0.12$ | $1.25 \pm 0.35$ | $28.3 \pm 3.51$ | 129 |

[a] Data are the mean ± SD of three independent experiments.
[b] $EC_{50}$, concentration required to reduce EBV DNA synthesis or VCA expression by 50%.
[c] $CC_{50}$, concentration required to reduce the growth of exponentially growing P3HR-1 cells by 50%.
[d] SI, selectivity index = $CC_{50}/EC_{50}$ (DNA synthesis).

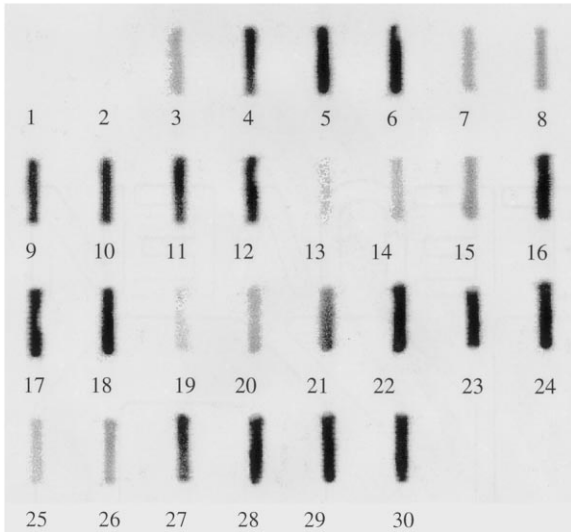


Fig. 3. Inhibition of EBV DNA synthesis in P3HR-1 cells by different *cyclo*Sal-phosphotriesters of aciclovir using a slot blot hybridization assay followed by chemiluminescence detection. Ramos cells, Namalwa cells and Raji cells served as controls for the sensitivity of the method used. The differences in the intensity of the blot signals correspond with the antiviral efficiency.

geal virus secretion over the treatment time, but there was no clear evidence of symptomatic benefit (Pagano et al., 1983; Andersson et al., 1986). Only marginal effects on the reduction of EBV replication in the oropharynx but without clinical benefit were seen with oral aciclovir (Van der Horst et al., 1991). Compared to HSV, aciclovir is less effective against EBV in vitro, but it is likely that even high doses used in EBV-related disorders are too low to influence the disease. In vitro assays have demonstrated that ganciclovir is more potent than aciclovir in inhibiting EBV and may be more effective in the treatment of this infection (Lin et al., 1984). However, in a clinical observation Kuo et al. (1995) reported a failure of currently used ganciclovir and high doses aciclovir as prophylactic measure against primary EBV infection, EBV reactivation and subsequent development of posttransplant lymphoproliferative disorders. In addition, a disadvantage of ganciclovir and cidofovir is the relative high toxicity in vitro (Meerbach et al., 1998).

Our data on the anti-EBV activity of the new series on compounds derived from aciclovir demonstrated a similar inhibitory effect of EBV replication and VCA expression as for the parent compound ACV. But there might be a benefit for these pro-nucleotides because of their monophosphate form in bypassing viral enzyme pathways in EBV-infected cells. In parallel with the anti-HSV results, there was no antiviral activity against EBV for the penciclovir *cyclo*Sal derivatives.

In conclusion, these results demonstrate that pro-nucleotides based on *cyclo*Sal derivatives of aciclovir are potent anti-EBV- and anti-HSV-1 agents and retain their antiviral activity also against TK-deficient HSV-1 strains.

## References

Alrabiah, F.A., Sacks, S.L., 1996. New antiherpesvirus agents. Their targets and therapeutic potential. Drugs 52, 17–32.

Anagnostopoulos, I., Hummel, M., 1996. Epstein–Barr virus in tumours. Histo-Pathol. 29, 297–315.

Andersson, J., Britton, S., Ernberg, I., Andersson, U., Henle, W., Sköldenberg, B., Tisell, A., 1986. Effect of acyclovir on infectious mononucleosis: a double-blind, placebo-controlled study. J. Infect. Dis. 153, 283–290.

Bacon, T.H., Boyd, M., 1995. Activity of penciclovir against Epstein–Barr virus. Antimicrob. Agents Chemother. 39, 1599–1602.

Benda, R., Petrovic, S., Rychterova, V., 1972. Transmission of herpesvirus along the trigeminal nerve in rabbits. Acta Virol. 16, 459–465.

Boyd, M.R., Bacon, T.H., Sutton, D., Cole, M., 1987. Antiherpesvirus activity of 9-(4-hydroxy-3-hydroxy-methylbut-1-yl)-guanine (BRL39123) in cell culture. Antimicrob. Agents Chemother. 31, 1238–1242.

Brandwein, M., Nuovo, G., Ramer, M., Orlowski, W., Miller, L., 1996. Epstein–Barr virus reactivation in hairy leukoplakia. Mod. Pathol. 9, 298–303.

Burkitt, D.B., 1958. Sarcoma involving jaws in African children. Br. J. Surg. 46, 218–223.

Cao, S., Cox, K., Esquivel, C.O., Berquist, W., Concepcion, W., Ojogho, O., Monge, H., Krams, S., Martinez, O., So, S., 1998. Posttransplant lymphoproliferative disorders and gastrointestinal manifestations of Epstein–Barr virus infection in children following liver transplantation. Transplantation 66, 851–856.

Chatis, P.A., Crumpacker, C.S., 1992. Resistance of herpesvirus to antiviral drugs. Antimicrob. Agents Chemother. 36, 1589–1595.

Colby, B.M., Shaw, J.E., Elion, G.B., Pagano, J.S., 1980. Effect of acyclovir [9-(2-hydroxyethoxymethyl)-guanine] on

Epstein–Barr virus DNA replication. J. Virol. 34, 560–568.

De Clercq, E., 1995. Trends in the development of new antiviral agents for the chemotherapy of infections caused by herpesviruses and retroviruses. Rev. Med. Virol. 5, 149–164.

Dubbs, D.R., Kit, S., 1964. Mutant strains of herpes simplex deficient in thymidine kinase-inducing activity. Virology 22, 493–502.

Elion, G.B., Furman, P.A., Fyfe, J.A., de Miranda, P., Beauchamp, L., Schaeffer, H.J., 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. USA 74, 5716–5720.

Emanuel, J.R., 1991. Simple and efficient system for synthesis of non-radioactive nucleic acid hybridization probes using PCR. Nucleic Acids Res. 19, 2790.

Englund, J.A., Zimmermann, M.E., Swierkosz, E.M., Goodman, J.L., Scholl, D.R., Balfour, H.H., 1990. Herpes simplex virus resistant to acyclovir. A study in a tertiary care center. Ann. Int. Med. 112, 416–422.

Epstein, M.A., Achong, B.G., Barr, Y.M., 1964. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet I, 702–703.

Francois, P., 1963. I.D.U. in ophthalmology. Annee. Ther. Clin. Ophthalmol. 14, 261–270.

Glaser, S.L., Lin, R.J., Stewart, S.L., Ambinder, R.F., Jarrett, R.F., Brousset, P., Pallesen, G., Gulley, M.L., Khan, G., O'Grady, J., Hummel, M., Preciado, M.V., Knecht, H., Chan, J.K.C., Claviez, A., 1997. Epstein–Barr virus-associated Hodgkin's disease: epidemiologic characteristics in international data. Int. J. Cancer 70, 375–382.

Greenspan, D., De Souza, Y.G., Conant, M.A., Hollander, H., Chapman, S.K., Lennette, E.T., Petersen, V., Greenspan, J.S., 1990. Efficacy of desciclovir in the treatment of Epstein–Barr virus infection in oral hairy leukoplakia. J. Aquired Immune Deficiency Syndromes 3, 571–578.

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

Kost, R.G., Hill, E.L., Tigges, M., Straus, S.E., 1993. Brief report: recurrent acyclovir-resistant genital herpes in an immunocompetent host. New Engl. J. Med. 329, 1777–1782.

Kuo, P.C., Dafoe, D.C., Alfrey, E.J., Sibley, R.K., Scandling, J.D., 1995. Posttransplant lymphoproliferative disorders and Epstein–Barr virus prophylaxis. Transplantation 59, 135–138.

Leoncini, L., Vindigni, C., Megha, T., Funto, I., Pacenti, L., Musaro, M., Renieri, A., Seri, M., Anagnostopoulos, J., Tosi, P., 1993. Epstein–Barr virus and gastric cancer: data and unanswered questions. Int. J. Cancer 53, 898–901.

Lin, J.-C., Shaw, J.E., Smith, M.C., Pagano, J.S., 1979. Effect of 12-*O*-Tetra-decanoylphorbol-13-acetate on the replication of Epstein–Barr Virus. I. Characterization of viral DNA. Virology 99, 183–187.

Lin, J.-C., Smith, M.C., Pagano, J.S., 1984. Prolonged inhibitory effect of 9-(1,3-dihydroxy-2-propoxymethyl)guanine against replication of Epstein–Barr virus. J. Virol. 50, 50–55.

Lin, J.-C., De Clercq, E., Pagano, J.S., 1991. Inhibitory effects of acyclic nucleoside phosphonate analogs, including (*S*)-1-(3-hydroxy-2-phosphonyl-methoxypropyl)-cytosine, on Epstein–Barr virus replication. Antimicrob. Agents Chemother. 35, 2440–2443.

Meerbach, A., Holy, A., Wutzler, P., De Clercq, E., Neyts, J., 1998. Inhibitory effects of novel nucleoside and nucleotide analogues on Epstein–Barr virus replication. Antiviral Chem. Chemother. 9, 275–282.

Meier, C., Knispel, T., Lorey, M., Balzarini, J., 1997. *Cyclo*Sal-pro-nucleotides: the design and biological valuation of a new class of lipophilic nucleotide prodrugs. Int. Antiviral News 5, 183–185.

Meier, C., 1998. Pro-nucleotides-recent advances in the design of efficient tools for the delivery of biologically active nucleoside monophosphates. Synlett, 233–242.

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 (*cyclo*Sal-NMP) of acyclic nucleoside analogues. Antiviral Chem. Chemother. 9, 389–402.

Meijer, C.J.L.M., Jiwa, N.M., Dukers, D.F., Oudejans, J.J., De Bruin, P.C., Walboomers, J.M.M., Van den Brule, A.J.C., 1996. Epstein–Barr virus and human T-cell lymphomas. Sem. Cancer Biol. 7, 191–196.

Niedobitek, G., 1996. The role of Epstein–Barr virus in the pathogenesis of Hodgkin's disease. Ann. Oncol. Suppl. 7 4, S11–S17.

Nyquist, A.C., Rotbart, H.A., Cotton, M., Robinson, C., Weinberg, A., Hayward, A.R., et al., 1994. Acyclovir-resistant neonatal herpes simplex virus infection of the larynx. J. Ped. 124, 967–971.

Okano, M., Matsumoto, S., Osato, T., Sakiyama, Y., Thiele, G.M., Purtilo, D.T., 1991. Severe chronic active Epstein–Barr virus infection syndrome. Clin. Microbiol. Rev. 4, 129–135.

Osato, T., Imai, S., 1996. Epstein–Barr virus and gastric carcinoma. Sem. Cancer Biol. 7, 175–182.

Pagano, J.S., Sixbey, J.W., Lin, J.-C., 1983. Acyclovir and Epstein–Barr virus infection. J. Antimicrob. Chemother. 12, Suppl. B, 113–121.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J., De Clercq, E., 1988. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods 20, 309–321.

Pavan-Langston, D., 1977. Use of vidarabine in ophthalmology: a review. Ann. Ophthalmol. 9, 835–839.

Rawls, W.E., 1973. Herpes simplex virus. In: Kaplan, A.S. (Ed.), The herpesviruses. Academic Press, New York, 291–325.

Reyes, M., Graber, J.M., Weatherall, N., Hodges-Savola, C., Reeves, W.C. and the Task Force on herpesvirus resis-

tance, 1998. Acyclovir-resistant herpes simplex virus: Preliminary results from a national surveillance system. Antiviral Res. 37, A44/16.

Savage, P., Waxman, J., 1997. Post-transplantation lymphoproliferative disease. Quart. J. Med. 90, 497–503.

Shibata, D., Weiss, L.M., Hernandez, A.M., Nathwani, B.N., Bernstein, L., Levine, A.M., 1993. Epstein–Barr virus-associated non-Hodgkin's lymphoma in patients infected with the human immunodeficiency virus. Blood 81, 2102–2109.

Su, B.I.-J., Hsieh, H.-C., Lin, K.-H., Uen, W.-C., Kao, C.-L., Chen, C.-J., Cheng, A.-L., Kadin, M.E., Chen, J.-Y., 1991. Aggressive peripheral T-cell lymphomas containing Epstein–Barr viral DNA: a clinicopathologic and molecular analysis. Blood 77, 799–808.

Van der Horst, C., Joncas, J., Ahronheim, G., Gustafson, N., Stein, G., Gurwith, M., Fleisher, G., Sullivan, J., Sixbey, J., Poland, S., Fryer, J., Champney, K., Schooley, R., Sumaya, C., Pagano, J.S., 1991. Lack of effect of peroral acyclovir for the treatment of acute infectious mononucleosis. J. Infect. Dis. 164, 788–792.

Wagstaff, A., Faulds, D., Goa, K.L., 1994. Aciclovir: a reappraisal of ist antiviral activity, pharmacokinetic properties and therapeutic efficacy. Drugs 47, 153–205.

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

zur Hausen, H., Schulte-Holthausen, H., Klein, G., Henle, W., Henle, G., Clifford, P., Santesson, L., 1970. EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature 228, 1056–1058.