The depolymerized fucosylated chondroitin sulfate from sea cucumber potently inhibits HIV replication via interfering with virus entry

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1. Introduction

Human immunodeficiency virus type-1 (HIV-1) is the etiological agent for the acquired immunodeficiency syndrome (AIDS), a public health issue and ranks among the greatest infectious disease scourges in history. It was estimated that there were more than 33.3 million people worldwide with HIV-1 infection or AIDS. Although 26 anti-HIV drugs, including nucleoside/nucleotide reverse transcriptase (RT) inhibitors (NRTIs), nonnucleoside RT inhibitors (NNRTIs), protease inhibitors (Pis), integrase inhibitors (INIs), and fusion (or entry) inhibitors (FIs) were approved by the FDA, more and more patients with HIV infection and/or AIDS are unable to use these drugs because of the serious adverse effect and emergence of HIV mutants having single or multiple resistance to the drugs used. Therefore, it is essential to develop more effective and less toxic anti-HIV drugs targeting various stages of HIV replication cycle.

In the marine environment, sulfated polysaccharides extracted from algae have been intensively studied for their various pharmacological activities including antioxidant, antitumor, and antiviral.

Tecogalan sodium, a sulfated polysaccharide isolated from the bacterium Arthrobacter, acts as an angiogenesis inhibitor by inhibiting the binding of basic fibroblast growth factor to its cellular receptor. Sulfated polysaccharides, like dextran sulfates and heparin, have also shown a potent inhibitory activity against several different viruses. The inhibitory effects of sulfated galactan on the replication of herpes simplex virus (HSV) have been reported. Sulfated polysaccharides from the sea alga Spirulina platensis inhibited the activity of human immunodeficiency virus (HIV). Since the inhibitory activities of algal polysaccharides against mumps and influenza virus were first reported by Gerber et al., polysaccharides have attracted more attention as antiviral agents. Since then, anti-HIV activities of polysaccharides from different origins were extensively reported. Many sulfated polysaccharides inhibited HIV replication in vitro. They prevent the attachment, entry, or reverse transcription. Recently, we isolated a new fucosylated chondroitin sulfate from the body wall of the sea cucumber Thelenota ananas which consisted of N-acetylgalactosamine, galuronic acid, fucose, and ester sulfate with about 1:1:3:7, respectively. Additionally, to study its biological activity, its low molecular weight fragment (FuCS-1) with approximately 12.5 kDa was prepared by free radical depolymerization.
2. Results and discussion

2.1. Anti-HIV-1 activities

The antiviral activities of FuCS-1 are summarized in Table 1. The CC_{50} of FuCS-1 against both C8166 cells and PBMC were >25,000 μg/mL (Fig. 2). In HIV-1_{RF} or HIV-1_{L10R/M46I/L63P/V82T/I84V}, acute infected C8166 cells, FuCS-1 inhibited p24 antigen production with EC_{50} values of 0.73 μg/mL and 1.14 μg/mL (Fig. 3A), respectively. FuCS-1 dramatically reduced p24 antigen by clinical isolated virus HIV-1_{KMD18} and HIV-1_{TC-2} with EC_{50} values of 23.75 μg/mL and 31.86 μg/mL, respectively (Fig. 3B). It potently inhibited p24 antigen by drug resistant strains, NNRTIs’ resistant strains (HIV-1_{A17}), protease inhibitors’ (PIs) resistant strains, (HIV-1_{RF/V82F/I84V}, HIV-1L10R/M46I/L63P/V82T/I84V) (Fig. 3C) and T-20-resistant strains (pNL4-3gp41_{36G/V38A,N42T}, pNL4-3gp41_{36G/V38A,N42D}, pNL4-3gp41_{36G/V38A,N42E}, and pNL4-3gp41_{36G/V38A,N42T}) in dose-dependent manners with EC_{50} values approximately 1 μg/mL, respectively (Table 2). It also inhibited HIV-2_{CBL-20} and HIV-2_{ROD} replication in C8166 with EC_{50} values of 71.76 μg/mL and 97.63 μg/mL, respectively (Fig. 3D)

Due to severe side effects of anti-HIV drugs and ascendance of drug resistance mutants, continuous development of new antiviral agents, particularly ones with low toxic and novel action mechanisms, is necessary. Polysaccharides are derived from marine plants or animals with various biological activities and low toxicity.26,27 They may offer more opportunities to find anti-HIV drugs or lead compounds. Our data indicated that FuCS-1 possesses low cytotoxicities against C8166 and PBMC. FuCS-1 inhibited potent anti-HIV activities. It not only inhibited replication of HIV-1 laboratory strains (HIV-1_{RF} and HIV-1_{L10R/M46I/L63P/V82T/I84V}), but also inhibited HIV-1 clinical strains (HIV-1_{KMD18}, HIV-1_{TC-2}). NNRTIs’ resistant strains, and PIs resistant strains. Interestingly, FuCS-1 showed strong inhibitory activities against all T-20-resistant strains with EC_{50} values about 1 μg/mL (Table 2). In spite of low potency compared with that against HIV-1, FuCS-1 also repressed the replication of HIV-2 (HIV-2_{ROD} and HIV-2_{CBL-20}) in vitro. These results indicated that FuCS-1 may have wide applications for anti-HIV treatment. Additionally, FuCS-1 was also effective in T-20-resistant strains and the EC_{50} were similar to those in HIV-1_{RF} and HIV-1_{B}. This result implied that FuCS-1 inhibits HIV with the mechanism different to

2.2. Mechanisms of action

It was reported that the mechanisms of inhibitory HIV replication of polysaccharides inhibited HIV replication are mainly by interfering with entry of HIV into the target cells or by inhibition of HIV RT activity.26,27 But, in this study, it was found that FuCS-1 could not inhibit HIV-1 RT activity, even at a concentration up to 1000 μg/mL (data no shown). Therefore, the mechanism of anti-HIV-1 activity of FuCS-1 may be different from those of these sulfated polysaccharides.

HIV fusion inhibitor T-20 and FuCS-1 might be further developed as an alternative entry inhibitor for treatment of patients with HIV-1/AIDS, in particular those infected with T-20-resistant variants.

Figure 2. In vitro cytotoxicity of FuCS-1 in various cells. Data are expressed as means ± standard deviations.

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>HIV strains</th>
<th>Assays</th>
<th>EC_{50} (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8166</td>
<td>HIV-1_{RF}</td>
<td>Syncytia</td>
<td>0.24</td>
</tr>
<tr>
<td>C8166</td>
<td>HIV-1_{RF}</td>
<td>p24</td>
<td>0.73</td>
</tr>
<tr>
<td>C8166</td>
<td>HIV-1_{RF}/H9</td>
<td>Co-cultivation</td>
<td>4.26</td>
</tr>
<tr>
<td>C8166</td>
<td>HIV-1_{A17}</td>
<td>p24</td>
<td>1.14</td>
</tr>
<tr>
<td>PBMC</td>
<td>HIV-1_{KMD18}</td>
<td>p24</td>
<td>23.75</td>
</tr>
<tr>
<td>PBMC</td>
<td>HIV-1_{TC-2}</td>
<td>p24</td>
<td>31.86</td>
</tr>
<tr>
<td>C8166</td>
<td>HIV-1_{A17}</td>
<td>p24</td>
<td>1.09</td>
</tr>
<tr>
<td>C8166</td>
<td>HIV-1 RF/V82F/I84V</td>
<td>p24</td>
<td>0.95</td>
</tr>
<tr>
<td>C8166</td>
<td>HIV-1 L10R/M46I/L63P/V82T/I84V</td>
<td>p24</td>
<td>1.12</td>
</tr>
<tr>
<td>C8166</td>
<td>HIV-2_{CBL-20}</td>
<td>Syncytia</td>
<td>71.76</td>
</tr>
<tr>
<td>C8166</td>
<td>HIV-2_{ROD}</td>
<td>Syncytia</td>
<td>97.63</td>
</tr>
</tbody>
</table>

EC_{50} is the effective concentration that inhibits 50% of viral production.

HIV fusion inhibitor T-20 and FuCS-1 might be further developed as an alternative entry inhibitor for treatment of patients with HIV-1/AIDS, in particular those infected with T-20-resistant variants.

2.2. Mechanisms of action

It was reported that the mechanisms of inhibitory HIV replication of polysaccharides inhibited HIV replication are mainly by interfering with entry of HIV into the target cells or by inhibition of HIV RT activity.26,27 But, in this study, it was found that FuCS-1 could not inhibit HIV-1 RT activity, even at a concentration up to 1000 μg/mL (data no shown). Therefore, the mechanism of anti-HIV-1 activity of FuCS-1 may be different from those of these sulfated polysaccharides.

Therefore, we investigated the mechanism of action for FuCS-1. As shown in Figure 4A, results of time addition assay indicated that FuCS-1 was added in 2 h post-infection, it would inhibit the HIV-1_RF p24 antigen by 100%. In contrast, it was added after 6 h post-infection, only less than 30% of HIV-1_RF p24 antigen was inhibited. In co-cultivation assay, uninfected C8166 cells were co-cultured with infected H9 cells in the presence of compound and FuCS-1 effectively blocked cell-to-cell fusion induced by HIV-1_{RF}, with EC_{50} values of 4.26 μg/mL and at concentration of 1.6 μg/mL, FuCS-1 inhibited more than 90% of syncytia formation (Fig. 4B). These results indicated that FuCS-1 functions against the early stage of HIV life cycles. To explore if the effect on HIV-1 replication was caused by the inhibition of viral entry, we also ana-
lyzed the capacity of FuCS-1 to inhibit HIV attachment and entry. Results showed that FuCS-1 caused about 99% inhibition of HIV-1 particle attachment (Fig. 5A) and about 99% inhibition of fusion (Fig. 5B) at the concentrations of 10 μg/mL. These results further supported that FuCS-1 potently inhibited HIV-1 IIIB attachment or entering the target cells. All of these results indicated FuCS-1 may be an entry inhibitor.

HIV-1 entry is initiated by the binding of viral gp120 glycoprotein to cell surface receptor CD4 molecule and a co-receptor (CXCR4/CCR5). HIV gp120 protein plays a crucial role in HIV entry into the target cells. Because the anti-HIV-1 activity of FuCS-1 was related to the inhibition of HIV-1 entry, the binding of FuCS-1 to HIV-1 gp120 was investigated by using BLI technology. As shown in Figure 6, FuCS-1 bound the recombinant HIV-1 gp120 protein potently in a dose-dependent manner. And the kinetic parameters ($k_{on}$ and $k_{off}$) and affinity ($K_D$) were $7.02 \times 10^{7} M^{-1} s^{-1}$, $3.04 \times 10^{-3} s^{-1}$, and 4.33 nM, respectively. The evidence in this study suggests that FuCS-1 can specifically bind to gp120. The interaction between FuCS-1 and gp120 might interfere with gp120 normal functions, so HIV could not successfully enter the target cells.

In conclusion, it was found that FuCS-1 significantly inhibited entry and replication of laboratory and clinical HIV-1 strains. In particular, it could potently block replication of T-20-resistant strains. The collective results of this study suggest that FuCS-1 might possess great potential to be further developed as a novel HIV-1 entry inhibitor for treatment of HIV/AIDS patients, particularly for those infected by T-20-resistant variants.

3. Experimental

3.1. Chemicals and reagents

The fucosylated chondroitin sulfate was extracted from the body wall of the sea cucumber Thelenota ananas. The extraction, preparation, and purification of the native fucosylated chondroitin sulfate were performed as previously described. The low molecular weight fragment (FuCS-1) of glycosaminoglycan from the sea cucumber Thelenota ananas was prepared by free radical depolymerization. The structure of the depolymerized fucosylated

Table 2

<table>
<thead>
<tr>
<th>Virus</th>
<th>Phenotype</th>
<th>EC$_{50}$ T-20 (μg/mL)</th>
<th>EC$_{50}$ FuCS-1 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNL4-3gp41(36G)/N42S</td>
<td>S</td>
<td>0.23</td>
<td>0.79</td>
</tr>
<tr>
<td>pNL4-3gp41(36G)/N18A,N42D</td>
<td>R</td>
<td>&gt;1.00</td>
<td>0.94</td>
</tr>
<tr>
<td>pNL4-3gp41(36G)/N38E,N42S</td>
<td>R</td>
<td>&gt;1.00</td>
<td>0.76</td>
</tr>
<tr>
<td>pNL4-3gp41(36G)/N38A,N42T</td>
<td>R</td>
<td>&gt;1.00</td>
<td>1.13</td>
</tr>
</tbody>
</table>

* Sensitive (S) or resistant (R) to T-20.
chondroitin sulfate had been characterized as our previous report. Purity of FuCS and FuCS-1 determined by HPLC was over 99% as our previous report, and average molecular weight of FuCS-1 was approximately 12.5 kDa. Structures of FuCS-1 are presented in Figure 1. FuCS-1 was dissolved in complete medium for all assays carried out in this study.

AZT, dextran sulfate (DS), and T-20 were purchased from Sigma. Recombinant HIV-1 gp120 IIIB was purchased from Immune Diagnostics, Inc. (Woburn, MA01801 USA). Amine Reactive Second-Generation (AR2G) Biosensors were purchased from ForteBio. p5F1, monoclonal antibody (McAb) against HIV-1 p24, was prepared by our laboratory. Human polyclonal anti-HIV-1 serum was kindly donated by Dr. Hiroo Hoshino (Gunma University School of Medicine, Japan).

3.2. Cells and virus

Cell lines (C8166, H9, and H9/HIV-1IIIB) were maintained in RPMI-1640 supplemented with 10% heat-inactivated newborn calf serum (Gibco). The cells used in all experiments were in log-phase growth. PBMC from healthy donors were isolated by Ficoll–Hypaque centrifugation and incubated in complete medium containing 5 μg/mL phytohemagglutinin (PHA) (Sigma) and 50 U/mL human recombinant IL-2 for 72 h prior to use for anti-viral assays. The laboratory-derived viruses (HIV-1IIIB, HIV-1RF, HIV-2_ROD, and HIV-2_CBL-20) and the drug resistant strains viruses (HIV-1_A17, HIV-1RF_V32F/184V, HIV-1L10R/M46I/L63P/V82T/I84V, pNL4-3gp41[36G/N42S]-pNL4-3gp41[36G/V38A/N42D], pNL4-3gp41[36G/V38A/N42D], and pNL4-3gp41[36G/V38A/N42D]) were obtained from the NIH AIDS Research and Reference Reagent Program and the MRC AIDS Reagent Project. The clinically isolated HIV-1_KMB18 and HIV-1_TC were obtained from a HIV-1 infected individual in Yunnan Province of China as
described. The 50% HIV-1 tissue culture infectious dose (TCID50) was determined and calculated by the Reed and Muench method. Virus stocks were stored in aliquots at −70 °C.28

3.3. MTT-based cytotoxicity assay

Cellular toxicity of compounds was assessed by MTT method as described previously. Briefly, cells were seeded on a microtiter plate in the absence or presence of various concentrations of compounds in triplicate and incubated at 37 °C in a humid atmosphere of 5% CO2 for 3 days. Twenty microliters of MTT reagent (5 mg/mL in PBS) was added to each well and incubated at 37 °C for 4 h, then 100 μL of 50% DMF-20% SDS was added. After the formazan was dissolved completely, the plates were read on a Bio-Tek ELX 800 ELISA reader at 595/630 nm (A595/630). The cytotoxic concentration that caused the reduction of viable cells by 50% (CC50) was calculated from dose to response curve.29

3.4. Syncytia assay

In the presence of 100 μL of various concentrations of FuCS-1, 4 × 10^5/mL of C8166 cells (50 μL) were infected with virus (HIV-2R5-20 and HIV-2ROD) (50 μL) at a multiplicity of infection (M.O.I) of 0.1. The final volume per well was 200 μL. Control assays were performed without the testing compounds in HIV-1-infected and uninfected cultures. AZT was included as positive control. Then, the plates were incubated in a humidified incubator at 37 °C and 5% CO2. After 3 days of culture, the cytopathic effect (CPE) was measured by counting the number of syncytia (multinucleated giant cell). Percentage inhibition of syncytia formation was calculated and 50% effective concentration (EC50) was calculated.30

3.5. Inhibition of HIV-1 p24 antigen production in acute infection

The inhibitory effect of compound on HIV-1 replication in vitro was further examined by quantification of p24 expression in C8166 cells.31 Briefly, C8166 cells were inoculated with HIV-1infected, HIV-1ef, HIV-1A17, HIV-1F1VR2F184V, HIV-1L10R,M46I,G63P/V32T/S45F, pNL4-3gp41/C36V/N342S, pNL4-3gp41/C36V/I38A/N42D, pNL4-3gp41/C36V/I38E/N42S, and pNL4-3gp41/C36V/I38A/N42T (M.O.I. = 0.2) in the absence or presence of various concentrations of compound at 37 °C for 2 h to allow for viral absorption. It was then washed three times with PBS. The cells were plated at 3 × 10^5 cells/well with or without various concentrations of compound and incubated at 37 °C in a humidified atmosphere of 5% CO2 for 4 days. AZT was included as positive control. HIV-1 p24 expression in cell-free supernatants was assayed by ELISA.

3.6. Inhibition of HIV-1 p24 antigen production in PBMC

Adequate numbers of PHA-activated normal PBMC were inoculated with HIV-1KMN018 at a multiplicity of infection of (M.O.I) 0.2 in the presence of various concentrations of compound. After 3 h of virus adsorption, the cells were washed twice with PBS and incubated with or without various concentrations of compound in culture medium supplemented with 50 U/mL human recombinant IL-2 at 1.2 × 10^5 cells/mL for 7 days. Half of the medium was changed with corresponding compound concentrations on the third day. At 7 days post-infection, HIV-1 p24 antigen in the culture supernatants was analyzed by ELISA. The inhibition of HIV-1 p24 antigen production in PBMC was calculated.32

3.7. Time addition assay

To determine the stage of the HIV replication cycle with which this anti-HIV compound interfered, a time-of-addition experiment was carried out. C8166 cells were exposed to HIV at a multiplicity of infection (M.O.I) of 0.2. To ensure that the virus replication steps were synchronized in the whole-cell population, infected cells were incubated for 2 h at 4 °C. After allowing adequate time for adsorption, the unabsorbed virus was removed by washing twice with complete medium. The temperature was then shifted to 37 °C, and FuCS-1 was added at different times (0, 15 min, 30 min, 2 h, 4 h, 8 h, 16 h, 24 h) after adsorption. T-20 and AZT were included as positive control. After 3 days of culture, HIV-1p24 expression was detected by ELISA.

3.8. Co-cultivation assay

C8166 cells (3 × 10^4) were co-cultured with 1 × 10^5 virus (HIV-1infected) infected H9 cells in the presence or absence of the compound with various concentrations at 37 °C in a humidified atmosphere of 5% CO2. T-20 was used as positive control. After 6 h incubation, the number of syncytia was scored under an inverted microscope.30

3.9. RT (reverse transcriptase) assay

HIV-1 RT activity was measured by ELISA RT kit (Roche) according to the protocol provided by the manufacturer. Samples were incubated with DIG-labeled-reaction mixture at 37 °C for 15 h. Anti-DIG-POD solution was added afterward followed by substrate ABTS. The absorbance at 405/490 nm (A405/490) was determined in the ELISA reader.33

3.10. Assay of HIV-1 particle attachment and entry

Assay of HIV-1 particle attachment and entry, HIV-1 entry was estimated from the concentration of intracellular virus RNA by real-time RT-PCR. C8166 cells were pretreated with different concentrations of FuCS-1 for 1 h. It was then inoculated with HIV-1infected and allowed to adsorb with virus for 2 h at 37 °C. HIV-1 bound on the cell surface was removed by trypsinization, and then washed three times with PBS. The attachment of HIV-1 to cells was monitored after 1 h of incubation with HIV-1infected at 4 °C, and then washed extensively with PBS to eliminate unbound HIV-1 particles. T-20 and DS were used as control. The amount of RNA in cell extracts was measured by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). HIV-1 RNA was amplified with a commercial HIV-1 PCR fluorogene diagnostic kit (PG Biotech). Total RNA from cultured cells was reverse-transcribed into cDNA. The PCR cycling conditions were 1 cycle at 42 °C for 30 min and 95 °C for 3 min, 5 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and then followed by 40 cycles of 5 s at 95 °C, 30 s at 60 °C, on an fast 7500 Sequence Detection System.34

3.11. Binding potency of compound to HIV-1 gp120

Gp120 was immobilized to the AR2G Biosensors as the ForteBio technical notes described. The sensor surface was activated with 20 mM EDC and 10 mM s-NHS. Next, gp120 (5 μg/mL in 10 mM acetic buffer, pH 5) was immobilized to the surface. Any remaining activated groups were quenched using 1 M ethanolamine pH 8.5. Subsequently, various concentrations of FuCS-1 were incubated with gp120. The interaction experiments were conducted at 30 °C in PBS (50 mM sodium phosphate at pH 7.2, 150 mM NaCl) containing 1 mg/mL BSA using an Octet Red 96 instrument (ForteBio). The final volume for all solutions was 200 μL. Assays were performed in black, solid 96-well, flat bottom plates and agitated, which was set to 1000 rpm. A 600–900 s biosensor washing step was utilized before the analysis of the ligand association from the biosensor to the analyte in solution, which was performed for about 100 s. Finally, the dissociation was allowed to proceed.
for 300 s. The dissociation wells were used only once to ensure the potency of the buffer. The correction of any systematic baseline drift was accomplished by subtracting the shift recorded for a sensor loaded with ligand but no analyte. The data were analyzed using Octet software version 7.0, and the binding curve was globally fitted using a 1:1 model. The kinetic parameters \( (k_{on} \text{ and } k_{off}) \) and affinity \( (K_D) \) were calculated.

3.12. Statistical analysis

Data were expressed as mean ± standard error values \((n = 3)\) and were analyzed using the analysis Sigma Plot version 11.0 of Statistical Analysis System (systat software Inc., USA).

Acknowledgments

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