Antiviral Research 96 (2012) 138–147

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Poly (4-styrenesulfonic acid-co-maleic acid) is an entry inhibitor against both HIV-1 and HSV infections – Potential as a dual functional microbicide

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ARTICLE INFO

Article history:
Received 14 March 2012
Revised 9 August 2012
Accepted 14 August 2012
Available online 30 August 2012

Keywords:
Poly (4-styrenesulfonic acid-co-maleic acid) (PSM)
Human immunodeficiency virus type 1 (HIV-1)
Herpes simplex virus
Antiviral activity
Microbicide
Entry inhibitor

ABSTRACT

Genital herpes is one of the most prevalent sexually transmitted diseases (STD) caused by herpes simplex viruses type 1 and 2 (HSV-1 and -2). HSV is considered as a major risk factor in human immunodeficiency virus type-1 (HIV-1) infection and rapid progression to acquired immunodeficiency syndrome (AIDS). Here, we reported the finding of a polymer of styrenesulfonic acid and maleic acid (PSM) which exhibited antiviral activity with low cytotoxicity. PSM exhibited *in vitro* inhibitory activity against HIV-1 pseudovirus and HSV-1 and -2. *In vivo* efficacy of PSM against HSV-2 (G) was also investigated. We found that both 1% and 5% PSM gels protected mice from HSV-2 vaginal infection and disease progression significantly. Mechanistic analysis demonstrated that PSM was likely an entry inhibitor that disrupted viral attachment to the target cells. In particular, PSM disrupted gp120 binding to CD4 by interacting with the gp120 V3-loop and the CD4-binding site. The *in vitro* cytotoxicity studies showed that PSM did not stimulate NF-κB activation and up-regulation of proinflammatory cytokine IL-1β and IL-8 in vaginal epithelial cells. In addition, PSM also showed low adverse effect on the growth of vaginal *Lactobacillus* strains. PSM is, therefore, a novel viral entry inhibitor and a potential microbicide candidate against both HIV-1 and HSV.

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1. Introduction

Herpes simplex virus infection is one of the most prevalent infections in many parts of the world with prevalence exceeding 90% in some settings (Gwanzura et al., 1998; Nahmias et al., 1990). HSV-2 (genital herpes) is transmitted through sexual contact and is common among persons infected with HIV-1 (Kapiga et al., 2007; Lama et al., 2006). In sub-Saharan Africa, the region hardest hit by the HIV-1 epidemic, the seroprevalence of HSV-2 reached from 50% to 90% among HIV-1 infected individuals (Auvert et al., 2001; Greenblatt et al., 1988; Mbizvo et al., 1996; McClelland et al., 2005; Mostad et al., 2000; Sanchez et al., 2002; Weiss, 2004). In the Americas and Europe, HSV-2 seroprevalence is 50% among HIV-1 infected men who have sex with men (Celum et al., 2008). It is well established that genital herpes is facilitating the perseverance of the global HIV-1 epidemic, primarily due to their shared route of transmission (Corey et al., 2004). A meta-analysis concluded that HSV-2 infection increases the risk of HIV-1 acquisition approximately 3-fold in both men and women, and that primary HSV-2 infection may have an even greater effect on HIV-1 susceptibility (Freeman et al., 2006). Among those who are infected with HIV-1, HSV-2 seropositivity and genital ulcer disease resulting from herpes infection have been associated with significantly higher HIV-1 plasma viral loads (Gray et al., 2004; Schacker et al., 2002) and was associated with a 4-fold increase in the likelihood of HIV-1 transmission among heterosexual HIV-1 discordant couples (Gray et al., 2001). A number of observational studies have found that HSV-2 reactivation, including asymptomatic shedding, also increases the concentration of HIV-1 in plasma and genital secretions (Celum et al., 2005; Schacker et al., 2002). Thus, co-infection with HSV-2 may contribute to faster HIV-1 disease progression and enhanced genital HSV-2 shedding among HIV-1-infected individuals in turn likely increases the risk of HSV-2 transmission. A recent study on HSV-2-infected individuals who were successfully treated with acyclovir found that the treatment failed to reduce the increased acquisition of HIV-1 due to HSV-2 infection (Zhu et al., 2009), further highlighting the need for dual preventative intervention of the infections.

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0166-3542/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2012.08.005

Sexual transmission has become the major route of the global HIV-1 epidemic, particularly in Africa and Southeast Asia (UNAIDS, 2010). Development of effective and safe microbicide is considered as an effective way to contain the AIDS pandemic, particularly in the resource-limiting regions that tend to bear the burden of both HIV-1 and HSV-2 infection (Balzarini and Van Damme, 2007; Klasse et al., 2008; Nikolic and Piguet, 2009). A recent clinical trial of a nucleoside reverse transcriptase inhibitor (NRTI)-based microbicide (CAPRISA) provides encouraging proof-of-concept that when properly used a microbicide can be effective as a prophylactic agent and reduce new infections (Abdool Karim et al., 2010). Due to their shared transmission route and pathological relatedness, it is imperative to develop antiviral agents that have dual activities against both HIV and HSV-2. In this report, we described a molecule, poly (4-styrenesulfonic acid-co-maleic acid) (PSM), which exhibited novel inhibitory activity against both HIV-1 and HSV-2 infections. PSM is a styrenesulfonic acid-maleic acid polyanion and its derivative compounds have previously been shown to prevent HIV-1 infection *in vitro* (Anderson et al., 2000; Qian et al., 2005; Vanessa et al., 2010). However, PSM exhibited lower cytotoxicity than its derivative homologs. We investigated the *in vitro* activities of PSM in inhibiting HIV-1 and HSV-1/2 infection and explored the mechanisms of action. We also evaluated the PSM efficacy in preventing genital herpes infection in HSV-2 genital challenge mouse model.

2. Materials and methods

2.1. Reagents, cell lines, plasmids, viruses and bacteria

PSM, Poly (4-styrenesulfonic acid) (PSS), heparan sulfate, dextran sulfate, DEAE-dextran, Nonoxynol-9 (N-9), azidothymidine (AZT) and *p*-nitrophenyl phosphate (*p*-NPP) were purchased from Sigma–Aldrich (St. Louis, USA). Nevirapine and acyclovir were obtained from National Institutes for Food and Drug Control in China (Beijing, China). Recombinant gp120s (rgp120s) and soluble CD4 (sCD4) were purchased from ImmunoDiagnostics, Inc. (Woburn, USA). Anti-HSV gD mAb (H170) was purchased from Santa Cruz (Santa Cruz, USA). Anti-gp120 monoclonal antibodies (mAbs) b12, 2G12, 447-52D and F105 were obtained from National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (USA). Normal human IgG control was from R&D Systems (Minneapolis, USA). Anti-CD4 mAb OKT4 and goat-anti-human IgG FITC were purchased from eBioscience (San Diego, USA). Alkaline phosphatase labeled goat-anti-human/mouse IgG and IRDye 800 IgG were purchased from Zymed (South San Francisco, USA) and Li-COR (Lincoln, USA), respectively. V3 peptides of ADA (KSIHIGPRRAFYTTG) and IIIB (KRIRIQRGPGRTFVT) were synthesized by GL Biochem Ltd. (Shanghai, China).

HEC-293T, Vero, VK2/E6E7, Ect1/E6E7, End1/E6E7, Caco-2, HEC-1-A cells and *Lactobacillus acidophilus* (ATCC#4356) were obtained from American Type Culture Collection (ATCC, USA). Other 7 strains of *Lactobacillus* were isolated from the vagina of a healthy woman. GHOST (3) X4/Hi5, MT-2 and CHO-WT were obtained from National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (USA).

NF-κB-luc reportor plasmid was from Clontech (California, USA). The HIV-1 Env or VSV-G pseudotyped viruses were produced by transient co-transfection of HEC-293T cells with pNL4-3 E^-R^- Luc and Env-encoding plasmids as reported (Connor et al., 1995; He et al., 1995), and the 50% tissue culture infective dose ($TCID_{50}$) of infectious pseudovirions was determined as reported previously (Johnson and Byington, 1990; Montefiori, 2009). HSV-1 (HF) and HSV-2 (G) were propagated and titrated on Vero cells as described previously (McLean et al., 1994).

2.2. In vitro antiviral activity assay

PSM antiviral activity against HIV-1 pseudovirus and VSV-G pseudovirus was determined as previously described (Montefiori, 2009). Serially diluted drug was mixed with 200 $TCID_{50}$ pseudotyped viruses in 96-well plate and incubated for 30 min. 10^4 GHOST (3) X4/Hi5 cells in 100 μl 10 μg/ml DEAE-dextran-containing medium were dispensed to each well. All drug dilutions were in triplicate. The level of HIV-1 infection was quantified by measurement of relative luminescence units (RLU) using a luciferase assay kit (Promega, Madison, USA) after 48 h incubation. The luminescence was determined by GloMax-96 Microplate Luminometer (Promega, Madison, USA). The half maximal effective concentration (EC_{50}) were calculated using CalcuSyn software (Chou and Hayball, 1991).

The anti-HSV-1/2 activity was performed as described (Fletcher et al., 2006), with modifications. 10^4 Vero cells were seeded onto 96-well plate and cultured for 24 h, followed by the addition of serial concentrations of PSM and 100PFU HSV-1/2. Cell monolayer was fixed in 10% formalin in phosphate buffered saline (PBS) and then stained with 0.8% crystal violet in 50% ethanol after 48 h incubation. Plaques were counted using an inverted microscope.

2.3. In vitro cytotoxicity of PSM

The *in vitro* cytotoxicity of the drugs was measured using a commercial CCK-8 kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. 10^4 cells per well were dispersed into 96-well plates and cultured for 24 h before a series of diluted drugs were added in triplicate, and the plates were kept in an CO_2 incubator for 6, 12, 24 and 48 h, respectively. Following this incubation, 10 μl CCK-8 working solution was added to each well, and the plates were incubated at 37 °C for 3 h. Absorbance at 450 nm was measured using a TECAN Infinite M200 microplate reader (Männedorf, Switzerland). The 50% cytotoxicity concentration (CC_{50}) was calculated using CalcuSyn software (Chou and Hayball, 1991).

2.4. Time-of-drug-addition assay for the anti-HIV-1 and HSV-2 activity of PSM

The time-of-drug-addition assay for PSM anti-HIV-1 activity was performed as described (Daelemans et al., 2005; Qiu et al., 2012).

To investigate the stages of inhibitory action of PSM on HSV-2 life cycle, 10^4 Vero cells per well were infected with HSV-2 (G) (MOI = 1). The anti-HSV-2 drugs, including dextran sulfate (100 μg/ml), acyclovir (50 μg/ml) and PSM (100 μg/ml), were added at various time points post viral inoculation. The infection was determined by HSV-2 gD quantification 24 h postinfection via In-cell Western assay.

2.5. In-cell Western

The wells in 96-well plate were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min. The cell layers were permeabilized by 5 washes in 0.1% Triton-X 100 in PBS with 5 min for each wash. Cell layers were blocked for 90 min and then incubated in 50 μl primary antibodies diluted into LI-COR Blocking Buffer (Lincoln, USA) (1:200) for 2 h. After washing 5 times with PBS-T buffer, the cell layers were inoculated in IRDye 800 goat-anti-mouse IgG with 1:2500 dilution for 1 h. The plate was washed 5 times finally and scanned in LI-COR Odyssey Infrared Imager.

2.6. Inhibition of cell–cell fusion

The effect of PSM in inhibiting fusion between MT-2 cells (expressing CD4 and CXCR4) and CHO-WT cells stably transfected with $gp160_{HXB2}$ was determined using a modified syncytium formation assay (Li et al., 2010a; Qiu et al., 2012). PSM concentration used in this experiment was below that of causing apparent cytotoxicity, determined using CCK-8 kit.

2.7. Interaction of PSM with HIV-1 viral gp120, and PSM effects on the interaction gp120s binding to sCD4 or $CD4^+$ T lymphocytes

The interaction of PSM with HIV-1 rgp120s and the effects of PSM on sCD4 binding to gp120 were analyzed by solid-phase enzyme-linked immunosorbent assay (ELISA) as described (Qiu et al., 2012).

PSM inhibition of rgp120 binding to $CD4^+$ human T lymphocytes was investigated by FACS. 1 μg $gp120_{ADA}$ or $gp120_{IIIB}$ was pre-incubated with 10^6 MT-2 cells in the presence or absence of PSM at 37 °C for 1 h. After washing twice, cells were stained with 1 μg/ml 2G12 at 4 °C, followed by incubation with goat-anti-human IgG-FITC (1 μg/ml). For nonspecific staining, cells were stained in parallel with isotype control mAb. The cells were then washed and fixed with 2% paraformaldehyde solution and analyzed with a FACSCalibur (BD, San Jose, US).

2.8. Inhibition of HIV-1 and HSV-2 viral attachment

PSM inhibition of HIV-1 viral attachment was examined as previously described (Qiu et al., 2012; Wu et al., 2003). p24 level in the clarified lysate was determined using p24 core antigen ELISA kit (Shanghai Qifa Biotech Ltd., Shanghai, China).

The inhibitory effect of PSM on HSV-2 viral attachment was determined in Vero cells. PSM were added into wells and then exposed to HSV-2 (G) (MOI = 1) at 37 °C for 2 h. Cells were then washed 5 times with PBS and cultured for 24 h. The cell layers were scraped off and lysed. HSV-2 entry was quantified by measuring amounts of HSV-2 gD in cell lysates by Western blot.

2.9. Western blot

Cells were lysed with RIPA lysis buffer (Santa Cruz, Santa Cruz, USA). The lysates were then centrifuged and the supernatants were collected and the total protein concentrations were determined using BCA protein assay kit (Pierce, Rockford, USA). After separated using SDS–PAGE, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA). The membranes were blocked and then inoculated in diluted primary antibody at room temperature. The blots were incubated in IRDye 800 goat-anti-mouse IgG with 1:10,000 dilution for 1 h after washing 4 times with PBS-T buffer. The membranes were washed 4 times after secondary antibody inoculation and visualized under LI-COR Odyssey Infrared Imager (Lincoln, USA).

2.10. Mice and vaginal HSV-2 challenge

Five-week old female BALB/c mice were subcutaneously injected with 2.5 mg Depo-Provera (Pfizer, New York, USA) 5 days before vaginal challenge. Gel formulation containing 1%, 5% PSM or placebo was formulated with additional hydroxyethyl cellulose to sterile PBS. The viscosity was controlled under 75,000–80,000 cps. The formulated gels contained 0.05% methyl 4-hydroxybenzoate as antiseptic. The mice were given 10 μl gel intravaginally 5 min before vaginal challenge with 8 μl inoculums containing 5×10^5 PFU HSV-2 (G). Gel containing no PSM served as a placebo. Vaginal swab samples were collected 2 days postinfection and stored at −80 °C. Mice were evaluated 14-days post-inoculation for symptomatic infection as described (0 = no sign of infection; 1 = slight genital erythema and edema; 2 = moderate genital inflammation; 3 = purulent genital lesions; 4 = hind limb paralysis; 5 = death) (Morrison et al., 1998). Animals that did not develop symptoms were defined as the infected individuals if vaginal swab samples contained HSV-2 after detection on Vero cell monolayers.

2.11. Inhibition of Lactobacillus growth

Lactobacillus was cultured in anaerobic chamber for 24 h. Cells were harvested via centrifugation and resuspended in MRS broth (OD_{600} = 0.1). To assess the inhibitory effect of PSM to vaginal *Lactobacillus*, 100 μl cell suspensions were seeded onto 96-well plate and serial concentrations of drugs were added. Ampicillin was set as positive control. The plate was maintained in GENbag anaer (BioMérieux, l'Etoile, France) and incubated at 37 °C. The minimum inhibitory concentration (MIC) of the compounds, defined as the highest concentration in the well with clear culture was determined.

2.12. Quantification of NF-κB activation and IL-1β and IL-8 expression

Quantification of NF-κB activation in cells exposed to PSM was detected by using NF-κB-luc reportor plasmid. The plasmid was transiently transfected into VK2/E6E7 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). After 24 h, PSM and N-9 (10 μg/ml) were added, and the cells were cultured for another 24 h. The level of NF-κB activation was quantified by RLU as described.

To determine IL-1β and IL-8 expression, cells were treated with various concentrations of PSM. N-9 (10 μg/ml) was used as positive control. After 6 h incubation, the supernatants were harvested and centrifuged to remove cellular debris and IL-1β and IL-8 concentrations were measured using an ELISA kit (eBioscience, San Diego, USA) according to the manufacturer's instructions.

2.13. Statistic

In vitro data were analyzed by Student's *t*-test. And the statistical significance of difference in proportion of HSV-2 infection or disease was determined by the Fisher exact test with no correction for multiple comparisons.

3. Results

3.1. PSM inhibited infections of HIV-1 and HSV-1/2 in vitro

The anti-HIV-1 activity of PSM was analyzed in a single-cycle infectivity assay on GHOST(3)-CD4 cells expressing both CXCR4 and CCR5 co-receptors (GHOST(3) X4/Hi5) by HIV-1NL4-3 luciferase reporter viruses pseudotyped with different Envs, including JR-FL, HXB2 and a panel of diverse clinical isolates derived from infected Chinese patients CNE6 (B' subtype, R5-tropic), CNE30 (B'C subtype, R5-tropic), CNE50 (B'C subtype, dual tropic) and CNE55 (CRF01-AE subtype, R5-tropic). PSM inhibited the infection of both JR-FL (R5-tropic) and HXB2 (X4-tropic) with EC_{50} values of 5.76 μg/ml and 0.77 μg/ml (Table 1), respectively. Its inhibitory activities against the clinical isolates ranged from EC_{50} values of 1.70 to 3.15 μg/ml (Table 1). The EC_{50} values for different strains were well below the concentrations which caused significant cytotoxicity of human epithelial cells (Fig. 4) and GHOST (3) X4/Hi5 (Table 2). The EC_{90}s for HXB2 and the clinical isolates were in the range of 2.55 to 14.27 μg/ml while JR-FL appeared to be somewhat insensitive to the molecule with an EC_{90} at 43.69 μg/ml. In contrast, at the

Table 1
PSM inhibited HIV-1 pseudovirus and HSV-1/2 infection *in vitro*.

Virus		Tropism	Subtype	EC_{50} ± SD (μg/ml)	EC_{90} ± SD (μg/ml)	IC_{50} ± SD
HIV-1	JR-FL	R5 tropic	B	5.76 ± 2.29	43.69 ± 6.21	7.93 ± 0.28 mg/ml for Ghost (3) X4/Hi5 cells
	HXB2	X4 tropic	B	0.77 ± 0.15	2.55 ± 0.28	
	CNE6	R5 tropic	B′	1.85 ± 0.26	7.57 ± 1.87	
	CNE30	R5 tropic	B′C	3.15 ± 1.02	14.27 ± 3.58	
	CNE50	Dual tropic	B′C	1.70 ± 0.32	5.13 ± 0.45	
	CNE55	R5 tropic	CRF01-AE	2.27 ± 0.27	7.44 ± 0.96	
VSVG pseudovirus				>60.00	>60.00	
HSV-1 (HF)				2.83 ± 1.01	4.97 ± 1.56	>10.00 mg/ml for Vero cells
HSV-2 (G)				0.27 ± 0.08	0.51 ± 0.12	

Table 2
PSM had low inhibitory effects on *Lactobacillus* growth *in vitro*.

Strain	MIC (PSM, mg/ml)	MIC (ampicillin, μg/ml)
Lactobacillus #1	12.5	0.63
Lactobacillus #2	>50	1.25
Lactobacillus #3	6.25	0.31
Lactobacillus #4	25	1.25
Lactobacillus #5	25	0.63
Lactobacillus #6	>50	0.63
Lactobacillus #7	6.25	0.31
L. acidophilus (ATCC#4356)	>50	1.25

maximal concentration of 60 μg/ml, PSM failed to significantly inhibit the infection by VSV-G pseudotyped virus. Additionally, we also considered the synergy effects of PSM and AZT (an NRTI) or nevirapine (an NNRTI). As shown in Supplementary Fig. 4, the combination of PSM with AZT or nevirapine exhibited synergism or strong synergism against JR-FL and HXB2, respectively.

PSM also exhibited anti-HSV-1/2 activity on Vero cells as measured by a plaque forming assay. PSM inhibited the infection of pathogenic HSV-2 (G) with an EC_{50} value at 0.27 μg/ml and inhibited HSV-1 (HF) infection with EC_{50} at 2.83 μg/ml. The EC_{90}s for HSV-1 and HSV-2 were 4.97 and 0.51 μg/ml, respectively. These results suggested that PSM was an excellent candidate as antiviral agents with a broad spectrum of antiviral activity against HIV-1 and HSV-1/2.

3.2. PSM inhibited HIV-1 and HSV-2 infection by blocking viral entry

A time-of-drug-addition assay was performed to investigate the PSM anti-HIV-1 inhibitory mechanism. PSM was added at various time points post viral exposure and the infection level was evaluated in comparison with the infection without PSM. Two RTIs, AZT (an NRTI) and nevirapine (an NNRTI), and an entry inhibitor, dextran sulfate, were included as reference drugs. PSM exhibited similar inhibitory profile as dextran sulfate, but was distinct from both RTIs (Fig. 1A). Virus infection was inhibited by ~90% when PSM was added 1 h postinfection and about 20% HIV-1 infection was detected when PSM was added at 2 h postinfection. When PSM was added at 3 to 5 h postinfection, increasing amounts of infection (40%) were detected. In contrast, AZT and nevirapine completely inhibited HIV-1 infection even added at 5 h postinfection. The observation above suggested that PSM functioned before viral reverse transcription and acted at an early stage of viral entry. This conclusion was further substantiated by PSM inhibition of the cell-cell fusion between CHO-WT cells expressing gp160-trimer and MT-2 cells expressing CD4 and CXCR4 in a dose-dependent manner (Fig. 1B).

The effect of PSM on HIV-1 attachment to $CD4^+$ cells was further investigated, using either $CD4^+$ GHOST cells or $CD4^-$ HEC-1-A (Fig. 1C). Heparan sulfate (HS) served as a positive control. On GHOST (3) X4-Hi5 cells and HEC-1-A cells, the attachment of both JR-FL and HXB2 virions was only slightly inhibited by PSM. Even at 500 μg/ml, a concentration significantly higher than the inhibitory dosage, no complete inhibition was observed.

Similar time-of-drug-addition analysis was performed for HSV-2 infection with acyclovir, a potent inhibitor of viral thymidine kinase, and dextran sulfate as reference drugs, and the infection was quantified by In-Cell-Western analysis, using an anti-gD mAb as primary antibody. As shown in Fig. 1D, significant amount of HSV-2 already escaped when both PSM and dextran sulfate were added 1 h postinfection, in contrast to the complete inhibition by acyclovir added as late as 8 h postinfection. HSV-2 attachment to Vero cells was analyzed by Western blot and showed that PSM inhibited HSV-2 attachment in a dose-dependent manner (Fig. 1E). Taken together, our findings demonstrated that PSM acted on the entry stage against both HIV-1 and HSV-2 infections.

3.3. PSM blocked rgp120 binding to sCD4 and cellular CD4

We further investigated the molecular basis of PSM inhibition, using solid-phase ELISA to analyze PSM effect on interaction of rgp120 with sCD4 or cellular CD4. It was shown that PSM blocked sCD4 binding to immobilized rgp120s in a dose dependent manner (Fig. 2A and B) and PSM also inhibited rgp120 binding to $CD4^+$ human T lymphocytes (MT-2) as measured by FACS (Fig. 2C and D). However, it was noted that even at the highest PSM concentration (500 μg/ml), the blocking of rgp120-CD4 interaction was partial.

3.4. PSM interacted with gp120 V3 loop and CD4-binding site (CD4bs)

gp120 regions for PSM binding were investigated by analyzing PSM interference on a panel of neutralizing mAbs specific for various gp120 regions. Our data showed that PSM inhibited CD4bs mAb b12 binding to $gp120_{ADA}$ and $gp120_{IIIB}$ in a dose-dependent manner (Fig. 3). F105, another CD4bs mAb was remarkably inhibited from binding $gp120_{IIIB}$ by PSM (Fig. 3). V3 specific mAb 447-52D's binding to the gp120s was significantly reduced by the presence of PSM in a dose-dependent manner. To validate the observation, linear synthetic V3 peptides derived from ADA, IIIB and JR-FL were used for analyzing PSM inhibition of 447-52D binding. It was shown that PSM inhibited 447-52D binding to the V3 peptides in a dose-dependent manner, consistent with the observation made with gp120s (Supplementary Fig. 1). 2G12, a broadly neutralizing mAb specific for high mannose carbohydrate moiety, was not affected, which ruled out the possibility that the inhibition was caused by nonspecific PSM binding to the antibody.

3.5. PSM had low in vitro toxicity to genital epithelial cells and vaginal Lactobacillus

The PSM cytotoxicity was investigated in four human genital tract epithelial cells and one colorectal epithelial cell line. As

shown in Fig. 4, low cytotoxicity was observed in Vk2/E6E7 (vagina), Ect1 (ectocervix), End1 (endocervix), HEC-1-A (uterus) and Caco-2 (colon) cells at the maximal concentration of 10 mg/ml after exposure for 6 or 12 h. High level reductions of cell viability were detected at PSM concentrations >300 μg/ml and prolonged exposures of 48hr. Ect1 cells were more sensitive to PSM than other epithelial cell lines after 48 h exposure. Endocervical cells remained >75% viable at PSM concentrations up to 10 mg/ml. Similarly, vaginal cells also remained >75% viable at concentrations up to ~5 mg/ml, but viability dropped to <50% after 48hr exposure to the highest dose of 10 mg/ml.

Lactobacillus is an important component of vaginal environment and its growth was examined in the presence of PSM. As shown in Table 2, PSM was shown to have low toxicity to the growth of *L. acidophilus* and clinical isolations of *Lactobacillus* from a health woman, suggesting that PSM could be compatible with the vaginal *Lactobacillus*.

3.6. PSM did not activate NF-κB and up-regulate IL-1β and IL-8 production

Whether PSM could activate NF-κB and up-regulate IL-1β and IL-8 production was evaluated. We did not find augmented NF-κB activity in VK2/E6E7 cells after 24 h PSM treatment. As a control, N-9, a surfactant, activated NF-κB expression significantly (Fig. 5B). Furthermore, PSM did not elevate IL-1β and IL-8 expression beyond their basal levels, as shown in Fig. 5A. N-9, serving as a control, showed significant up-regulation of both IL-1β and IL-8 in VK2/E6E7 cells.

We further investigated the effect of PSM on the tight junction protein expression using human vaginal epithelial cells, HEC-1A

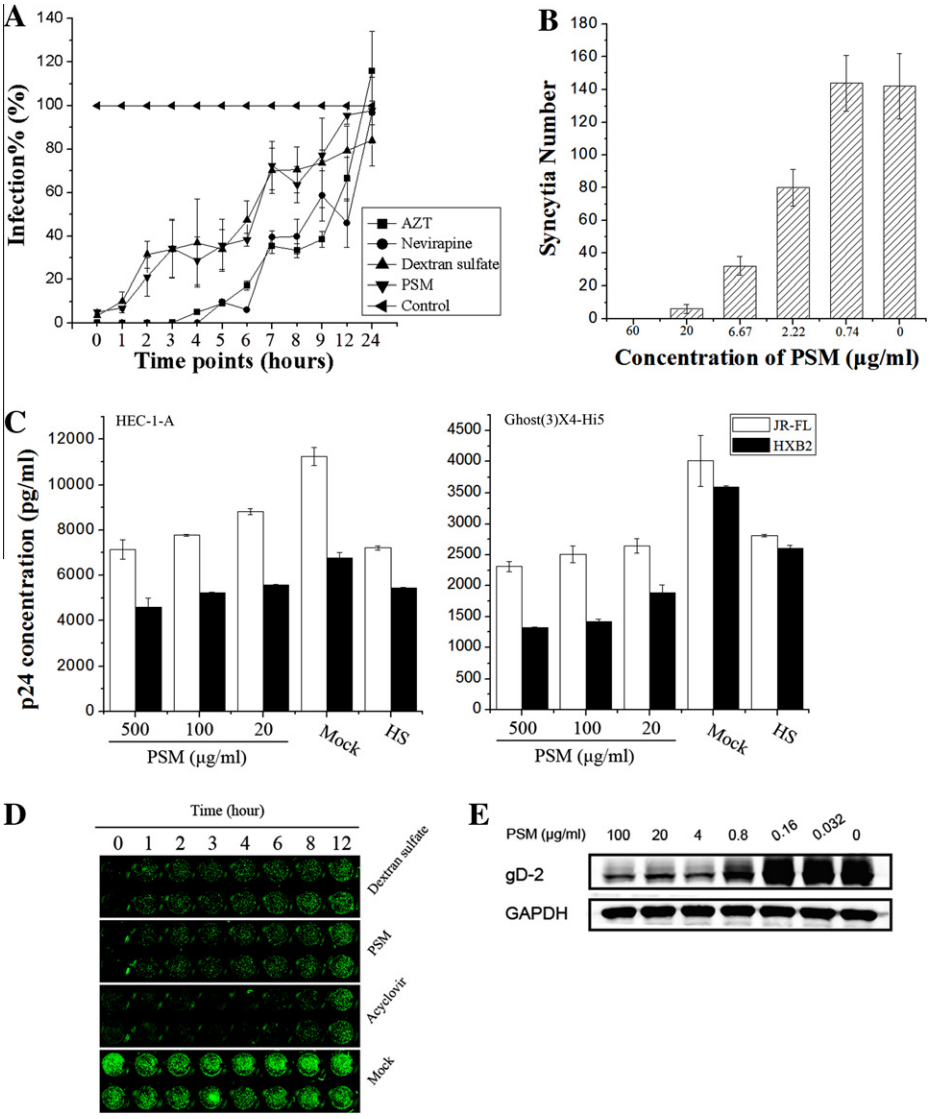

Fig. 1. PSM inhibited HIV-1 and HSV-2 infection by blocking viral entry. (A) Time-of-drug addition assay for evaluating the anti-HIV-1 mechanism of PSM. GHOST (3) X4/Hi5 cells were infected with JR-FL pseudotyped virus, and the test compounds were added at different time points postinfection as indicated. AZT (0.5 μg/ml), nevirapine (2 μg/ml) and dextran sulfate (300 μg/ml) were used as controls. luciferase activity was measured after 48 h. (B) PSM inhibition of cell-cell fusion between CHO-WT cells and MT-2 cells. The syncytia were counted after 24 h. (C) The effect of PSM on inhibition of HIV-1 viral attachment. HEC-1-A or GHOST cells were incubated with 100 ng p24 equivalent pesudovirus in the presence or absence of PSM for 2 h. The effect of PSM on the HIV-1 virion attachment was determined though p24 level detection. Heparan sulfate (HS) (100 μg/ml) was used as control. (D) Time-of-drug addition assay for evaluating the anti-HSV-2 mechanism of PSM. Vero cells were exposed to HSV-2 (G) (MOI = 0.1) and dextran sulfate (100 μg/ml), PSM (100 μg/ml) and acyclovir (50 μg/ml) were added at different time points. Viral replication was represented by gD-2 expression determined via In-cell Western 14 h postinfection. (E) PSM inhibition of HSV-2 viral attachment to Vero cells. Vero cells were exposed to HSV-2 (G) (MOI = 0.1) in the presence of PSM for 2 h. The cells were then washed 5 times with PBS and cultured for 24 h. The amounts of gD-2 were measured by Western blot. Data are means of triplicate determinations with standard error.

and Caco-2 and showed that PSM did not significantly modulate the expressions of ZO-1, E-cadherin and occludin and had minimal effects on the occluding expression (unpublished data). PSM did not disrupt the integrity of the epithelial monolayers either, as judged by a Lucifer Yellow leakage assay (Supplementary Fig. 2).

3.7. In vivo activity of PSM in preventing vaginal HSV-2 infection

To evaluate *in vivo* efficacy, PSM was formulated in hydroxyethyl cellulose gels and tested in a HSV-2 murine infection model through vaginal challenge. The disease and infection were determined as described. As shown in Fig. 6A, using 1% PSM gel, 80% (12/15) of the mice were protected from the disease and 46.7% (7/15) of the mice were protected from the infection. 5% PSM gel led to significantly better protection with 93.3% (14/15) protected from the disease and 73.3% (11/15) protected from the infection. In contrast, 10 out of 11 mice were infected in the placebo group. In Fig. 6B, average score of each group were shown, which demonstrated that 1% and 5% PSM were able to protect the mice against HSV-2 infection and disease progression significantly.

4. Discussion

PSM is a copolymer consisting of alternating styrenesulfonic acid and maleic acid at the ratio of 1:1 and is characterized by the rich contents of anionic groups and aromatic rings. PSM derives its anionic charges from two chemical groups: carboxyl groups (pKa 1.9 and 6.9) of maleic acid and styrenesulfonic groups (pKa 0.7), making PSM more negatively charged compared with its derivate – poly (4-styrenesulfonic acid) (PSS) (See in Supplementary Table 1). However, PSM showed lower cytotoxicity than PSS, demonstrating that PSM have higher Treatment Indexes. In current study, we demonstrated that PSM effectively inhibited infections of both HIV-1 and HSV-1/2 in *in vitro* assays. PSM inhibited infection of HIV-1 pseudovirus not only laboratory strains but also clinical isolates representing B, B', B'C and CRF01-AE subtypes derived from Chinese patients. The EC_{50} values for the tested HIV-1 isolates ranging from 0.77 to 5.76 μg/ml, in the same potency as many previously reported polyanions (Li et al., 2010a,b; Scordi-Bello et al., 2005). PSM also exhibited good strong synergism against HIV-1 with AZT and nevirapine. The PSM inhibition of HSV-2 is more potent, with an EC_{90} value at sub-microgram level (0.51 μg/ml) and was less active against HSV-1 with EC_{90} at about 5 μg/ml. We also demonstrated in an *in vivo* vaginal challenge model that 1% and 5% PSM gel could protect mice from HSV-2 vaginal infection and disease progression significantly.

It is known that NF-κB could trigger the expression of many proinflammatory factors, including cytokines, chemokines, and adhesion molecules (Barnes and Karin, 1997), which could facilitate the HIV-1 infection through recruitment and activation of T lymphocytes, macrophages, dendritic cells and other susceptible target cells. In addition, NF-κB could also activate HIV-1 LTR directly (Mallardo et al., 1996; Perkins, 1997), and enhance HIV-1 replication. Additionally, IL-1β and IL-8 were considered as the sensitive indicators of mucosal toxicity (Fichorova et al., 2004). PSM showing no effects on the activation of cellular NF-κB and up-regulation of IL-1β and IL-8 production of genital epithelial cells demonstrated that it did not cause immuno-ativation in epithelium.

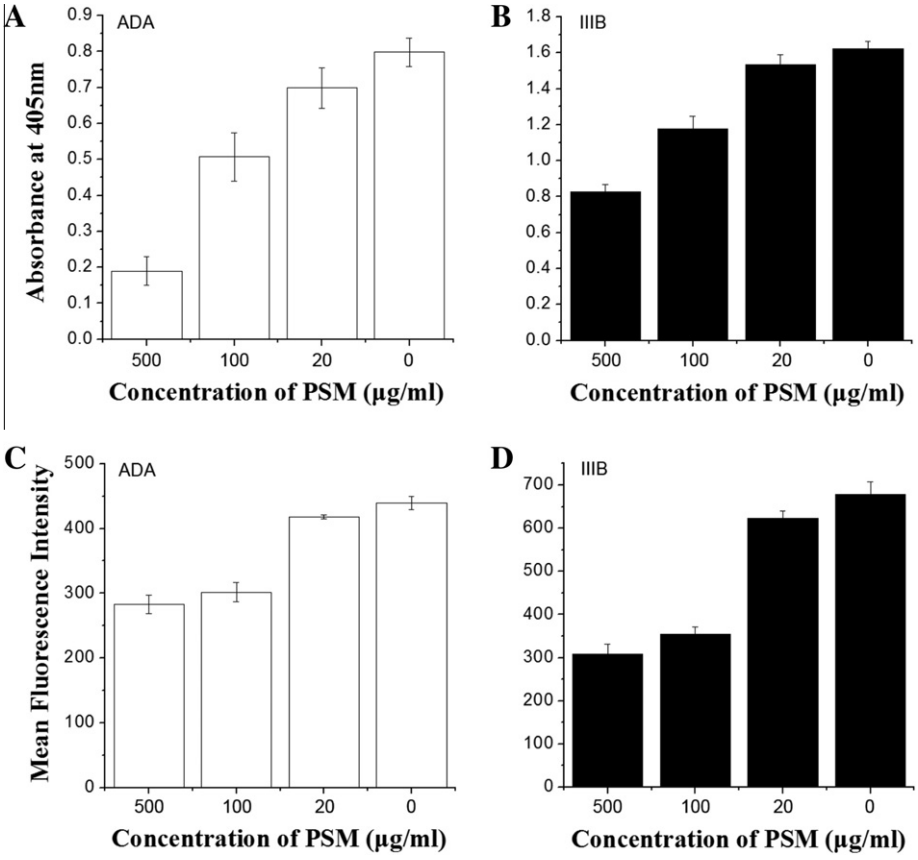

Fig. 2. PSM inhibition of the rgp120 binding to sCD4 and human T lymphocytes. PSM inhibition of the rgp120s binding of to sCD4 was determined by solid-phase ELISA. 400 ng $gp120_{ADA}$ (A) or $gp120_{IIIB}$ (B) was coated into 96-well plate and various concentrations of PSM and 2 μg/ml sCD4 were added to the wells. Bound sCD4 was detected using anti-CD4 mAb OKT4. PSM inhibition of rgp120 binding to human T lymphocytes was detected using FACS analysis. The MT-2 cells were coincubated with $rgp120_{ADA}$ (C) or $rgp120_{IIIB}$ (D) and serial concentrations of PSM. The amount of bound rgp120s on the surface of MT-2 cells was determined by gp120-specific mAb 2G12 and FITC-goat-anti-human IgG. Data are means of triplicate determinations with standard error.

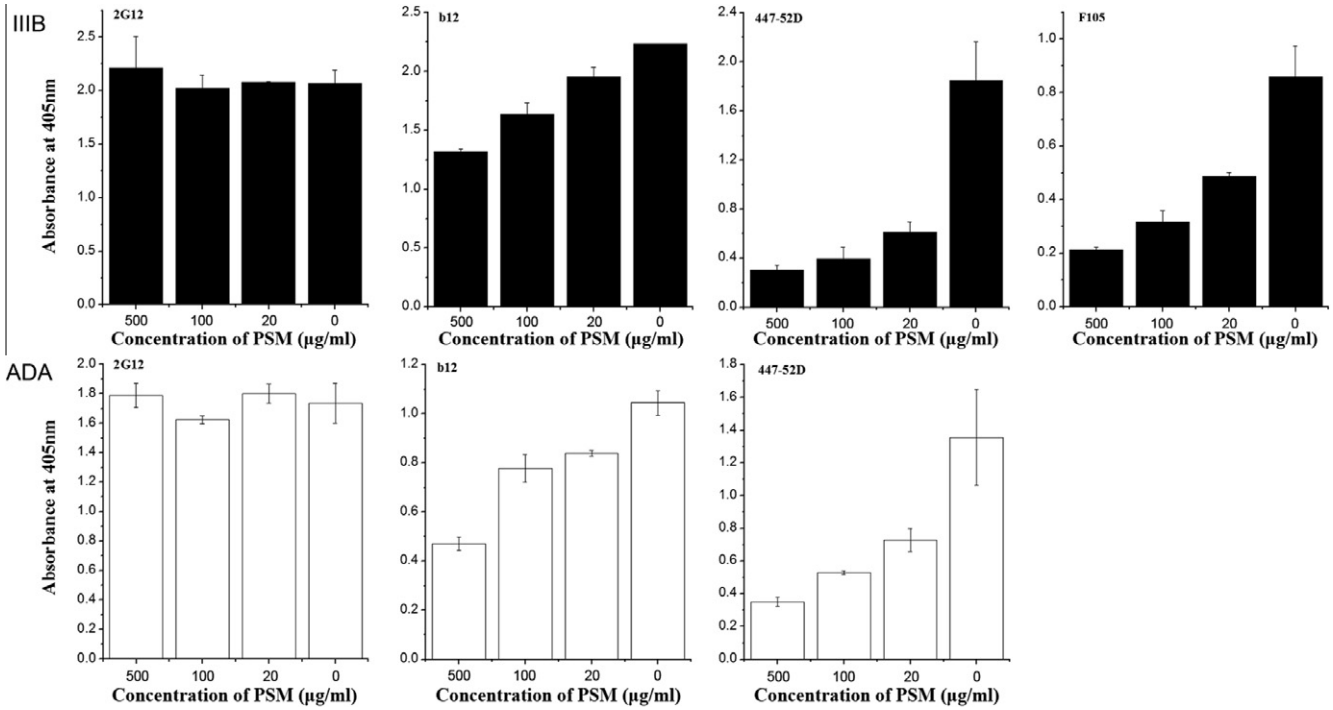

Fig. 3. The effect of PSM on the interaction between rgp120 and anti-gp120 mAbs. rgp120s derived from ADA, IIIB and YU-2 were coated onto the plate. Serial concentrations of PSM were added to the plate together with mAbs specific for gp120 (clone 2G12, b12, 447-52D and F105) for 1 h at 37 °C. The effect of PSM blocking on the antibody binding was determined by solid-phase ELISA. Data are means of triplicate determinations with standard error.

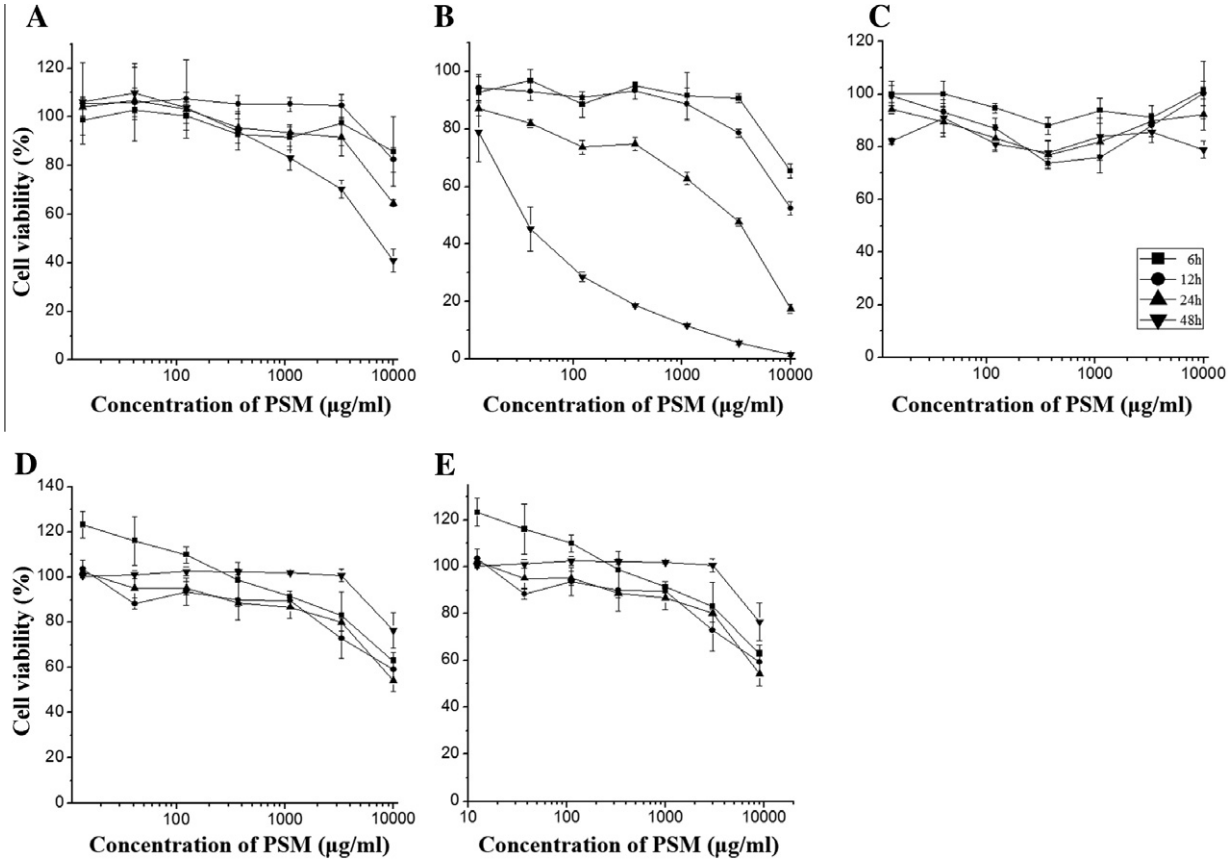

Fig. 4. The effect of PSM on cell viability of human genital tract and colonal epithelial cell lines. (A) Vk2/E6E7; (B) Ect1/E6E7; (C) End1/E6E7; (D) HEC-1-A; (E) Caco-2; these cells were exposed to PSM for 6, 12, 24 and 48 h and then the cell viability was determined by CCK-8 colorimetric assay. Data are means of triplicate determinations with standard error.

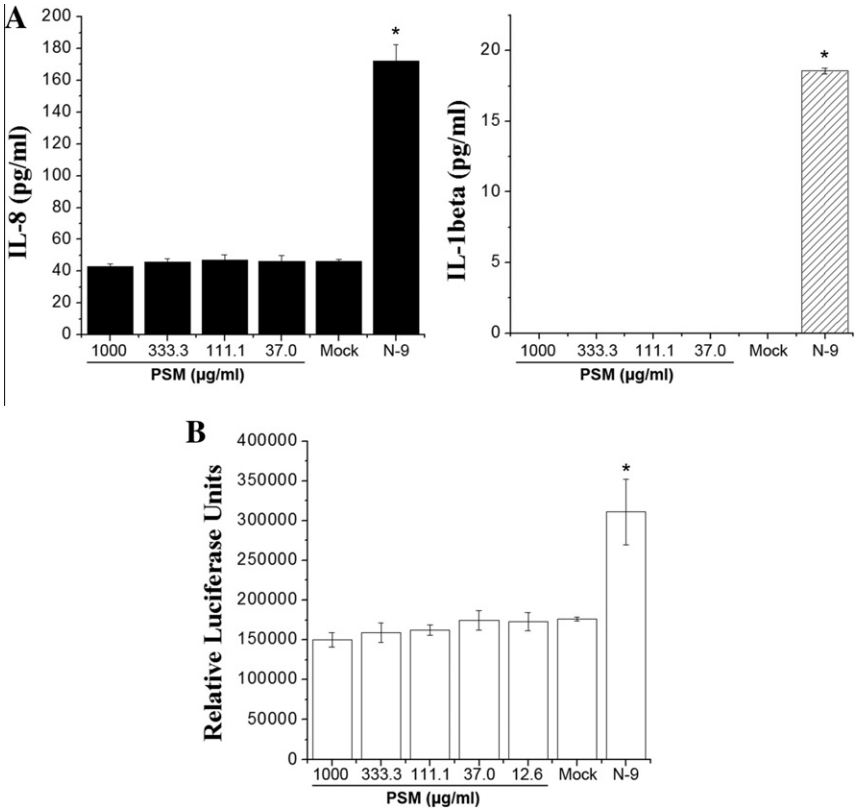

Fig. 5. PSM showed no ability to activate NF-κB and up-regulate IL-1β and IL-8 production significantly. (A) IL-1β and IL-8 secretions of VK2/E6E7 cells after PSM incubation. Cells were seeded into 24-well plates and induced with various doses of PSM and controls. After incubation for 6 h, the supernatants were harvested and IL-1β and IL-8 concentrations were measured by ELISA assay. (B) Quantification of NF-κB activation in VK2/E6E7 cells post-incubation with various concentration of PSM. Luciferase reportor plasmid operated by NF-κB response element was transfected into VK2/E6E7 cells. The levels of NF-κB activation was evaluated through measurement of RLU. N-9 (10 μg/ml) was set as the positive control. Each panel shows the means of standard deviations from three independent experiments. $^{*}P < 0.05$ for comparison with mock.

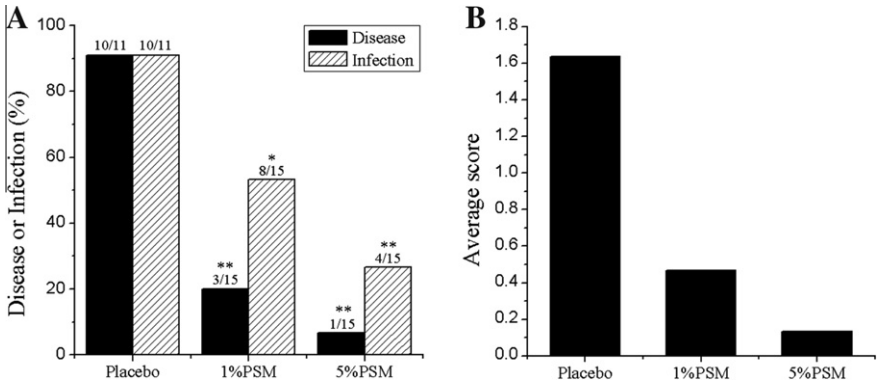

Fig. 6. PSM efficacy against vaginal HSV-2 inoculation in murine model. Female BALB/c mice injected with 2.5 mg Depo-Provera 5 days before vaginal challenge were then given 5%, 1% PSM and placebo gel intravaginally 5 min before vaginal challenge with HSV-2 (G). Mice were evaluated 14 days post-inoculation for symptomatic infection. Symptomatic infection was ruled out through detection of HSV-2 infectious particles in vaginal swabs collected 2 days post-inoculation. (A) Infection and Disease occurrence ratio. (B) Score evaluation as described. $^{*}P < 0.05$ and $^{**}P < 0.01$ for comparison with placebo.

A healthy vaginal microbiome aids in the prevention of bacterial vaginosis, yeast infections and other pathogens by maintaining an acidic pH (<4.5). It is critical that a microbicide should not disturb the normal growth of the vaginal flora. As a predominant component of the vaginal microbiome (Antonio et al., 1999), *L. acidophilus* has been reported to inhibit HIV-1 viral replication by producing hydrogen peroxidase (Sewankambo et al., 1997). Our results showed that PSM did not affect the growth of *L. acidophilus* implying that it might be compatible with the vaginal microbiome. In addition, PSM showed no significant inhibitory effect on the growth of seven *Lactobacillus* clones isolated from vaginal swabs, consistent with the results obtained from *L. acidophilus*.

The inhibitory activities of PSM appear to be mediated by blocking the entry steps of both HIV-1 and HSV-2. This conclusion is supported by the time-of-drug-addition and the cell-cell fusion inhibition analyses for HIV-1 (Fig. 1A and B) and time-of-drug-addition and In-cell Western analyses (Fig. 1D and E) for HSV-2. The observations are consistent with known mechanisms of other sulfated polysaccharides (Baba et al., 1988; Li et al., 2010a; Mondor et al., 1998; Nishimura et al., 1998; Parish et al., 1990; Roehr, 2009). Based on the PSM inhibition of 447-52D binding to gp120

(Fig. 3), it appeared that the V3 sequence was probably the major determinant mediating the interaction with PSM. PSM did not efficiently inhibit 447-52D binding to JR-FL V3 polypeptides (Supplementary Fig. 1), which may account for the insensitivity of JR-FL to PSM inhibition. However, whether the V3 binding is correlated with PSM inhibitory activity remains to be investigated. We also found that PSM efficiently interfered CD4bs mAb F105 binding to $gp120_{IIIB}$, and b12, another CD4bs mAb recognizing a distinct epitope (Zhou et al., 2010) was also inhibited by PSM in a dose-dependent manner. We speculate that PSM binds to the gp120 region overlapping or proximal to the CD4 binding sites. This type of interaction would directly or indirectly disrupt CD4-gp120 binding. Several studies have reported that polyanions inhibited HIV-1 infection by disrupting the interaction between gp120 and CD4, by either binding to CD4 (Lederman et al., 1989; Parish et al., 1990) or to gp120 (Crublet et al., 2008; Moulard et al., 2000). Lynch et al. (Lynch et al., 1994) reported that dextran sulfate and other polyanions prevented HIV-1 infection by disrupting the interaction between gp120 and CD4 molecules and suggested that the polyanion binding to CD4 is the predominant mechanism of inhibition. Crublet et al. (Crublet et al., 2008) mapped four gp120 domains that interacted with heparan sulfate, V2, V3, a bridging sheet induced by CD4 binding and a sequence at the C-terminus of gp120. Therefore, it is plausible that different polyanions may exhibit distinct binding specificities though polyanions are generally considered nonspecific. The differing binding specificities may depend on the number of charged groups, the size and the structure of the polyanion or the topology of the binding domains involved. Viral attachment to both the $CD4^+$ cells and $CD4^-$ endometrial cells was not remarkably affected by PSM treatment. Studies by Monder et al. (Mondor et al., 1998) reported that HIV-1 viral attachment was minimally influenced by the presence of cellular CD4 and suggested that nonspecific cellular adhesion molecules may play a vital role in mediating initial virus-cell interaction. To obtain a better view of the structural relationship, we employed bioinformatics methods to analyze and predict the PSM binding sites on the viral envelope protein (Supplementary Fig. 3). The complete structure of $gp120_{JR-FL}$ including V3 loops was reconstructed by homology modeling using published data (Huang et al., 2005). The docking analysis predicted that the most stable and energy-optimized status was PSM in complex with the stem and partial tip of V3-loop (Supplementary Fig. 3A and B), consistent with the PSM blocking of 447-52D binding. Another potential binding site on gp120 was predicted to be proximal to the CD4 binding site (Supplementary Fig. 3C and D).

HSV entry into host cells marks the most critical step in viral pathogenesis and involves a cascade of complex events. Five viral glycoproteins have been implicated in the entry process: gB, gC, gD, gH, and gL (Campadelli-Fiume et al., 2007; Spear, 2004). The initial interaction, or binding to cells, is mediated via interactions of gC and/or gB with heparan sulfate proteoglycans (HSPGs), followed by the membrane fusion step requiring essential participation from gB, gD, gH and gL forming a multiglycoprotein complex (Atanasiu et al., 2007). The use of heparan sulfate as cellular receptor may explain the observation that HSV-2 attachment to cells was significantly inhibited by PSM since the positively charged PSM will bind the negatively charged HS moiety as an antagonist to the virus.

Genital herpes is highly prevalent in many populations, particularly in those of low economic settings (Konda et al., 2005). Due to the shared route of transmission, genital herpes is a risk factor for HIV-1 sexual transmission (Corey et al., 2004; Kapiga et al., 2007; Lama et al., 2006). Genital herpes increases the risk of HIV-1 acquisition through sexual contact approximately 3-fold (Freeman et al., 2006). A recent study showed that even HSV-2 infected patients successfully treated by acyclovir were still more susceptible to HIV infection by sexual contact (Zhu et al., 2009). Although it is not clear what the incidence rates are for HIV-1 and HSV-2 co-transmission, it can be postulated that high incidence may exist in view of the high rates of co-infections in many populations (Corey et al., 2004). Therefore, it would be preferable to develop a preventative topical microbicide that is effective for both pathogens. A dual functional inhibitory drug will offer convenience, reduced cost and advantages in preventing infections of both HIV-1 and HSV-2 simultaneously. The dual activities of PSM in inhibiting both HIV and HSV-2 infection, combined with its low cytotoxicity to human genital epithelial cells and non-disruption on vaginal microfloral growth, deserve further investigation as a topical anti-viral agent.

Acknowledgements

We thank Dr. Linqi Zhang at Comprehensive AIDS Research Center, Tsinghua University, China for the generous gift of Plasmids pNL4-3 E^-R^- Luc and HIV-1/VSV-G Env, Dr. Qihan Li at Institute of Medical Biology, Chinese Academy of Medical Sciences for HSV-1 strain HF and Dr. Erguang Li at the School of Medicine, Nanjing University, China for HSV-2 strain G. This study was supported by the National Science Foundation (Grant No. 30870124), a grant from the Committee of Sciences and Technologies of Shanghai Municipal Government (Grant No. 1052nm05600), the Major Research and Development Project from the Ministry of Health (Grant No. 2008ZX10001-015 and 2012ZX10001-007-009-001), and an International Collaborative Research Grant from the Ministry of Sciences and Technology (Grant No. 2009DFA31260).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.08.005.

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