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Spectrum of antiviral activity of *o*-(acetoxyphenyl)hept-2-ynyl sulphide (APHS)

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Abstract

Since some antiviral drugs have a broad spectrum of action, the aim of this study was to assess whether *o*-(acetoxyphenyl)hept-2-ynyl sulphide (APHS), a recently described inhibitor of human immunodeficiency virus type 1 (HIV-1) replication, has an effect on the replication of other retroviruses, (−) and (+) RNA viruses and DNA viruses. APHS did not affect the replication of feline immunodeficiency virus, HIV-2 and a HIV-1 strain resistant to non-nucleoside reverse transcriptase inhibitors (NNRTI). APHS could also not inhibit the replication of the RNA viruses, respiratory syncytium virus or mouse hepatitis virus. In contrast, APHS did inhibit the replication of wild-type herpes simplex virus type 1 (HSV-1) as well as acyclovir-resistant HSV-1 and HSV-2 mutant. These results suggest that APHS is a NNRTI of HIV-1 replication, but not HIV-2 replication, and that APHS is an inhibitor of both HSV-1 and HSV-2 replication.

Keywords: APHS; Antiviral; Antiherpetic; HIV; HSV; Drug resistance

1. Introduction

Some antiviral compounds have a broad spectrum of action while others are more specific [1]. The pyrophosphate analogue phosfonoformic acid (foscarnet, PFA) is a broad-spectrum DP inhibitor that is active against several DNA and RNA viruses, including herpes simplex virus type 1 (HSV-1) and human immunodeficiency virus type 1 (HIV-L) and acts by interfering with the exchange of pyrophosphate from deoxynucleoside triphosphate during viral replication by binding to a site on the HSV-1 DP or HIV-1 RT [2,3]. Acyclic nucleoside phosphonates are chain terminators which are active against HIV, feline immunodeficiency virus (FIV), HSV, adenovirus, papillomaviruses and hepatitis B virus [4–6]. Receptor antagonists such as bicyclams inhibit cell-virus fusion and can inhibit HIV, simian immunodeficiency virus (SIV) and FIV replication [1]. Most of the non-nucleoside reverse transcriptase inhibitors (NNRTI) are very specific; for instance they inhibit the replication of HIV-1, but not that of HIV-2, SIV or FIV [7].

We have previously found that the non-steroidal anti-inflammatory drug *o*-(acetoxyphenyl)hept-2-ynyl sulphide (APHS) can inhibit HIV-1 replication by interfering with the reverse transcription process [8]. The aim of this study was to determine the antiviral specificity of APHS against viruses representative of different classes. HIV-1, HIV-2 and FIV were selected from the retroviridae family. Retroviruses are RNA viruses, which possess a reverse transcriptase (RT) enzyme, which is a RNA- and DNA-dependent DNA polymerase (DP) that transcribes the viral RNA into proviral DNA [9–12]. This proviral DNA can be inserted into the cellular genome by the viral integrase. Once the virus is integrated into the cellular genome it can stay latent for many years until the cell is stimulated and viral transcription starts.

Respiratory syncytium virus (RSV) is a negative (−) stranded RNA virus, which belongs to the family paramyxoviridae [13]. The RSV core contains an RNA replicase,

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which is an RNA-dependent RNA polymerase. When released into the cell cytoplasm, this RNA polymerase makes a complementary copy of the genome, which is (+) stranded. This complementary strand acts as a template for genome synthesis. Simultaneously, a series of (+) strands are produced, which act as mRNAs.

Mouse hepatitis virus (MHV) is a positive (+) stranded RNA virus, which belongs to the family coronaviridae [14]. The MHV genome serves as an mRNA for a polyprotein from which, by proteolytic cleavages, the various subunits of the viral RNA-dependent RNA polymerase are derived. The coronaviral RNA polymerase directs the synthesis of both genome-length and subgenomic negative-stranded RNAs, which in turn serve as templates for the synthesis of genomic RNA and subgenomic mRNAs, respectively.

HSV-1 is a double-stranded DNA virus, which belongs to the family herpesviridae and subfamily α-herpesviridae [15]. After virus entry into the cytoplasm, the nucleocapsid is transported to the nuclear pores where the viral DNA is released into the nucleus. The HSV-1 DNA forms a circular molecule, which acts as the template for replication. Early gene expression results in the expression of enzymes involved in nucleic acid metabolism such as thymidine kinase (TK) and proteins essential for DNA synthesis such as DNA replicase, which is a DNA-dependent DP. The DP catalyses the viral DNA synthesis. New progeny virus DNA is synthesised off the DNA genome of the input parental virus. New progeny DNA acts as a template for the synthesis of more genomes for new virus particles and for transcription of late virus mRNA that encode mainly viral structural proteins.

2. Materials and methods

2.1. Cells

Donor peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood from HIV-1, HIV-2, and hepatitis B-seronegative donors and obtained on Ficoll-Isopaque density gradients. To prepare a PBMC mixed batch, PBMC isolated from six donors were pooled together in RPMI 1640 medium (Gibco, Invitrogen, Paisley, Scotland) supplemented with 10% dimethyl sulphoxide (DMSO; Merck, Darmstadt, Germany), 20% foetal calf serum (FCS; Invitrogen) and 10 μg/ml gentamicin (Invitrogen) and frozen at −140 °C. Cells were thawed and cultured for 4 days prior to the experiment in RPMI 1640 medium supplemented with 10% FCS, 10 μg/ml gentamicin and 2 μg/ml lectin from *Phaseolus vulgaris* (PHA, Sigma Chemie, Zwijndrecht, The Netherlands) at 37 °C and 5% CO_2.

Crandell feline kidney (CrFK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Paisley, Scotland), supplemented with 5% foetal calf serum (FCS; Invitrogen) and antibiotics. Primary feline thymocytes were isolated from specific-pathogen-free cats (Harlan, Zeist, The Netherlands), stimulated with concanavalin A at 2.5 μg/ml and cultured in RPMI 1640 medium containing 10% FCS supplemented with 100 IU/ml of recombinant IL-2 (Roche Diagnostics GmbH, Mannheim, Germany). Human epithelial (HEp-2) cells were cultured as monolayers in Iscove's modified Dulbecco's medium (IMDM; Invitrogen), supplemented with 5% FCS and 10 μg/ml gentamicin (Invitrogen) and maintained at 37 °C and 5% CO_2. Mouse L cells were cultured in DMEM containing 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin (DMEM/10).

Human foreskin fibroblasts (HFF) were cultured as monolayers in IMDM supplemented with 10% FCS and 10 μg/ml gentamicin and maintained at 37 °C and 5% CO_2. The HSV inducible reporter cell line baby hamster kidney (BHKICP6LacZ-5) cells [16] were obtained as frozen aliquots of 7.5×10^5 cells/vial from Diagnostic Hybrids, Inc., Athens, OH. BHKICP6LacZ-5 cells contain the *Escherichia coli lacZ* gene placed behind the inducible HSV-1 early promoter ICP6. The *lacZ* gene encodes β-galactosidase. Upon HSV-1 or HSV-2 infection there is induction of β-galactosidase activity. There is no constitutive expression from this promoter in uninfected cells, activation of the promoter appears to be specific for HSV and expression from the promoter occurs within hours after infection. Cells were thawed immediately before usage in minimal essential medium (MEM; Invitrogen) containing 7% FCS and 10 μg/ml gentamicin.

2.2. Virus

The following reagents were obtained from NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-$1_{Ba\text{-}L}$ (subtype B) strain from Dr. Suzanne Gartner, Dr. Mikulas Popovic and Dr. Robert Gallo; HIV-$2_{CDC310319}$ (subtype B isolate) strain from Dr. Stefan Wiktor and Dr. Mark Rayfield [17]; and NNRTI-resistant HIV-1_{IIIB} (A17 variant) strain from Dr. Emilio Emini [18]. HIV-$1_{Ba\text{-}L}$ and HIV-$2_{CDC310319}$ were propagated and titrated in PBMC. HIV-1_{IIIB} A17 was propagated in the H9 cell line and titrated in PBMC. HIV-1_{IIIB} A17 contains the mutations K103N and Y181C in the viral RT domain. A virus stock of the Dutch isolate FIV UT-113 was prepared from a culture of persistently infected CrFK cells. RSV serotype A was propagated and titrated in HEp-2 cells. RSV stock contained 2.4×10^7 PFU (plaque-forming units)/ml. MHV-A59 was propagated in Moloney sarcoma virus-transformed Sac(−) cells and plaque titrated on mouse L cells as described previously [19]. The MHV stock contained 5.3×10^8 PFU/ml.

The HSV-1 strain KOS and the KOS-derived acyclovir (ACV)-resistant HSV-1 strains PAA^{r}5, F891C, PFA^{r}2, and PFA^{r}5, which contain mutations in the DP gene [20–22] were kindly provided by Dr. D.M. Coen (Harvard Medical School, Boston, MA, USA). The HSV-1 ACV-sensitive strain McIntyre was generously provided from Dr. A. Linde (Swedish Institute for Infectious Disease Control, Solna, Sweden). The ACV-resistant HSV-1 clinical isolate 98.14742-PE/1, which contains mutations in the TK gene [23], was a gift of Dr.

M. Aymard (Université Claude Bernard, Lyon, France). The ACV-resistant HSV-1 clinical isolate 97-12961, which contains mutations in the TK gene, was selected from our own collection. This strain was obtained from a bone marrow transplant recipient suffering from severe HSV infections despite ACV prophylaxis. Viruses were isolated on human diploid embryonic lung fibroblasts. The HSV-2 strain O24, which contain mutations in the TK gene, was a gift from Dr. N. Goyette (University of Laval, Quebec, Canada). HSV stocks were grown in Vero cells (African green monkey kidney) and the infectious titre was determined by plaque assay in Vero cells as described previously [24].

2.3. Compounds

APHS was synthesised at the Department of Metal-Mediated Synthesis of the University of Utrecht. Synthesis details have been described elsewhere [25]. APHS was diluted in aliquots in 100% ethanol, topped with argon gas and stored at −20 °C. The concentration of ethanol during incubations never exceeded 0.1%. At this concentration, ethanol did neither affect virus replication nor cellular viability (data not shown). Zidovudine (AZT; Sigma) was diluted in DMSO. The concentration of DMSO during incubations never exceeded 0.001% and no effect on HIV-1 replication or cellular viability was observed at this concentration (data not shown). Efavirenz (EFV; Merck) and ACV (Sigma) were diluted in water.

2.4. Determination of cellular viability

CrFK cells grown in 96-well plates to confluent monolayers were incubated with fivefold dilutions of the antiviral drugs (ranging from 0.024 to 100 μM) and after 6 days of incubation at 37 °C and 5% CO_2, a cellular viability assay was performed on these cells. HEp-2 cells were seeded in 96-wells plates in the presence of APHS for 4, 5 and 6 days. Concentrations of HEp-2 cells were determined by microscopic observation in order to have a confluent monolayer at the day of measurement. Afterwards a cellular viability assay was performed on these cells. HFF cells were seeded at a concentration of 6×10^4 cells/ml and incubated for 2 days at 37 °C and 5% CO_2. Subsequently, the medium was removed and replaced by medium containing increasing concentrations of APHS. After 24 h of incubation the medium was aspirated and the HFF monolayer was overlaid with 6×10^3 BHKICP6LACZ-5 cells/ml and the culture was incubated for 24 h at 37 °C and 5% CO_2. Afterwards, a cellular viability assay was performed on these cells. PHA-stimulated PBMC were washed twice and incubated at a density of 5×10^5 cells/ml with increasing concentrations of AZT, APHS or EFV for 7 days at 37 °C and 5% CO_2.

The metabolic activity of the above-mentioned cells was assessed by a cellular viability assay as described previously [26]. Shortly, 150 μg/ml tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma) is added to the cells. During the subsequent 2 h incubation period metabolic active cells convert MTT into blue formazan crystals. Subsequently, 3/4 of the supernatant was gently removed and substituted by stop buffer consisting of 90% 2-propanol, 10% triton X-100 and 0.4% HCl (Merck, Darmstadt, Germany). When all formazan crystals were dissolved optical density was measured at 550 nm.

2.5. FIV infection of CrFK cells and primary feline thymocytes

CrFK cells and primary feline thymocytes (5×10^3 and 5×10^4 cells/well, respectively) grown in 96-well plates to confluent monolayers were preincubated for 1 h with fourfold dilutions of APHS (ranging from 0.024 to 100 μM) and subsequently infected with 100 50% tissue culture infective doses of FIV UT-113/well. After 2 h, cells were rinsed twice with phosphate buffered saline (PBS) and the cultures were maintained in the presence of different dilutions of APHS. After 6 days of incubation FIV replication was measured by quantification of the p24 viral core antigen in the cell culture supernatant by an FIV p24 enzyme-linked immunoabsorbent assay as described previously [27].

2.6. HIV infection of PBMC

PHA-stimulated PBMC were washed twice and incubated for 7 days at a density of 5×10^5 cells/ml with HIV-$1_{Ba\text{-}L}$, HIV-$2_{CDC310319}$ and HIV-1_{IIIB} A17 at a multiplicity of infection (MOI) of 0.0001 (input virus), in the presence of AZT, APHS or EFV and 10 U/ml recombinant IL-2, at 37 °C and 5% CO_2. To correct for the input virus, an extra control consisting of medium containing the same amount of input virus as added to the cells was included in the experiment. The amount of p24 in this control was subsequently subtracted from the p24 values of the samples in order to obtain the exact amount of p24 produced by the cells. After 2 or 7 days incubation, samples of the supernatants were collected, inactivated by addition of Empigen (Calbiochem, La Jolla, CA, USA) and by heat inactivation at 56 °C for 30 min. p24-core antigen concentration was determined by an enzyme-linked immunoabsorbent assay (ELISA) (AMPAK™, DAKO, Cambridgeshire, UK) as described previously [28,29]. The optical density values were converted into p24 concentration (ng/ml) with the use of a calibration curve made by serial dilutions of recombinant p24 protein (NIBSC, UK) that was submitted to the same treatment as the samples.

2.7. RSV infection of HEp-2 cells

HEp-2 cells were recovered from the culture flasks by trypsinisation. Concentrations of HEp-2 cells were determined by microscopic observation in order to have a confluent monolayer at the day of measurement. HEp-2 cells were incubated with APHS and infected with different MOI

of RSV in order to have a good infection at the day of measurement. After 4, 5 and 6 days of incubation at 37 °C and 5% CO_2, HEp-2 cell monolayers were washed three times with PBS and cells were recovered by trypsinisation. The cells were washed with PBS^{2+} (PBS containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+}) and incubated with murine-anti-RSV monoclonal antibody conjugated to fluorescein isothiocyanate (FITC) (DAKO, Cambridgeshire, UK) for 15 min at 37 °C. Afterwards, cells were washed and resuspended in PBS^{2+}. Percentage of fluorescent cells was analysed by FACSCalibur flow cytometer (Becton Dickinson, California, USA) with computer assisted evaluation data (CellQuest Pro).

2.8. MHV infection of LR7 cells

Mouse L cells were seeded at a concentration of 5×10^4 cells/well and preincubated for 1 h with five-fold dilutions of APHS (ranging from 0.009 to 143 μM) in DMEM/10. Afterwards the medium was discarded and the cells were incubated for 1 h with MHV-A59 at a MOI of 0.05 in the presence or absence of APHS in DMEM. Subsequently the cells were washed and incubated with APHS in DMEM/10. After 24 h, the cytopathic effect (CPE) of the virus and the cytotoxicity of the compounds were scored by microscopic observation.

2.9. ELVIRA® HSV (enzyme-linked virus inhibitor reporter assay)

This assay was performed as described previously [30]. Briefly, HFF cells were seeded at a concentration of 6×10^4 cells/ml and incubated for 2 days at 37 °C and 5% CO_2. Afterwards the medium was removed and the cells were infected with 70–300 PFU/ml of HSV in the presence of increasing concentrations of APHS or ACV. Infection was enhanced by centrifugation of the cells for 1 h at room temperature. The culture was then incubated for 1 day at 37 °C and 5% CO_2. Subsequently, the medium was aspirated and the HFF monolayer was overlaid with 6×10^3 BHKICP6LacZ-5 cells and the culture was incubated at 37 °C and 5% CO_2. After 24 h of incubation, the supernatant was aspirated and the cells were lysed. β-Galactosidase activity was detected in the lysates using chlorophenol red-β-D-galactopyranoside (CPRG) (Roche, Almere, The Netherlands).

2.10. Determination of IC_{50} and TC_{50}

The ability of each compound to inhibit HSV-1 replication and cellular viability were expressed as the 50% inhibitory concentration (IC_{50}) and the 50% toxic concentration (TC_{50}), respectively. IC_{50} and TC_{50} were calculated using the computer software program CalcuSyn for Windows (Biosoft, Cambridge, UK) according to the method of Chou et al. [31,32]. This program uses the median-effect equation to produce dose–effect curves:

$$f_a = 1/[1 + (D_m/D)^m] \quad (1)$$

where f_a represents fraction affected by the dose (reduction of viral replication or cellular viability at a certain drug concentration expressed in decimals), D_m the median-effect dose (same as IC_{50} or TC_{50}), D the dose of the drug and m the sigmoidicity coefficient of the dose–effect curve. Data was accepted when the linear correlation coefficient of the median-effect plot based on experimental data was >0.90.

3. Results

3.1. Effect of APHS on HIV replication

Since APHS can inhibit the replication of HIV-1, we investigated whether APHS also could inhibit HIV-2 replication. To assess the effect of APHS on HIV-2 replication, virus replication was determined by quantifying the levels of the HIV capsid protein p24 in culture supernatants after 7 days of incubation. Results are shown in Table 1 and Fig. 1. AZT, EFV and APHS did inhibit the replication of the wild-type strain HIV-$1_{Ba\text{-}L}$ with IC_{50}'s similar to those described previously [33]. AZT did inhibit HIV-$2_{CDC310319}$ replication with an IC_{50} similar to the IC_{50} for HIV-$1_{Ba\text{-}L}$. As expected for a first generation NNRTI, EFV did not inhibit HIV-$2_{CDC310319}$ replication. APHS also did not inhibit HIV-$2_{CDC310319}$ replication. Because it was previously suggested that APHS might be an NNRTI [8], HIV-1_{IIIB} A17, a HIV-1 strain highly resistant to all NNRTIs, was also included in these series of experiments. Replication of HIV-1_{IIIB} A17 was inhibited by AZT with an IC_{50} similar to the IC_{50} for HIV-$1_{Ba\text{-}L}$. In contrast, HIV-1_{IIIB} A17 showed a 1000-fold resistance against EFV and APHS could not inhibit the replication of this NNRTI-resistant virus. All concentrations used in this assay were not cytotoxic as determined by the cellular viability assay (data not shown). The TC_{50} of APHS for these cells was 105 ± 15 μM [8].

3.2. Effect of APHS on FIV replication

To determine the effect of APHS on the replication of the animal retrovirus FIV, virus replication was assessed by quantifying the levels of the FIV capsid protein p24 in culture supernatants 6 days after infection. Results are shown in

Table 1
The effect of AZT, EFV and APHS on HIV-$1_{Ba\text{-}L}$, HIV-$2_{CDC310319}$, and HIV-1_{IIIB} A17 replication in PBMC

	IC_{50}[a] (μM)		
	HIV-$1_{Ba\text{-}L}$	HIV-$2_{CDC310319}$	HIV-1_{IIIB} A17
AZT	0.004 ± 0.0004	0.006 ± 0.001	0.005 ± 0.001
EFV	0.00004 ± 0.00001	>1000	0.04 ± 0.006
APHS	23 ± 8	>100	>100

Results are the mean ± S.E.M. of at least three independent experiments.
[a] IC_{50} represents 50% inhibitory concentration.

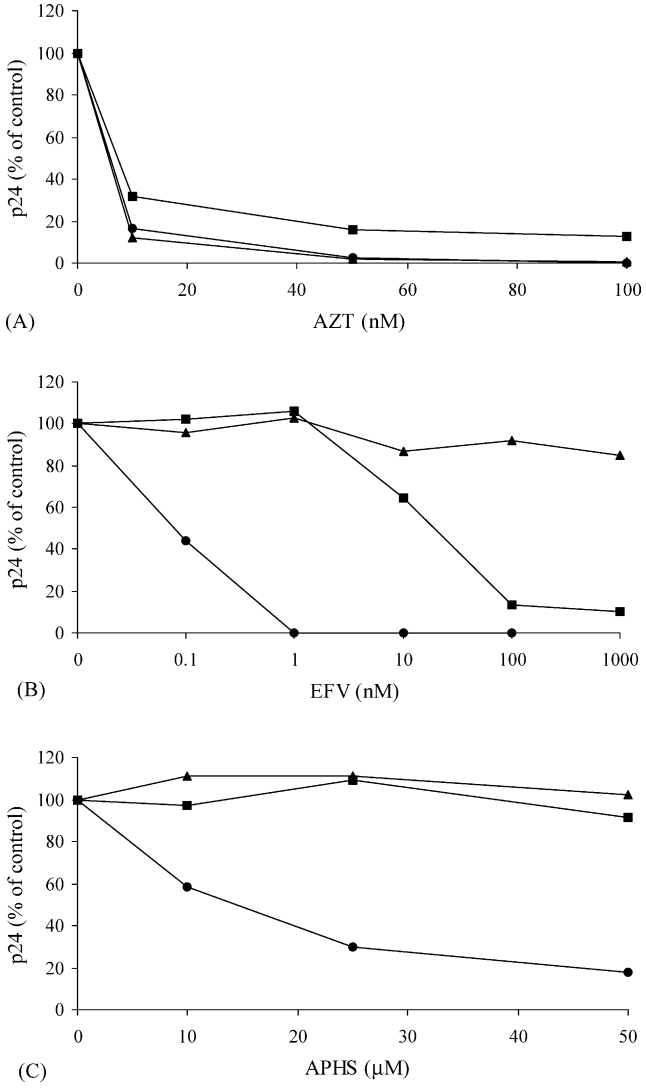

Fig. 1. Effect of AZT (A), EFV (B) and APHS (C) on the replication of HIV-$1_{Ba\text{-}L}$ (●), HIV-$2_{CDC310319}$ (▲) and HIV-1_{IIIB} A17 (■) in PBMC. Compounds were administered simultaneously with HIV and after 7 days HIV production was determined by quantifying p24 antigen concentration in culture supernatant (shown as percentage of the control, which is HIV-infected PBMC not treated with any compound). Results are representative of three independent experiments.

Table 2. APHS did not show any effect on FIV replication in both CrFK cells and primary feline thymocytes, while the known FIV inhibitor PMEA could inhibit FIV replication in a dose-dependent manner in both CrFK cells and primary feline thymocytes (Fig. 2). The IC_{50} of PMEA for the inhibition of FIV replication was similar to the IC_{50} described previously [5].

3.3. Effect of APHS on RSV and MHV replication

To determine whether APHS could inhibit the replication of the (−) stranded RNA virus RSV, virus replication was quantified by determining the percentage of HEp-2 cells with RSV adherent to their surface. The results are shown in Fig. 3. RSV infection was measured 4 (Fig. 3A), 5 (Fig. 3B) and 6 (Fig. 3C) days after infection. On day 6 after infection 80% of the cells were infected by RSV. RSV infection was also confirmed by microscopic evaluation of syncytium formation (data not shown). All APHS concentrations tested showed no inhibition of RSV replication in HEp-2 cells. In this assay, cellular viability significantly decreased at concentrations above 10 μM and the TC_{50} of APHS for these cells was 25 ± 3 μM.

To assess the effect of APHS on the replication of the (+) stranded RNA virus MHV, virus replication was assessed by CPE scoring after 24 h of incubation. CPE were visible in every sample except for the negative control, which indicates a good infection but no effect of APHS (data not shown).

Table 2
Effect of APHS and PMEA on FIV replication on CrFK cells and primary feline thymocytes

	Thymocytes		CrFK	
	PMEA	APHS	PMEA	APHS
IC_{50}[a] (μM)	0.3 ± 0.2	>100	1.6 ± 0.5	>100
TC_{50}[b] (μM)	7 ± 2	87 ± 4	30 ± 2	>100
SI[c] (TC_{50} /IC_{50})	22	0	19	0

Results are the mean ± S.E.M. of at least three independent experiments.
[a] IC_{50} represents 50% inhibitory concentration.
[b] TC_{50} represents 50% toxic concentration.
[c] SI represents the selectivity index.

3.4. Effect of APHS on HSV replication

Since some antiviral drugs act against both HIV and HSV, it was determined whether APHS could also be active against the double-stranded DNA virus HSV. Therefore, viral replication of the two reference HSV-1 strains McIntyre and KOS in presence of different concentrations of APHS was studied. APHS was capable of inhibiting the replication of both strains in a dose-dependent manner. The IC_{50} of APHS for the McIntyre and KOS strain was 17 ± 3 93 ± 31 μM,

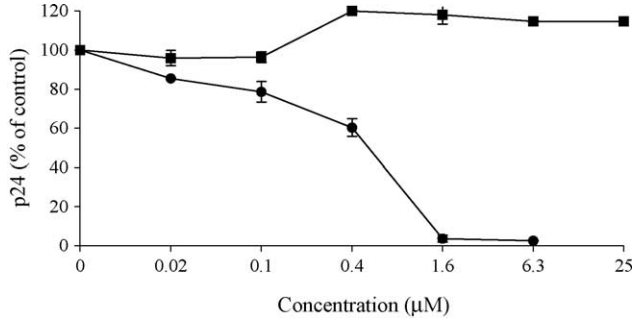

Fig. 2. Effect of APHS and PMEA on FIV replication in primary feline thymocytes. To determine FIV replication, increasing concentrations of APHS (■) or PMEA (●) were administered to FIV-infected thymocytes and after 6 days FIV production was determined by quantifying p24 antigen concentration in culture supernatant (shown as percentage of the control, which is FIV-infected thymocytes not treated with the compounds). Results are representative of three independent experiments.

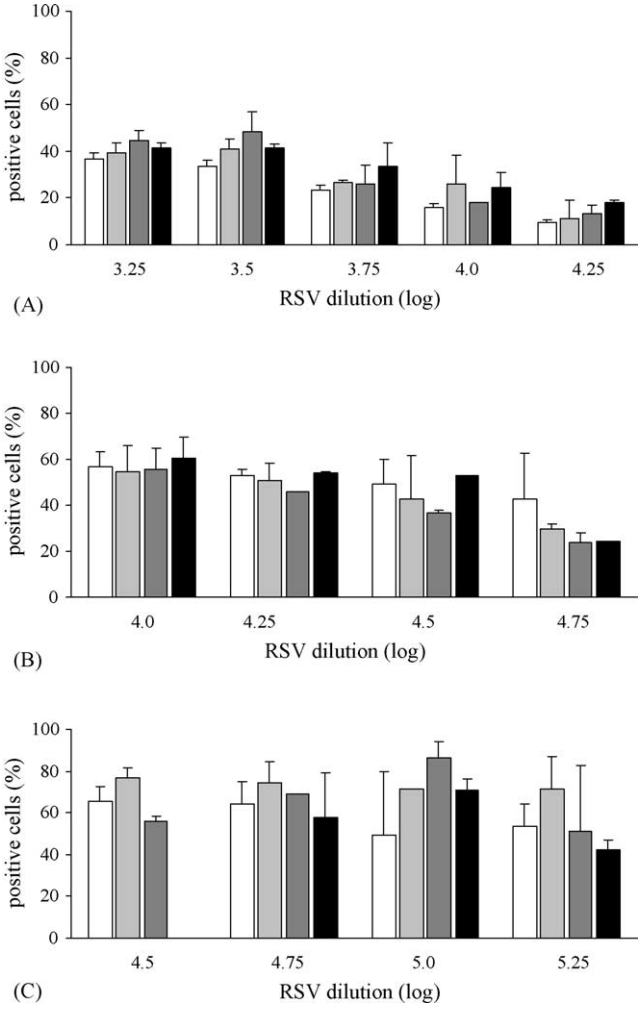

Fig. 3. Effect of APHS on RSV adhesion to HEp2 cells. HEp2 cells were infected by RSV in the presence of several concentrations of APHS for 4 (A), 5 (B) and 6 (C) days. The concentrations of APHS that were used were 0 (□), 0.1 (▭), 1 (▩), and 10 μM (■). The amount of HEp-2 cells with RSV adherent to their surface (percentage of positive cells) was determined by incubating the cells with a FITC-labelled anti-RSV antibody and measuring the fluorescence by FACS. Results are representative of three independent experiments. Uninfected control cells did not contain positive cells (0%).

respectively. The TC_{50} of APHS for the cells that were used in the assay was 266 ± 27 μM. Thus, APHS inhibits the replication of HSV-1. Next, the anti-viral activity of APHS on ACV-resistant HSV strains was compared with that of ACV. While the IC_{50} of ACV on HSV-1 KOS replication was 7.7 μM, the KOS-derived DP mutant HSV-1 strains PAA^{r}5, F891C, PFA^{r}2 and PFA^{r}5 and the TK mutant HSV strains 98.14742-PE/1, O24, and 97-12961 showed a 2–13-fold decrease in susceptibility to ACV when compared with the parental HSV-1 KOS strain (Table 3). These results correlate well with previous studies [30]. The replication of the DP mutant HSV-1 strains PAA^{r}5, F891C, PFA^{r}2 and PFA^{r}5, the TK mutant HSV-1 strains 98.14742-PE/1 and 97-12961 and the TK mutant HSV-2 strain O24 was inhibited by APHS at concentrations ranging from 24 to 191 μM. The IC_{50} values of APHS for all ACV-resistant HSV strains tested were statistically similar to the IC_{50} value of APHS obtained for the wild-type KOS strain suggesting that APHS can inhibit ACV-resistant HSV.

Table 3
The effect of APHS and ACV on the replication of ACV-resistant HSV strains in HFF

HSV strain	Mutations[a]	APHS, IC_{50}[b] (μM)	ACV, IC_{50}[b] (μM)
KOS	WT	93 ± 31	7.7 ± 3
PAA^{r}5[c]	DP	108 ± 47	29 ± 9
F891C[c]	DP	26 ± 8	25 ± 8
PFA^{r}2[c]	DP	116 ± 7	16 ± 2
PFA^{r}5[c]	DP	191 ± 46	69 ± 16
98.14742-PE/1	TK	94 ± 28	81 ± 26
O24[d]	TK	24 ± 1	31 ± 18
97-12961	TK	97 ± 9	98 ± 32

Results are the mean ± S.E.M. of at least three independent experiments.
[a] The HSV strains do not contain mutations (WT), contain mutations in the HSV DNA polymerase gene (DP) or contain mutations in the HSV thymidine kinase gene (TK).
[b] IC_{50} represents 50% inhibitory concentration.
[c] This HSV strain is also resistant to foscarnet.
[d] This HSV strain is a HSV-2 strain.

4. Discussion

Since APHS can inhibit the replication of HIV-1 by interfering with its reverse transcription process [8], we hypothesise that APHS could also be active against other retroviruses. Surprisingly, APHS did not show any effect on HIV-2 or FIV replication. This result seems to indicate that APHS specifically interferes with the RT of HIV-1 or with other HIV-1 proteins involved in the reverse transcription process. It is known that the NNRTIs currently used in the clinic are only active against HIV-1 and not against HIV-2, SIV, FIV, or other retroviruses apparently because only HIV-1 RT offers the required allowance for interactions of the NNRTIs with their binding pocket, i.e., stacking interactions with the aromatic amino acids (aa) Tyr-181, Tyr-188, Trp-229 and Tyr-318; electrostatic interactions with Lys-101, Lys-103 and Glu-138; van der Waals interactions with Leu-100, Val-106, Tyr-181, Gly-190, Trp-229, Leu-234 and Tyr-318; and hydrogen bonding with the main-chain peptide bonds [7]. In addition, although the aa in the NNRTI-specific pocket of HIV-1 RT display higher similarity to the corresponding FIV RT aa than to HIV-2 RT aa, the wild-type FIV and the FIVp66/HIVp51 chimeric enzyme showed no susceptibility to NNRTI [33]. Although it has been previously shown that APHS could inhibit the replication of several NRTI-resistant HIV-1 strains [34], APHS could not inhibit the replication of a HIV-1 strain highly resistant to all NNRTIs. These results taken together come in agreement with previous studies, which suggested that APHS is an NNRTI.

APHS did not inhibit the replication of RSV and MHV. This is not surprising since the mechanism of replication of these viruses is totally different from the mechanism of

replication of HIV-1. Interestingly, APHS did inhibit the replication of the HSV-1 wild-type strains McIntyre and KOS. This result is supported by previous studies that showed that HSV replication can be inhibited by non-nucleoside agents [35,36]. Although very speculative, APHS might be interacting with regions of the HSV DP, that share structural and functional similarity with those in HIV-1 RT, but not in the HIV-2 or FIV RT. Subsequently, several HSV-1 strains containing a point mutation in the DP gene that confers resistance to ACV and PFA were also tested. None of the DP mutant HSV-1 strains showed a significant level of resistance to APHS. This result strongly suggests that APHS is inhibiting HSV-1 replication in a different way from ACV and PFA. In addition to mutations in DP, mutations in TK also confers resistance to ACV. ACV needs to be phosphorylated by TK in order to become an active antiherpetic agent. Therefore, the antiviral activity of APHS on HSV strains, which contained mutations in the viral TK gene but not in the viral DP gene, was determined. The two TK mutant HSV-1 strains and the HSV-2 strain showed no significant level of resistance to APHS. Thus, APHS is able to inhibit the replication of ACV-resistant HSV strains with resistance conferring mutations in either the DP or TK gene. This finding makes APHS a potential candidate lead compound for the development of an antiherpetic drug.

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