3-Hydroxyphthalic anhydride-modified human serum albumin as a microbicide candidate inhibits HIV infection by blocking viral entry

Lin Li1, Jiayin Qiu1, Lu Lu2,3, Shengli An4, Pengyuan Qiao3, Shibo Jiang2,3† and Shuwen Liu1*†

1School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China; 2Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Shanghai Medical College and Institute of Medical Microbiology, Fudan University, Shanghai 200032, China; 3Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY 10065, USA; 4Department of Biostatistics, School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou 510515, China

*Corresponding author. Tel: +86-20-61648538; Fax: +86-20-61648655; E-mail: liusw@smu.edu.cn
†These authors made equal contributions to this study.

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Objectives: We recently demonstrated that both 3-hydroxyphthalic anhydride (HP)- and maleic anhydride-modified chicken ovalbumin (OVA) could effectively inhibit HIV-1 infection. But because OVA may cause allergy in some human subjects, here we replaced OVA with human serum albumin (HSA) in designing a new anti-HIV-1 agent, HP-HSA, and then tested its anti-HIV-1 activity and cytotoxicity.

Methods: The in vitro anti-HIV-1 activities of HP-HSA were detected by measuring p24 production and luciferase activity. The cytotoxicities of HP-HSA on target cells and human vaginal and cervical epithelial cells and the effect of HP-HSA on human peripheral blood mononuclear cell (PBMC) proliferation were evaluated by XTT assay. The effect of HP-HSA on interferon-γ secretion by PBMCs was detected by enzyme-linked immunospot (ELISPOT) assay.

Results: We found that HP-HSA exhibited broad and potent antiviral activity against infection by the HIV-1 strains tested, including drug-resistant strains. HP-HSA displayed no or low cytotoxicity on human vaginal and cervical epithelial cells and the cells used for testing HIV-1 infectivity. In addition, HP-HSA had no significant effect on proliferation or interferon-γ secretion by normal or phytohaemagglutinin-stimulated human PBMCs. A time-of-addition assay indicated that HP-HSA was an HIV-1 entry inhibitor.

Conclusions: Because of its broad and potent anti-HIV-1 activity, low cytotoxicity and low immunogenicity to humans, HP-HSA has great potential for further development as a microbicide to prevent the sexual transmission of HIV.

Keywords: HIV-1 entry inhibitor, antiviral activity, HIV-1 sexual transmission

Introduction

Current antiretroviral (ARV) drug-based microbicides, such as those based on a nucleoside reverse transcriptase inhibitor (NRTI), may be helpful in stopping the spread of HIV/AIDS in women.1 However, these microbicides may now be challenged by the rapid emergence of HIV resistance, as a result of their widespread use during the past decade. HIV entry inhibitors constitute another class of ARV-based microbicides, which interfere with the entry of HIV virions into target cells.2,3 Because HIV entry inhibitors block the first step of the HIV life cycle, they should be more suitable than others as candidate microbicides to prevent HIV infection.

Recently, our group has demonstrated that 3-hydroxyphthalic- and maleic anhydride-modified ovalbumin (HP-OVA and ML-OVA, respectively) exhibited broad anti-HIV-1 activity by blocking HIV entry.4,5 Our previous studies also showed that the combination of HP-OVA and some ARV-based candidate microbicides could increase anti-HIV-1 activities,6 suggesting that such combinations could be developed into an ideal microbicide.

However, the clinical application of anhydride-modified OVAs in humans may cause allergy and other immune responses because OVA comes from chicken egg white.7 Here we replaced OVA with human serum albumin (HSA) for the preparation of 3-hydroxyphthalic anhydride-modified HSA (HP-HSA). Because HSA constitutes about one-half of the human blood serum protein, HP-HSA applied in the human vagina is expected to be less immunogenic and allergenic than HP-OVA, a product containing the animal protein OVA. Here we evaluated the in vitro antiviral activity of HP-HSA and studied its putative mechanism of action.

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Materials and methods

Reagents
Different virus strains, MT-2 cells, TZM-bl cells and zidovudine were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. VK2/E6E7 cells, Ect1/E6E7 cells and End1/E6E7 cells were purchased from ATCC (Manassas, VA, USA). CEMX174 5.25M7 cells were kindly provided by Dr C. Cheng-Mayer (The Rockefeller University, New York, NY, USA). 8-Hydroxyphthalic anhydride (HP), HSA, phytohaemagglutinin (PHA) and XTT were purchased from Sigma (St Louis, MO, USA).

Viral infectivity assay
The inhibitory effects of HP-HSA on infection by different laboratory-adapted, primary and drug-resistant HIV-1 strains were measured as previously described. Briefly, graded concentrations of HP-HSA were incubated with virus target cells (1 x 10^5 cells/mL of MT-2 cells, TZM-bl cells or peripheral blood mononuclear cells (PBMCs)) and 100 TCID50 (50% tissue culture infective dose) of different HIV-1 strains at 37°C. At various times post-infection (4, 3 or 7 days), the p24 antigen levels or luciferase activities were assayed.

The effective concentration for 50% inhibition (EC50) was calculated using CalcuSyn software.9

Cytotoxicity assay
The in vitro cytotoxicities of HP-HSA on HIV-1 target cells (MT-2 cells, TZM-bl cells and PBMCs) and human vaginal and cervical epithelial cells (VK2/E6E7 cells, Ect1/E6E7 cells and End1/E6E7 cells) were measured by XTT assay as described previously. The 50% cytotoxic concentrations (CC50s) were calculated using CalcuSyn software.10

Effect of HP-HSA on proliferation and function of PBMCs
The proliferation of PBMCs was determined as previously described. In brief, spontaneous or PHA-stimulated PBMCs were incubated with HP-HSA for 72 h and then measured by XTT assay. The effects of HP-HSA on the production of interferon-γ (IFN-γ) by PBMCs stimulated by PHA or not were detected using an enzyme-linked immunospot (ELISPOT) kit (Mabtech, Mariemont, OH, USA) following the manufacturer’s protocols. The spots of IFN-γ-producing cells were counted with the ELISPOT reader system (Carl Zeiss, Germany).

Effect on HIV-1 entry by time-of-addition assay
For the HIV-1 IIIB or BaL strain, 40 or 400 nM HP-HSA was added to MT-2 or TZM-bl cells, respectively, at different intervals post-infection (0, 0.5, 1, 2, 4, 6 and 8 h). Zidovudine at 200 nM or 2 μM, respectively, was included as a control. At 3 or 4 days post-infection, p24 antigen or luciferase activity was measured as described previously.

Results and discussion
We previously demonstrated that HP-OVA and ML-OVA exhibited high anti-HIV-1 activity and low cytotoxicity in vitro, which indicated that they had promising potential to be developed as microbicide candidates. However, as OVA is a protein of non-human origin, if used repeatedly in human beings it might induce anti-OVA antibodies, which could neutralize the antiviral activity of products containing OVA, or may cause allergic reactions. Therefore, we replaced OVA with HSA in preparing a new microbicide candidate, HP-HSA. Since HSA is one of the most common human blood proteins, HP-HSA is expected to be much safer than HP-OVA and ML-OVA as a topical microbicide.

Table 1. Inhibitory activities of HP-HSA against infection by different HIV-1 strains

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Inhibitory activity [mean (95% CI)]a</th>
<th>EC50 (nM)</th>
<th>EC90 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory-adapted HIV-1 strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIIB (clade B, X4)</td>
<td></td>
<td>0.851 (0.577–1.125)</td>
<td>2.483 (0.515–4.451)</td>
</tr>
<tr>
<td>MN (clade B, X4)</td>
<td></td>
<td>11.455 (7.897–15.014)</td>
<td>69.806 (40.792–98.819)</td>
</tr>
<tr>
<td>RF (clade B, X4R5)</td>
<td></td>
<td>1.974 (1.041–2.907)</td>
<td>6.636 (1.990–11.283)</td>
</tr>
<tr>
<td>Bal. (clade B, R5)</td>
<td></td>
<td>1.454 (0.000–3.692)</td>
<td>6.295 (2.263–10.327)</td>
</tr>
<tr>
<td>Primary HIV-1 isolates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>92US567 (clade B, R5)</td>
<td></td>
<td>56.534 (36.478–76.590)</td>
<td>83.687 (70.141–97.233)</td>
</tr>
<tr>
<td>93IN101 (clade C, R5)</td>
<td></td>
<td>133.074 (123.357–142.790)</td>
<td>318.182 (293.730–342.633)</td>
</tr>
<tr>
<td>BCF02 (clade O, R5)</td>
<td></td>
<td>161.436 (17.936–304.936)</td>
<td>260.505 (58.743–462.268)</td>
</tr>
<tr>
<td>Drug-resistant HIV-1 strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zidovudine resistant</td>
<td></td>
<td>34.350 (9.046–59.654)</td>
<td>160.328 (57.224–263.432)</td>
</tr>
<tr>
<td>A17</td>
<td></td>
<td>0.830 (0.232–1.429)</td>
<td>3.496 (0.000–8.228)</td>
</tr>
<tr>
<td>NL4-3Δ36G [b]</td>
<td></td>
<td>51.271 (0.000–117.729)</td>
<td>99.115 (35.708–162.522)</td>
</tr>
<tr>
<td>NL4-3Δ36GIV38A [b]</td>
<td></td>
<td>6.717 (0.752–12.682)</td>
<td>18.733 (4.296–33.169)</td>
</tr>
<tr>
<td>NL4-3Δ36GIV38EN42S [b]</td>
<td></td>
<td>3.354 (1.549–5.159)</td>
<td>10.497 (6.607–14.386)</td>
</tr>
</tbody>
</table>

aThe data presented are from one representative experiment out of three.

[b]NL4-3Δ36G is an enfuvirtide-susceptible strain, which is the parent strain used for the generation of enfuvirtide-resistant mutants, including NL4-3Δ36GIV38A and NL4-3Δ36GIV38EN42S. NL4-3Δ36GIV38EN42S is also resistant to T1249.
HP-HSA as a microbicide targeting HIV entry

Notably, the anti-HIV-1 activity of HP-OVA was related to the percentages of HP-modified and unmodified lysine and arginine residues. HSA contains 59 lysines and 27 arginines, while OVA has only 20 lysine and 15 arginine residues. In theory, HP-HSA is expected to have more potent anti-HIV-1 activity than HP-OVA because there are more alkaline amino acid residues in one HSA molecule than in an OVA molecule. Indeed, HP-HSA exhibited highly potent antiviral activities against infection by distinct laboratory-adapted and primary HIV-1 strains, particularly the R5 viruses (Table 1); these activities were much more potent than those mediated by anhydride-modified OVAs. One possible reason that early generations of microbicide candidates, such as Carraguard, have failed to prevent HIV-1 acquisition in clinical trials may be their relatively low efficacy against HIV-1 R5 isolates. Thus, HP-HSA has obvious advantages over those anionic polymer-based microbicide candidates.

The rapid emergence of HIV-1 variants with multidrug resistance remains one of the greatest challenges facing the future of microbicide development. Here we demonstrated that HP-HSA exhibited high antiviral activities against HIV-1 variants resistant to NRTIs (e.g. zidovudine), non-nucleoside reverse transcriptase inhibitors (e.g. strain A17) and HIV-1 fusion inhibitors [e.g. strains NL4–3G62V38A and NL4–3G62V38E/N42S] (Table 1). These results indicate that HP-HSA is capable of preventing the sexual transmission of HIV-1 strains that are resistant to currently used antiretrovirals.

The control protein, unmodified HSA, had no inhibitory activity against any of the tested HIV-1 strains, even at concentrations up to 10 μM (data not shown). It is worth noting that HP-HSA is at least 10 times more potent than HP-OVA and ML-OVA against most HIV-1 isolates. Currently, tenofovir gel is one of the most promising ARV-based candidate microbicides. Our previous studies have shown that tenofovir has highly potent antiviral activity against a broad spectrum of HIV-1 strains, with EC50s in the range of 0.020–0.231 μg/mL. We have also demonstrated that combining HP-OVA with tenofovir resulted in synergistic and complementary anti-HIV-1 effects. These results suggest that HP-HSA might be even more suitable for working cooperatively with these ARV-based microbicide candidates against both drug-susceptible and -resistant HIV-1 strains.

An ideal microbicide must be not only effective but also safe to use. Tests showed that HP-HSA had very low cytotoxicities in vitro and the selectivity indexes (SIs) were >10000 on cells susceptible to HIV-1 infection and reproductive tract epithelial cells (Table S1, available as Supplementary data at JAC Online). Naive CD4+ T lymphocytes in human beings play an important role in the immune system. One advantage of HP-HSA is that the protein originates from human serum, which is expected to have much less deleterious effects than animal proteins on the function of immune cells in the blood circulation. By XTT and ELISPOT assay, we confirmed that HP-HSA had no significant effects on the proliferation and IFN-γ secretion of normal or PHA-stimulated PBMCs (Figure S1, available as Supplementary data at JAC Online). These results suggest that HP-HSA, when used as a topical microbicide in humans, may not cause significant adverse effects on the human immune system.

In the time-of-addition assay, HP-HSA exhibited significantly decreased inhibitory activity against both X4 and R5 viruses when it was added 0.5–2 h post-HIV-1 infection (Figure 1). However, the positive control, zidovudine (an NRTI) continued to exert its full effect, even when it was added to cells 8 h post-infection. These results illustrate that HP-HSA exhibited antiviral activities at an early stage of viral entry with a window of ~2 h. The ability of HP-HSA to target the viral entry step and block HIV-1 transmission at the initial stage of viral infection makes it an excellent candidate for prophylactic use as an anti-HIV-1 microbicide candidate.

In short, HP-HSA is a broad-spectrum HIV-1 entry inhibitor with low cytotoxicity and easy preparation, thus having a low cost of production. Those properties, together with strong anti-HIV-1 efficacy, especially against drug-resistant strains, suggest that HP-HSA has the potential to be developed further as a safe and effective microbicide. Combination of an HP-HSA-based microbicide and an ARV-based microbicide against drug-resistant strains, makes it an excellent candidate for prophylactic use as an anti-HIV-1 microbicide candidate.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Time-of-addition assay. (a) HP-HSA (40 nM) was added at different times post-infection by HIV-1_M18 in MT-2 cells (zidovudine at 200 nM was used as a control). (b) HP-HSA (400 nM) was added at different times post-infection by HIV-1_M18 in TZM-bl cells (zidovudine at 2 μM was used as a control). The assay was done in triplicate and the data are presented as means±SEM from one representative experiment out of three conducted.

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*Table 1.* Notably, the anti-HIV-1 activity of HP-OVA was related to the percentages of HP-modified and unmodified lysine and arginine residues. HSA contains 59 lysines and 27 arginines, while OVA has only 20 lysine and 15 arginine residues. In theory, HP-HSA is expected to have more potent anti-HIV-1 activity than HP-OVA because there are more alkaline amino acid residues in one HSA molecule than in an OVA molecule. Indeed, HP-HSA exhibited highly potent antiviral activities against infection by distinct laboratory-adapted and primary HIV-1 strains, particularly the R5 viruses (Table 1); these activities were much more potent than those mediated by anhydride-modified OVAs. One possible reason that early generations of microbicide candidates, such as Carraguard, have failed to prevent HIV-1 acquisition in clinical trials may be their relatively low efficacy against HIV-1 R5 isolates. Thus, HP-HSA has obvious advantages over those anionic polymer-based microbicide candidates.
may have a synergistic antiviral effect against HIV-1 sexual transmission.

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**Transparency declarations**
None to declare.

**Supplementary data**
Table S1 and Figure S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**