APHS can act synergically with clinically available HIV-1 reverse transcriptase and protease inhibitors and is active against several drug-resistant HIV-1 strains in vitro

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Objectives: The use of multiple drug combinations in current anti-human immunodeficiency virus (HIV) therapy allows lower dosages of individual drugs and results in enhancement of the therapeutic effect due to synergic interactions between different drugs. We have shown that o-(acetoxyphenyl)hept-2-ynyl sulphide (APHS), a recently developed non-steroidal anti-inflammatory drug, shows anti-HIV activity in a dose-dependent manner. The first aim of this study was to investigate whether APHS can act synergically with the clinically available reverse transcriptase and protease inhibitors (RTIs and PIs, respectively) in vitro. Because of the increasing prevalence of RTI- and PI-resistant HIV-1 strains, the second aim of this study was to assess the antiviral activity of APHS against drug-resistant HIV-1 strains in vitro.

Materials and methods: HIV-infected peripheral blood mononuclear cells (PBMC) were treated for 7 days with different combinations of APHS and RTIs or PIs. The MT-2 cell line was infected with different HIV-1 strains and treated with APHS for 5 days.

Results: APHS showed synergic interactions with the RTIs zidovudine, lamivudine and efavirenz and with the PIs indinavir and ritonavir. The 50% inhibitory concentration (IC50) of APHS in this assay dropped from 13 µM when used alone, to 5 µM after combination with an RTI or PI. In combination with APHS the IC50 of the RTI and PI drugs tested also dropped. APHS inhibits the replication of HIV-1 strains resistant to zidovudine, lamivudine, stavudine, didanosine, zalcitabine and ritonavir.

Conclusions: These results indicate that APHS can be combined with RTIs and PIs and can inhibit several NRTI and PI-resistant HIV-1 strains.

Keywords: o-(acetoxyphenyl)hept-2-ynyl sulphide, Calcusyn, peripheral blood mononuclear cells, MT-2 cell line

Introduction

Several steps in viral replication are susceptible to the action of anti-human immunodeficiency virus (HIV) agents. Current anti-HIV therapy uses two classes of drugs: reverse transcriptase (RT) inhibitors (RTIs) and protease inhibitors (PIs). The first class of drugs comprises two subgroups: (i) nucleoside/nucleotide RTIs (NRTIs) such as zidovudine, lamivudine, didanosine, stavudine, zalcitabine, abacavir and tenofovir disoproxil fumarate; and (ii) non-nucleoside RTIs (NNRTIs) such as efavirenz, delavirdine and nevirapine. Examples of PIs are indinavir, ritonavir, saquinavir, amprenavir and nelfinavir. NRTIs prevent elongation of the DNA chain, which results in DNA chain termination. NNRTIs bind to an allosteric site of RT adjacent to the polymerase active site, rendering it inactive. PIs bind to the viral protease and in

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this way they prevent processing of virus proteins and virus maturation. Although use of these drugs rapidly reduces HIV RNA levels, long-term treatment leads to the emergence of drug resistance and toxicity. HIV RT lacks proofreading activity, which results in a high mutation rate of $4 \times 10^{-5}$ mutations per bp. Since the estimated average of HIV-1 production is $10.5 \times 10^9$ virions per day, replication-competent, drug-resistant viruses are rapidly selected.

Current anti-HIV therapy consists of combinations of three or more antiretroviral drugs, the so-called highly active antiretroviral therapy (HAART). This therapy is more efficient in suppressing viral production than single drug therapy. Retroviral therapy (HAART). This therapy is more efficient in suppressing viral production than single drug therapy. Also, synergic interaction, defined as a combined effect greater than expected from the additive effect of the individual drugs, has been shown for different combinations of anti-HIV drugs. However, drug combination can also have undesirable side-effects such as emergence of cross-resistance mutations, combined toxicity and, as a consequence, poor adherence to the treatment and frequent alteration in the panel of agents used.

Intervention at more than a single step in the HIV replication cycle will probably be more efficient in suppressing viral replication and avoiding resistance. Therefore, in the last few years several anti-oxidative compounds, anti-proliferative compounds, anti-inflammatory compounds and compounds that target new viral and cellular factors involved in virus replication have been proposed as anti-HIV agents. In addition to having good antiviral activity, new drugs should also be pharmacologically compatible with other anti-HIV drugs, show minimal toxicity and inhibit HIV strains that are resistant to clinically available drugs.

We have previously found that $o$-(acetoxyphenyl)hept-2-ynyl sulphide (APHS) (Figure 1), a selective non-steroidal anti-inflammatory drug (NSAID), can inhibit the replication of several HIV-1 strains ($Ba-L$, $HXB2$ and $AT$) in primary cells (peripheral blood mononuclear cells, monocyte-derived macrophages and peripheral blood lymphocytes) in vitro by interfering with the reverse transcription process. APHS 50% inhibitory concentration (IC$_{50}$) for HIV-1 was 6 $\mu$M, whereas its 50% toxic concentration for primary cells was 105 $\mu$M.

The aim of this study was to investigate whether APHS shows synergic interactions with the clinically available drugs zidovudine, lamivudine, efavirenz, indinavir and ritonavir, and whether APHS can inhibit the replication of NRTI and PI-resistant HIV-1 strains.

**Materials and methods**

**Isolation of primary cells**

Donor peripheral blood mononuclear cells (PBMCs) 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, PBMCs isolated from six donors were pooled together in RPMI 1640 medium (Gibco/Invitrogen, Paisley, UK) supplemented with 10% dimethyl sulphoxide (DMSO; Merck, Darmstadt, Germany), 20% fetal calf serum (FCS; Invitrogen) and 10 $\mu$g/mL gentamicin (Invitrogen) and frozen at $-140^\circ$C. Cells were thawed and cultured for 4 days before the experiment in RPMI 1640 medium supplemented with 10% FCS, and 10 $\mu$g/mL gentamicin containing 2 $\mu$g/mL phytohaemagglutinin (PHA) lectin from *Phaseolus vulgaris* (Sigma Chemie, Zwijndrecht, The Netherlands) at $37^\circ$C and 5% CO$_2$.

**Compounds**

APHS was supplied by Dr L. J. Marnett. Synthesis details are described by Kalogutkar et al. APHS was diluted in aliquots in 100% ethanol, topped with argon gas and stored at $-20^\circ$C. The concentration of ethanol during incubations never exceeded 0.1%. At this concentration, ethanol did not affect HIV-1 replication or cellular viability (data not shown). Zidovudine (Sigma), indinavir (Merck) and ritonavir (Abbott Laboratories S.A., Baar, Switzerland) were diluted in DMSO and lamivudine (GlaxoSmithKline, Middlesex, UK) and efavirenz (Merck) were diluted in water. 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).

**HIV-1 infection of PBMCs**

PHA-stimulated PBMCs were washed twice to remove PHA and incubated for 7 days at a concentration of $5 \times 10^5$ cells/mL with HIV-1$_{Ba-L}$ at a multiplicity of infection (MOI) of 0.0025, in the presence of APHS and/or other drugs and 10 U/mL recombinant interleukin-2 (IL-2) (Roche Diagnostics Nederland B. V., Almere, the Netherlands), at $37^\circ$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.

![Chemical structure of $o$-(acetoxyphenyl)hept-2-ynyl sulphide (APHS).](image-url)

Figure 1. Chemical structure of $o$-(acetoxyphenyl)hept-2-ynyl sulphide (APHS).
Combinations of APHS with other anti-HIV compounds

**p24-core antigen quantification by ELISA**

After 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 ELISA (AMPK, DAKO, Cambridgeshire, UK) as described previously.39,40 The absorbance 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.

**Determination of viability of PBMCs**

After 7 days incubation, the metabolic activity of PBMCs from the HIV-1 infection model was assessed by a cellular viability assay, as described previously.41 Briefly, 150 µg/mL tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma) was added to the cells. During the subsequent 2 h incubation period, metabolically active cells convert MTT into blue formazan crystals. Afterwards, three-quarters 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). When all formazan crystals were dissolved absorbance was measured at 550 nm using 630 nm as reference.

**Determination of IC50 of the compounds**

The ability of each compound to decrease p24 antigen was expressed as IC50. IC 50 was calculated using the computer software program CalcuSyn for Windows (Biosoft, Cambridge, UK) according to the method of Chou & Talalay.18,42 This program uses the median-effect equation to produce dose–effect curves:

\[ f_a = \frac{1}{1 + \left( \frac{D_m}{D} \right)^m} \]  
(Equation 1)

where \( f_a \) represents the fraction affected by the dose (reduction of p24 at a certain drug concentration expressed in decimals), \( D_m \) is the median effect dose (same as IC50), \( D \) is the dose of the drug and \( m \) is the sigmoidicity coefficient of the dose–effect curve. Data were accepted when the linear correlation coefficient of the median-effect plot based on experimental data was >0.90.

**Combination of HIV-1 inhibitors**

In the combination experiments, PBMCs were infected as described above and seeded in the presence of multiple-diluted fixed ratios of a combination of APHS and the clinically available drugs zidovudine, lamivudine, efavirenz, indinavir and ritonavir, in duplicate wells, according to the chequerboard design (Table 1). Before the experiments were carried out, IC50 values of all drugs were determined as described before in order to choose the appropriate dosage ranges for the combinations with APHS. Concentrations of APHS, zidovudine, lamivudine, efavirenz, indinavir and ritonavir were in the range 0.75–24 µM, 0.63–10 nM, 0.63–10 nM, 37.5–600 pM, 0.63–10 nM and 0.88–14 nM, respectively. After 7 days of incubation, p24 antigen in culture supernatant was quantified by ELISA and cellular viability was assessed by a cytotoxicity assay. \( f_a \) was calculated for each drug combination after p24 quantification. Using the CalcuSyn program, dose–effect curves were plotted for each drug combination, i.e. each dose was plotted against the corresponding \( f_a \) value. Using the multiple drug-effect equation, which is based on the median-effect equation and the isobologram method, IC50 was calculated for both drugs alone and in combination at their equipotent ratio (IC50 APHS/IC50 drug). Data were accepted when the linear correlation coefficient of the median-effect plot based on experimental data was >0.90. The combination index (CI), a quantitative measure of drug interaction, was calculated for each \( f_a \) at three different drug ratios (1:2, 1:1 and 2:1) based on the IC50 values of each drug used alone [e.g. 1:1 drug ratio represents the equipotent ratio (IC50 APHS:IC50 drug)], according to the following equation:

\[ CI_x = \left( \left( \frac{D_1}{D_x} \right)^{1/2} \times \left( \frac{D_2}{D_x} \right)^{1/2} + \frac{D_1}{D_x} \times \frac{D_2}{D_x} \right) \]  
(Equation 2)

<table>
<thead>
<tr>
<th>↓</th>
<th>0</th>
<th>0.125 × IC50</th>
<th>0.25 × IC50</th>
<th>0.5 × IC50</th>
<th>1 × IC50</th>
<th>2 × IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>0</td>
<td>0.125 × IC50</td>
<td>1:1</td>
<td>1:2</td>
<td>2:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25 × IC50</td>
<td>1:1</td>
<td>2:1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 × IC50</td>
<td>1:2</td>
<td>1:1</td>
<td>2:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 × IC50</td>
<td>1:2</td>
<td>1:1</td>
<td>2:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 × IC50</td>
<td>1:2</td>
<td>1:1</td>
<td></td>
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</tr>
</tbody>
</table>
where CI\textsubscript{x} stands for the combination index at x\% inhibition, (D\textsubscript{x})\textsubscript{1} and (D\textsubscript{x})\textsubscript{2} are the doses of drug 1 and 2 used when inhibiting x\% in combination and (D\textsubscript{x})\textsubscript{1} and (D\textsubscript{x})\textsubscript{2} are the doses of drug 1 and 2 alone, inhibiting x\% (calculated from equation 1). A drug combination is additive when CI \approx 1, synergic when CI < 1 (combined effect is greater than the additive effect) and antagonistic when CI > 1 (combined effect is less than the additive effect). As the range of CI \ll 1, the degree of synergic interaction gets stronger. 18 CI values were calculated using the mutually non-exclusive assumption.

Drug susceptibility assay

MT-2 cells (Medical Research Council, London, UK) were cultured in RPMI 1640 medium supplemented with 10% FCS and 10 \( \mu \)g/mL gentamicin, and passaged once a week until a maximum of 20 passages. The cells were maintained at 37°C, 5% CO\textsubscript{2}. One day before the experiments, the cells were passaged 1:1 in a concentration of 1 \( \times \) 10\textsuperscript{6} cells/mL.

HXB2 is the molecular clone of the first laboratory HIV-1 isolate. The genes that contain the drug resistance mutations were excised from the clinical isolates and cloned into the genetic background of HXB2. Thus all HIV-1 strains tested have the same genetic background. 41 + 215Y is a zidovudine-resistant HIV-1 strain as described by Jeeninga \textit{et al.} This strain contains one amino acid (aa) change at codon 41 (Met is substituted by Leu) and one aa change at codon 215 (Thr is substituted by Tyr). 184V is a lamivudine-resistant HIV-1 strain as described by Schuurman \textit{et al.} This strain contains one aa change at codon 184 (Met is substituted by Val). Strain 3096 is an RTI-resistant HIV-1 strain as described by de Jong \textit{et al.} This strain contains an insertion of two aa between codons 68 and 69 of RT as well as an aa change at codon 67. Phenotypic resistance analysis showed high levels of resistance to zidovudine, lamivudine and stavudine, and moderate levels of resistance to didanosine and zalcitabine. Strain 4602 is a ritonavir-resistant HIV-1 strain as described by Nijhuis \textit{et al.} It contains the following four mutations: 36I, 54V, 71V and 82T.

The susceptibility of these HIV-1 strains to APHS was analysed in a cell-killing assay (MTT assay) as described previously. Briefly, MT-2 cells in a concentration of 0.4 \( \times \) 10\textsuperscript{6} cells/mL were seeded with or without HIV-1 wild-type or drug-resistant mutants at an MOI of 0.002 or 0.006 and in the presence of increasing concentrations of APHS and maintained at 37°C, 5% CO\textsubscript{2}. After 5 days of incubation, a cellular viability assay was carried out as described above. In this case, the amount of formazan reflects the number of cells protected by the drug against killing by the virus and is used as a read out for drug susceptibility. The IC\textsubscript{50} values for the wild-type and for each of the virus mutants were determined using a computer software program.

Table 2. IC\textsubscript{50} of APHS and clinically available anti-HIV drugs alone and after combination at their equipotent ratio

<table>
<thead>
<tr>
<th>Combination</th>
<th>IC\textsubscript{50}a</th>
<th>IC\textsubscript{50}b</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>APHS (( \mu )M)</td>
<td>drug (nM)</td>
</tr>
<tr>
<td>APHS/zidovudine</td>
<td>15 ( \pm ) 7c</td>
<td>3 ( \pm ) 1</td>
</tr>
<tr>
<td>APHS/lamivudine</td>
<td>10 ( \pm ) 4</td>
<td>10 ( \pm ) 3</td>
</tr>
<tr>
<td>APHS/efavirenz</td>
<td>14 ( \pm ) 3</td>
<td>1 ( \pm ) 0.3</td>
</tr>
<tr>
<td>APHS/indinavir</td>
<td>12 ( \pm ) 4</td>
<td>11 ( \pm ) 1</td>
</tr>
<tr>
<td>APHS/ritonavir</td>
<td>15 ( \pm ) 3</td>
<td>6 ( \pm ) 2</td>
</tr>
</tbody>
</table>

\textsuperscript{a}IC\textsubscript{50} represents the 50% inhibitory concentration.

\textsuperscript{b}Combinations were carried out at the equipotent ratio (1:1 or IC\textsubscript{50} APHS/IC\textsubscript{50} drug).

\textsuperscript{c}Values represent mean \( \pm \) S.E.M. of at least three independent experiments.

\textsuperscript{*}P < 0.05 (according to Student’s t-test).
alone varied from 10 to 15 µM (mean 13 µM). When used alone, the IC_{50}s of zidovudine, lamivudine, efavirenz, indinavir and ritonavir were 3, 10, 1, 11 and 6 nM, respectively. IC_{50}s of these clinically available drugs were comparable to those previously described in the literature. The IC_{50} of APHS dropped to 4, 5 and 6 µM (mean 5 µM) when used in combination with another drug. The IC_{50}s of zidovudine, lamivudine, efavirenz, indinavir and ritonavir dropped to 2, 3, 0.2, 5 and 3 nM, respectively. Although a definite trend was evident, the only statistically significant decreases in IC_{50}s were observed for indinavir when used in combination with APHS and for APHS when used in combination with efavirenz (P < 0.05 according to the Student’s t-test).

In order to investigate possible synergic interactions between APHS and RTIs or PIs, CIs were determined for each combination of drugs at three different drug ratios. These drug ratios were based on the IC_{50}s of the individual drugs. Additive interaction between multiple drugs is indicated when CI = 1, synergic interaction is indicated when CI < 1 and antagonistic interaction is indicated when CI > 1. For each combination, CI values for three drug ratios were determined at the calculated 50%, 75% and 95% of HIV-1 inhibition levels.

Results are shown in Table 3. The combination of APHS with zidovudine yielded moderate synergic to nearly additive interactions at the 1:2 drug ratio. At the 1:1 drug ratio, moderate to synergic interactions were observed. Synergic interactions were found at 50% and 75% of inhibition at the 2:1 drug ratio. For 95% of inhibition, no conclusions could be drawn due to the high sample variation. Thus, the combined effect of APHS with zidovudine was additive to synergic. The combination of APHS and lamivudine at the 1:2 drug ratio showed slight synergic interactions for 50% of inhibition but strong synergic interactions for 75% and 95% of inhibition. At the 1:1 drug ratio, moderate synergic interactions were observed for 50% of inhibition but strong synergic interactions were observed for 75% and 95% of inhibition. The 2:1 drug ratio showed slight antagonism interactions at 50% of inhibition, but synergic to strong synergic interactions were found at higher effect levels. So, the combination of APHS and lamivudine yielded synergic to strong synergic interactions at higher levels of inhibition in all drug ratios tested. The combination of APHS and efavirenz showed nearly additive interactions for 50% and 75% of inhibition but synergic interactions were observed for 95% of inhibition at the 1:2 drug ratio.
ratio. At the 1:1 drug ratio, synergic interactions for 50% and 75% of inhibition were observed but strong synergic interactions were observed for 95% of inhibition. At the 2:1 drug ratio, sample variation was too high for conclusions to be drawn. Thus, the combination APHS/efavirenz showed synergic interactions at higher levels of inhibition for the 1:2 and 1:1 drug ratios. The combination of APHS and indinavir yielded moderate synergic interactions at the 1:2 drug ratio. At the 1:1 drug ratio, the combination showed synergic to nearly additive interactions at the lowest percentage of inhibition levels. At 95% of inhibition no conclusions could be drawn due to sample variation. At the 2:1 drug ratio, slight synergic interactions were observed for 50% of inhibition but synergic interactions were observed for 75% and 95% of inhibition. In conclusion, the combination of APHS and indinavir showed synergic to additive interactions for all drug ratios tested. For the combination of APHS and ritonavir at the 1:2 drug ratio, slight synergic interactions were observed for 50% of inhibition, synergic interactions were observed for 75% of inhibition and slight antagonistic interactions were observed for 95% of inhibition. At the 1:1 drug ratio, moderate antagonistic interactions were observed for 50% of inhibition, nearly additive interactions were observed for 75% of inhibition and slight synergic interactions were found for 95% of inhibition. At the 1:2 drug ratio, slight synergic interactions were found for 75% of inhibition and synergic interactions were observed for 95% of inhibition. At 50% of inhibition, synergic interactions were observed for 50% of inhibition but nearly additive interactions at the lowest percentage of inhibition levels. At 95% of inhibition no conclusions could be drawn due to sample variation. At the 2:1 drug ratio, slight synergic interactions were observed for 50% of inhibition but synergic interactions were observed for 75% and 95% of inhibition. In conclusion, the combination of APHS and indinavir showed synergic to additive interactions for all drug ratios tested. For the combination of APHS and ritonavir at the 1:2 drug ratio, slight synergic interactions were observed for 50% of inhibition, synergic interactions were observed for 75% of inhibition and slight antagonistic interactions were observed for 95% of inhibition. At the 1:1 drug ratio, moderate antagonistic interactions were observed for 50% of inhibition, nearly additive interactions were observed for 75% of inhibition and slight synergic interactions were found for 95% of inhibition. At the 2:1 drug ratio, slight synergic interactions were found for 75% of inhibition and synergic interactions were observed for 95% of inhibition. At 50% of inhibition, no conclusions could be drawn due to sample variation. Thus, the combination of APHS and ritonavir resulted in synergic to moderate antagonistic interactions.

Drug susceptibility assay

To determine the susceptibility of wild-type HIV-1 and several NRTI- or PI-resistant HIV-1 strains for APHS, a drug susceptibility assay was carried out. This assay gives information not only about antiviral activity of APHS but also about its cytotoxicity patterns. The IC50 of APHS for wild-type HIV-1 and drug-resistant strains are depicted in Table 4. The IC50 of APHS for the wild-type strain HXB2 was 10 µM. The IC50 of APHS for the zidovudine-resistant strain 41 + 215Y was 2 µM. The IC50 of APHS for the lamivudine-resistant strain 184V was 5 µM. The IC50 of APHS for the lamivudine-, lamivudine-, stavudine-, didanosine- and zalcitabine-resistant strain 3096 was 4 µM. The IC50 of APHS for the ritonavir-resistant strain 4602 was 5 µM. APHS did not show any significant toxicity for MT-2 cells at the concentrations tested and therefore it can be concluded that APHS was capable of inhibiting the replication of all four HIV-1 strains tested.

Discussion

We have previously shown that APHS, a novel selective NSAID derived from aspirin, can inhibit HIV-1 replication in vitro by interfering with the reverse transcription process.37

It is important to investigate the interaction between new drugs and currently used drugs, especially the determination of the possible synergic interactions and the possible adverse effects arising from the combination. In this study, there was a clear trend towards a decrease in IC50 of the drugs when used in combination than when used alone. This means that the same effect can be obtained with lower doses of the individual drugs and, in this way, toxic side-effects are also minimized.

There are several methods described in the literature to determine synergic interaction.46 We used the computer program CalcuSyn,42 which is based on the median-effect principle, as described before.18 This program is simple to use, is not limited by the number, effect and interactions of the drugs tested, and only requires a minimum of data points to analyse drug interaction. CalcuSyn is often used in combination studies of anti-HIV agents.45,47–49 It has been shown that the results obtained by this method are comparable to those of other methods such as MacSynergy, especially if more than one drug ratio is employed.20,48

Although combinations of APHS with the RTIs zidovudine, lamivudine and efavirenz showed, for all drug ratios, slight antagonistic to synergic interactions at the 50% inhibition level, moderate synergic to strong synergic interactions were observed at the 95% inhibition level. The fact that synergic interactions between APHS and RTIs become stronger at higher percentages of inhibition is important for in vivo therapeutics when a higher percentage of inhibition is desired. The strongest synergic interactions were found between APHS and lamivudine. Moderate antagonistic to synergic interactions were found between APHS and the PIs indinavir and ritonavir. No correlation was found between the percentage of inhibition level and the degree of interaction between APHS
and PI. Slightly different effects were observed at different drug ratios for all combinations tested. This reflects the importance of choosing the most effective drug ratio for a combination of antiviral drugs.

The strong synergic interactions between APHS and RTIs are probably because APHS also inhibits the reverse transcription process. There are many studies that report a benefit arising from the combination of NRTIs and NNRTIs, Although the mechanism for synergy seen with the use of NNRTIs and NRTIs is not known, it is conceivable that the interaction of the NNRTI with the RT at the NNRTI-binding site, which has been shown to cause conformational distortion of the catalytic aspartate triad, may allow improved incorporation of the NRTI into the growing DNA molecule, leading to more efficient chain termination or decreased rates of phosphorolytic removal of the terminator. APHS can also interact synergically with PIs. In acutely infected cell cultures, individual cells are present at different stages of the HIV-1 life cycle. Several other RTIs have shown synergic interactions with PIs. Combination of antiviral compounds with different targets can inhibit HIV-1 replication in a greater proportion of cells at different stages of viral replication, resulting in an increased viral suppression. Importantly, no strong antagonism was found between APHS and RTIs or PIs. Although in vivo studies will have to be conducted, these data indicate that APHS can safely be used in combination with RTIs and PIs.

New drugs should not only be pharmacologically compatible with currently available drugs but also be able to inhibit the replication of drug-resistant HIV strains. Some compounds with this characteristic are being developed. In this study, APHS inhibited the replication of both wild-type and several NRTI and PI-resistant HIV-1 strains. Since the mutations tested are very common in vivo, APHS may prove to be very useful against HIV-1 strains that have acquired resistance to clinically available anti-HIV-1 drugs.

Since APHS is a derivative of aspirin and since previous studies in a rat air pouch model indicated that APHS concentrations up to 100 mg/kg are not toxic, it is reasonable to believe that it will be safe to use APHS in vivo. In conclusion, since previous studies have shown that APHS has minimal toxicity in vivo and a good anti-HIV activity, and since this study showed that APHS acts synergically with clinically available RTIs and PIs and is able to inhibit both wild-type and RTI- and PI-resistant HIV-1 strains, APHS is a very promising candidate for anti-HIV therapy in vivo.

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