ADS-J1 Inhibits Semen-Derived Amyloid Fibril Formation and Blocks Fibril-Mediated Enhancement of HIV-1 Infection

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Semen-derived enhancer of viral infection (SEVI) is composed of amyloid fibrils that can greatly enhance HIV-1 infectivity. By its cationic property, SEVI promotes viral sexual transmission by facilitating the attachment and internalization of HIV-1 to target cells. Therefore, semen-derived amyloid fibrils are potential targets for microbicide design. ADS-J1 is an anionic HIV-1 entry inhibitor. In this study, we explored an additional function of ADS-J1: inhibition of SEVI fibril formation and blockage of SEVI-mediated enhancement of viral infection. We found that ADS-J1 bound to an amyloidogenic peptide fragment (PAP_{248–286} comprising amino acids 248 to 286 of the enzyme prostatic acid phosphatase), thereby inhibiting peptide assembly into amyloid fibrils. In addition, ADS-J1 binds to mature amyloid fibrils and antagonizes fibril-mediated enhancement of viral infection. Unlike cellulose sulfate, a polyanion that failed in clinical trial to prevent HIV-1 sexual transmission, ADS-J1 shows no ability to facilitate fibril formation. More importantly, the combination of ADS-J1 with several antiretroviral drugs exhibited synergistic effects against HIV-1 infection in semen, with little cytotoxicity to vaginal epithelial cells. Our results suggest that ADS-J1 or a derivative may be incorporated into a combination microbicide for prevention of the sexual transmission of HIV-1.

According to the latest report on the global AIDS epidemic, published in 2013, 2.3 million new HIV-1 infections were reported worldwide in 2012, and sexual contact remains the major mode of transmission. Therefore, the development of effective preventive measures is of great importance. Recently, an oral antiretroviral (ARV)-based preexposure prophylactic (PrEP) was approved by the U.S. Food and Drug Administration (FDA) (1), but concerns have been raised about oral application, such as the emergence of potential ARV resistance, systemic toxicity (2), suboptimal local drug concentration at mucosal sites (3, 4), and poor adherence, among others. Microbicides, such as the topical PrEP strategy, are an alternative form of treatment to prevent HIV-1 sexual transmission, both for women to take full control to protect themselves and for men who have sex with men, who continue to have disproportionate and increasing levels of HIV infection. The advantages of microbicides include higher drug levels at vaginal or rectal sites without systemic exposure. After 20 years of experience in microbicide development, candidate microbicides have been focused on more potent ARV drugs instead of agents with non-specific mechanisms, as well as on combination drug formulations with multipurpose prevention technologies (MPT) instead of a single ARV drug, to increase the potential level of efficacy (5).

Semen is an important carrier of HIV-1 during sexual intercourse. Accumulating evidence shows that semen harbors distinct endogenous amyloid fibrils, termed semen-derived enhancers of viral infection (SEVI), which can greatly enhance HIV-1 infection and other sexually transmitted infection (STI) pathogens (6–10). A proteolytic cleavage product of prostatic acid phosphatase (the peptide PAP_{248–286}, comprising amino acids 248 to 286 of the enzyme) is the best characterized amyloid fibril in semen. Due to its strong positive charge, SEVI can neutralize the negative charges on the viral membrane surface and can target the cell membrane, capture virus, and promote viral attachment to target cells to drastically enhance HIV-1

infection (11). Due to the presence of SEVI, semen impairs the antiviral activities of a panel of candidate microbicides (12). Thus, seminal amyloid fibrils are a potential target for microbicides. Although the mechanism of the generation of amyloid fibrils during sexual intercourse remains elusive, it is accepted that semen harbors amyloidogenic peptides, endogenous amyloid fibrils, or a mixture of both (7, 10, 13–15). Agents that show the ability to (i) inhibit peptide self-assembly into amyloid fibrils, (ii) antagonize fibril-mediated enhancement of HIV-1 infection, or (iii) destroy mature fibrils will provide the properties required for the design of a combination microbicide.

Several agents targeting semen-derived amyloid fibrils have been evaluated (16–21). Amyloid-binding molecules bind amyloid and abrogate SEVI-mediated enhancement of viral infection (16, 18). The aminoquinoline surfen interferes with the interactions between SEVI and target cells and between SEVI and virus (19). Metal ions and nonnatural amino acid inhibitors have been shown to inhibit SEVI fibril formation (20, 21). Epigallocatechin-3-gallate (EGCG) degrades mature SEVI fibrils and abrogates fibril-mediated enhancement of viral infection (17). However, most of these agents exhibit

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monopharmaceutical effects. Agents that show efficacy against both amyloidogenic peptide to inhibit fibril formation and SEVI to block its mediation of enhanced viral infection might have greater preventive potential against HIV-1 sexual transmission.

Polyanions were previously shown to bind to SEVI and antagonize SEVI-mediated enhancement of HIV-1 infection (11). However, we also reported that polyanions might actually interact with an amyloidogenic peptide fragment to facilitate the process of SEVI fibril formation; therefore, the application of polyanions should be carefully reevaluated (22). ADS-J1 is an anionic agent that was first discovered by our group and demonstrated to show inhibitory effects on HIV-1 infection by targeting gp41 (23, 24). Based on its anionic property and a molecular weight that is different from those of other polyanions, we hypothesized that ADS-J1 might bind to amyloidogenic monomeric peptides and SEVI fibrils, resulting in inhibition of peptide aggregation and abolishment of SEVI-mediated enhancement of HIV-1 infection, respectively. More importantly, unlike cellulose sulfate, a polyanion that failed in clinical trial, ADS-J1 might not facilitate SEVI fibril formation. Therefore, in this study, we investigated the dual mechanism of action of ADS-J1 to inhibit SEVI fibril formation and to block SEVI-mediated enhancement of HIV-1 infection. Moreover, combined with other ARV drugs, ADS-J1 displays synergistic effects against HIV-1 infection in semen while at the same time showing little or no toxicity to cervical cells, suggesting that ADS-J1 is a potential lead candidate for microbicide formulation.

MATERIALS AND METHODS

Materials. Thioflavin T (ThT), Congo red, D-galactose 6-sulfate, N-acetyl-D-galactosamine 6-sulfate (Fig. 1B and C), and [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-(phenylamino)carbonyl-2H-tetrazolium hydroxide] (XTT) were purchased from Sigma (St. Louis, MO). The Proteostat amyloid plaque detection kit was purchased from Enzo Life Sciences (Plymouth Meeting, PA). PAP_{248–286} and the semenogelin 1 peptide, comprising amino acids 86 to 107 (SEM1_{86–107}), were synthesized by Scilight-Peptide (Beijing, China) and GL Biochem (Shanghai, China) (Fig. 1D), respectively. Lyophilized peptides (>95% purity) were dissolved at a concentration of 10,000 µg/ml in phosphate-buffered saline (PBS) and stored at –20°C. Fibril formation was initiated by agitation at 37°C for 1 to 3 days at 1,400 rpm with an Eppendorf Thermomixer. Polyclonal rabbit antibody against PAP_{248–286} was produced by AbMax Biotechnology Co., Ltd. (Beijing, China). Semen (SE) samples were obtained from healthy members of the laboratory with written informed consent; the protocol was approved by the Human Ethics Committee of Southern Medical University, China. Ejaculates were liquefied for 30 min at room temperature as soon as collected. Seminal fluid (SE-F), representing the cell-free supernatant of SE, was collected by centrifugation of 1 ml SE for 15 min at 10,000 rpm and stored in 1-ml aliquots at –20°C. MT-2 cells, TZM-bl cells, plasmid encoding enhanced green fluorescent protein (EGFP)-Vpr, anti-p24 monoclonal antibody (183-12H-5C), TMC120, zidovudine (AZT), tenofovir, maraviroc, HIV-1 IIB, and HIV-1 US4 (GS007) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. CXCR4-tropic NL4-3 and CCR5-tropic SF162 infectious clone plasmids were kindly provided by Jan Münch of Ulm University, Ulm, Germany. Viruses were prepared as previously described (11). Briefly, viruses were produced by calcium phosphate-mediated transfection of 293T cells with DNA proviral expression plasmids. Viruses were collected 2 days later. ADS-J1 (Fig. 1A) was purified by high-performance liquid chromatography (HPLC) from the crude material provided by Ciba Specialty Chemicals Corp. (High Point, NC).

Monitoring of semen-derived amyloid fibril formation. Fibril formation of PAP_{248–286} or SEM1_{86–107} was monitored by ThT and Congo red assays, as previously reported (22). Fibrils were generated by incubating 3,000

µg/ml PAP_{248–286} or SEM1_{86–107} in the presence or absence of ADS-J1 or sulfated sugars at the concentrations indicated below and then agitating at 1,400 rpm at 37°C with an Eppendorf Thermomixer. At the time points indicated below, aliquots were withdrawn from each sample for ThT and Congo red staining that was performed similarly to previously described methods (22). To monitor amyloid fibril formation in semen, 5 µl ADS-J1 at different concentrations or PBS was incubated with 95 µl whole SE-F, the mixtures were agitated, and samples were withdrawn at different time points to detect fibril formation by ThT staining as described above.

Observation of amyloid fibrils by TEM. At different time points, peptide suspensions (500 µg/ml) were adsorbed for 2 min onto glow-discharged carbon-coated grids. The grids were subsequently stained with 2% phosphotungstic acid for 2 min, and fibrils were visualized with an H-7650 transmission electron microscope (TEM) (Hitachi Limited, Tokyo, Japan).

Native polyacrylamide gel electrophoresis and Western blot analyses. Ten microliters of PAP_{248–286} monomers (500 µg/ml) was incubated with 10 µl of serially diluted ADS-J1 (1,000, 500, 250, 125, and 62.5 µg/ml) at 37°C for 30 min. Then, the samples were centrifuged at 5,000 rpm for 15 min, and the supernatant was mixed with an equal volume of 50% glycerol. Samples were run on acidic continuous native polyacrylamide (10%) gels (22). Gels were either stained with Coomassie blue or subjected to Western blot analysis to specifically recognize PAP_{248–286}. To determine the antagonizing effect of Polybrene on the binding of ADS-J1 to PAP_{248–286}, 5 µl of serially diluted ADS-J1 (1,000, 500, 250, 125, and 62.5 µg/ml) was first incubated with 5 µl of Polybrene (1,000 µg/ml) at 37°C for 30 min. Then, 500 µg/ml PAP_{248–286} (10 µl) was added, and the mixtures were incubated at 37°C for 30 min. The mixtures were centrifuged at 5,000 rpm for 15 min, and the PAP_{248–286} remaining in the supernatant was analyzed as described above.

ITC analysis of the binding of ADS-J1 to PAP_{248–286}. The binding of ADS-J1 to PAP_{248–286} was measured by a NANO isothermal titration calorimeter (ITC) (TA Instruments), with stirring at 300 rpm at 37°C. The titration of ADS-J1 with PAP_{248–286} involved 20 injections of 2.5 µl of PAP_{248–286} solution (~900 µg/ml, 200 µM) at intervals of 2 min into a sample cell containing 300 µl of ADS-J1 solution (~210 µg/ml, 200 µM). Blank ITC experiments were done to correct heat-of-dilution effects. The data were analyzed with NANO ITC software to determine the enthalpy (ΔH) and dissociation constant (*K_d*).

Detection of HIV-1 binding to SEVI and amyloid fibrils in semen by confocal microscopy. EGFP-labeled virions were prepared as described elsewhere (25). SEVI (1,000 µg/ml) and SE-F (1:100 diluted) were incubated in the presence or absence of 500 µg/ml ADS-J1 at 37°C for 30 min. Mixtures were stained with Proteostat as recommended by the manufacturer and incubated with EGFP-labeled virions (10 ng p24) for 30 min. Proteostat and EGFP were excited by using a 561-nm and a 488-nm laser line, respectively, and the emissions were collected using appropriate beam splitters. To acquire images, Plan Apochromat oil immersion lenses on a Nikon A1 confocal microscope (Nikon, Japan) equipped with NIS-Elements Viewer 4.20 software (Nikon, Japan) were used.

Virus pulldown assay. The effect of ADS-J1 on the binding of HIV-1 to SEVI or amyloid fibrils in semen was determined using a virus pulldown assay (26). Briefly, SEVI at 1,000 µg/ml or whole SE-F was mixed with an equal volume of ADS-J1 at the concentration indicated below for 30 min at 37°C. Then, samples were collected and centrifuged at 5,000 rpm for 15 min to remove the supernatant. The pellets were dissolved in the original volume of fresh medium, and 100 µl HIV-1 SF162 (100 ng/ml p24) was added. Mixtures were incubated for 30 min at 37°C. Then, the samples were centrifuged at 12,000 rpm for 5 min to pellet the fibrils and bound virions. The proportion of p24 in the pellet was evaluated.

HIV-1 infection assays. To observe the enhancing effect of SEVI or amyloid in semen, 3,000 µg/ml PAP_{248–286} or whole SE-F was agitated to allow fibril formation in the presence or absence of 1,000 or 500 µg/ml ADS-J1. Sixty-microliter amounts of samples were collected at different time points and centrifuged at 12,000 rpm for 5 min to remove the remaining free ADS-J1. The pellets were dissolved in medium (60 µl) and incubated with 2 ng of p24 antigen of viruses (60 µl) at room temperature for 10 min, followed by

FIG 1 Models of ADS-J1 interacting with PAP₂₄₈₋₂₈₆ and SEVI. (A to C) Structures of the anionic molecules ADS-J1 (A), D-galactose 6-sulfate (B), and N-acetyl-D-galactosamine 6-sulfate (C). (D) Amino acid residues of the amyloidogenic peptides, with the cationic residues highlighted in bold. (E) Cartoons depicting the binding of ADS-J1 to PAP₂₄₈₋₂₈₆ (top) and amyloid fibrils (bottom). (Top) ADS-J1 inhibits PAP₂₄₈₋₂₈₆ aggregation. The sulfated groups of ADS-J1 bind to the positive residues (highlighted by amino acid residue) in PAP₂₄₈₋₂₈₆, which might hinder access to the sites vital for peptide aggregation. (Bottom) ADS-J1 blocks SEVI-mediated enhancement of HIV-1 infection. The coating of SEVI fibrils with ADS-J1 might interfere with the direct interaction between HIV-1 (large round particles) and SEVI fibrils, thereby blocking SEVI-mediated enhancement of viral infection. Branches on the amyloid fibril represent positive residues.

the addition of 100 μ l of the mixture to 1×10^4 TZM-b1 cells that had been cultured overnight. After a 3-h incubation period, unbound viruses were removed, and cells were replenished with fresh medium. Luciferase activity was measured 3 days postinfection. SEVI was tested at a final concentration of 50 μ g/ml, and SE-F was used at a final dilution of 1:100.

To determine the antagonizing effects of ADS-J1 on SEVI- or seminal fibril-mediated enhancement of HIV-1 infection, 1,000 μ g/ml SEVI or whole SE-F was incubated with an equal volume of ADS-J1 at 800, 400, 200, 100, 50, 25, 13, or 6.25 μ g/ml at 37°C for 15 min. Then, the mixtures were centrifuged at 5,000 rpm for 15 min to remove the remaining free ADS-J1. Pellets were dissolved in 60 μ l of medium and incubated with 2 ng of p24 of viruses (60 μ l) at room temperature

for 10 min, after which 100 μ l of mixture was used for the infection experiments as described above. SEVI was tested at a final concentration of 50 μ g/ml, and SE-F was used at a final dilution of 1:100.

Cytotoxicity assays. The potential cytotoxic effects of ADS-J1 on various cells were detected by XTT assay as previously described (22). Briefly, 100- μ l amounts of serial dilutions of ADS-J1 or the mixtures of ADS-J1 and various ARV drugs in SE-F were added to 100- μ l amounts of cells in 96-well plates. After incubation at 37°C for 48 h, the culture medium was removed, and 125 μ l of XTT solution (200 μ g/ml) containing phenazine methosulfate (PMS) was added. After 4 h, the absorbance at 450 nm was measured with an enzyme-linked immunosorbent assay (ELISA) reader.

Measurement of the anti-HIV-1 activity of the ARV agents individually and in combination in semen. The inhibitory activities of ADS-J1 alone, other ARV agents alone, including maraviroc, tenofovir, AZT, and TMC120, and the combinations of ADS-J1 with each of these ARV drugs in SE-F against infection caused by 100 50% tissue culture infective doses (TCID₅₀) of HIV-1 SF162 in TZM-bl cells were determined as previously described (27), except that drugs, alone or in combination, were incubated in SE-F first. To avoid cytotoxicity of SE-F to cells, SE-F was used at a final dilution of 1:100 (8). The effective 50% and 90% inhibitory concentrations (IC₅₀ and IC₉₀, respectively) were calculated using the CalcuSyn software. Cooperative effects were analyzed by using the Chou-Talalay method. The combination index (CI) values were calculated by using the CalcuSyn program (28). Compounds were examined at fixed molar ratios individually and in combination. The CI value reflects the nature of the interaction between compounds. CI values of <1, 1, and >1 indicate synergy, additivity, and antagonism, respectively. A CI of <0.10 indicates very strong synergism, 0.10 to ~0.30 indicates strong synergism, 0.30 to ~0.70 indicates synergism, 0.70 to ~0.85 indicates moderate synergism, and 0.85 to ~0.90 indicates slight synergism (27). Dose reductions were calculated as the ratio between the IC₅₀s of the compound used alone and in combination.

RESULTS

ADS-J1 inhibits SEVI fibril formation. ADS-J1 was first examined for its ability to inhibit SEVI fibril formation by the peptide PAP_{248–286}. Fibril growth curves were generated by agitating PAP_{248–286} with or without ADS-J1, and fibril signals were detected by ThT and Congo red. Of note, ADS-J1 at 500 µg/ml could completely inhibit SEVI fibril formation at 3,000 µg/ml (the molar ratio between ADS-J1 and PAP_{248–286} was 0.7:1), while PAP_{248–286} alone showed typical fibril growth (Fig. 2A and B). Decreasing the dose of ADS-J1 resulted in the inhibition of SEVI fibril formation in a dose-dependent manner, as characterized by delayed lag phase and less yield of fibril formation *in vitro*, which was indicated by the decreased magnitude of fluorescence plateau values (Fig. 2D). As observed by TEM, PAP_{248–286} started to form amyloid fibrils at 4 h. At 12 h, mature fibrils could be detected. As the time proceeded to 48 h, bundles of fibrils were found in the solution with PAP_{248–286} alone. However, in the presence of ADS-J1 (at 1,000 and 500 µg/ml), fibrils could not be detected at 48 h following incubation (Fig. 2C). Next, we investigated whether ADS-J1 could inhibit amyloid fibril formation in semen. We agitated SE-F and found that it showed increased binding with ThT within 8 h, demonstrating the formation of amyloid fibrils in SE-F. However, when incubated with 1,000 or 500 µg/ml ADS-J1, SE-F did not induce an increase in the fluorescence intensity, suggesting that little seminal fibril formed. Therefore, ADS-J1 could also inhibit fibril formation in semen (Fig. 2E).

Because SEVI fibrils have been shown to enhance HIV-1 infection, a viral infection assay was used to investigate amyloid fibril formation in the presence or absence of ADS-J1. PAP_{248–286} was incubated with or without ADS-J1, and fibrils were allowed to form. At different time points, each sample was pelleted by centrifugation to remove the free ADS-J1 in the supernatant, which showed antiviral activity. The abilities of the pellets to enhance CCR5-tropic HIV-1 SF162 and CXCR4-tropic HIV-1 NL4-3 infection were determined. As shown by the results in Fig. 3A and B, PAP_{248–286} alone efficiently enhanced HIV-1 infection in a time-dependent manner, consistent with the formation and accumulation of amyloid fibrils within the elapsed time. However, in the presence of ADS-J1 (at ADS-J1/PAP_{248–286} molar ratios of 1.4:1 and 0.7:1), PAP_{248–286} completely lost the ability to enhance HIV-1 infection at the time intervals tested. Similar findings could be observed when testing viral infection using the laboratory-adapted strain HIV-1 IIIB and the clinical isolate

HIV-1 US4 (GS007) (unpublished data). We also tested the ability of SE-F to enhance HIV-1 infection in the presence of ADS-J1 at different time points during the process of fibril formation. SE-F agitated alone showed a time-dependent enhancement of various HIV-1 strains, while in the presence of ADS-J1 at a concentration as high as 500 µg/ml (~460 µM), SE-F, which was diluted 1:100 before the infection, showed no enhancement of HIV-1 infection compared to the results for the virus control (Fig. 3C and D and unpublished data). Next, we wanted to determine the effective concentration of ADS-J1 to inhibit seminal fibril formation. Serial dilutions of ADS-J1 (from 500 µg/ml down to 15.625 µg/ml) were tested for inhibition of fibril formation in whole SE-F. As shown by the results in Fig. 3E, ADS-J1 at 62.5 µg/ml (~58 µM) could completely inhibit seminal fibril formation as evidenced by the ThT staining method. Consistent with these findings, ADS-J1 at 0.625 µg/ml (~0.53 µM) could still block 1% semen-mediated enhancement of viral infection after 8 h of agitating SE-F (Fig. 3F).

ADS-J1 binds to PAP_{248–286} via electrostatic interaction. We hypothesized that negatively charged ADS-J1 bound to cationic PAP_{248–286}, thus preventing self-aggregation of PAP_{248–286}. We applied ITC to detect the binding of ADS-J1 to PAP_{248–286}. ADS-J1 bound to PAP_{248–286} with a binding constant (K_d) of 3.62×10^{-7} M (Fig. 4A). Native gels were applied to analyze the potential interaction between ADS-J1 and PAP_{248–286}. As expected, with the addition of increasing amounts of ADS-J1, a gradual reduction of peptide in the supernatant was detected after centrifugation, suggesting that PAP_{248–286} formed aggregated complexes with ADS-J1 (Fig. 4B, left). Next, we wanted to apply a competitive binding assay to demonstrate that PAP_{248–286} interacts with ADS-J1 through electrostatic interaction. Polybrene is considered a cationic polymer and is widely used to increase the efficiency of retrovirus infection *in vitro*, due to its ability to neutralize the charge repulsion between virions and sialic acid on the cell surface (29). We incubated ADS-J1 with Polybrene. If ADS-J1 interacted with Polybrene via electrostatic interaction, then the ability of ADS-J1 to bind to PAP_{248–286} could be impaired. Since Polybrene is a polymer, the concentration we used in this assay was per weight. Our results showed that after binding to Polybrene at 1,000 µg/ml, ADS-J1 at 1,000 µg/ml lost the ability to interact with PAP_{248–286}, resulting in detection of PAP_{248–286} in the same quantity as in the mock-treated PAP_{248–286} solution (Fig. 4B, right). These results revealed that ADS-J1 bound to PAP_{248–286} through electrostatic interaction.

ADS-J1 also binds to amyloid fibrils and antagonizes SEVI-mediated enhancement of HIV-1 infection. Due to its positive charge, SEVI binds to viruses and enhances HIV-1 infection. Therefore, ADS-J1 might also bind to SEVI and antagonize SEVI-mediated enhancement of viral infection. Using a virus pulldown assay, we found that SEVI fibrils alone bound more than 40% of the input virus, whereas 1,000 µg/ml SEVI fibrils in the presence of 500 µg/ml ADS-J1 markedly lost the ability to bind to virus (Fig. 5A). Similarly, SE-F alone bound approximately 30% of the input virus; however, in the presence of 500 µg/ml of ADS-J1, SE-F bound only 15% of the input virus (Fig. 5B).

Using Proteostat, a dye that specifically recognizes amyloid in semen (10), we found that both SEVI and endogenous amyloid in SE-F could bind to EGFP-labeled HIV-1 (Fig. 5C and D). However, in the presence of ADS-J1, amyloid-virus complexes could hardly be found (Fig. 5C and D), demonstrating that the binding of ADS-J1 to amyloid fibrils inhibited the binding of amyloid fibrils to HIV-1.

FIG 2 Inhibition of the formation of semen-derived amyloid fibrils by ADS-J1. PAP₂₄₈₋₂₈₆ at a concentration of 3,000 µg/ml was agitated in the presence or absence of ADS-J1 at 1,000 or 500 µg/ml. The molar ratios between ADS-J1 and PAP₂₄₈₋₂₈₆ were 1.4:1 and 0.7:1, respectively. (A and B) The formation of amyloid fibrils was monitored by ThT (A) or Congo red staining (B) OD490-650, optical density at 490 to 650 nm. (C) The amyloid fibril structures were visualized by negative-stain TEM at different time points (4, 12, and 48 h). Scale bar, 1 µm (magnification of ×5,000). (D) ADS-J1 (500, 250, and 125 µg/ml) was incubated with 3,000 µg/ml PAP₂₄₈₋₂₈₆, and the mixtures were agitated. Fibrils were detected by ThT staining at different time points. (E) SE-F was agitated in the presence or absence of ADS-J1. ThT fluorescence was measured at timed intervals. Data are represented as the average values ± standard deviations (SD) from triplicate measurements. The results shown in panels A, B, and D are representative of two independent experiments.

The viral infection experiment confirmed that HIV-1 infection of 50 µg/ml SEVI-treated cells was enhanced from 14- to 100-fold relative to the infection of mock-treated cells using various viral strains (Fig. 6A and B) (unpublished data). However, when exposed to ADS-J1, SEVI lost its ability to enhance HIV-1 infection in a dose-dependent manner (Fig. 6A and B and unpublished data). ADS-J1 at 5 µg/ml could completely antagonize the enhancement of viral infection against HIV-1 SF162, NL4-3, and IIIB mediated by 50 µg/ml SEVI. We also

assessed whether ADS-J1 inhibited the activity of endogenous SEVI in semen. As shown by the results in Fig. 6C and D, in the absence of ADS-J1, treating cells with 1% SE-F enhanced HIV-1 infection. The addition of ADS-J1, however, decreased the enhancement of viral infection mediated by semen-derived amyloid fibrils. Pretreatment of whole SE-F with 50 µg/ml ADS-J1 was effective to block semen-mediated enhancement of viral infection against HIV-1 SF162, NL4-3, and IIIB (Fig. 6C and D and unpublished data). ADS-J1 inhibited SEVI-medi-

FIG 3 Inability of PAP₂₄₈₋₂₈₆ or SE-F incubated with ADS-J1 to enhance HIV-1 infection. (A to D) In the presence or absence of ADS-J1 (1,000 or 500 µg/ml), 3,000 µg/ml PAP₂₄₈₋₂₈₆ (A and B) or whole SE-F (C and D) was agitated to allow fibril formation. At the indicated time points, samples were collected and centrifuged to pellet the fibrils, which were then examined for their ability to enhance CCR5-tropic HIV-1 SF162 (A and C) and CXCR4-tropic HIV-1 NL4-3 (B and D) infection. SEVI was tested at a final concentration of 50 µg/ml, and SE-F was tested at a final dilution of 1:100. (E) SE-F was agitated in the presence or absence of ADS-J1 at different concentrations. ThT fluorescence was measured at timed intervals. (F) At the time point of 8 h, mixtures were centrifuged to remove the free ADS-J1. The pellets were dissolved in medium and incubated with 2 ng p24 of HIV-1 SF162. The mixtures were then used to infect TZM-bl cells with the final SE-F dilution of 1:100. The results shown in this figure are the average values $\pm$ SD of triplicate measurements from one of two independent experiments that yielded equivalent results. RLU/s, relative light units per second.

A

B

Polybrene (1000 $\mu\text{g/ml}$)	-	-	-	-	-	-	-	+	+	+	+	+	+
PAP ₂₄₈₋₂₈₆ (500 $\mu\text{g/ml}$)	+	+	+	+	+	+	+	+	+	+	+	+	+
ADS-J1 ($\mu\text{g/ml}$)	-	1000	500	250	125	62.5	-	1000	500	250	125	62.5	-

FIG 5 Antagonizing effect of ADS-J1 on the binding of virus to SEVI. (A and B) SEVI, at 1,000 μg/ml (A), and SE-F (B) were incubated with ADS-J1 at the indicated concentrations. Then, HIV-1 SF162 virions (10 ng of p24) were added to the mixture. The absolute amounts of p24 in the pellets and supernatant were determined by ELISA. Values are the means $\pm$ SD of triplicate measurements from one of the two independent experiments that yielded similar results. (C and D) SEVI, at 1,000 μg/ml (C), and SE-F (D) in the presence or absence of ADS-J1 were first stained with Proteostat dye (red) and then mixed with EGFP-labeled HIV-1 (10 ng of p24, green). Images were acquired 20 min later on a laser-scanning confocal microscope. ADS-J1 abrogates the ability of SEVI and seminal amyloid to bind to viral particles. Scale bar, 5 μm.

ated enhancement of infection by HIV-1 US4 (GS007) to a lesser extent (unpublished data).

ADS-J1 also inhibits the formation of semenogelin-derived amyloid fibril. Besides PAP-derived amyloid-forming peptides, se-

men contains various amyloidogenic peptides derived from other precursor proteins (6–8, 14). SEM1_{86–107} is a well-defined amyloidogenic peptide derived from semenogelin (14). We thus investigated whether ADS-J1 could also inhibit amyloid formation induced by

FIG 4 Electrostatic interaction between ADS-J1 and PAP_{248–286}. (A) ITC analysis of ADS-J1 binding to PAP_{248–286}. ADS-J1 dissolved in PBS at a concentration of 200 μM (~210 μg/ml) was injected into the chamber containing 200 μM (~900 μg/ml) PAP_{248–286}. The experiments were done at 37°C. Data acquisition and analysis were performed using MicroCal Origin software. Top, ITC data are plotted as heat signal versus time; bottom, the integrated heat responses per injection are plotted. (B) The interaction of ADS-J1 with PAP_{248–286} was measured by acidic native polyacrylamide gel electrophoresis. (Left) ADS-J1 at the indicated concentrations was incubated with PAP_{248–286} at 500 μg/ml, and then the remaining peptides in the supernatants were electrophoresed in 10% native polyacrylamide continuous gels. Gels were either stained with Coomassie blue (top gels) or subjected to immunoblotting with PAP_{248–286} polyclonal antibody (bottom gels). (Right) The competitive binding assay showed that cationic Polybrene bound to ADS-J1 and prevented ADS-J1 binding to PAP_{248–286}. The experiment was performed as described above, except that ADS-J1 was first incubated with 1,000 μg/ml Polybrene. Bar charts show the quantification of the peptide levels in the Western blots by using Quantity One image analysis software.

FIG 6 Antagonizing effect of ADS-J1 on SEVI-mediated enhancement of HIV-1 infection. SEVI, at 1,000 µg/ml (A and B), or whole SE-F (C and D) was incubated with ADS-J1 at various concentrations. The mixtures were centrifuged, and the pellets were mixed with 2 ng p24 antigen of CCR5-tropic HIV-1 SF162 (A and C) or CXCR4-tropic HIV-1 NL4-3 (B and D). Cells were assayed for luciferase activity. SEVI was tested at a final concentration of 50 µg/ml, and SE-F was used at a final dilution of 1:100. Shown are the mean values $\pm$ SD of triplicate measurements from one of two independent experiments that yielded similar results.

SEM₁₈₆₋₁₀₇. As shown by the results in Fig. 7, SEM₁₈₆₋₁₀₇ displayed characteristics of amyloid fibril formation. As expected, ADS-J1 at 500 µg/ml could completely block amyloid fibril formation mediated by SEM₁₈₆₋₁₀₇ at 3,000 µg/ml (the molar ratio between ADS-J1 and SEM₁₈₆₋₁₀₇ is 0.375:1).

Unlike polyanions, ADS-J1 shows no ability to facilitate fibril formation. Previously, we showed that polyanionic chemicals could facilitate the formation of amyloid fibrils (22). As shown by the results in Fig. 8A, in line with our previous findings, cellulose sulfate facilitated the formation of SEVI fibrils; however, at the same concentration, ADS-J1 was ineffective in promoting fibril formation, while it still inhibited SEVI fibril formation. These results suggested that ADS-J1 exerted no ability to promote fibril formation.

Of note, different from ADS-J1, other smaller anionic chemicals could not inhibit SEVI fibril formation. Two smaller sulfated mono-sugars, galactose sulfate and galactosamine sulfate (Fig. 1B and C), could not inhibit PAP₂₄₈₋₂₈₆ amyloid fibril formation, even at 1,000 µg/ml (the molar ratios between the sugars and PAP₂₄₈₋₂₈₆ were 5.4:1 and 4.7:1, respectively) (Fig. 8B). However, ADS-J1 completely inhibited PAP₂₄₈₋₂₈₆ amyloid fibril formation under these assay conditions even at a molar ratio of 0.7:1 between ADS-J1 and PAP₂₄₈₋₂₈₆.

FIG 7 Inhibition of the formation of semenogelin-derived amyloid fibril by ADS-J1. SEM₁₈₆₋₁₀₇, at 3,000 µg/ml, was agitated in the presence or absence of ADS-J1 at 1,000 or 500 µg/ml. The molar ratios between ADS-J1 and SEM₁₈₆₋₁₀₇ were 0.75:1 and 0.375:1, respectively. The formation of amyloid fibrils was monitored by ThT staining. The results shown are representative of two independent experiments.

FIG 8 Inability of ADS-J1 to facilitate fibril formation. (A) ADS-J1 did not facilitate the process of fibril formation. PAP₂₄₈₋₂₈₆, at 3,000 $\mu\text{g/ml}$, was agitated at 37°C and 1,200 rpm in the presence or absence of ADS-J1 or cellulose sulfate at 100 $\mu\text{g/ml}$. Fibril growth was revealed by determining ThT fluorescence. The results shown in this figure are representative of two independent experiments. (B) Sulfated monosugars with smaller sizes failed to inhibit SEVI fibril formation. PAP₂₄₈₋₂₈₆, at 3,000 $\mu\text{g/ml}$, was agitated in the presence or absence of various anionic chemicals at 1,000 $\mu\text{g/ml}$. The molar ratios between the sugars and PAP₂₄₈₋₂₈₆ were 5.4:1 and 4.7:1. The formation of amyloid fibrils was monitored by ThT staining.

These contrasting effects of anionic agents in preventing fibril formation revealed that chemical properties were important for the agents to block fibril formation.

Combinations of ADS-J1 with ARV-based candidate microbicides display synergistic and additive effects against HIV-1 infection in semen. ADS-J1 has been found to inhibit HIV-1 entry into target cells by targeting gp41 and gp120 (23, 30, 31). Here, we found that ADS-J1 inhibited amyloid fibril formation and antagonized fibril-mediated enhancement of HIV-1 infection. We determined the overall complementary effects of ADS-J1 combined with ARV-based candidate microbicides on infection by HIV-1 SF162 in semen. The drugs used for the combination study included maraviroc, tenofovir, TMC120, and AZT, which are either candidate microbicides widely tested in the clinical phase or effective agents used in clinics (32, 33). As shown by the data in Table 1, the observed 50% competitive index (CI_{50}) values of ADS-J1 combined with these ARVs ranged from 0.302 to 0.692, and dose reductions ranged from 1.6- to 53.4-fold. These results suggested that ADS-J1 shows promise as a potential lead compound for incorporation into a candidate combination microbicide. Of note, the drug combinations in SE-F at

the concentrations tested in the assay had no cytotoxicity on TZM-bl cells (data not shown).

ADS-J1 shows little *in vitro* cytotoxicity to reproductive tract epithelial cells. Safety is important when evaluating a potential candidate microbicide. Therefore, we assessed the cytotoxicity of ADS-J1 to endometrial cells, including HEC-1-A, HEC-1-B, Ect1/E6E7 cervical epithelial cells, VK2/E6E7 vaginal epithelial cells, and TZM-bl target cells. Based on the XTT colorimetric assay results, ADS-J1 had low *in vitro* cytotoxicity to vaginal epithelial cells (unpublished data), with 50% cytotoxicity concentration (CC_{50}) values ranging from 1,800 to 3,520 $\mu\text{g/ml}$. The CC_{50} of ADS-J1 on TZM-bl was above 4,000 $\mu\text{g/ml}$.

DISCUSSION

Using a computer-assisted virtual screening method, ADS-J1, an HIV-1 entry inhibitor, was discovered by our group, and it was found to have antiviral activity at low μM levels (23, 30). In this study, we reexplored its function in the context of HIV-1 sexual transmission. We provided multiple lines of evidence for the potential impact of ADS-J1 on semen-derived amyloid fibrils. First, ADS-J1 inhibited PAP₂₄₈₋₂₈₆ aggregation *in vitro* (Fig. 2A to D), and it also inhibited amyloid fibril formation in semen (Fig. 2E and 3E). Therefore, upon exposure to ADS-J1, PAP₂₄₈₋₂₈₆ and semen lost the ability to enhance HIV-1 infection, even after 48 h of incubation, whereas PAP₂₄₈₋₂₈₆ and semen alone showed enhancement of viral infection after only 4 h of incubation (Fig. 3). Second, ADS-J1 interacts with mature amyloid fibrils and blocks the viral binding activity of SEVI or semen (Fig. 5) and the subsequent enhancement of viral infection (Fig. 6). Third, besides inhibiting PAP-derived amyloid fibrils, ADS-J1 could also inhibit semenogelin-derived amyloid fibril formation (Fig. 7). Thus, ADS-J1 shows a wide spectrum of inhibition of cationic seminal amyloid fibril formation.

The ability of ADS-J1 to inhibit SEVI fibril formation might be attributable to the electrostatic interaction between them. PAP₂₄₈₋₂₈₆ is a highly cationic peptide, while ADS-J1 is highly negatively charged due to the presence of sulfated groups. Strong electrostatic interaction likely exists when they are exposed to each other. The results from ITC and Western blot analysis confirmed that ADS-J1 bound to PAP₂₄₈₋₂₈₆ (Fig. 4). The competitive binding assay demonstrated that the cationic polymer Polybrene could nullify the ability of ADS-J1 to interact with PAP₂₄₈₋₂₈₆ (Fig. 4B).

In a similar manner, ADS-J1 shields the positive charges on mature fibrils, leading to a deficiency in the binding of SEVI to virus and the subsequent enhancement of HIV-1 infection (Fig. 1E, 5, and 6). It was interesting that ADS-J1 at 500 $\mu\text{g/ml}$ was partially effective in the virus binding assay (Fig. 5B), while ADS-J1 at 62.5 $\mu\text{g/ml}$ could totally inhibit semen-mediated enhancement of viral infection (Fig. 3F). We supposed that, besides amyloid fibrils, other components in semen might also bind to virus. Therefore, the dual functions of ADS-J1 of inhibiting semen-derived amyloid fibrils and blocking HIV-1 entry make it a plausible candidate for incorporation into combination microbicide formulations. ADS-J1 might readily bind to amyloidogenic peptide to inhibit seminal fibril formation, which might be a spontaneous process under physiologic conditions (34), and it also might target mature fibrils to prevent the augmented viral infection (Fig. 1E).

An interesting finding from this study is that different anionic chemicals showed distinct biological properties. Our previous studies showed that polyanions such as cellulose sulfate might, at relatively low concentrations, serve as templates to enrich peptide monomer

TABLE 1 Combination index and dose reduction values for inhibition of HIV-1 SF162 infection by combining ADS-J1 with ARVs in semen

Drug combination, % inhibitory concn (molar ratio)		Mean value for ^b :					
		ADS-J1			ARVs		
		Concn (nM)		Dose reduction	Concn (nM)		Dose reduction
CI ^a	Alone	Mixture	Alone		Mixture		
ADS-J1:maraviroc (41.5:1)							
50	0.437	1,056.82	41.59	25.41	2.61	1.04	2.51
90	0.347	3,369.02	179.54	18.76	15.29	4.49	3.41
ADS-J1:tenofovir (20:1)							
50	0.302	9,418.39	262.73	35.85	47.90	13.14	3.65
90	0.458	29,411.10	1,166.57	25.21	139.44	58.33	2.39
ADS-J1:TMC120 (53.4:1)							
50	0.692	1,072.63	74.71	14.36	2.40	1.49	1.61
90	0.511	3,672.60	151.94	24.17	6.47	3.04	2.13
ADS-J1:AZT (33.3:1)							
50	0.379	1,245.33	23.33	53.38	2.16	0.78	2.77
90	0.581	8,926.80	274.76	32.49	16.64	9.16	1.82

^a CI, combination index.^b Data are the means of two independent experiments performed in triplicate.

and, hence, accelerate amyloid fibril formation, which would account for the poor performance of polyanions in clinical trials (22). However, ADS-J1 could still inhibit fibril formation, even at the concentration typically allowing polyanions to facilitate the formation of amyloid fibrils (Fig. 8A). ADS-J1 shows no ability to enhance seminal fibril formation.

In addition, it was previously thought that any anionic molecule might inhibit PAP_{248–286} aggregation due to possible formation of a coating on PAP_{248–286}. However, two sulfated monosugars showed no inhibitory activity against PAP_{248–286} aggregation (Fig. 8B). One possible reason for the different activities of ADS-J1 and monosugars might be the molecular size (Fig. 1A to C). ADS-J1 might bind to PAP_{248–286} and hide the site essential for PAP_{248–286} aggregation (Fig. 1E) (35). Monosugars might also bind to PAP_{248–286}. However, they might not shield the site against PAP_{248–286} self-aggregation. Other biochemical and biological properties might also contribute to the different activities of anionic molecules. Further investigation of the exact mechanism underlying SEVI fibril formation and the structure-activity relationship of ADS-J1 that inhibits it should be pursued.

ADS-J1 is a well-defined HIV-1 entry inhibitor and, as shown here, a SEVI inhibitor. Apart from any specific mechanism of action, it is because of its anionic properties that ADS-J1 does not facilitate fibril formation, instead demonstrating inhibitory effects on a wide spectrum of cationic seminal amyloid fibrils (Fig. 7). Next, we also demonstrated that combining ADS-J1 with maraviroc, tenofovir, TMC120, and AZT resulted in synergism against infection by HIV-1 SF162 in semen, which is the most common vehicle for HIV transmission (Table 1). The synergism that results from combining ADS-J1 with ARV drugs can augment antiviral potency and increase barriers against the development of drug resistance. Moreover, we evaluated the potential safety of ADS-J1 as a candidate microbicide. Our results showed that ADS-J1 displayed little cytotoxicity to vaginal epithelial cells (unpublished data). The effective concentration of ADS-J1 for inhibiting seminal fibrils was around 62.5 µg/ml (Fig. 3F and 6C and D), and ADS-J1 only showed cytotoxicity to various cells at over 1,782 µg/ml. However, long-term observation of the potential

harmful effect of ADS-J1 on the mucosal epithelium is warranted. The design and synthesis of ADS-J1 derivatives without azo moieties, which are carcinogenic, is ongoing.

In conclusion, apart from its antiviral activity, ADS-J1 was found here to possess a dual mechanism of action on semen-derived amyloid fibrils and to show cooperative effects against infection by HIV-1 in semen when combined with ARV drugs. Based on its multipharmaceutical effects on HIV-1 sexual transmission, ADS-J1 might represent a lead product for the design of combination microbicide candidates.

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