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Antiviral Research 58 (2003) 253–263
Antiviral Research
www.elsevier.com/locate/antiviral

Aspirin-like molecules that inhibit human immunodeficiency virus 1 replication

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Received 4 July 2002; accepted 19 December 2002

Abstract

Some anti-inflammatory molecules are also known to possess anti-human immunodeficiency virus (HIV) activity. We found that *o*-(acetoxyphenyl)hept-2-ynyl sulfide (APHS), a recently synthesized non-steroidal anti-inflammatory molecule can inhibit HIV-1 replication. The aim of this study was to clarify the mechanism of action of APHS. When administered during the first steps of the infection, APHS was capable of inhibiting the replication of several HIV-1 strains (macrophage-tropic and/or lymphocytotropic) in a dose-dependent manner in both peripheral blood mononuclear cells (PBMC), monocyte-derived macrophages and peripheral blood lymphocytes with 50% inhibitory concentration values of approximately 10 μM. The 50% toxic concentration of APHS varied between 100 and 200 μM in the different primary cells tested. APHS did not affect HIV-1 replication once the provirus was already inserted into the cellular genome. APHS also did not inhibit HIV-1 entry into the host cells as determined by quantification of gag RNA inside PBMC 2 h after infection. However, APHS did inhibit gag DNA synthesis during reverse transcription in primary cells, which indicates that APHS may target the reverse transcription process.

Keywords: Human immunodeficiency virus; Non-steroidal anti-inflammatory drugs; Reverse transcription; Real-time Taqman polymerase chain reaction; Peripheral blood mononuclear cells

1. Introduction

HIV-1 inhibitors currently used in the clinic target the viral reverse transcriptase (RT) or the viral protease. Treatment of HIV-1-infected individuals with a combination of three or more of these antiretroviral drugs, the so-called highly active antiretroviral therapy (HAART), has proven to be a most successful treatment (Hammer et al., 1994). Indeed, this strategy has been shown to decrease the HIV-1 viral load, increase the amount of $CD4^+$ lymphocytes and delay disease progression (Henry et al., 1998; Palella et al., 1998). Furthermore, it has several advantages over single drug therapy, like providing an additive or synergistic antiviral effect (Snyder et al., 2000). Notwithstanding, no drug or combination of drugs is still able to completely block HIV-1 replication or to clear HIV-1 from the long-lived reservoirs of latently infected cells (Ramratnam et al., 2000). Furthermore, absence of a proofreading mechanism for the RT allows emergence of 4×10^{-5} mutations per base pair per cycle (Mansky, 1996). Since the estimated average of HIV-1 production is 10.5×10^9 virions per day (Perelson et al., 1996), replication-competent HAART-resistant viruses will eventually be selected. Also, long-term toxicity of current drugs and poor adherence to the treatment are important causes of therapy failure (Max and Sherer, 2000).

Therefore, new medicines and strategies are needed to eliminate HIV. Besides more effective and less toxic new RT inhibitors (RTIs) and protease inhibitors (PIs) several new compounds that target viral entry, virus–cell fusion, viral assembly and disassembly, proviral DNA integration and viral mRNA transcription are in development (De Clercq, 2000). Cellular factors involved in HIV entry (like CD4,

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doi:10.1016/S0166-3542(03)00006-8

CCR5 and CXCR4) and HIV transcription (such as NF-κB and Sp1) have also been described as targets for anti-HIV therapy (Baba, 1997). Also the anti-proliferation drug hydroxyurea possesses anti-HIV activity in vitro (Gao et al., 1993). Since it is known that inflammatory molecules such as cytokines and oxygen radicals are involved in the pathogenesis of HIV-1 infection, many anti-inflammatory compounds including platelet-activating factor receptor antagonists, aspirin and indomethacin have been shown to possess anti-HIV potential (Bourinbaiar and Lee-Huang, 1995; Kopp and Ghosh, 1994; Serradji et al., 2000).

o-(Acetoxyphenyl)hept-2-ynyl sulfide (APHS) is an aspirin-like molecule that belongs to the group of selective non-steroidal anti-inflammatory drugs (NSAIDs) (Kalgutkar et al., 1998a). This new generation of selective NSAIDs preferentially acetylates and irreversibly inactivates COX-2 and maintains good anti-inflammatory activity. Moreover, concentrations of APHS up to 100 mg/kg showed no toxicity in the rat air pouch model (Kalgutkar et al., 1998a).

The aim of this study was to determine whether APHS possesses anti-HIV-1 activity and, if so, by which mechanism.

2. Materials and methods

2.1. Isolation of primary cells

Donor peripheral blood mononuclear cells (PBMC) were isolated from heparinized 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 sulfoxide (DMSO; Merck, Darmstadt, Germany), 20% fetal calf serum (FCS; Invitrogen) and 10 μg/ml gentamycin (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 gentamycin and 2 μg/ml lectin from *Phaseolus vulgaris* (PHA, Sigma Chemie, Zwijndrecht, The Netherlands) at 37 °C and 5% CO_2.

Peripheral blood lymphocytes (PBLs) were isolated after incubation of PBMC in tissue culture flasks for 1 h. Monocytes adhered to the flask and PBL-containing supernatant was collected, seeded in culture flasks with medium (RPMI-1640 supplemented with 10% FCS and 10 μg/ml gentamycin) at a concentration of 2×10^6 cells/ml, stimulated with 2 μg/ml PHA and incubated for 4 days at 37 °C and 5% CO_2.

To obtain monocytes, PBMC were washed twice and monocytes were purified by countercurrent centrifugal elutriation. Cells were >98% monocytes by criteria of cell morphology on May-Grünwald-Giemsa-stained cytosmears and by non-specific esterase staining using α-naphtylacetate (Sigma) as substrate. Monocytes were cultured in suspension at a concentration of 2×10^6 cells/ml in Teflon flasks (Nalgene, Rochester, NY) in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated human AB serum negative for anti-HIV antibodies, 10 μg/ml gentamycin, and 10 μg/ml ciprofloxacin (Sigma) for 7 days at 37 °C and 5% CO_2. As previously described, HIV-1 infection of non-adherent macrophages, especially when using a low multiplicity of infection, appears much more reproducible than infection of macrophages that were first allowed to adhere (Boven et al., 1999).

2.2. Compounds

APHS and derivatives were supplied by Dr. L.J. Marnett. Synthesis details have been described by Kalgutkar et al. (1998b). All compounds tested were diluted in aliquots in 100% ethanol, topped with argon gas and stored at −20 °C. The concentration of ethanol during the incubations never exceeded 0.1%. At this concentration, ethanol did not affect HIV-1 replication or cellular viability (data not shown). Zidovudine (AZT; Sigma) was diluted in DMSO. The concentration of DMSO during the incubations never exceeded 0.001% and no effect on HIV-1 replication or cellular viability was observed at this concentration (data not shown). Dextran sulphate (DS; Sigma) was diluted in 100% ethanol. The concentration of ethanol during the incubations with DS never exceeded 0.1%.

2.3. HIV-1 infection of primary cells

Monocyte-derived macrophages (MDMs) and PHA-stimulated PBL or 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-1_{AT} or HIV-1_{HXB2} (multiplicity of infection (MOI): 0.0025), in the presence of AZT, APHS or related compounds and 10 U/ml recombinant IL-2 (Roche Diagnostics GmbH, Mannheim, Germany), 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.

2.4. p24-core antigen quantification by ELISA

After 2 or 7 days incubation, samples of the supernatants were collected, inactivated by addition of Empigen (Calbiochem, La Jolla, CA) 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 (McKeating et al., 1991; Moore et al., 1990). 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.

2.5. Determination of viability of primary cells

MDM and PHA-stimulated PBMC or PBL were washed twice and incubated at a density of 5×10^5 cells/ml with increasing concentrations of AZT, APHS or related compounds for 7 days at 37 °C and 5% CO_2. Afterwards the metabolic activity of these cells was assessed by a cellular viability assay as described previously (Pauwels et al., 1988). Shortly, 150 μg/ml tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolliumbromide (MTT; Sigma) was added to the cells. During the subsequent 2 h incubation period, metabolic active cells converted MTT into blue formazan crystals. Afterwards, 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.

2.6. Infection model with the monocytic cell line U1

U1 cells (AIDS Reagents, Rockville, MA) were incubated with 100 ng/ml phorbol myristate acetate (PMA; Sigma) or with medium (negative control) for 30 min. Afterwards the cells were washed and seeded at a density of 1×10^6 cells/ml in RPMI-1640 medium containing 10% FCS and 10 μg/ml gentamycin either with increasing concentrations of APHS or with 30 mM *N*-acetyl-L-cysteine (NAC; Sigma), which served as a positive control. After 2 days of incubation, samples of the supernatant were collected, inactivated by addition of Empigen and by 30 min incubation at 56 °C. The concentration of p24 was determined by ELISA (AMPAK™) as described above.

2.7. Determination of IC_{50} and TC_{50} of the compounds

The ability of each compound to decrease p24 antigen and cellular viability was 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 and Talalay (Chou and Hayball, 1996; Chou and Talalay, 1984). This program uses the median-effect equation to produce dose–effect curves:

$$f_a = \frac{1}{1 + (D_m/D)^m} \quad (1)$$

where f_a represents fraction affected by the dose (reduction of p24 or cellular viability at a certain drug concentration expressed in decimals), D_m is the median effect dose (same as IC_{50} or TC_{50}), D is the dose of the drug and m is 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. The selectivity index (SI), which represents the concentration range where a drug is effective without being toxic, was calculated by the ratio TC_{50}/IC_{50}.

2.8. PBMC infection with HIV-$1_{Ba\text{-}L}$ or HIV-1_{HXB2} for real-time Taqman PCR

The ability of HIV-$1_{Ba\text{-}L}$ to enter the cells in the presence of APHS was tested using DS as positive control (entry assay). The amount of HIV-1_{HXB2} reverse transcription products in the presence of APHS was also tested, using AZT as positive control (reverse transcription assay).

Before infection, PHA-stimulated PBMC from the PBMC batch were pre-incubated with different concentrations of APHS, DS or AZT for 24 h. Afterwards, cells were washed and 1×10^6 PBMC were incubated for 40 min with HIV-$1_{Ba\text{-}L}$ or HIV-1_{HXB2} at an MOI of 0.0025 and with appropriate concentrations of APHS, AZT, DS or medium in the presence of 1/20 concentrated M buffer (Roche) and 4 U/ml DNase (Roche) to digest possible contaminating viral DNA associated with the virions. Afterwards, PBMC were washed and resuspended in medium with or without compounds. Cells were treated with 1 mg/ml Pronase (Roche) to remove virus particles bound to the cells. After 5 min of incubation at 37 °C and 5% CO_2, the cells were washed three times, resuspended in medium with or without the compounds and incubated at 37 °C and 5% CO_2. At different time points, 1.0×10^6 cells were added to 900 μl Nuclisens lysis buffer (Organon Teknika, Boxtel, The Netherlands) containing 40 μl silica to bind total nucleic acids (Nuclisens isolation kit). For the entry assays, samples were collected 2 h after the start of the infection; and for the reverse transcription assays, samples were collected 2, 7 and 10 h after the start of the infection.

2.9. HIV RNA/DNA extraction

HIV RNA and DNA were extracted from samples of infected cells according to a method described before (Boom et al., 1990). Briefly, after 10 min of mixing the silica and lysis buffer, tubes were shortly centrifuged at 13,000 rpm and the supernatant was discarded. Free non-nucleic acid components were removed by washing the silica particles twice with Nuclisens washing buffer, twice with 70% ethanol and once with acetone. The silica pellet was dried for 10 min at 56 °C. Total nucleic acids were eluted by resuspending the pellet in 100 μl nuclease-free water. After mixing the tubes for 10 min at 56 °C, tubes were shortly centrifuged and the supernatant was transferred to a new tube and stored at −20 °C.

2.10. RT-PCR using random hexamers

Extracted RNA was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) to obtain cDNA. The PCR mixture consisted of 10 μl of extracted RNA, 1× RT buffer, 5.5 mM $MgCl_2$, 0.5 mM dNTP, 2.5 μM Random hexamers, 0.4 U/μl RNase inhibitor, 1.25 U/μl MultiScribe RT (Taqman reverse transcription reagents kit, Applied Biosystems, Forster City, CA) and nuclease-free water. Amplification was performed in a GeneAmp PCR system 2400 (Perkin-Elmer) with a cycle program of 10 min at 25 °C, 30 min at 48 °C and 5 min at 95 °C.

2.11. Real-time 5′ nuclease (Taqman) PCR

Real-time Taqman PCR was based on cleavage of a probe, hybridized to the target sequence by 5′–3′ nuclease activity of Taq DNA polymerase during amplification, as described previously (Holland et al., 1991). Briefly, target sequence-specific forward and reverse primers prime the amplification reaction. The probe contains a reporter dye at the 5′ end and a quencher dye at the 3′ end. When both dyes are attached to the probe, reporter dye emission is quenched. The probe is displaced from the target during each polymeration cycle. Once separated from the quencher, the reporter dye emits its characteristic fluorescence, which can then be measured. The amount of fluorescence measured is proportional to the amount of PCR product.

HIV-1 gag cDNA and HIV-1 (−) strong stop and gag DNA were amplified and quantified by real-time Taqman PCR as described elsewhere (Pereira et al., submitted for publication). Sets of primers/probes were designed according to a previous study by Zack et al. (1990). To quantify the amount of gag RNA in the samples, a cDNA standard curve was prepared from five-fold dilutions of extracted RNA from an HXB2 viral batch, of which the RNA amount was previously determined. To quantify (−) strong stop and gag DNA in the samples, a DNA standard curve was prepared from five-fold dilutions of DNA extracted from U1 cells, which contain two copies of the HIV-1_{HXB2} genome per cell. Albumin was also quantified and used for normalization of all samples.

2.12. Cell-free RT assay

The HIV-1 RNA template (positions +1 to +368, with +1 being the capped G residue) was synthesized in vitro with T7 RNA polymerase as described previously (Beerens and Berkhout, 2000). In the reverse transcription assay, 10 ng of the RNA template was incubated with 1.5 μg calf liver tRNA (6 pmol total tRNA, of which approximately 1.2 pmol $tRNA^{lys3}$, Boehringer) or 20 ng of a DNA primer complementary to the primer binding site (PBS) (positions +182 to +202) in 12 μl annealing buffer (83 mM Tris–HCl, pH 7.5, 125 mM KCl) at 85 °C for 2 min and 65 °C for 10 min, followed by cooling to room temperature over a 1 h period. Reverse transcription was initiated by the addition of 6 μl RT buffer (9 mM $MgCl_2$, 30 mM DTT, 150 μg/ml actinomycine D, 30 μM dATP, dGTP and dTTP and 1.5 μM dCTP), 0.5 μl [α-^{32}P]dCTP and 0.5 U HIV-1 RT (MRC) in the presence or absence of APHS (10, 30, 300 and 1000 μM) or 10% ethanol. The reaction was incubated for 30 min at 37 °C. The cDNA product was precipitated in 0.3 M sodiumacetate, pH 5.2 and 80% ethanol at −20 °C, dissolved in formamide loading buffer and analyzed on a denaturing 6% polyacrylamide-urea sequencing gel.

3. Results

3.1. Effect of APHS on HIV-1 replication in primary cells

To assess whether APHS was capable of inhibiting HIV-1 replication, HIV-1 infection of PBMC was performed as described above. HIV-1 production was assessed by quantifying the levels of the HIV-1 capsid protein p24 in culture supernatants. The IC_{50} of AZT in PBMC was 3 nM (data not shown). This value fell within the reported range published in the Physicians' Desk Reference 2000 (PDR, 2000). APHS was capable of inhibiting HIV-$1_{Ba\text{-}L}$ production in PBMC in a dose-dependent manner (Fig. 1), while mock-infected cells showed undetectable levels of p24 (data not shown). At 30 μM, APHS inhibited HIV-$1_{Ba\text{-}L}$ replication by 80%. When the MOI was increased from 0.0025 to 0.01, the IC_{50} increased by a factor of 1.4 (data not shown). APHS was also capable of inhibiting HIV-1 replication in MDM and PBL in a dose-dependent manner. Results are shown in Table 1. The IC_{50} of APHS in PBMC infected with HIV-$1_{Ba\text{-}L}$, HIV-1_{AT} or HIV-1_{HXB2} was 6, 7 and 11 μM, respectively. The IC_{50} of APHS in MDM infected with HIV-$1_{Ba\text{-}L}$ and in PBL infected with HIV-1_{HXB2} was 12 μM.

3.2. Effect of APHS on viability of primary cells

To determine whether APHS could affect cellular viability, a cellular viability assay was performed as described above. The cytotoxicity of APHS was determined by quantifying cellular metabolic activity. The TC_{50} of AZT in PBMC was higher than 80 μM (data not shown). This value fell

Table 1
The effect of APHS on cellular viability and HIV-$1_{Ba\text{-}L}$, HIV-1_{HXB2}, HIV-1_{AT} replication in PBMC, MDM and PBL

Cells	HIV-1 strain	IC_{50}[a] (μM)	TC_{50}[b] (μM)
PBMC	Ba-L	6 ± 1	105 ± 15
	AT	7 ± 5	
	HXB2	11 ± 7	
MDM	Ba-L	12 ± 4	235 ± 127
PBL	HXB2	12 ± 4	147 ± 71

Results are the average of 10 independent experiments.
[a] IC_{50} represents 50% inhibitory concentration.
[b] TC_{50} represents 50% toxic concentration.

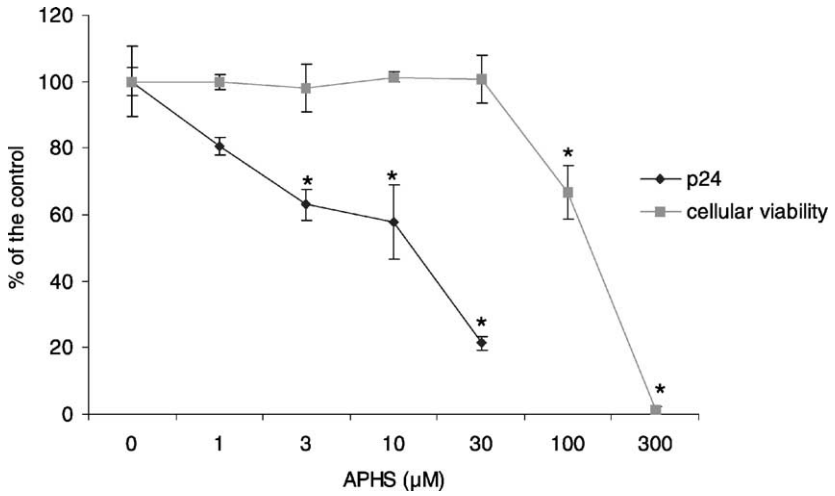


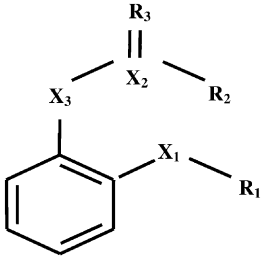
Fig. 1. The effect of APHS on HIV-$1_{Ba\text{-}L}$ replication in PBMC and on PBMC viability. To determine HIV-1 replication, APHS was administered simultaneously with HIV-1, and after 7 days HIV-1 production was determined by quantifying p24 antigen concentration in culture supernatant (shown as percentage of the control (HIV-infected PBMC not treated with APHS)). To determine cellular viability PBMC were incubated with APHS for 7 days after which cellular viability was determined by measuring PBMC metabolic activity (shown as percentage of control (PBMC not treated with APHS)). Results are representative of 10 independent experiments. $^{*}P < 0.05$ (according to Student's *t*-test).

within the reported range published in the Physicians' Desk Reference 2000 (PDR, 2000). The effect of APHS on PBMC viability is shown in Fig. 1. APHS was cytotoxic at concentrations above 30 μM. Table 1 shows the effect of APHS on PBMC, MDM and PBL. The TC_{50} of APHS in PBMC, MDM and PBL were 105, 235 and 147 μM, respectively.

3.3. Activity of APHS and related compounds

Since APHS was designed to be a potent and selective COX-2 inhibitor, a possible correlation between HIV-1 inhibition capacity and COX-2 inhibition capacity was studied further by using several compounds related to APHS, with and without COX-2 activity (Table 2). The following compounds are not clinically available: APHS-d1, a sulfone derivative of APHS without anti-COX-2 activity; APHS-d2, a heptyl sulfide derivative of APHS which is as potent as APHS but less selective against COX-2 than APHS; APHS-d3, a phenol hydrolysis product of APHS without COX-2 activity and APHS-d4, a methylsulfide analog of APHS which is more selective than APHS but 100-fold less potent. Also commercially available COX-2 inhibitors

Table 2
Effect of APHS, four APHS derivatives, aspirin, indomethacin and ibuprofen on HIV-$1_{Ba\text{-}L}$ replication and cellular viability in PBMC



	X_1	X_2	X_3	R_1	R_2	R_3	IC_{50}[a] (HIV-1)	TC_{50}[b]	SI[c] (HIV-1)
APHS	S	C	O	$CH_2C{\equiv}C(CH_2)_3CH_3$	CH_3	O	6 ± 1	105 ± 15	18
APHS-d1	SO_2	C	O	$CH_2C{\equiv}C(CH_2)_3CH_3$	CH_3	O	4 ± 1	345 ± 131	86
APHS-d2	S	C	O	$(CH_2)_6CH_3$	CH_3	O	5 ± 1	205 ± 168	41
APHS-d3	S	H	O	$CH_2C{\equiv}C(CH_2)_3CH_3$	–	–	28 ± 4	3 ± 1	<0.1
APHS-d4	S	C	O	CH_3	CH_3	O	223 ± 15	340 ± 151	2
Aspirin	–	C	O	CO_2H	CH_3	O	527 ± 210	8977 ± 8486	17
Indomethacin							21 ± 2	71 ± 10	3
Ibuprofen							44 ± 37	219 ± 41	5

Results are the average of ten independent experiments.
[a] IC_{50} represents 50% inhibitory concentration.
[b] TC_{50} represents 50% toxic concentration.
[c] The SI represents the TC_{50}/IC_{50} ratio.

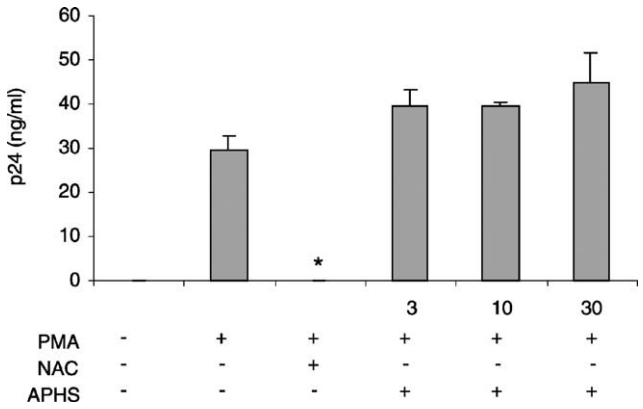


Fig. 2. The effect of APHS or NAC on HIV-1 production by the chronically HIV-1-infected U1 cell line. By incubating the cells with PMA (100 ng/ml) HIV-1 production was stimulated. Afterwards NAC (30 mM) or APHS (3, 10 and 30 μM) were added and after 2 days HIV-1 production was determined by quantifying the p24 antigen concentration (ng/ml) in culture supernatant. Results are the average of three independent experiments. $^{*}P < 0.05$ (according to Student's *t*-test).

were tested: aspirin, which is 60 times less potent and 100 times less selective than APHS; indomethacin, which is as potent but less selective than APHS and ibuprofen, which is less potent and less selective than APHS. The IC_{50} and TC_{50} of these compounds were determined in PBMC and the results are shown in Table 2. APHS and compounds APHS-d1 and -d2 showed the highest SI ratios (18, 86 and 41, respectively). Compounds APHS-d3 and -d4 showed lower SI ratios (lower than 0.1 and 2, respectively). Interestingly, aspirin could also inhibit HIV-1 replication but with an IC_{50} of approximately 100 times higher than APHS and a SI of 17. Indomethacin and ibuprofen showed SI values of 3 and 5, respectively. Since compounds APHS-d1 and -d3 are not COX-2 inhibitors and since the clinically used COX-2 inhibitors tested did not show good anti-HIV activity, the anti-HIV-1 activity of APHS does not seem to be related with its COX-2 inhibition activity.

3.4. Effect of APHS on HIV-1 production in U1 cells

To further elucidate the mechanism of action of APHS, a chronic infection model consisting of U1 cells that were incubated with increasing concentrations of APHS was performed. U1 is a clone of the U937 cell line, which is chronically infected with HIV-1 and shows minimal constitutive expression of HIV-1. When stimulated with cytokines or PMA, the production of HIV-1 by these cells increases 20-fold. HIV-1 production was assessed by quantifying the levels of the HIV-1 capsid protein p24 in culture supernatants of PMA-treated cells (Fig. 2). NAC was used as a positive control because it blocks PMA-induced HIV-1 expression. APHS was not capable of inhibiting HIV-1 production in this model. This supports the fact that APHS does not inhibit the post-integration steps of the HIV-1 life cycle.

3.5. Effect of APHS on HIV-1 entry into target cells

Since APHS seems to work at a step prior to transcription, a series of experiments was performed in order to determine the exact point in the HIV-1 life cycle that is being inhibited by APHS. The first set of experiments consisted in testing the effect of APHS on the entry of HIV-1 virions into PBMC. The amount of gag cDNA, which represents the viral RNA that entered into the target cells, was quantified. DS, a known entry inhibitor (Esté et al., 1997), was used as a positive control. The entry of HIV-1 virions was also investigated in PBMC that were pre-incubated with APHS and DS 24 h prior to infection. None of the drug concentrations used were cytotoxic to PBMC after 24 h incubation. Similarly to what was observed in the

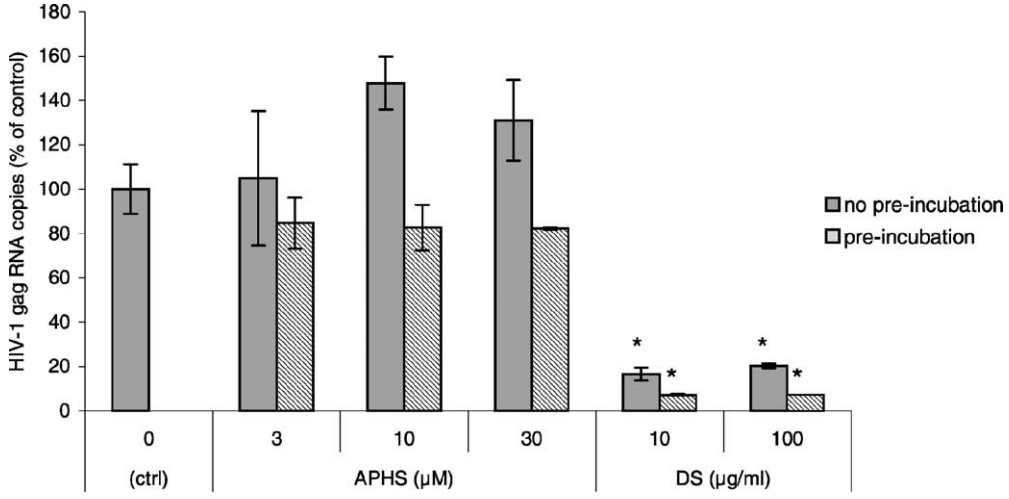


Fig. 3. Number of HIV-1_{Ba-L} gag RNA copies in PBMC treated with 10 or 30 μM APHS or 10 or 100 μg/ml DS as compared to the untreated control, at 2 h after infection. The results of PBMC that were pre-incubated with APHS and DS 24 h prior to infection and PBMC that were not pre-incubated are shown. Results are the average of three independent experiments. $^{*}P < 0.05$ (according to Student's *t*-test).

cytotoxic assay (Fig. 1), APHS concentrations above 30 μM proved to be significantly cytotoxic after 24 h incubation and were therefore not included in this assay. To measure the amount of HIV-$1_{Ba\text{-}L}$ RNA that has entered the cells 2 h after infection, viral gag RNA was quantified by real-time Taqman PCR. The results are shown in Fig. 3. DS inhibited viral entry by approximately 80% at all concentrations used ($P < 0.04$). The inhibition of viral entry by DS was further improved when PBMC were first pre-incubated with the compound. PBMC that were treated with APHS showed no inhibition of viral entry. Also, the amounts of viral RNA copies were not significantly decreased, when PBMC were pre-incubated with APHS prior to infection.

3.6. Effect of APHS on cellular HIV-1 reverse transcription

The effect of APHS on reverse transcription was also investigated using the RTI AZT as a positive control. None of the drugs at the concentrations used were cytotoxic to PBMC

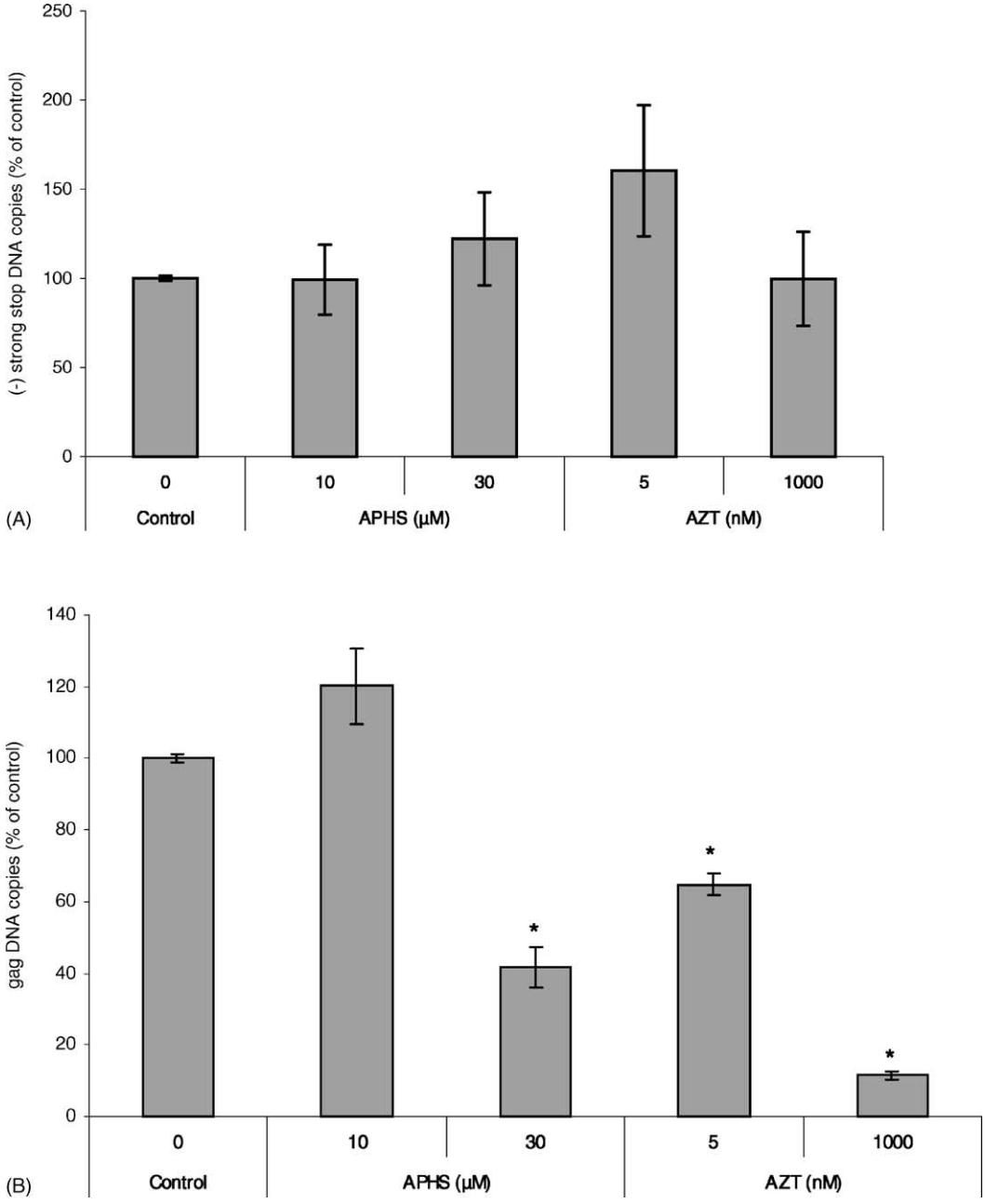


Fig. 4. Number of HIV-1_{HXB2} (−) strong stop (A) and gag (B) DNA copies in PBMC at 10 h after infection. PBMC were either untreated (control) or treated with 10 or 30 μM APHS or 5 or 1000 nM AZT (shown as percentage of the control). Results are the average of three independent experiments. $^{*}P < 0.05$ (according to Student's *t*-test).

after 24 h incubation (not shown). PBMC were pre-incubated for 24 h with different concentrations of APHS and AZT. HIV-1_{HXB2} DNA extracted from these cells was subjected to real-time Taqman PCR in order to quantify different parts of the HIV-1 genome which represent different stages of reverse transcription: (−) strong-stop DNA is produced before the first template switch and gag DNA is produced after the first template switch.

The effect of APHS and AZT on the synthesis of (−) strong stop and gag DNA on completion of reverse transcription are shown in Fig. 4. Since in our assay, reverse transcription was completed between 7 and 10 h the results at 10 h are shown here. APHS- or AZT-treated PBMC did not show a significant decrease in (−) strong-stop DNA copy numbers as compared to control untreated PBMC (Fig. 4A). It has previously been reported that (−) strong-stop DNA production is relatively insensitive to AZT. AZT preferentially terminates viral DNA synthesis after the first template switch and in this way (−) strong-stop DNA can be present for at least 6 days after infection in AZT-treated cultures (Arts and Wainberg, 1994). It is also known that partial reverse transcription is initiated in the virion before entry into the host cell resulting in an excess of early versus late transcripts inside the virion (Trono, 1992). A small but not significant increase in (−) strong-stop DNA copies was observed for PBMC treated with 30 μM APHS or 5 nM AZT.

The gag cDNA levels after completion of reverse transcription in APHS- or AZT-treated PBMC as compared to untreated PBMC is shown in Fig. 4B. At 10 h after infection, no significant differences could be detected between untreated PBMC and PBMC treated with 10 μM of APHS. However, 30 μM APHS significantly inhibited gag production by about 60%. A 35 or 90% reduction in gag cDNA copies was observed in PBMC treated with 5 or 1000 nM AZT, respectively.

3.7. Cell-free RT assay

To test whether APHS can inhibit RT directly, we performed a cell-free RT assay. We used an in vitro synthesized HIV-1 RNA template encompassing the complete untranslated leader region (+1 to +368) in combination either with the natural $tRNA^{lys3}$ primer or with a DNA primer complementary to the PBS to initiate reverse transcription (Fig. 5). The primers were heat-annealed at 85 °C, and reverse transcription was initiated by the addition of dNTPs and HIV-1 RT enzyme. Extension of the DNA primer produced a full-length cDNA product of 202 nt (lane 1), whereas extension of the tRNA primer produced a 257 nt product (lane 7). Shorter cDNAs represent RT pauses due to stable RNA secondary structure in the HIV-1 template (Beerens et al., 2000). Concentrations of APHS that ranged from 10 to 300 μM did not affect cDNA formation, while at 1000 μM APHS decreased the amount of reverse transcription product. However, addition of APHS from the stock solution in ethanol raised the ethanol concentration during reverse transcription to 5% for the 1000 μM sample. Therefore, control reactions were performed in the presence of 10% ethanol (lanes 6 and 12). Indeed, ethanol by itself inhibited the reverse transcription. This indicates that the effect observed with 1000 μM APHS is probably due to the ethanol in the sample.

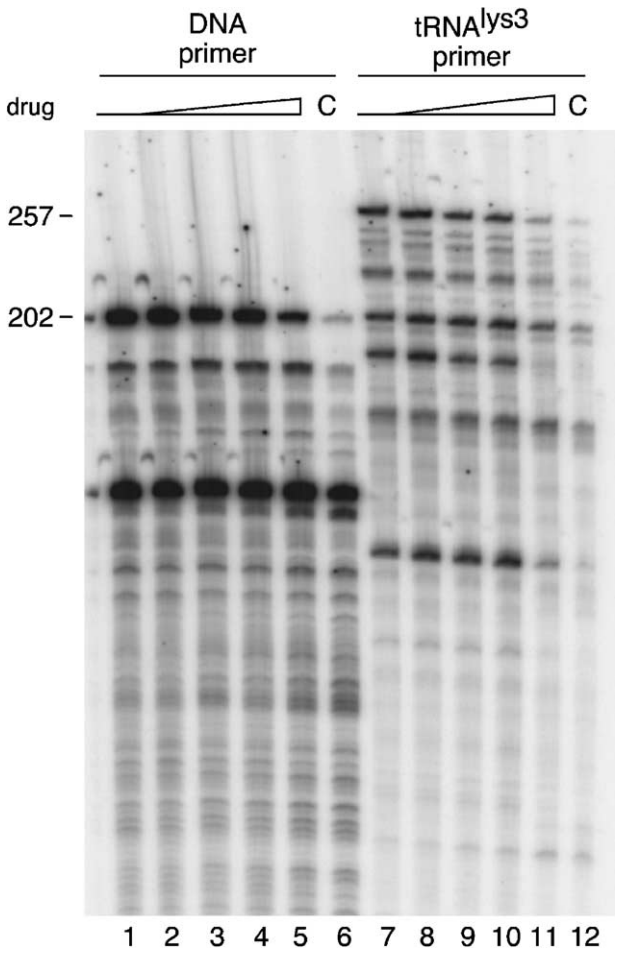


Fig. 5. Reverse transcription in the presence of APHS. The DNA primer complementary to the PBS (lanes 1–6) or the natural $tRNA^{lys3}$ primer (lanes 7–12) were heat-annealed onto the in vitro synthesized HIV-1 RNA template, and reverse transcription was performed by the addition of dNTPs and HIV-1 RT enzyme (lanes 1 and 7). An increasing amount of APHS (10, 100, 300 and 1000 μM) was added to the reverse transcription reaction (lanes 2–5 and 8–11). Control reactions in the presence of 10% ethanol were performed in lanes 6 and 12. Results are representative of three independent experiments.

4. Discussion

In this study, it was demonstrated that APHS inhibited the replication of several HIV-1 strains in human primary cells (PBMC, MDM and PBL) at non-toxic concentrations. Since it was previously shown that cell proliferation is not required for productive HIV-1 infection of MDM (Schmidtmayerova

et al., 1997), it can be concluded that the antiviral activity of APHS is not mediated by inhibition of cell proliferation. The other NSAIDs tested did not show better anti-HIV activity than APHS, which suggests that anti-inflammatory activity is not necessary for anti-HIV activity. Furthermore, one of two APHS derivatives that had no anti-inflammatory activity did inhibit HIV-1 replication slightly better than APHS did.

APHS did inhibit HIV-1 replication before provirus integration into the host cell genome occurred. HIV-1 life cycle kinetics was then used to investigate the possible effect(s) of APHS on entry of HIV-1 into host cells and on reverse transcription. It has been estimated that all virions, bound to the host cell, will have entered the cell 2 h after infection (Reddy and Yin, 1999). In the entry assay, entry of HIV-1 virions was measured at 2 h after infection. DS, which inhibits binding of virions to the host cell by interacting with gp120 (Esté et al., 1997), significantly decreased the amount of viral gag RNA entering the host cell. APHS did not have a significant effect on entry of HIV-1 virions into host cells.

The RTI AZT significantly decreased the amount of gag DNA but not of (−) strong-stop DNA, which is produced before the first template switch. Indeed, previous studies have shown that AZT is preferentially incorporated into the growing DNA chain after the first template switch (Arts and Wainberg, 1994). At 30 μM APHS was able to decrease the production of gag DNA, which is a late product of the reverse transcription. This observation suggests that APHS may target a step between entry and reverse transcription or the reverse transcription itself. But since APHS does not inhibit the production of (−) strong stop DNA, which is an early product of the reverse transcription, it can be concluded that APHS is targeting the reverse transcription itself. However, at 10 μM, the IC_{50} of APHS in primary cells as described in Section 3.1, APHS did not inhibit gag DNA production in the real-time Taqman PCR assay. This discrepancy is probably due to experimental differences between the two assays. Perhaps longer incubation times may enable APHS to protect more cells. Also the method used for quantification of HIV replication differs between the two assays. The same phenomenon was observed for AZT. The IC_{50} of AZT in the PBMC was 3 nM, while in the real-time Taqman PCR 5 nM AZT showed only 40% protection against HIV. The effect of APHS could not be studied at concentrations higher than 30 μM because of toxicity for the cells. Compounds are being developed by systemic variation of structural groups of APHS in order to find a compound related to APHS, which may have a more favorable selectivity index. Thus, APHS is inhibiting HIV-1 replication by interfering with the RT enzyme or with some viral or cellular factor(s) involved in the reverse transcription process.

The most critical cellular factor involved in reverse transcription is the deoxynucleoside-5′-triphosphate (dNTP) pool. APHS could have a mechanism of action similar to hydroxyurea, which is known to inhibit the cellular enzyme ribonucleotide reductase, thus reducing the intracellular levels of dNTPs and impeding HIV DNA synthesis (Gao et al., 1993). It is also known that the virus requires no cellular factors after infection, except for a nucleotide pool, to achieve initiation of reverse transcription (Harrich and Hooker, 2002). Since we know that APHS could not affect the replication of the first product of the reverse transcription, it is reasonable to assume that APHS is not affecting dNTP levels in the cell.

Many viral factors including nef, tat, vif, vpr, matrix protein, nucleocapsid protein and integrase are involved in reverse transcription (Harrich and Hooker, 2002) and are potential targets for APHS. APHS can inhibit the production of the reverse transcription gag DNA but not of the (−) strong-stop DNA indicating that APHS inhibits the elongation process and not the initiation of reverse transcription. This mechanism of action is similar to that of nucleoside RT inhibitors (NRTIs) but not to that of non-nucleoside RT inhibitors (NNRTIs) (Hooker et al., 2001). Moreover, the structure of APHS is very different from a typical NRTI. Interestingly, a previous study demonstrated the potential of aspirin in the synthesis of AZT and 3′-deoxy-3′-fluorothymidine (FLT) prodrugs (Zahran et al., 1996). Our findings suggest that APHS may target the reverse transcription process in a different way from that of the known RTI.

APHS showed no inhibition of RT activity in the primer extension assay. This result can be interpreted in two different ways: APHS does not inhibit RT directly but it affects some cellular or viral factor involved in reverse transcription process or APHS may have to be metabolized by the cell in order to become active and, in this way, its anti-RT activity can only be detected in cell-based systems. This last option is reinforced by the fact that the NRTIs AZT and dideoxyinosine (ddI) have to be phosphorylated by cellular enzymes to become active. These NRTIs are only able to terminate chain elongation in cell-free assays when added in the triphosphate form (St Clair et al., 1987). We do not yet know how the cell metabolizes APHS into its antivirally active form.

The fact that APHS is also an anti-inflammatory compound may be useful in anti-HIV therapy because it targets not only virus replication but also inflammatory processes. This can be beneficial in reaching areas like the brain where inflammation is thought to account for severe neurodegeneration associated with HIV-1 infection (Kaul et al., 2001).

Acknowledgements

This work was supported by grants from the The Netherlands Organization for Scientific Research Program STIGON (014-80-107) to Dr. Hans S.L.M. Nottet and from the National Institutes of Health (CA89450) to Dr. Lawrence J. Marnett. Cândida da Fonseca Pereira is a Marie

Curie fellow. We are grateful to L. de Graaf and P.C. Arts for technical support and Dr. C.A. Boucher for critical reading of the manuscript. The following reagent was obtained through the NIH AIDS Research and Reference Program, Division of AIDS, NIAID, NIH: U1/HIV-1 cell line from Dr. Thomas Folks.

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