Evaluation of Novel Acyclic Nucleoside Phosphonates against Human and Animal γ-Herpesviruses

Revealed an Altered Metabolism of Cyclic Prodrugs upon EBV Reactivation in P3HR-1 Cells

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ABSTRACT

Acyclic nucleoside phosphonates (ANPs), such as HP MPC, are an important group of broad-spectrum antiviral agents with activity against DNA viruses. In this report, we present the in vitro potency of novel ANPs against γ-herpesviruses, including Kaposi’s sarcoma-associated herpesvirus, Epstein-Barr virus (EBV), and three animal γ-herpesviruses. HPMP-5-azaC, 3-deaza-HPMPA and their cyclic derivatives emerged as highly potent anti-γ-herpesvirus agents. Interestingly, cyclic prodrugs of ANPs exhibited reduced activities against EBV P3HR-1 strain, but not against EBV Akata strain. Cell culture metabolism studies with HPMPC and cyclic HPMPC revealed that these differences were attributed to an altered drug metabolism in P3HR-1 cells after EBV reactivation and more specifically to a reduced hydrolysis of cyclic HPMPC by cyclic CMP phosphodiesterase. We did not correlate this effect to phosphodiesterase downregulation, nor to functional mutations. Instead, altered cAMP levels in P3HR-1 cells indicated a competitive inhibition of the phosphodiesterase by this cyclic nucleotide. Finally, both HPMPC and HPMP-5-azaC emerged as highly effective inhibitors in vivo through significant inhibition of murine γ-herpesvirus replication and dissemination. In the current need of potent anti-γ-herpesvirus antivirals, our findings underlined the requirement of appropriate surrogate viruses for antiviral susceptibility testing and highlighted HPMP-5-azaC as a promising compound for future clinical development.

INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) are lymphotropic viruses characterized by their ability to induce various tumors and lymphoproliferative diseases, particularly in immunocompromised patients (1,2). The lack of a permissive replication system and an appropriate in vivo animal model system has hampered the study of both KSHV and EBV (3,4). Experiments involving the lytic cycle require reactivation of KSHV and EBV in latently-infected B-cells using phorbol esters or IgG (5-7). Alternatively, the use of closely related animal γ-herpesviruses, such as murine γ-herpesvirus 68 (MHV-68), herpesvirus saimiri (HVS) and rhesus rhadinovirus (RRV) can overcome these difficulties, and are commonly used as surrogate viruses to study EBV and KSHV pathogenesis. These viruses are capable of replicating to high titers and form plaques in different cell types (8-10). Moreover, infection of laboratory
mice with MHV-68 has been generally used as a small animal model offering relevant aspects to KSHV and 
EBV (9,11). Major features of $\gamma$-herpesvirus pathogenesis are similar in humans and mice, including the 
initial acute respiratory infection and the establishment of viral latency in B-cells (12).

The antiviral drug class of acyclic nucleoside phosphonates (ANPs) encompasses HPMPC 
(cidofovir), formally licensed for the treatment of CMV retinitis in AIDS patients, as well as PMEA 
(adenosine) and PMPA (tenofovir) which are both active against hepatitis B virus (HBV) and human 
immunodeficiency virus (HIV) infections. These nucleotide analogs contain a phosphonate group that is 
linked to the acyclic nucleoside moiety through a stable P-C bond, which cannot be cleaved off by cellular 
esterases (13). ANPs need to be phosphorylated by cellular kinases to their diphosphate forms to become 
biologically active and their selectivity is based on inhibition of reverse transcriptase and/or viral DNA 
polymerase (14).

New derivatives of ANPs containing a 3-hydroxy-2-(phosphonomethoxy)propyl (HPMP) group or a 
2-phosphonomethoxyethyl (PME) group as acyclic nucleoside moiety have been developed. These analogs 
are derivatives of diaminopurine (i.e., HPMPDAP and PMEDAP), or diaminopyrimidine (i.e., HPMPO-
DAPy, PMEDAPy), in which the base moiety is linked via an ether linkage to the aliphatic phosphonate 
side chain through an oxygen atom at the C-6 position of the pyrimidine ring. A new generation of ANPs 
encompasses HPMP derivatives with a 5-azacytosine moiety, with HPMP-5-azaC as the leading compound.
In addition, cyclic prodrugs of ANPs have been developed, such as cyclic HPMPC, which was reported to 
exhibit similar antiviral activity compared to HPMPC, but reduced nephrotoxicity (15). These novel ANPs 
have been shown to exert anti-herpesvirus activity and yield great potential for the treatment of various 
DNA-virus and retrovirus infections (13,16,17). However, their potency against $\gamma$-herpesviruses was not 
investigated so far.

Although no antiviral drugs are currently licensed for KSHV or EBV infections, several anti-herpetic 
agents have been shown to inhibit these viruses in vitro, particularly those that target the viral DNA 
polymerase, such as acyclovir, ganciclovir, foscarinet and HPMPC (18-22). Besides the inhibition of virus 
lytic replication, HPMPC elicit antitumor activity by induction of apoptosis, and this effect was also 
demonstrated against nasopharyngeal carcinoma (associated with EBV infection) xenografts in nude mice
(23,24). On the other hand, maribavir (MBV), a benzimidazole riboside, has potent antiviral activity against EBV and is in the late-stage of clinical development for HCMV diseases. The mechanism of action remains partially unclear, but it was recently shown to involve the inhibition of the viral protein kinase BGLF4, and thus independent of the viral DNA polymerase (25). The risk of developing virus-associated diseases is higher in immunocompromised patients with high EBV and KSHV loads, and therefore reducing viral loads by a combination of therapy with antivirals may have positive effects on the onset and disease progression (26,27). The use of antivirals for the treatment of infectious mononucleosis (IM) in immunocompetent patients is still debatable because the symptoms of IM are subtle in onset and the disease has a long incubation period (4-6 weeks) resulting in a late diagnosis (28). However, in patients with severe EBV-induced infectious mononucleosis, antiviral therapy may be considered as an adjunct to corticosteroid treatment (29). New and promising therapeutic approaches to EBV and KSHV-associated malignancies are under investigation and consist on the induction of virus replication followed by administration of viral DNA polymerase inhibitors, as well as targeting viral latency (30,31).

In this study, we evaluated the in vitro activity and selectivity of various ANPs, including cyclic HPMP analogs, against γ-herpesvirus replication. Interestingly, this study revealed notable differences in the anti-EBV activities between the non-cyclic and cyclic forms of ANPs in P3HR-1 cells, but not in Akata cells. Drug metabolism studies with HPMPC and cyclic HPMPC were performed in these cell lines, and the involvement of cyclic AMP and the cellular 2′-3′-cyclic nucleotide 3′-phosphodiesterase (CNP, EC.3.1.4.37) in the altered drug metabolism in induced P3HR-1 cells was investigated. Finally, the antiviral efficacy of a potent ANP, HPMP-5-azaC, was examined in a mouse model for γ-herpesvirus infection.

MATERIALS AND METHODS

Cells and viruses

KSHV-infected BCBL-1 cells (NIH AIDS Research & Reference Reagent Program) and JSC-1 cells (ATCC CRL-2769), and EBV-infected P3HR-1 cells (ATCC HTB-62) and Akata 2000 cells (kindly provided by P. J. Farrell, Imperial College Faculty of Medicine, St. Mary’s Campus, London, UK) were cultured in RPMI-1640 media (Life technologies Europe BV, Gent, Belgium). Murine fibroblasts (NIH/3T3 cells; ATCC
CRL-1685), owl monkey kidney cells (OMK; ATCC CRL-1556), and rhesus monkey fibroblasts (RF; kindly provided by S. Wong, Oregon Health and Science University, Beaverton, USA) were grown in Dulbecco’s modified eagle’s medium (DMEM). All media were supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 1% HEPES. Cultures were incubated at 37°C and 5% CO₂. The following viral strains were used: MHV-68 (clone G2.4; provided by A. A. Nash, Edinburgh, UK), HVS (strain C-488; ATCC VR-1414) and RRV (strain 17577; kindly provided by S. Wong, Oregon Health and Science University, Beaverton, USA) were grown respectively in NIH/3T3, OMK, and RF cells.

Compounds

The sources of the compounds were as follows: (S)-1-[(3-hydroxy-2-(phosphonomethoxy)propyl)]cytosine (HPMPC), cyclic HPMPC, and 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA), Gilead Sciences, Foster City, CA. The following ANPs (and their cyclic analogs) were synthesized at the Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic: HDP-HPMPC [hexadecyloxypropyl-HPMPC; HPMP-5-azaC [1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine]; HPMPA [(S)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine]; 3-deaza-HPMPA [(S)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-3-deazaadenine]; 7-deaza-HPMPA [(S)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-7-deazaadenine]; HPMPDAP [(S)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine]; HPMPO-DAPy [(R)-{2,4-diamino-3-hydroxy-6-[2-(phosphonomethoxy)propoxy]}pyrimidine]; PMEDAP [9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine]; PMEO-DAPy [2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]pyrimidine]. The compound structures were previously published (32).

[5-³H]-HPMPC (MT-833, specific activity 26.0 Ci/mmol) and [5-³H]-cyclic HPMPC (MT-1713, specific activity 23 Ci/mmol) were custom-synthesized by Moravek Biochemicals (Brea, California, USA).

KSHV and EBV antiviral assays
Cells were seeded in 48-well plates at a density of $3 \times 10^5$ cells/ml (BCBL-1) or $10^6$ cells/ml (JSC-1, Akata and P3HR-1). Virus replication was induced by addition of 20 ng/ml 12-O-tetradecanoylphorbol 13-acetate (TPA, Sigma) to the cells or 0.1% of rabbit anti-human IgG (A0423, Dako) to Akata cells. After 24h induction, cells were washed and resuspended in fresh medium in the presence of varying concentrations of antiviral drugs. At day 5 post-induction, total DNA was extracted (QIAamp DNA kit, Qiagen, Benelux B.V, Venlo, Netherlands) and viral DNA was quantified by real-time quantitative PCR using ABI Prism 7500 Sequence Detection System (Life technologies). The sequence of the PCR primers for the detection of the target genes of KSHV (ORF73) and EBV (BNRF1) has been described elsewhere (18). The 50% and 90% effective concentrations (EC$_{50}$ and EC$_{90}$) were calculated by regression analysis. These EC$_{50}$ and EC$_{90}$ values are the concentrations required to reduce KSHV and EBV DNA synthesis in TPA-induced cells by 50% and 90%, respectively.

Cytopathic effect (CPE) reduction assays

NIH/3T3, OMK and RF cells were grown in 96-well plates and infected with MHV-68, HVS and RRV, respectively, at a multiplicity of infection of approximately 0.02 PFU/cell. Following 2h of virus adsorption at 37°C, serial dilutions of test compounds were added in duplicate. Viral infection and dilutions of the drugs were performed in DMEM containing 2% FCS. At day 5 (MHV-68 and HVS) or 8 (RRV) post infection (p.i.), CPE was evaluated microscopically and EC$_{50}$ values were determined as the compound concentrations required to reduce the virus-induced CPE by 50%.

Cytotoxicity assays

The cytotoxicity of the antiviral compounds was determined based on the inhibition of cell growth. In 48-well plates, uninduced BCBL-1 and P3HR-1 cells were grown in culture medium in the presence or absence of serial dilutions of the test compounds. After 3 days of incubation, cells were counted with a Coulter counter (Analis, Namur, Belgium). In 96-well plates, NIH/3T3, OMK and RF cells were grown at a density of $3 \times 10^3$ cells/well. The next day, varying concentrations of the compounds were added. The cells were counted after 3 days of incubation. The cytostatic concentrations (CC$_{50}$) were defined as the concentration of
compound required to reduce cell growth by 50%. Selectivity indices were determined by calculation of the
CC_{50}/EC_{50} ratio.

Intracellular metabolism of HPMPC and cyclic HPMPC

P3HR-1 and Akata cells were seeded at a density of 1.5 x 10^6 cells/ml and grown in the presence or absence
of 20 ng/ml TPA or 0.1% IgG. The next day, 10 μCi [5-^{3}H]-HPMPC or [5-^{3}H]-cyclic HPMPC was added to
the medium. Unradiolabeled compound was added to obtain a final concentration of HPMPC or cyclic
HPMPC of 10 μM. After 24, 72 or 120h, cell extracts were obtained as previously described (33). Briefly,
cells were washed three times with ice-cold serum-free medium, centrifuged at 13,000 rpm for 10 minutes
and cell pellets were extracted with 300 μl ice-cold 67% methanol in water. Extracts were incubated 10
minutes on ice and centrifuged at 13,000 x rpm for 10 minutes. Supernatants and cell pellets were stored at -20°C until further use. For HPLC analysis, 200 μl of the supernatants was injected onto an anion-exchange
Partisphere SAX column (dimensions: 4.6 mm x 125 mm) from Whatman (Maidstone, UK). The buffer
composition and gradient system were the same as described (33). One minute fractions of the eluate were
collected, mixed with Hisafe 3 cocktail (Perkin Elmer, Waltham, MA) and analyzed for radioactivity in a
scintillation counter. The different metabolites were identified based on their retention time.

To determine incorporation of [5-^{3}H]-HPMPC and [5-^{3}H]-cyclic HPMPC into total cellular DNA,
the methanol-insoluble pellets were digested in 500 ml 5N sodium hydroxide during 24 h incubation at
37°C. After neutralization with 500 ml 5N hydrochloride, digested samples were mixed with Hisafe 3
cocktail and analyzed for total radioactivity.

Western blot

Blots were performed, using a 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP, EC 3.1.4.37)-specific
mouse monoclonal antibody [(1:500), ab6319, Abcam, Cambridge, MA] and goat anti-β-actin [(1:500),
sc1615, Santa Cruz, California, USA] as the primary antibodies and polyclonal goat anti-mouse HRP
[(1:10,000); P0447, Dako, Belgium] or polyclonal rabbit anti-goat horseradish peroxidase [(1:10,000);
P0449, Dako] as secondary antibodies. Films were scanned and quantification of the bands was obtained by applying ImageJ software. The area under the curve of CNP was normalized with that of β-actin.

**Sequencing**

After extracting total RNA from P3HR-1 and Akata cells with Trizol reagent (Life technologies) and RNeasy mini kit (Qiagen), cDNA was obtained using first strand cDNA synthesis kit (GE Healthcare). The entire CNP mRNA was amplified by PCR using FastStart high-fidelity DNA polymerase (Roche Applied Science, Mannheim, Germany) following the manufacturer’s instructions. PCR products were purified with QIAquick® purification kit (Qiagen), and the amplicons were sequenced using cycle sequencing kit BigDye® terminator kit version 3.1 on an ABI 3730 sequencing system (Applied Biosystems), and a set of primers spanning the entire coding region of the CNP gene. The sequencing results were computer assembled and compared with the sequence of the reference sequence (NM_033133.4) using the software SeqScape® version 2.7 (Applied Biosystems).

**Quantification of cyclic AMP (cAMP)**

P3HR-1, Akata, JSC-1 and BCBL-1 cells were induced with 20 ng/ml TPA or 0.1% IgG. After 24, 72 and 120h, the concentration of cAMP was quantified in the cell lysates using a cAMP (direct) enzyme immunoassay kit according to the manufacturer's instructions (Enzo life sciences, Antwerp, Belgium).

**Animal studies**

All animal work was approved by the KU Leuven Ethics Committee for Animal Care and Use (Permit number: P097-2010). All animal experiments were conducted in accordance with the Belgian and European guidelines for the protection of vertebrate animals used for experimental and other scientific purposes.

BALB/c mice (age: 4 weeks) were inoculated intranasally with 10,000 PFU MHV-68 under anesthesia using ketamine/xylazine in saline. HPMPC and HPMP-5-azaC were administered intraperitoneally (i.p.) at a dose of 25 mg/kg of body weight/day for 5 consecutive days (starting 2h p.i.). At day 6 and 12 p.i., five mice per group were sacrificed by injection of pentobarbital, and lungs, mediastinal
lymph nodes (MLNs) and spleen were harvested. Tissue sections were stored (i) at -20°C in PBS for DNA extraction, (ii) at -80°C in RNAlater (Ambion) for RNA extraction, and (iii) were fixed in 10% buffered formalin. Tissues were embedded in paraffin and 5 µm sections were stained with hematoxylin-eosin for histopathological examination.

Quantification of viral DNA load and relative ORF73 and gB expression in the tissues were performed as previously described (34). Briefly, tissues were homogenized and DNA was extracted using QIAamp DNA Mini kit (Qiagen) and was quantified by qPCR. RNA was extracted by a combination of Trizol® reagent (Invitrogen) and RNeasy mini kit (Qiagen). One-step reverse transcription (RT) qPCR was performed using TaqMan RNA-to-CT 1-Step Kit (Applied Biosystems) for relative quantitation of RNA.

**RESULTS**

*In vitro antiviral activity of ANPs against γ-herpesviruses*

The activity and selectivity of ANPs against KSHV, EBV, MHV-68, HVS and RRV are summarized in Table 1 and Table 2. Overall, HPMP derivatives were potent inhibitors of KSHV replication in BCBL-1 cells. With the exception of cyclic HPMPO-DAPy, EC₅₀ values ranged from 0.3 to 5.1 µM. The highest anti-KSHV activity was found for 3-deaza-HPMPA. HPMP-5-azaC displayed the highest selectivity with a SI >714 and an EC₉₀ value of 9.6 µM. In contrast, PMEA, PMEDAP and PMEO-DAPy demonstrated relatively weak antiviral activity and no selectivity against KSHV in BCBL-1 cells.

When compared to HPMPC (EC₅₀ of 1.9 µM), HDP-HPMPC and 3-deaza-HPMPA were more active against EBV P3HR-1 strain, with EC₅₀ values of 0.3 µM and 0.7 µM, respectively. HPMPC, HPMP-5-azaC, cyclic HPMP-5-azaC, HPMPA and 7-deaza-HPMPA depicted similar anti-EBV activity with EC₅₀s ranging from 1.9 to 5.3 µM. Weak or no inhibition of EBV replication was observed for HPMPDAP, HPMPO-DAPy and cyclic HPMP derivatives, with the exception of cyclic HPMP-5-azaC. PMEA, PMEDAP and PMEO-DAPy showed moderate EC₅₀ values in the range of 5 to 13 µM.

Regarding the inhibition of MHV-68 replication, HPMPC, HPMP-5-azaC, HPMPA and their cyclic forms exhibited EC₅₀ values in the range of 1.4 to 4.6 µM and SIs between 10 and 272. The other drugs
(except for cyclic 7-deaza HPMPA, cyclic HPMPDAP and cyclic HPMPO-DAPy) were even more inhibitory against MHV-68 replication than HPMPC and HPMPA, with EC$_{50}$ values < 1 µM.

HVS was selectively inhibited by HPMPC (SI of >143), its 5-azaC derivative (SI of 348), HPMPA (SI of 99) and its 3-deaza derivative (SI of 119), as well as by their respective cyclic forms. Poor activity and selectivity were obtained for the other ANP derivatives.

The replication of RRV was markedly inhibited by HPMPC, HPMP-5-azaC, 3-deaza and 7-deaza HPMPA, as well as their cyclic derivatives, with EC$_{50}$ values ranging from 0.2 to 1.8 µM. Moderate activity but high selectivity were found for HPMPDAP, HPMPO-DAPy and their cyclic forms. Poor activity and no selectivity were determined for PMEA, PMEDAP and PMEO-DAPy against this virus.

Among the ANPs tested against the five γ-herpesviruses, the highest selectivity indices were found for HPMPC, HPMP-5-azaC, 3-deaza-HPMPA and their cyclic derivatives. Compared to BCBL-1, P3HR-1, OMK and RF cells, NIH/3T3 appeared to be more susceptible to the cytostatic effects of ANPs, and therefore, relatively lower SIs were calculated against MHV-68 in NIH/3T3 cells.

Reduced activities of cyclic HPMP derivatives against EBV in P3HR-1 cells

Activity of cyclic and non-cyclic forms of ANPs in different EBV and KSHV positive B-cell lines. Unlike what was seen with KSHV, the prodrugs of HPMPC, HPMPA, 3-deaza-HPMPA, 7-deaza-HPMPA, HPMPDAP and HPMPO-DAPy, were 10- to 50-fold less active against EBV compared to their non-cyclic forms, e.g. the EC$_{50}$ values of HPMPC and cyclic HPMPC were 2 and 41 µM in P3HR-1 cells, respectively. Among the cyclic HPMP derivatives, cyclic HPMP-5-azaC was found to be an exception, showing a similar EC$_{50}$ value compared to HPMP-5-azaC against the EBV P3HR-1 strain (1 and 3 µM, respectively).

To determine whether these differences in antiviral activity reflected a particular feature of EBV and/or the P3HR-1 cell line, we evaluated the inhibitory activity of several ANPs and their cyclic forms in the EBV positive Akata cell line, as well as in the KSHV positive JSC-1 cell line (Figure 1). No discrepancies in antiviral activities between non-cyclic versus cyclic forms of ANPs were noticed in Akata cells, e.g. the EC$_{50}$ values determined for HPMPC and cyclic HPMPC were 5.4 and 8.7 µM, respectively. In addition, we found similar activities for the cyclic and non-cyclic compounds against KSHV in JSC-1 cells. Thus, the
reduced antiviral activity of cyclic HPMP derivatives was only observed against the EBV P3HR-1 strain. We hypothesized that an impaired conversion of the cyclic forms into their parent counterparts, e.g. cyclic HPMPC into HPMPC, was responsible for the reduced activity of the cyclic prodrugs against EBV P3HR-1 strain.

Metabolism of HPMPC and cyclic HPMPC in P3HR-1 and Akata cells. After cellular uptake, cyclic HPMPC is efficiently converted into its parent form HPMPC by the phosphodiesterase CNP (35). Further metabolic conversion of HPMPC leads to the production of three metabolites: HPMPC monophosphate (HPMPCp), HPMPC diphosphate (HPMPCpp) and the choline adduct (HPMPCp-choline) that is the intracellular reservoir form of HPMPC (Figure 2A). We studied the metabolism of HPMPC and cyclic HPMPC in the two EBV-infected cell lines. A kinetic study was performed to determine the concentrations of drug metabolites after 24, 72 and 120h incubation with 10 µM of [5-3H]-HPMPC or [5-3H]-cyclic HPMPC in P3HR-1 and Akata cells that were induced to the EBV lytic cycle by either TPA or IgG (Figure 2C). A representative chromatogram of HPMPC and cyclic HPMPC metabolism at 120h is shown in Figure 2B. Under the assay conditions used, cyclic HPMPC coeluted with HPMPCp-choline (retention time: 1 to 3 min). As a consequence, the radioactivity in the first 3 min of the chromatograms obtained from cells incubated with cyclic HPMPC contains a mixture of cyclic HPMPC and HPMPCp-choline.

The metabolic profiles of HPMPC versus cyclic HPMPC showed interesting differences in induced P3HR-1 cells, as well as between P3HR-1 and Akata cells (Figure 2C). The total amount of radioactivity of all metabolites after incubation with HPMPC was markedly increased in P3HR-1 cells compared to Akata cells (7 to 18 pmol/10⁶ cells versus 1 to 3 pmol/10⁶ cells). Also, after addition of HPMPC or cyclic HPMPC to Akata cells, similar levels of total radioactivity were seen at 24, 72 and 120h. In contrast, following incubation of P3HR-1 cells with cyclic HPMPC, the total radioactivity was 2- to 4-fold reduced compared to the cells incubated with HPMPC (e.g. concentrations at 72h after addition of cyclic HPMPC or HPMPC were 4 pmol/10⁶ cells or 18 pmol/10⁶ cells, respectively).

We observed another striking dissimilarity in the metabolism of cyclic HPMPC in induced P3HR-1 at the three designated time points. As shown in Figure 2C, the concentrations of metabolites that were
formed in these cells were low when cyclic HPMPC was added, as compared to addition of HPMPC. After 72 and 120h of incubation with cyclic HPMPC, the relative proportion of drug metabolites did not increase. Unlike P3HR-1 cells, Akata cells were found to produce relatively similar concentrations of drug metabolites when cells were incubated with HPMPC versus incubation with cyclic HPMPC. Taken together, these findings pointed towards an impaired metabolism of cyclic HPMPC in P3HR-1 cells, in particular the conversion of cyclic HPMPC into HPMPC.

A similar experiment was conducted in latently-infected P3HR-1 and Akata cells, which were incubated with [5-3H] HPMPC or [5-3H] cyclic HPMPC during 72h, since the altered drug metabolism was the most pronounced at this time point in activated cells. Both latently EBV-infected cell lines had comparable amounts of total radioactivity after incubation with either HPMPC or cyclic HPMPC, i.e. 3 to 4 pmol/10⁶ cells (Figure 2D). Although, the metabolite concentrations in P3HR-1 cells were slightly lower after addition of cyclic HPMPC as compared to HPMPC, we did not consider that the metabolism of cyclic HPMPC was altered such as in induced P3HR-1 cells. The drug metabolites produced in Akata cells incubated with both compounds accounted for similar relative concentrations. The amount of the active metabolite HPMPCpp was shown to be slightly lower in both cell lines when incubated with cyclic HPMPC as compared to HPMPC.

We further examined the incorporation of radioactivity into total cellular DNA after incubating the cells with cyclic HPMPC or HPMPC. As shown in Figure 2E, the incorporated radioactivity after 72h incubation of P3HR-1 cells with cyclic HPMPC was 4-fold lower compared to the conditions receiving HPMPC. In contrast, in Akata cells, DNA incorporation levels were comparable after incubation with cyclic HPMPC or HPMPC. Both latently-infected cell lines showed somewhat reduced incorporation of HPMPC into DNA after addition of cyclic HPMPC, but no differences in drug incorporation were observed in P3HR-1 versus Akata cells. Hence, the altered drug metabolism was exclusively observed in induced P3HR-1 cells.

**Characterization of CNP expression in EBV- and KSHV-infected cells.** The cellular CMP phosphodiesterase CNP has been reported to convert cyclic HPMPC into HPMPC (35). To examine whether reduced expression of CNP may be responsible for the altered cyclic HPMPC metabolism in induced P3HR-1 cells,
we performed Western blot analysis to determine the protein levels of this enzyme in different B-cell lines, including P3HR-1 and Akata, as well as two KSHV positive cell lines, BCBL-1 and JSC-1. Relative quantification of the band intensities showed no significant differences in the amount of CNP between the different cell lines, neither in latently-infected cells, nor in cells induced to the virus lytic cycle (Figure 3). Even though there was a trend towards an elevated CNP protein level in JSC-1 cells, this difference was not significant.

Sequence analysis of CNP cDNA derived from different B-cell lines. We performed genotypic analysis on the cDNA for CNP obtained from P3HR-1, Akata, BCBL-1 and JSC-1 cells to identify any mutations that could potentially affect the enzymatic activity. In one allele of the coding sequence of CNP derived from P3HR-1 cells, a missense mutation was identified at nucleotide position 620, resulting in a Q207R amino acid change. This mutation was mapped to the CNPase domain of the protein, but was previously reported as a single nucleotide polymorphism (reference SNP: rs34353668). Therefore, this amino acid change is not expected to affect the enzymatic activity.

cAMP levels in latently-infected cells and in cells induced to the virus lytic cycle. Mendel et al. previously demonstrated that cyclic HPMPC is an efficient substrate for CNP and that it competes with the natural substrates cAMP and cCMP (35). Therefore, we hypothesized that enhanced conversion of cAMP might compete with cyclic HPMPC for hydrolysis by CNP in P3HR-1 cells, but not in Akata cells. Therefore, we performed an ELISA assay to quantify the intracellular cAMP levels in latently-infected cells and in induced P3HR-1, Akata, BCBL-1 and JSC-1 cells at 24, 72 and 120h (Figure 4). The levels of cAMP were comparable in latently-infected Akata cells, BCBL-1 cells and JSC-1 cells and similar levels were also found after induction of the virus lytic cycle, with mean levels ranging from 5 to 20 pmoles/10^6 cells. In contrast, cAMP levels in latently-infected P3HR-1 cells were as high as 70 pmoles/10^6 cells, but after EBV reactivation, the cAMP levels decreased to approximately 10 pmoles/10^6 cells.

The reduction of intracellular cAMP concentration, as observed after reactivation of EBV in P3HR-1 cells, may be the result of increased cAMP efflux from the cells and/or its increased hydrolysis. We
quantified the extracellular cAMP levels in P3HR-1 cells, yet observed no differences in cAMP levels between the latent and lytic state (approximately 10 pmol/10^6 cells/ml) (Figure 4). Thus, the decrease in the intracellular cAMP level after EBV reactivation in P3HR-1 cells appears to result from enhanced degradation of cAMP to AMP by phosphodiesterases. Supernatants of BCBL-1, JSC-1 and latently-infected Akata cells showed comparable cAMP levels, ranging from 1 to 4 pmol/10^6 cells/ml. The high cAMP levels obtained in IgG-induced Akata cells were due to interference of the IgG with the ELISA assay, as reported by the manufacturer. To confirm this, we included an IgG control sample consisting of medium and IgG, showing similar cAMP levels as those found in the supernatants of Akata cells in the lytic state.

**Efficacy of HPMPC and HPMP-5-azaC treatment in MHV-68-infected mice**

In this study, we showed that HPMP-5-azaC possessed potent *in vitro* activity and selectivity against all five γ-herpesviruses tested, and moreover, its cyclic derivative was highly active against EBV in P3HR-1 cells. Therefore, we evaluated the *in vivo* efficacy of HPMPC and HPMP-5-azaC in BALB/c mice intranasally infected with MHV-68. Drug efficacy was evaluated at two time points to determine the inhibition of acute MHV-68 replication in the lungs (at day 6 p.i.), as well as the prevention of establishment of viral latency in the spleen at an early time point (at 12 day p.i.). Both time points were selected based on previous kinetic experiments in which latent (ORF73) and lytic (gB) MHV-68 gene expression was determined in this mouse model (34). Infected mice were treated i.p. with 25 mg per kg per day of HPMPC or HPMP-5-azaC during 5 consecutive days. Animals were sacrificed at day 6 and day 12 p.i. and viral DNA loads were recorded in lungs, spleen and MLNs. A significant decrease in MHV-68 DNA copies was observed in the lungs of HPMPC- and HPMP-5-azaC-treated mice at day 6 p.i and day 12 p.i. (Figure 5A). At day 12 p.i., infected untreated animals showed high viral DNA copies in MLNs (ranging from 10^3 to 10^6 copies) and spleen (10^4 to 10^5 copies/mg). In contrast, no viral DNA was detected in these tissues in mice treated with HPMPC or HPMP-5-azaC.

We further evaluated the impact of antiviral treatment on the lytic and latent stages of MHV-68 infection at the end (day 6 p.i.) and after (day 12 p.i.) drug treatment, by determining the ORF73 (latent) and gB (lytic) gene expression levels in lungs and spleen tissues. Compared to the untreated infected control,
drug treatment resulted in a 300-fold (HPMPC) and 20-fold decrease (HPMP-5-azaC) in gB expression at
day 6 p.i. in lung tissue (Figure 5A). Both drugs reduced the levels of the ORF73 transcript by 300-fold at
this time point. No gB expression was found in the lungs of HPMPC-treated mice at 12 days p.i., whereas
two out of five HPMP-5-azaC-treated mice showed low gB expression levels. ORF73 expression was
reduced by approximately 10-fold in the lungs at day 12 p.i. of treated mice, compared to the untreated
controls. In the spleen tissue, the gB and ORF73 transcripts were not detected in drug-treated mice.

Histological examination revealed the presence of an inflammatory response in the lungs of infected
mice at day 6 p.i. that was characterized by an increased interstitial cellularity in perivascular and
peribronchial locations (Figure 5B). At day 12 p.i., the inflammation in the lungs was dominated by
mononuclear inflammatory cells. Following HPMPC and HPMP-5-azaC treatment, lungs of infected mice
showed few inflammatory cells at day 6 p.i. which were increased in number by day 12 p.i. Enlargement of
the MLNs and splenomegaly were observed in infected mice, but not in drug-treated mice. Large, cell-rich
and poorly delineated follicles, as well as tingible body macrophages (black arrows) were observed in the
spleen of infected mice, but were not seen in the spleen of HPMPC- and HPMP-5-azaC-treated mice.

DISCUSSION

In this report, we evaluated the effects of ANPs, including some novel derivatives, on the lytic state of γ-
herpesvirus replication. Based on the anti-proliferative activity of HPMPC, further investigations are
required to determine whether these compounds also have an effect on KSHV-infected tumors cells or EBV-
transformed cells (24). These studies are of interests since there is a need for new and potent compounds to
clear latently-infected cells to prevent re-occurrence of viral replication. The broad-spectrum antiviral
activity of ANPs depends on the nature of the acyclic side chain and the base moiety (14). We showed that
modification of the acyclic side chains from a PME group to a HPMP group resulted in an enhancement of
antiviral activity against γ-herpesviruses, with the exception of MHV-68. In contrast, substitution of an
adenine, as in HPMPA, to a cytosine, as in HPMPC, had no effect on anti-γ-herpesvirus activity. In addition,
the 5-azacytosine analog showed similar in vitro antiviral potency as the parent compound HPMPC.
Modification of the adenine base in HPMPA and PMEA resulted in different profiles of antiviral activity
against γ-herpesviruses. Substitution of the purine base to a diaminopurine, such as in HPMPDAP, caused a significant decrease in anti-EBV, anti-HVS and anti-RRV activity, but did not modulate the activity against KSHV or MHV-68. Opposed to HPMPDAP, PMEDAP showed slightly higher activity against γ-herpesviruses than PMEA. The diaminopyrimidine counterpart HPMPO-DAPy possessed a slightly decreased inhibitory activity against γ-herpesviruses (except for MHV-68), compared to HPMPDAP. The inhibitory effect of PMEO-DAPy on EBV, KSHV and MHV-68 replication was comparable to that of PMEDAP, however PMEO-DAPy had lower potency against HVS and RRV (Table 1 and Table 2). The divergent anti-γ-herpesvirus activity of particular ANPs, mostly adenine derivatives, could not be linked to the genetic relationship among the γ-herpesviruses tested. Hence, this study highlights the requirement of different animal γ-herpesviruses such as MHV-68, HVS and RRV for antiviral susceptibility testing, in addition to KSHV and EBV, in order to consider them as appropriate surrogate viruses in drug-related studies.

Cyclic prodrugs of ANPs, including cyclic HPMPC and cyclic HPMPA, have been described to possess similar activity as their non-cyclic forms when evaluated against α- and β-herpesviruses, i.e. herpes simplex virus type 1 (HSV-1), HSV-2, varicella zoster virus and human cytomegalovirus (36). Previously, Lin and colleagues investigated the inhibitory effects of HPMPA and cyclic HPMPA on the replication of EBV in P3HR-1 cells (21). They reported that cyclic HPMPA showed a 19-fold decrease in anti-EBV activity compared to HPMPA. Remarkably, in our study not only cyclic HPMPA, but all cyclic HPMP derivatives, with the exception of cyclic HPMP-5-azaC, showed consistently diminished inhibitory effect on EBV replication in P3HR-1 cells compared to their parent compounds (ranging from 10- to 50-fold increase in EC50 values). On the contrary, we did not observe differences in antiviral activities of these compounds against the EBV Akata strain. These antiviral data agree with our finding that the induced P3HR-1, but not the induced Akata cells, showed reduced HPMPC incorporation into cellular DNA when incubated with cyclic HPMPC compared to HPMPC. Of note, diminished anti-MHV-68 activities were also observed for the cyclic prodrugs of 7-deaza-HPMPA, HPMPDAP and HPMPO-DAPy in comparison with their parent drugs (Table 2) and these findings must be further investigated to explain differences in anti-MHV-68 activity.
Diminished incorporation of HPMPC in induced P3HR-1 cells receiving cyclic HPMPC was the direct consequence of an altered metabolism of cyclic HPMPC compared to HPMPC in this cell line. The activation of HPMPC and cyclic HPMPC is the net result of different processes that involve drug uptake, intracellular hydrolysis of cyclic HPMPC, intracellular phosphorylation of HPMPC and efflux of cyclic HPMPC and/or HPMPC from the cells (15). Interestingly, we observed that in induced P3HR-1 cells, cyclic HPMPC was less efficient (than HPMPC) in delivering HPMPC metabolites including HPMPCpp, the active form that leads to DNA incorporation. In induced Akata cells, HPMPC and cyclic HPMPC had comparable metabolism, and consequently similar levels of drug incorporation into cellular DNA. The different behavior between the P3HR-1 and Akata cells was only observed after virus induction, since the latently-infected cells showed a similar metabolic profile when exposed to the compounds. Thus, we demonstrated an enhanced ability of P3HR-1 cells to metabolize HPMPC after induction of the EBV lytic cycle, but a decreased ability to metabolize cyclic HPMPC, when compared to Akata cells.

The reduced concentrations of HPMPC and its phosphorylated metabolites in induced P3HR-1 cells incubated with cyclic HPMPC, suggested that these cells display impaired conversion of cyclic HPMPC into HPMPC. It was previously reported that this conversion is mediated by the intracellular cyclic CMP phosphodiesterase CNP (37). In the current study, we demonstrated that induction of EBV replication in P3HR-1 cells did not lead to absence or diminished expression of CNP. The enzyme was equally expressed in different B-cell lines and, in addition, its expression was independent from the virus that reactivated (EBV or KSHV), as well as from the inducing agent (TPA or IgG). The enzymatic activity of CNP was not considered to be affected by the Q207R substitution that we identified in the CNP gene of P3HR-1 cells, since this mutation was previously described as a genetic polymorphism. Hence, the reduced capacity for CNP-mediated hydrolysis of cyclic HPMPC appears not be an intrinsic feature of the P3HR-1 cells. Thus, other factors such as altered competition with natural CNP substrates (i.e. cAMP and cCMP) may be involved.

Infections with viruses such as HSV are known to alter the intracellular levels of cyclic nucleotides, such as decreasing cAMP levels (38). We observed that latently-infected P3HR-1 cells contain notably higher levels of cAMP compared to other γ-herpesvirus-infected B-cells. Previously, high cAMP levels were
associated with maintenance of viral latency in Burkitt’s lymphoma cells (39). The contribution of the cAMP/PKA signaling pathway in EBV latency was attributed to inhibition of the transactivating immediate early gene BZLF-1, as well as to regulation of the activity and transcription of the latency C promoter (Cp) (39-41). Cp drives the transcription of six EBNA genes and is active in the EBV type III latency program, such as in the EBV P3HR-1 strain, but inactive in the EBV type I latency program (expressing only EBNA1), such as in the EBV Akata strain (42). Thus, activation and stimulation of Cp is important to control the expression of the different latency programs in EBV-infected cells, and this promoter is regulated by viral and cellular factors, such as cAMP.

Because of the inhibitory effect of cAMP on EBV replication in P3HR-1 cells, cAMP levels might need to be reduced in order for the virus to replicate more efficiently. Indeed, rapid cAMP degradation was triggered upon EBV reactivation. Reduction of intracellular cAMP levels is accomplished by its export across the plasma membrane, as well as by phosphodiesterase-mediated degradation (43). Since we were not able to correlate the decrease in intracellular cAMP to an increase of cAMP efflux in P3HR-1 cells, degradation by phosphodiesterases is most likely responsible for the rapid elimination of cAMP in these cells. Also, we do not exclude an indirect effect of TPA treatment on cAMP levels, since phorbol esters have been shown to influence cAMP phosphodiesterase activity in certain cell types through activation of protein kinase C (44).

Taken together, these data strongly indicate that the increased conversion of cAMP in induced P3HR-1 cells could competitively counteract cyclic HPMPC hydrolysis by CNP (Figure 6). Mendel et al. showed that cAMP, cCMP and cyclic HPMPC compete with each other for the active site of this enzyme and that the catalytic efficiency is 10- to 20-fold higher for cAMP and cCMP than for cyclic HPMPC (37). Additionally, we may assume that the cyclic forms of other ANPs, being cAMP and cCMP analogs, are also hydrolyzed by cAMP and/or cCMP converting phosphodiesterases besides CNP. Moreover, P3HR-1 cells are derived from the Jijoye cell line and, in contrast to Jijoye cells, P3HR-1 cells carry a deletion in the EBV genome. We can speculate that the presence of this mutation might also influence the metabolism of cyclic prodrugs. While it was not investigated here, additional studies with Jijoye cells might help to clear this point.
Cyclic HPMP-5-azaC was the only cyclic HPMP derivative for which the antiviral activity against EBV in P3HR-1 cells was comparable to that of the non-cyclic form. Interestingly, HPMP-5-azaC is known to possess a different pharmacologic profile than HPMPC since HPMP-5-azaC displays markedly higher phosphorylation to its active metabolite, and higher consecutive incorporation into DNA (33). Although diminished conversion of cyclic HPMP-5-azaC in P3HR-1 cells might also occur, this might be compensated by the efficient activation of HPMP-5-azaC leading to higher incorporation of HPMP-5-azaC into DNA, at levels comparable to those of cyclic HPMPC and other HPMP derivatives.

We also demonstrated that, in vivo, HPMP-5-azaC has comparable anti-MHV68 efficacy as HPMPC. The MHV-68 replication was greatly reduced in the lungs of infected mice treated i.p. with HPMPC or HPMP-5-azaC. Although drug treatment is only effective against the lytic cycle of the virus, ongoing productive replication is essential for maintaining high levels of latently-infected cells. In line with this, levels of the ORF73 transcript were markedly reduced in drug-treated mice. Until 12 days p.i., MHV-68 did not spread from the lungs to other organs, such as MLNs and spleen in the mice treated with HPMPC or HPMP-5-azaC. This prolonged effect may be attributed to the long intracellular half-life of some ANP metabolites, such as the choline adduct of the cytosine derivatives (33,45). In this report, we focused on the efficacy of HPMP-5-azaC to inhibit (i) virus replication in the lungs and (ii) establishment of latent infection in the spleen, rather than on the progression of infection during long-term latency. As virus still resides in the lungs of drug-treated mice, we expect MHV-68 to reactivate at later time points. Similar observations were made in previous studies examining the efficacy of HPMPC or nucleoside analogs (45,46).

In conclusion, we have performed a comprehensive investigation of the anti-γ-herpesvirus activity of several novel ANPs. HPMPC, HPMP-5-azaC, 3-deaza-HPMPA and their cyclic derivatives emerged as the most potent anti-γ-herpesvirus agents. Additionally, we observed that cyclic HPMP derivatives have reduced anti-EBV activity in induced P3HR-1 cells, and this effect appeared to be specific for this EBV-infected cell line. Our findings further suggested that the regulation of the different virus latency patterns and reactivation among two EBV-infected cell lines might be indirectly involved in the altered metabolism of cyclic HPMPC, and likely of other cyclic prodrugs. Finally, the in vitro and in vivo data presented here...
demonstrate that HPMP-5-azaC may be an attractive candidate for the development of anti-γ-herpesvirus drugs, and of DNA viruses in general.

ACKNOWLEDGEMENTS

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FUNDINGS

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REFERENCES


FIGURE LEGENDS
Figure 1. Inhibitory effects of HPMP derivatives against KSHV and EBV replication in different B-cell lines. EC50 values of ANPs against KSHV in BCBL-1 and JSC-1 cells, and against EBV in P3HR-1 and Akata cells, were plotted as the mean of at least three independent experiments ± SD. The EC50 was defined as the concentration required for 50% inhibition of viral DNA load.

Figure 2. Metabolism of [5-3H]-HPMPC and cyclic [5-3H]-HPMPC in P3HR-1 and Akata cells after 120h. (A) Activation pathway for HPMPC and cyclic HPMPC. CNP is the cellular 2',3'-cyclic-nucleotide 3'-phosphodiesterase. (B) Representative HPLC chromatogram showing the metabolism of 10 µM [5-3H]-HPMPC (●) and [5-3H]-cyclic HPMPC (○) in induced P3HR-1 and Akata cells after 120h exposure to the compound. The arrows point at the different metabolites of HPMPC and cyclic HPMPC. Please note the differences between values on the y-axis. (C) At the designated time points, intracellular concentrations of HPMPC, HPMPCp, HPMPCpp, HPMPp-choline and a mixture of cyclic HPMPC and HPMPCp-choline were determined in P3HR-1 and Akata cells after EBV reactivation. The means of two independent experiments were plotted. (D) Intracellular concentrations of HPMPC metabolites in latently-infected cells at 72h after addition of HPMPC or cyclic HPMPC were plotted as the means of two independent. (E) Incorporation of HPMPC into DNA of P3HR-1 and Akata cells in the latent and lytic stage of the virus after 72h of incubation.

Figure 3. Expression of CNP in EBV and/or KSHV positive cells. Western blot of CNP in induced and latently infected B-cells at 120h (upper panel) and band intensity relative to actin (lower panel).

Figure 4. Intracellular and extracellular cAMP levels in P3HR-1, Akata, BCBL-1 and JSC-1 cells. Cells were cultured for 24, 72 or 120h in the presence or absence of the inducing agent (TPA or IgG), and cAMP levels were measured by ELISA. The intracellular cAMP levels represent the amount of cAMP in 10^6 cells and the extracellular levels were expressed as the amount of cAMP in 1 ml of medium containing 10^6 cells. Each bar represents the mean ± SEM of at least two independent experiments. **p < 0.01, *** p<0.001.
Figure 5. Analysis of MHV-68 infection in different organs of untreated and treated mice at acute and latent stages. (A) DNA copy numbers were detected in the lungs, MLNs and spleen of mice infected by the intranasal route with 10^4 PFU MHV-68 and treated with HPMPC and HPMP-5-azaC (i.p.) for 5 consecutive days. Each group contained five mice. Values are given as the mean log viral copy number per mg of tissue ± SD. The level of MHV-68 gB and ORF73 expression, normalized to the endogenous control GAPDH, was measured relatively to the infected control by the comparative Ct method (ΔΔCt method). Statistical significance was calculated using the Mann-Whitney U test: *P<0.05, **P<0.01. (B) Hematoxylin and eosin-staining of lung tissues at days 6 and 12 p.i. of uninfected mice, MHV-68 infected mice, HPMPC-treated mice and HPMP-5-azaC-treated mice. Histopathology of the spleen at day 12 p.i. is shown in the right panels and arrows indicate the presence of prominent macrophages in the spleen of infected mice. Magnification: x20 and x100.

Figure 6. Proposed mechanism for reduced conversion of cyclic HPMPC in P3HR-1 cells after EBV reactivation. Cyclic HPMPC (cHPMPC) and cAMP are hydrolyzed by the phosphodiesterase CNP and act competitively for the active site of the enzyme (37). In addition, cAMP is known to be hydrolyzed by other phosphodiesterases (PDE) (47). Cyclic nucleotides are also subject to cellular efflux by membrane proteins such as the multidrug resistance proteins MRP4, MRP5 and MRP8 (48). In latently-infected P3HR-1 cells, high intracellular cAMP levels suggest low hydrolysis of this cyclic nucleotide by CNP and other PDE. Upon EBV reactivation, hydrolysis of cAMP by CNP and PDE is considerably increased. The cAMP that is bound to CNP competitively inhibits conversion of cyclic HPMPC by CNP.
Figure 2
Figure 3

Relative CNP intensity

Latent
Induced

P3HR-1
Akata
BCBL-1
JSC-1
P3HR-1
Akata
BCBL-1
JSC-1

CNP

Downloaded from https://jvi.asm.org/ on November 6, 2017 by guest
Figure 4
Figure 6
Table 1. Inhibitory effects of ANPs on the replication of human γ-herpesviruses.

<table>
<thead>
<tr>
<th>Compound</th>
<th>KSHV</th>
<th>EBV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antiviral activity (µM)</td>
<td>EC50 (µM)</td>
</tr>
<tr>
<td></td>
<td>EC90</td>
<td>CC50</td>
</tr>
<tr>
<td>HPMPC</td>
<td>1.3 ± 0.29</td>
<td>2.2 ± 1.3</td>
</tr>
<tr>
<td>Cyclic HPMPC</td>
<td>2.4 ± 1.0</td>
<td>3.7 ± 3.7</td>
</tr>
<tr>
<td>HDPM-HPMPC</td>
<td>0.7 ± 0.1</td>
<td>59 ± 18</td>
</tr>
<tr>
<td>HPMP-5-azaC</td>
<td>0.7 ± 0.4</td>
<td>9.6 ± 7.1</td>
</tr>
<tr>
<td>Cyclic HPMP-5-azaC</td>
<td>3.1 ± 0.8</td>
<td>4.6 ± 11</td>
</tr>
<tr>
<td>HPMPA</td>
<td>0.7 ± 0.7</td>
<td>≥ 96</td>
</tr>
<tr>
<td>Cyclic HPMPA</td>
<td>0.7 ± 0.4</td>
<td>≥ 112</td>
</tr>
<tr>
<td>3-deaza-HPMPA</td>
<td>0.3 ± 0.2</td>
<td>3.0 ± 1.7</td>
</tr>
<tr>
<td>Cyclic 3-deaza-HPMPA</td>
<td>1.8 ± 0.4</td>
<td>3.9 ± 3.2</td>
</tr>
<tr>
<td>7-deaza-HPMPA</td>
<td>5.0 ± 0.3</td>
<td>12 ± 6.6</td>
</tr>
<tr>
<td>Cyclic 7-deaza-HPMPA</td>
<td>1.4 ± 5.6</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>HPMPDAP</td>
<td>0.9 ± 0.3</td>
<td>31 ± 22</td>
</tr>
<tr>
<td>Cyclic HPMPDAP</td>
<td>2.0 ± 1.3</td>
<td>7.3 ± 6.7</td>
</tr>
<tr>
<td>HPMPO-DAPy</td>
<td>5.1 ± 1.4</td>
<td>17 ± 0</td>
</tr>
<tr>
<td>Cyclic HPMPO-DAPy</td>
<td>111 ± 20</td>
<td>≥ 500</td>
</tr>
<tr>
<td>PMEA</td>
<td>44 ± 15</td>
<td>≥ 172</td>
</tr>
<tr>
<td>PMEDAP</td>
<td>16 ± 7.6</td>
<td>≥ 174</td>
</tr>
<tr>
<td>PMEO-DAPy</td>
<td>12 ± 5.0</td>
<td>≥ 159</td>
</tr>
</tbody>
</table>

EC50 and EC90: concentration required to reduce KSHV or EBV DNA synthesis in TPA-stimulated BCBL-1 or P3HR-1 cells by 50% or 90%, respectively. CC50: concentration required to reduce the growth of uninduced BCBL-1 or P3HR-1 cells by 50%. SI: Selectivity index (ratio of CC50 to EC50). The values represent the mean ± SD of at least three independent experiments. Cyclic forms of ANPs are highlighted in grey.
Table 2. Inhibitory effects of ANPs on the replication of animal γ-herpesviruses.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MHV-68 EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>HVS EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>RRV EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>MHV-68 CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>HVS CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>RRV CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>MHV-68 SI</th>
<th>HVS SI</th>
<th>RRV SI</th>
</tr>
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<tr>
<td>HPMPC</td>
<td>1.6 ± 0.2</td>
<td>98 ± 25</td>
<td>61</td>
<td>3.5 ± 2.2</td>
<td>&gt;500</td>
<td>&gt;143</td>
<td>0.2 ± 0.1</td>
<td>578 ± 295</td>
<td>2627</td>
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<tr>
<td>Cyclic HPMPC</td>
<td>2.5 ± 1.2</td>
<td>680 ± 199</td>
<td>272</td>
<td>1.4 ± 0.7</td>
<td>&gt;500</td>
<td>&gt;357</td>
<td>0.2 ± 0.0</td>
<td>&gt;500</td>
<td>&gt;2941</td>
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<td>HPMP-5-azaC</td>
<td>1.8 ± 1.4</td>
<td>93 ± 36</td>
<td>52</td>
<td>2.1 ± 1.8</td>
<td>732 ± 130</td>
<td>348</td>
<td>0.2 ± 0.01</td>
<td>539 ± 125</td>
<td>2695</td>
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<td>Cyclic HPMP-5-azaC</td>
<td>4.6 ± 2.9</td>
<td>195 ± 114</td>
<td>42</td>
<td>1.5 ± 0.4</td>
<td>275 ± 53</td>
<td>183</td>
<td>1.1 ± 0.8</td>
<td>313 ± 164</td>
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<td>HPMPA</td>
<td>3.0 ± 3.3</td>
<td>30 ± 13</td>
<td>10</td>
<td>4 ± 2.3</td>
<td>396 ± 241</td>
<td>99</td>
<td>0.3 ± 0.3</td>
<td>353 ± 3</td>
<td>1177</td>
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<tr>
<td>Cyclic HPMPA</td>
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<td>63 ± 25</td>
<td>45</td>
<td>8.4 ± 4.2</td>
<td>&gt;500</td>
<td>&gt;60</td>
<td>0.3 ± 0.0</td>
<td>182 ± 109</td>
<td>607</td>
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<tr>
<td>3-deaza-HPMPA</td>
<td>0.2 ± 0.1</td>
<td>17 ± 13</td>
<td>85</td>
<td>1.0 ± 0.3</td>
<td>119 ± 63</td>
<td>119</td>
<td>0.3 ± 0.03</td>
<td>116 ± 76</td>
<td>387</td>
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<tr>
<td>Cyclic-3-deaza-HPMPA</td>
<td>0.4 ± 0.3</td>
<td>49 ± 7</td>
<td>123</td>
<td>2.8 ± 1.4</td>
<td>538 ± 289</td>
<td>192</td>
<td>1.4 ± 2.1</td>
<td>190 ± 32</td>
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<tr>
<td>7-deaza-HPMPA</td>
<td>0.7 ± 0.7</td>
<td>4.6 ± 1.3</td>
<td>7</td>
<td>56 ± 33</td>
<td>&gt;500</td>
<td>9</td>
<td>0.7 ± 0.3</td>
<td>46 ± 13</td>
<td>66</td>
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<tr>
<td>Cyclic 7-deaza-HPMPA</td>
<td>13 ± 6</td>
<td>137 ± 46</td>
<td>11</td>
<td>88 ± 74</td>
<td>&gt;500</td>
<td>6</td>
<td>1.8 ± 0.0</td>
<td>637 ± 320</td>
<td>354</td>
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<tr>
<td>HPMPDAP</td>
<td>0.9 ± 0.3</td>
<td>25 ± 14</td>
<td>28</td>
<td>28 ± 6</td>
<td>&gt;500</td>
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<td>&gt;500</td>
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<tr>
<td>Cyclic HPMPDAP</td>
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<td>7.7 ± 6.3</td>
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<td>67 ± 13</td>
<td>&gt;500</td>
<td>&gt;3</td>
<td>3.3 ± 1.7</td>
<td>&gt;500</td>
<td>&gt;152</td>
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<tr>
<td>HPMPO-DAPy</td>
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<td>1.0 ± 1.0</td>
<td>10</td>
<td>68 ± 17</td>
<td>&gt;500</td>
<td>&gt;7</td>
<td>5.2 ± 0.64</td>
<td>&gt;500</td>
<td>&gt;96</td>
</tr>
<tr>
<td>Cyclic HPMPO-DAPy</td>
<td>6.7 ± 0</td>
<td>211 ± 57</td>
<td>31</td>
<td>215 ± 10</td>
<td>&gt;500</td>
<td>&gt;2</td>
<td>15 ± 3.7</td>
<td>476 ± 238</td>
<td>32</td>
</tr>
<tr>
<td>PMEA</td>
<td>0.7 ± 0</td>
<td>12 ± 5</td>
<td>17</td>
<td>172 ± 81</td>
<td>&gt;500</td>
<td>&gt;3</td>
<td>238 ± 55</td>
<td>586 ± 187</td>
<td>2</td>
</tr>
<tr>
<td>PMEDAP</td>
<td>0.1 ± 0.03</td>
<td>2.8 ± 1.4</td>
<td>28</td>
<td>59 ± 7.1</td>
<td>&gt;500</td>
<td>&gt;8</td>
<td>73 ± 7.0</td>
<td>&gt;500</td>
<td>&gt;7</td>
</tr>
<tr>
<td>PMEO-DAPy</td>
<td>0.1 ± 0.08</td>
<td>2.3 ± 1.5</td>
<td>23</td>
<td>413 ± 208</td>
<td>583 ± 102</td>
<td>1</td>
<td>242 ± 121</td>
<td>242 ± 87</td>
<td>1</td>
</tr>
</tbody>
</table>

EC<sub>50</sub> concentration required for 50% inhibition of CPE. CC<sub>50</sub> concentration required to reduce the growth of NIH/3T3, OMK and RF cells by 50%. SI. Selectivity index (ratio of CC<sub>50</sub> to EC<sub>50</sub>). Cyclic forms of ANPs are highlighted in grey.