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In vitro anti-HIV and -HSV activity and safety of sodium rutin sulfate as a microbicide candidate

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Abstract

Sodium rutin sulfate (SRS) is a sulfated rutin modified from the natural flavonol glycoside rutin. Here, we investigated its in vitro anti-HIV and -HSV activities and its cytotoxic profile. Fifty percent inhibitory concentration (IC_{50}) values of SRS against HIV-1 X4 virus IIIB, HIV-1 R5 isolates Ada-M and Ba-L were 2.3 ± 0.2, 4.5 ± 2.0 and 8.5 ± 3.8 μM with a selectivity index (SI) of 563, 575 and 329, respectively. Its IC_{50} against primary R5 HIV-1 isolate from Yunnan province in China was 13.1 ± 5.5 μM, with a SI of 197. In contrast, unsulfated rutin had no activity against any of the HIV-1 isolates tested. Further study indicated that SRS blocked viral entry and virus–cell fusion likely through interacting with the HIV-1 envelope glycoprotein. SRS also demonstrated some activity against human herpes simplex virus (HSV) with an IC_{50} of 88.3 ± 0.1 μM and a SI of 30. The 50% cytotoxicity concentration (CC_{50}) of SRS was >3.0 mM, as determined in human genital ME180, HeLa and primary human foreskin fibroblast cells. Minimum inhibitory concentration of SRS for vaginal lactobacilli was >3.0 mM. These results collectively indicate that SRS represents a novel candidate for anti-HIV-1/HSV microbicide development.

Keywords: Microbicide; HIV-1; HSV; Sodium rutin sulfate; Rutin; Viral entry

1. Introduction

Sexual transmission is playing a major role in the spread of HIV worldwide. More than 90% of new HIV infections are spread through unprotected intercourse. Women, especially young women are biologically more vulnerable to HIV and other genital infections than men. Women are approximately twice as likely as men to contract HIV infection during vaginal intercourse (European Study Group, 1992). The presence of other untreated sexually transmitted pathogens (such as human herpes simplex virus) greatly increases the likelihood of acquiring and/or transmitting HIV infection (Fleming and Wasserheit, 1999). Because of gender inequality, condom use often depends on the consent of male sexual partner, and consequently condoms are not always a feasible option for many women, although effective when used correctly and consistently. Development of a new prevention method that women can control is urgently needed (Shattock and Moore, 2003). The use of microbicides (chemical barrier) by women has been proposed as a method to control the sexual transmission of HIV-1 and other STDs (Population Council and International Family Health, 2002). There has been significant progress in microbicide research and development in the past 15 years. Over 30 compounds or formulations are currently undergoing pre-clinical or clinical studies.

Abbreviations: AIDS, acquired immune deficiency syndrome; AZT, 3′-azido-3′-deoxythymidine; CC_{50}, 50% cytotoxic concentration; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IC_{50}, 50% inhibitory concentration; IL-2, interleukin-2; MIC, minimum inhibitory concentration; NO, nitric oxide; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; SI, selectivity index; SRS, sodium rutin sulfate; $CCID_{50}$, 50% cell culture infectious dose

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Discovery of novel compounds for use as potential microbicide candidates is a long-term task (Alliance for Microbicide Development, 2006).

Natural products are an important resource of leading compounds in research and the development of anti-HIV drugs (Vlietinck et al., 1998; Matthee et al., 1999). Calanolide A, a non-nucleoside reverse transcriptase inhibitor, isolated from tropical rainforest tree *Calophyllum lanigerum*, has been evaluated in phase I clinical trials (Cos et al., 2004). The novel HIV-1 maturation inhibitor, 3-O-(3′, 3′-dimethylsuccinyl)-betulinic acid (DSB), derived from natural product betulin, has reached phase II clinical trials (Yu et al., 2005). Several other natural products, such as carageenan and praneem polyherbal, have been registered as potential microbicide candidates, and reached phases III and II clinical trials (Alliance for Microbicide Development, 2006). Rutin or quercetin rutinoside, a flavonol glycoside, is an antioxidant and NO scavenger. Its sulfated compound, sodium rutin sulfate (SRS), also named as rutin deca(H-) sulfate sodium (Wang et al., 2006), possesses anti-inflammatory activity in a model of rat colitis (Cruz et al., 1998). SRS demonstrated good safety following 2-year oral administration in rats (Habs et al., 1984). Anti-HIV activity of SRS has been found by our group and others (Mu et al., 1998; Chen et al., 2004; Ben et al., 2006). Recent studies, however, showed that SRS has poor bioavailability when administered orally in animal models (He and Zeng, 2005; Andlauer et al., 2001) and a short half-life in blood (Wang et al., 2006). Therefore, SRS is not a good candidate for the treatment of AIDS patients by oral administration. Nevertheless, SRS may be a good candidate for topical microbicide development. In this study, we have evaluated in vitro anti-HIV-1 and -HSV activity of SRS. Its potential safety and mode of action have also been addressed.

2. Materials and methods

2.1. Chemicals

Sodium rutin sulfate (Mr 1631), its chemical structure shown in Fig. 1A, was provided by CONBA Pharmaceutics Group (Hangzhou, Zhejiang, China). Rutin (purity 99%, Mr 610.5) was donated by Chengdu Supermen Plant & Chemical Development Co., Ltd. (Chengdu, China). 3′-Azido-3′-deoxythymidine (AZT) was purchased from Sigma (St. Louis, MO).

2.2. Cells

C8166, H9 and H9/HIV-1_{IIIB} were obtained from the Centralized Facility for AIDS Reagents, NIBSC, UK. ME180 and HeLa cells were from ATCC (Rockville, MD). TZM-bl cell line was from the AIDS Research and Reference Reagent Program, NIAID, NIH. NP-2/CD4, NP-2/CD4/CXCR4 and NP-2/CD4/CCR5 cells for determining viral co-receptor usage were provided by Dr. Takebe Yutaka (National Institute of Infectious Diseases, Japan, Tokyo). The primary human foreskin fibroblast cells were prepared as described previously (Tao et al., 2005; Amit et al., 2004).

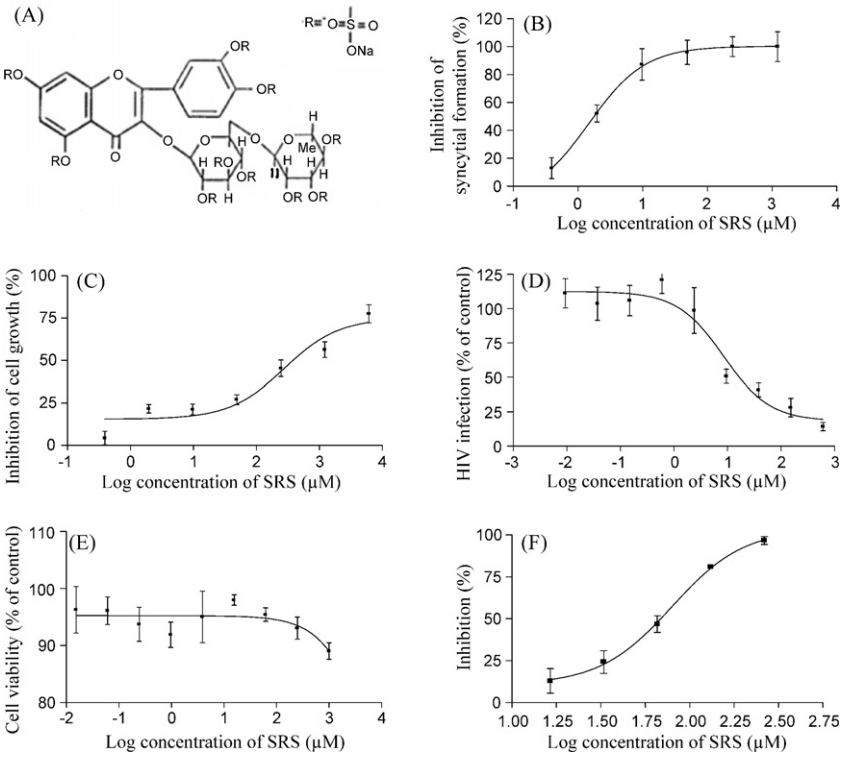

Fig. 1. Representative dose–response curves of SRS anti-HIV-1 and -HSV-1 activity and cytotoxicity. (A) Chemical structure of sodium rutin sulfate (SRS; $C_{27}H_{20}O_{26}R_{10}$, Mr 1631). Inhibition of HIV-1_{IIIB} infection in C8166 cells (B) and HIV-$1_{Ba\text{-}L}$ infection in TZM-bl cells (D). Cytotoxicity of SRS to C8166 (C) and TZM-bl cells (E). (F) SRS anti-HSV-1 activity in vero cells.

2.3. Viruses

HIV-1_{IIIB}, HIV-$1_{Ba\text{-}L}$ and HIV-$1_{Ada\text{-}M}$ were from the Centralized Facility for AIDS Reagents, NIBSC. The primary R5 HIV-1 viral strain was isolated from an HIV-1-infected patient in Yunnan province as described by Lane JR (Lane, 1999), and co-receptor usage was determined using NP-2 cells as described by Soda et al. (1999).

2.4. Measurement of anti-HIV activity

2.4.1. Syncytial formation analysis

Anti-HIV-1_{IIIB} activity was carried out as described previously (Zheng et al., 2000; Ben et al., 2006). Briefly, serially diluted compounds in RPMI 1640 medium were added to a 96-well plate in triplicate, and then 3×10^4 C8166 cells and 200 $CCID_{50}$ of HIV-1_{IIIB} were added to each well. After incubation at 37 °C for 72 h, syncytial cells from five different fields of each well were scored under an inverted microscope (100×). The % inhibition of syncytium formation was calculated.

2.4.2. Luciferase gene reporter assay

Anti-HIV-$1_{Ba\text{-}L}$ activity in TZM-bl cells was performed as described previously (Wei et al., 2002; Derdeyn et al., 2000). Infected cells were harvested at 48 h post-infection, lysed, and assayed using the LucLite Luciferase Assay Kit (Packard) in a Fusion Universal Microplate Analyzer (Packard).

2.4.3. P24 ELISA

Anti-HIV-$1_{Ada\text{-}M}$ and anti-primary HIV-1 isolate activity was analyzed by measuring supernatant p24 antigen according to the Manufacturer's protocol (Perkin-Elmer).

2.5. Time-of-addition experiment

Time-of-addition experiment was conducted as described by De Clercq et al. (1992). In brief, C8166 cells were pre-incubated with HIV-1_{IIIB} (MOI = 1.0) for 1 h at 4 °C to allow HIV-1 attach to cells without fusion, and subsequently washed three times with ice-cold medium to remove unbound viruses. Cells were then rapidly warmed to 37 °C to allow the viral replication cycle to proceed. Compounds at a concentration 50–100-fold their IC_{50} (102 μM of SRS or 134 μM of AZT) were added at different time points (0, 10, 30, 45, 60, 120, 240, 360, 480 and 600 min post-infection). Viral p24 antigen production was determined at 72 h post-infection.

2.6. Cell fusion assay

Cell fusion assay was carried out as described previously with modification (Zheng et al., 1998; Walker et al., 1987). 3×10^4 C8166 cells and 1×10^4 H9/HIV-1_{IIIB} cells per well were mixed and co-cultured in a 96-well plate in the presence or absence of serially diluted compounds (SRS or AZT) for 24 h. The number of syncytia in each well was counted under microscope.

2.7. SRS pre-treatment

Pre-treatment experiments were conducted as described with modification (Baba et al., 1988; Motakis and Parniak, 2002). In treatment 1, 3×10^4 C8166 cells, ∼200 $CCID_{50}$ of HIV-1_{IIIB} and serially diluted SRS were co-cultured in a 96-well plate at 37 °C for 1.5 h, washed three times at 400 × *g* centrifugation, and cultured for another 72 h before syncytia were counted under the microscope. In treatment 2, HIV-1_{IIIB} was pre-treated with serially diluted SRS at 37 °C for 1.5 h, washed three times at >20,000 × *g* centrifugation. ∼1000 CCID50 of SRS-pre-treated HIV-1_{IIIB} and 3×10^4 C8166 cells per well in a 96-well plate were co-cultured for 1.5 h, washed three times at 400 × *g* centrifugation, and cultured for another 72 h before syncytia were counted. In treatment 3, C8166 cells were pre-treated with serially diluted SRS for 3 h, washed three times at 400 × *g* centrifugation. ∼200 $CCID_{50}$ of HIV-1_{IIIB} and 3×10^4 SRS-pre-treated C8166 cells per well in a 96-well plate were co-cultured for 1.5 h, washed three times at 400 × *g* centrifugation, and cultured for another 72 h before syncytia were counted.

2.8. Anti-HSV assay

Anti-HSV activity was measured by plaque reduction assay as described by Talarico et al. (2004) and Schmidtke et al. (2001). Vero cells ($2–4 \times 10^5$ $well^{-1}$) in a 24-well plate were incubated at 37 °C for 24 h. Confluent Vero cell monolayers were then infected with 200 $CCID_{50}$ of HSV-1 in the presence or absence of serially diluted compounds. After adsorption at 37 °C for 1 h, the inoculum was aspirated and washed with 1 ml PBS. Then, 1 ml of 0.4% agar in RPMI 1640 complete medium was added. Culture was incubated for 3 days until plaques appeared. Monolayers were fixed with 1 ml of 2% formalin, and stained with 700 μl of 0.4% crystal violet for 20 min. Plaques were counted under the microscope after removal of the agar overlay. The IC_{50} was calculated as the compound concentration required to reduce the virus plaque number by 50%.

2.9. Measurement of cytotoxicity

Compound cytotoxicity to cervical epithelial cells, C8166 cells, PBMC and primary cultured foreskin fibroblast cells was determined by MTT assay as described (Zheng et al., 1995).

2.10. Toxicity analysis to human vaginal lactobacilli

Lactobacillus dehrueckii was isolated from the vagina of a healthy woman and grown in Lactobacilli MRS broth (Kang et al., 2001). A microdilution assay was used for assessing compound toxicity to *L. dehrueckii* growth (Tao et al., 2005). Briefly, lactobacilli were added into a 96-well plate at 10^6 CFU/ml per well in the presence of serially diluted compound. After the cells were incubated for 24 h at 37 °C in an anaerobic Gas Pak pouch (BioMerieux, Marcy, France), the minimum inhibitory concentration (MIC) was defined as the lowest compound concentration that allowed no detectable growth. Penicillin was used as a positive control.

3. Results

3.1. SRS has strong anti-HIV-1 activity against multiple viral isolates

To determine the anti-HIV-1 activity of SRS, a panel of HIV-1 strains, including R5 HIV-1_{Ba-L}, R5 HIV-1_{Ada-M}, X4 HIV-1_{IIIB}, and a primary HIV-1 isolate, were used in this study. The results are summarized in Table 1. The representative dose–response curves and cytotoxicity data are shown in Fig. 1B–E. As can be deduced from Table 1 and Fig. 1, SRS potently inhibited the infection of multiple viral isolates. In contrast, the non-sulfated compound rutin did not show any anti-HIV-1 activity against IIIB and other HIV-1 isolates (data not shown). In addition, SRS was active against primary R5 virus from Yunan province in China. Taken together, SRS, but not the non-sulfated rutin, demonstrated very good anti-HIV activity against multiple HIV-1 isolates.

3.2. SRS demonstrates anti-HSV activity

As sexually transmitted HSV can increase the likelihood of acquiring and/or transmitting HIV infection, plaque reduction assay was conducted to test whether SRS can also inhibit the replication of HSV. As shown in Fig. 1F, our data indicate that SRS can indeed inhibit HSV-1 replication. Its IC_{50} against HSV-1 was 88.3 ± 0.1 μM, with a CC_{50} of 2645.0 ± 204.3 μM and a SI of 30 (Fig. 1F).

3.3. SRS inhibits HIV-1 infection by targeting viral entry

Further experiments were conducted to understand the anti-HIV mechanism of SRS. As shown in the time-of-addition experiments (Fig. 2A), SRS almost completely inhibited HIV-1 replication when added between 0 and 1 h post-HIV infection, and its anti-HIV-1 activity dramatically decreased when SRS was added at later time points. As a control, AZT, a reverse transcriptase inhibitor, showed anti-HIV-1 activity when added as late as at 8 h post-infection. These data together suggest that SRS inhibits HIV-1 infection by targeting viral early life cycle before reverse transcription.

The interaction between HIV-1 envelope glycoprotein and host cell receptor/co-receptor can induce syncytium formation/cell–cell fusion. SRS strongly inhibited HIV-1 induced syncytium formation (Table 1). To determine whether the inhibition of syncytial formation by SRS was due to blockade of interaction between envelope glycoprotein and cellular receptor/co-receptor, cell fusion assay was carried out. As seen in Fig. 2B, inhibition of syncytium formation by SRS was dose-dependent, while AZT did not show any activity of inhibiting syncytium formation in cell–cell fusion assays, indicating that SRS prevented syncytium formation/cell fusion likely through blocking the interaction between envelope glycoprotein and cell receptor/co-receptor.

Table 2
Effect of SRS pre-treatments on anti-HIV-1 activity

Code	Pre-treatments	IC_{50} (μM), $M \pm$ S.D.[a]
1	C8166 cells and HIV-1 virus	2.3 ± 0.2
2	HIV-1 virus	14.1 ± 3.8
3	C8166 cells	>122.6

[a] Mean ± S.D. for three independent experiments.

To further examine whether SRS interacts with viral envelope glycoprotein or/and cellular receptor/co-receptor, SRS pre-treatment experiments were subsequently carried out and described in Fig. 2C and Table 2. Pre-treatment of virus plus cells or virus alone with SRS potently inhibited HIV-1 infection, with an IC_{50} of 2.3 ± 0.2 and 14.1 ± 3.8 μM, respectively. Pre-treatment of target cells alone had no anti-HIV-1 activity. Taken together, the above experiments provide strong evidences that SRS inhibits HIV-1 infection likely by interacting with viral envelope glycoprotein but not with cellular receptors.

3.4. SRS has low toxicity to human genital cells and vagina lactobacilli

To prevent sexual transmission of HIV-1, microbicides are mostly applied vaginally. Lactobacilli are the dominant member in vaginal flora, playing a key role in vaginal health, and often used for evaluating the safety of microbicide application. *L. dehrueckii* isolated from normal human vagina was used in the present study. The minimum inhibition concentration of SRS to the bacterium was >3048 μM (Table 3).

Human genital cells, including primary human foreskin fibroblast cells, cell lines ME-180 and HeLa, are useful for eval-

Table 1
In vitro anti-HIV-1 activities of SRS

Compound	Virus	Cells	IC_{50} (μM), $M \pm$ S.D.[a]	CC_{50} (μM), $M \pm$ S.D.[a]	SI[b]	Assay
Laboratory adapted HIV-1 strain						
SRS	HIV-1_{IIIB}	C8166	2.3 ± 0.2	1295.4 ± 269.8	563	Syncytial formation assay
Rutin			>330	2880.2 ± 158.2	<8.7	
AZT			1.9 ± 0.2	2905.2 ± 604.1	1528	
SRS	HIV-1_{Ba-L}	TZM-b1	8.5 ± 3.8	2800.0 ± 337.3	329	Luciferase assay
SRS	HIV-1_{Ada-M}	PBMC	4.5 ± 2.0	2589.5 ± 204.5	575	p24 ELISA
Primary HIV-1 isolate						
SRS	R5 virus	PBMC	13.1 ± 5.5	2589.5 ± 204.5	197	p24 ELISA

[a] Mean ± S.D. for three independent experiments.
[b] The selectivity index (SI) was defined as the ratio of 50% cytotoxic concentration to 50% inhibitory concentration (CC_{50}/IC_{50}).

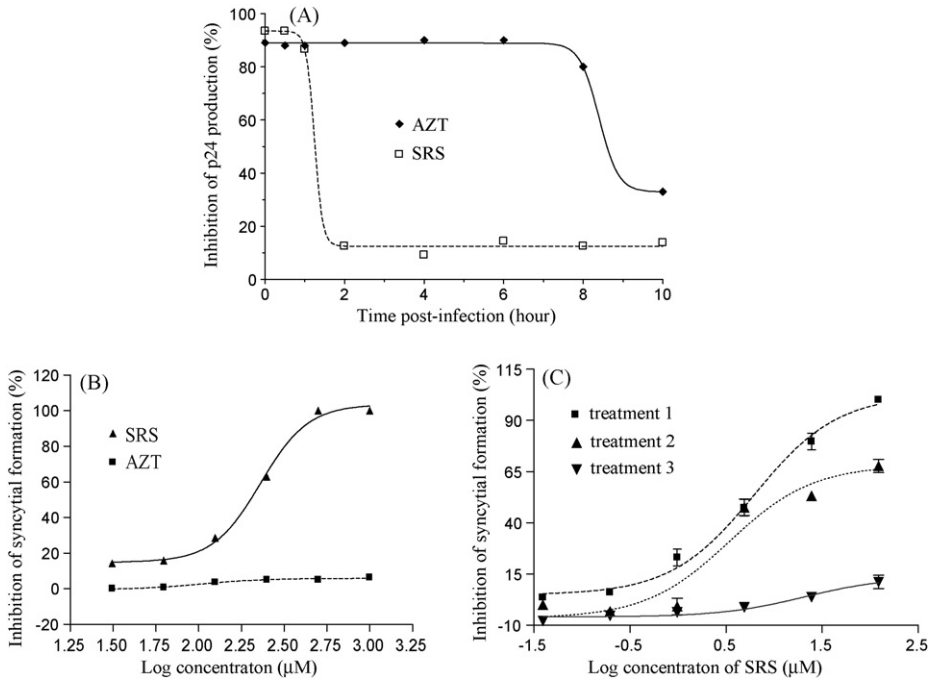

Fig. 2. Anti-HIV-1 mechanism of SRS. (A) Time-of-addition experiment. C8166 cells were infected with HIV-1_{IIB}. At the indicated time point post-infection, 102 μM SRS or 134 μM AZT was then added. Antiviral activity was measured at 72 h post-infection by p24 ELISA. (B) Inhibition of cell–cell fusion. C8166 cells and H9/HIV-1_{IIIB} cells were co-cultured for 1 day in the presence of serially diluted SRS or AZT. Syncytial formation was scored under microscope. (C) Dose–response curves of SRS anti-HIV-1_{IIIB} activity in different pre-treatments.

Table 3
Toxicity of SRS to various human cells and vaginal lactobacilli

Human cells or bacteria	CC_{50} (μM)[a]
ME180	>3048.1
HeLa	>3048.1
Primary foreskin fibroblast cell	>3048.1
PBMC	2589.5 ± 204.5
C8166	1481.4 ± 355.6
Vaginal lactobacillus (*L. dehrueckii*)	>3048.1[b]

[a] Means for three independent experiments.
[b] Minimum inhibitory concentration for lactobacilli.

uating the cytotoxicity of microbicide candidates. From our data, the CC_{50} of SRS in these cells was >3048 μM (Table 3). These results together indicate that SRS has very low toxicity to both lactobacilli and genital cells.

4. Discussion

Several plant-derived leading compounds with anti-HIV activity have been discovered and investigated in the past decade (Vlietinck et al., 1998; Matthee et al., 1999; Lee, 2004). Among them, flavonoids have been shown to inactivate HIV-1 through irreversibly binding to gp120 (Mahmood et al., 1993). Rutin, a natural water-insoluble ubiquitous flavonoid, is used in several drugs for treating hemorrhages, allergies and hypertonia, and for adjuvant medication of infectious diseases (Habs et al., 1984). SRS is a water-soluble sulfated rutin. In the present study, SRS demonstrated broad spectrum of anti-HIV activity against R5 and X4 viruses including laboratory-adapted and primary isolate. The selectivity indexes ranged from 197 to 575 (Table 1 and Fig. 1). Further experiments showed that SRS blocks the entry of HIV-1 into host cells without interacting with cell membrane, suggesting that SRS may inhibit HIV-1 by binding to envelope glycoprotein (Table 2 and Fig. 2). In addition, SRS also demonstrated some anti-HSV activity with an IC_{50} of 88.3 ± 0.1 μM and a SI of 30. In contrast, non-sulfated rutin showed no antiviral activity. Since one SRS molecule carries 10 sulfate anionic ions which are similar to polysaccharide sulfate, SRS is considered as a polyanionic compound. Previous studies by other investigators indicate that sulfated polysaccharides, such as dextran sulfate, preferably binds to the V3 loop of X4 gp120 rather than that of R5 gp120 (Moulard et al., 2000). Although SRS is a polyanionic compound, no significant difference was observed in terms of its anti-R5 and -X4 activity. Further investigation is underway to uncover the precise SRS binding sites on envelope glycoprotein. It is worthy to note that, although SRS efficiently blocks HIV-1 infection and envelope glycoprotein-mediated cell–cell fusion, higher concentration was needed to achieve similar level of inhibition in the cell–cell fusion assay. This may reflect the methodology difference in the two systems. In cell–cell fusion assay, envelope glycoproteins were expressed on the cell surface (H9/IIIB), whereas in an antiviral infection assay, envelope glycoproteins were on both virion and host cell C8166. In addition, it is probable that the density and/or susceptibility of envelope glycoproteins on H9 and C8166 cell surface and on virion are not exactly the same (Reeves et al., 2002; Pontow et al., 2004; Bachrach et al., 2005), and this might also contribute to the difference in the antiviral activity of SRS observed in the two assays.

Recently, pharmacokinetics study of SRS in rats after intravenous administration showed that its clearance, volume of distribution and terminal half-life were 1.333 ml/min/kg, 240 ml/kg and 125 min, respectively (Wang et al., 2006). Low oral bioavailability of SRS has been observed in dogs, and bi-directional transport study in Caco-2 cell model showed that the apparent permeability (P_{app}) in the secretory direction was 1.4–4.5-fold greater than the corresponding absorptive P_{app} at concentrations in the range of 50.0–2000 μM. Several efflux transporters participate in the absorption and efflux of SRS, and SRS is likely a substrate of P-glycoprotein (He and Zeng, 2005). Lack of significant systemic absorption is a preferable characteristic for microbicide candidates (Science Working Group of the Microbicide Initiative, 2003); therefore, SRS could be used as a microbicide. According to the recommendations for the non-clinical development of topical microbicides (Lard-Whiteford et al., 2004), we evaluated the cytotoxicity of SRS to human genital cells and to vaginal lactobacilli, respectively. The CC_{50} of SRS in ME180, HeLa and primary foreskin fibroblast cells are >3048 μM, and the MIC of SRS to vaginal lactobacilli is >3048 μM (Table 3). These results suggest that SRS is safe to genital tissues and to vaginal bacterial flora.

In summary, SRS possesses good anti-HIV and -HSV activities and low toxicity to human genital cells and vaginal lactobacilli. Further investigation of SRS could lead to its development as a topical microbicide.

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