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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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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.

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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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.