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Fejerlectin, a Lectin-like Peptide from the Skin of *Fejervarya limnocharis*, Inhibits HIV-1 Entry by Targeting Gp41

Weichen Xiong, Chenliang Zhou, Shuwen Yin, Jinwei Chai, Baishuang Zeng, Jiena Wu, Yibin Li, Lin Li,* and Xueqing Xu*

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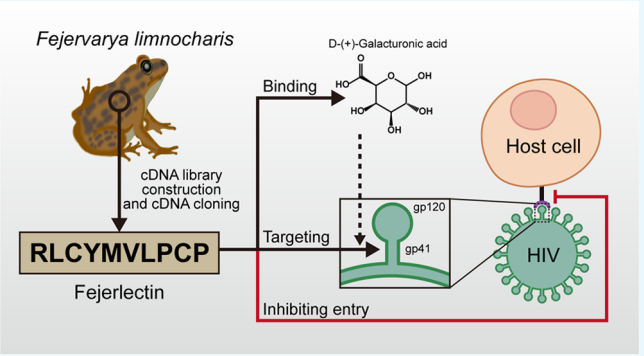
ABSTRACT: Human immunodeficiency virus type 1 (HIV-1) is mainly transmitted by sexual intercourse, and effective microbicides preventing HIV-1 transmission are still required. Amphibian skin is a rich source of defense peptides with antiviral activity. Here, we characterized a lectin-like peptide, fejerlectin (RLCYMVLPCP), isolated from the skin of the frog *Fejervarya limnocharis*. Fejerlectin showed significant hemagglutination and D-(+)-galacturonic acid-binding activities. Furthermore, fejerlectin suppressed the early entry of HIV-1 into target cells by binding to the N-terminal heptad repeat of HIV-1 gp41 and preventing 6-HB formation and Env-mediated membrane fusion. Fejerlectin is the smallest lectin-like peptide identified to date and represents a new and promising platform for anti-HIV-1 drug development.



INTRODUCTION

Human immunodeficiency virus (HIV), which infects about 2 million people annually, remains a serious global public health problem.[1] The entry of HIV-1 into host cells relies on the membrane fusion mediated by its envelope protein (Env), which comprises the exposed surface subunit gp120 and the transmembrane subunit gp41.[2] Gp120 first binds to the receptor CD4 receptor and then interacts with the CCR5 or CXCR4 co-receptors on the host cells.[3] Subsequently, gp41 undergoes conformational changes that result in fusion of the viral envelope to the host cell membrane.[4] As the critical components in the first stages of HIV-1 infection, gp120 and gp41 have become important targets for the development of HIV-1 entry inhibitors.[5]

Lectins are a group of carbohydrate-binding proteins that recognize and reversibly bind to glycosyl ligands.[6] Gp120 and gp41 are highly glycosylated and, consequently, are important lectin targets, and many lectins have significant anti-HIV-1 activity.[7] For example, BanLec, a lectin isolated from bananas (*Musa acuminata*), significantly inhibits HIV-1 infection by recognizing mannose-rich structures and binding gp120.[8] Similarly, the β-galactose-specific lectin from the polychaete marine worm *Chaetopterus variopedatus* has also been shown to exert anti-HIV-1 activity by blocking HIV-1 entry into cells.[9] However, lectins tend to weigh over 10 kDa, resulting in unavoidable toxicity or immunogenicity, limiting their clinical development and application.[10−12] Therefore, the discovery of small, specific lectins would be highly desirable. While small-size lectins such as retrocyclin-1, *Selenocosmia huwena* lectin-I, and odorranalectin have been discovered, only a few have exhibited anti-HIV capacity.[13−15]

Peptides secreted by amphibian skin have been extensively studied due to their bioactivity and low cytotoxicity.[16] Among them, caerin 1.1, maximin 3, and dermaseptin S4 have been found to suppress HIV proliferation by direct inactivation.[17−19] However, no amphibian lectin has yet shown anti-HIV activity. Here, we identify and describe a novel lectin-like peptide, which we call fejerlectin, from the skin of *Fejervarya limnocharis* frogs. A series of structural analyses and pharmacological investigations demonstrate that fejerlectin is the smallest lectin-like peptide with potent agglutination and anti-HIV-1 activity identified to date.

RESULTS

Identification and Characterization of Fejerlectin. Using polymerase chain reaction (PCR)-based cDNA cloning, we first obtained the complete nucleotide sequence encoding the fejerlectin precursor from a skin-derived *F. limnocharis* cDNA library. The nucleotide sequence has been deposited in the GenBank database under the accession code MW368972. As shown in Figure 1A, its precursor deduced from the 306 bp

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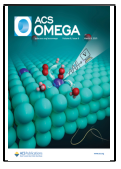

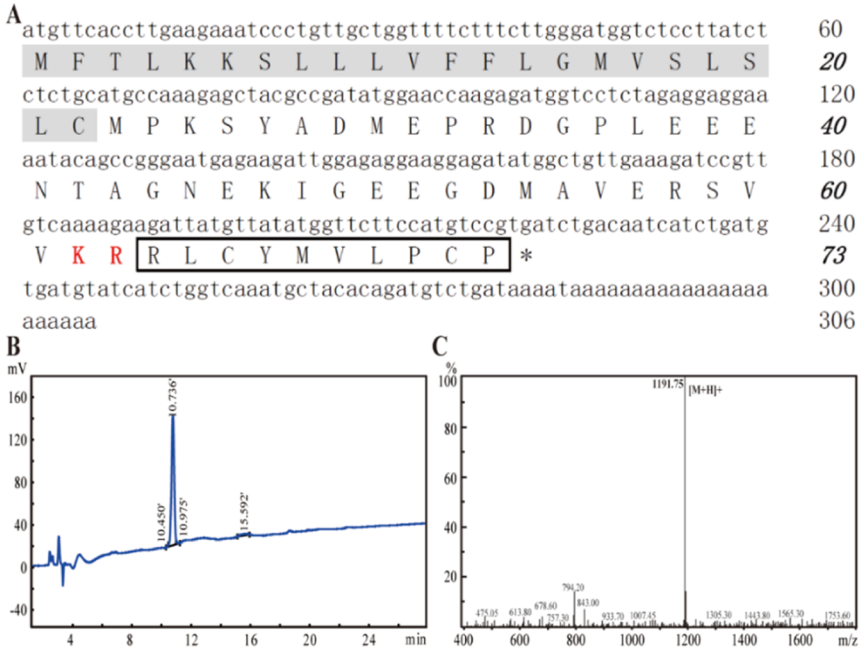

Figure 1. Identification and characterization of fejerlectin. (A) cDNA and the deduced amino acid sequence of fejerlectin. The signal peptide is shaded in gray and is followed by an acidic spacer domain with KR residues at the end (in red bold). The stop codon is indicated with an asterisk (*), and the sequence of mature fejerlectin is boxed. (B) Purity of synthesized fejerlectin detected by HPLC. (C) Molecular weight of synthesized fejerlectin confirmed by mass spectrometry.

nucleotide sequence comprised 73 amino acid residues and contained the typical primary structure characteristic of amphibian defense peptides, with a signal peptide region, a N-terminal acidic spacer domain followed by a well-known KR protease cleavage site, and a mature peptide at the C-terminus.[20] Thus, the amino acid sequence of mature fejerlectin was predicted to be RLCYMVLPCP and contain one intramolecular disulfide bridge formed by two cysteines. The NCBI BLAST search did not find any peptide similar to the putative fejerlectin, suggesting that this peptide represented a new amphibian peptide family. Its theoretical isoelectric point and molecular weight were 8.01 and 1193.54 Da, respectively. Finally, the synthesized peptide was purified by high-performance liquid chromatography (HPLC) and confirmed by mass spectrometry, which was then used in subsequent experiments (Figure 1B,C).

Hemagglutination (HA) Activity of Fejerlectin. The HA activity of fejerlectin is shown in Table 1. Fejerlectin could strongly agglutinate intact mice erythrocytes at a minimum concentration of 2.5 μM (8-fold dilution). The tested temperatures and pH did not affect its HA activity, indicating that fejerlectin was relatively stable under these conditions. Consistent with this, the HA activity of fejerlectin was also stable for 3 h in human plasma. Ethylenediaminetetraacetic acid (EDTA) treatment or addition of metal cations such as Ca^{2+} and Mg^{2+} had no effect on fejerlectin activity, suggesting that fejerlectin did not depend on metal cations to exert its lectin-like activity.

Given the fejerlectin precursor's structural similarity with amphibian defense peptides, its microbe-binding, microbe-killing, microbe-agglutinating, histamine-releasing, and mast cell degranulating activities, which are related to innate immunity, were investigated. Although fejerlectin did not show direct antibacterial activity (Figure S1), it could concentration-dependently bind and agglutinate bacteria

Table 1. HA Activity of Fejerlectin under Different Conditions[a]

test		HA units						
		1	2	4	8	16	32	64
	control	−	−	−	−	−	−	−
	fejerlectin	+	+	+	+	−	−	−
temperature (°C)	25	+	+	+	+	−	−	−
	37	+	+	+	+	−	−	−
	50	+	+	+	+	−	−	−
pH	6.5	+	+	+	+	−	−	−
	7.5	+	+	+	+	−	−	−
	8.5	+	+	+	−	−	−	−
serum stability (h)	1	+	+	+	+	−	−	−
	2	+	+	+	+	−	−	−
	3	+	+	+	+	−	−	−
metal ion	EDTA	+	+	+	+	−	−	−
	Ca^{2+}	+	+	+	+	−	−	−
	Mg^{2+}	+	+	+	+	−	−	−

[a]+, hemagglutinating; −, not hemagglutinating.

(Figure 2B,C). In addition, fejerlectin at 125 μM promoted histamine release and degranulation of mast cells by 35.41 ± 4.4 and 26.93 ± 6.92%, respectively.

Carbohydrate-Binding Specificity of Fejerlectin. To investigate the carbohydrate-binding specificity of fejerlectin, a hemagglutination inhibition test was carried out. Of the 25 tested monomeric sugars, only D-(+)-galacturonic acid inhibited its HA and microbe-agglutinating activities (Figure 2A,C and Table S1), demonstrating that D-(+)-galacturonic acid might be the specific target of fejerlectin. Isothermal titration calorimetry (ITC) analysis further displayed that fejerlectin could bind D-(+)-galacturonic acid with the K_D value of 4.39×10^{-6} M (Figure 2D and Table S2). In agreement, the binding reaction between fejerlectin and D-(+)-galacturonic

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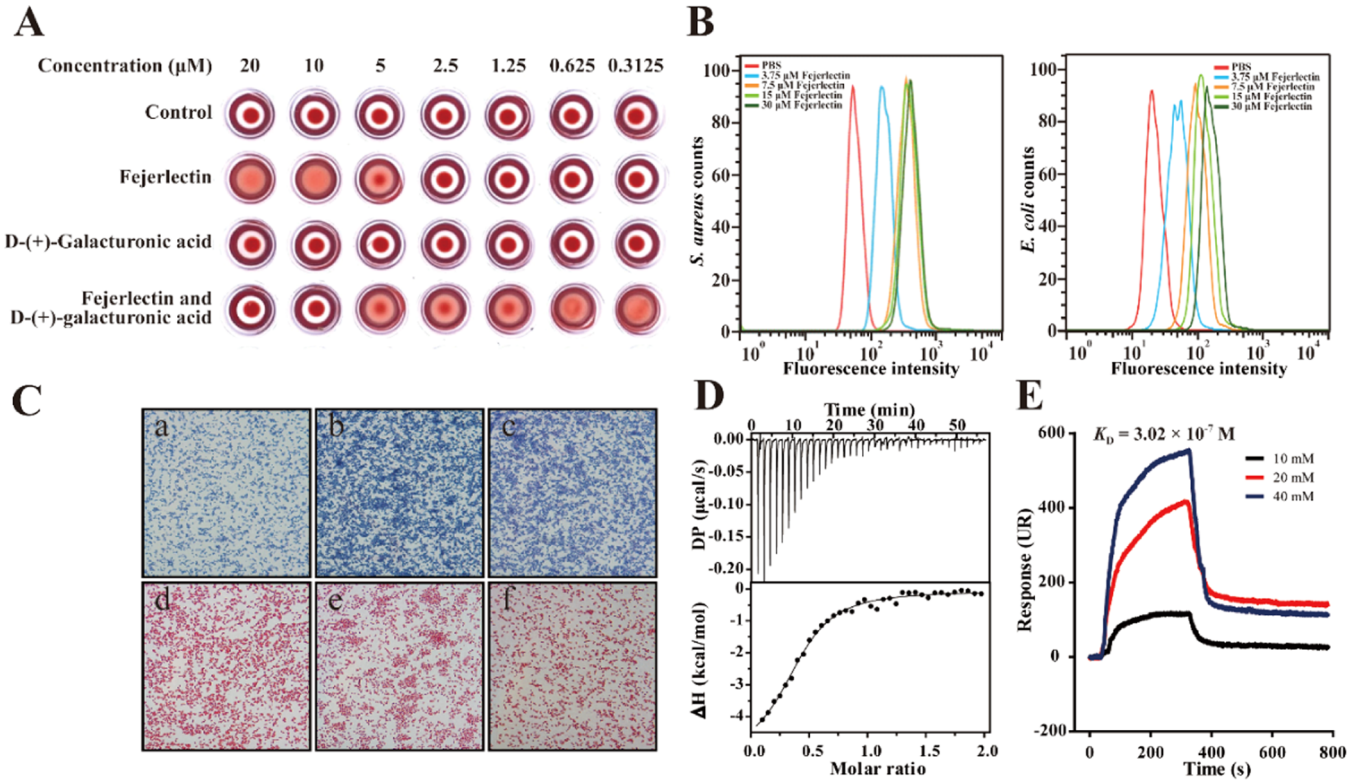

Figure 2. Binding reaction of fejerlectin with D-(+)-galacturonic acid. (A) Effects of D-(+)-galacturonic acid on the HA activity of fejerlectin. The second row shows the HA activity of fejerlectin at final concentrations between 20 and 0.3125 μM. The third row shows the HA activity of different concentrations of D-(+)-galacturonic acid (from left to right: 8, 4, 2, 1, 0.5, 0.25, and 0.125 mM). The fourth row shows the HA activity of 20 μM fejerlectin incubated with D-(+)-galacturonic acid at concentrations corresponding to the third row. (B) Flow cytometry of the binding reaction between fejerlectin and bacteria. *Staphylococcus aureus* and *Escherichia coli* were incubated with fluorescein isothiocyanate (FITC)-fejerlectin (3.75, 7.5, 15, and 30 μM) at 37 °C for 15 min before flow cytometry analysis. (C) Bacterial agglutination induced by fejerlectin. *S. aureus* and *E. coli* diluted to 2×10^8 cells/mL in Tris-buffered saline (TBS) were incubated with bovine serum albumin (BSA) (a, d), 5 μM fejerlectin (b, e), or 5 μM fejerlectin premixed with an equal volume of 4 mM D-(+)-galacturonic acid (c, f) for 1 h at room temperature and then stained with Gram dye. (D) Isothermal titration calorimetry (ITC) analysis of binding reaction of fejerlectin with D-(+)-galacturonic at 25 °C. The top panels displayed thermo changes of each injection at different time points, while the bottom panel presented the change of enthalpy as a function of ligand/target molar ratio. (E) Surface plasmon resonance imaging (SPRi) analysis of D-(+)-galacturonic acid binding to fejerlectin immobilized on a gold chip. Data were fit using a single-site binding model using the MicroCal Origin software package.

acid was further demonstrated by surface plasmon resonance imaging (SPRi) experiment (Figure 2E). Taken together, all of those results further clarified that D-(+)-galacturonic acid was the specific target of fejerlectin.

Suppression of Infection by HIV-1 Infectious Clones. As shown in Figure 3A–C, like positive maraviroc (MVC), AMD3100, and AZT controls, fejerlectin possessed highly potent anti-HIV-1 activity against infection with HIV-1 infectious clones including HIV-1_{SF162} (R5), HIV-1_{NL4-3} (X4), and HIV-$1_{81A \text{ and } NL4-3}$ (X4R5) with IC_{50} values of 2.17 ± 1.03, 1.43 ± 0.34, and 2.80 ± 0.37 μM, respectively (Figure 3A–C). Next, the potential cytotoxic effect of fejerlectin on TZM-bl cells was assessed to demonstrate that its anti-HIV-1 activity was not attributable to cytotoxicity. The 50% cytotoxicity concentrations (CC_{50}) value of fejerlectin against TZM-bl cells was 182.49 ± 9.35 μM, which was much higher than the IC_{50} value of anti-HIV-1 activity (Figure 3D). These results indicate that fejerlectin has potent and broad-spectrum antiviral activity.

Effects on Early-Stage HIV-1 Infection. To understand on which stage of HIV-1 infection fejerlectin acts, a time-of-addition assay was performed. As shown in Figure 4, the three positive control drugs had strong inhibitory activity in early-stage HIV-1 infection. The anti-HIV activity of maraviroc and AMD3100 but not AZT significantly decreased 8 h after infection (Figure 4A,B). By contrast, AZT still had high antiviral activity when added 8 h after infection (Figure 4A–C). When 25 μM fejerlectin was added to the target cells before viral infection or 0.5–8 h post-infection, it also displayed obvious anti-HIV-1 activity. However, as the time of viral infection increased, its antiviral activity gradually reduced. In particular, when added 6 or 8 h after infection, the anti-HIV activity of fejerlectin against the three tested pseudotyped viruses decreased to less than 50% (Figure 4A–C). These results suggest that fejerlectin might exert its antiviral activities at an early stage of HIV-1 infection and be an HIV-1 entry inhibitor.

Effects on Single-Cycle Infection by HIV-1 Env-Pseudotyped Viruses. To further identify whether fejerlectin acts as an HIV entry/fusion inhibitor, a single-round entry assay was performed with maraviroc and AMD3100 as positive controls. Fejerlectin potently inhibited infection by all tested HIV-1 Env-pseudotyped viruses with EC_{50} values against HIV-1_{JR-FL} and HIV-1_{HXB2} of 4.20 ± 0.96 and 9.63 ± 2.30 μM, respectively (Figure 5A,B). Moreover, VSV-G-pseudotyped virus expressing VSV-G Env as a negative control was used to examine the specificity of fejerlectin on HIV-1 Env. As shown in Figure 5C, fejerlectin had no inhibitory activity against VSV-

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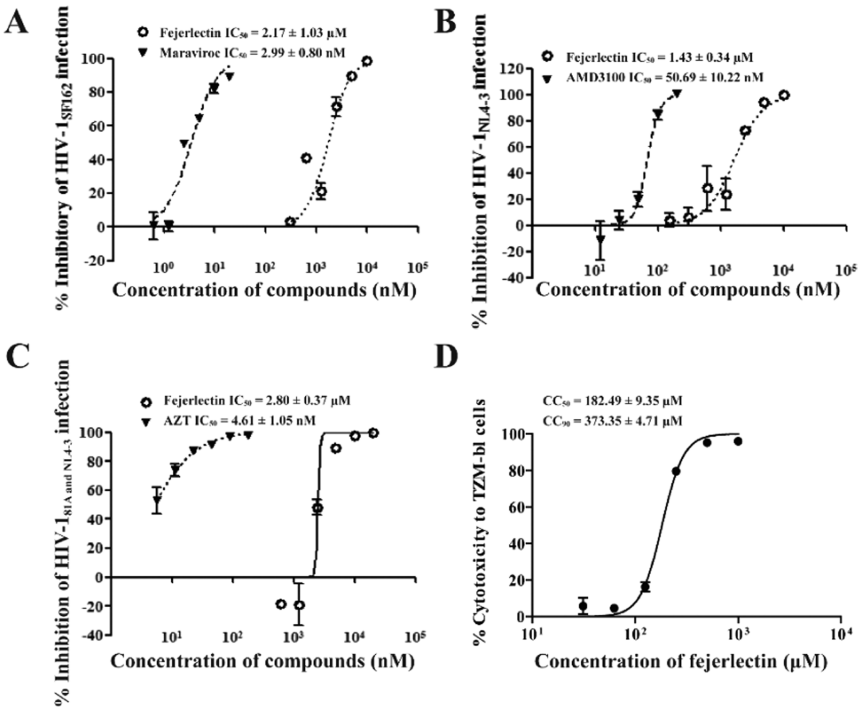

Figure 3. Anti-HIV-1 activity of fejerlectin and its cytotoxicity to host cells. The inhibitory activities of fejerlectin against HIV-1 infectious clones including HIV-1_{SF162} (A), HIV-1_{NL4-3} (B), and HIV-$1_{81A\ and\ NL4-3}$ (C). Maraviroc, AMD3100, and AZT were used as positive controls, respectively. (D) *In vitro* cytotoxicity of fejerlectin on TZM-bl cells. Experimental data are expressed as mean ± standard deviation (SD) (n = 3).

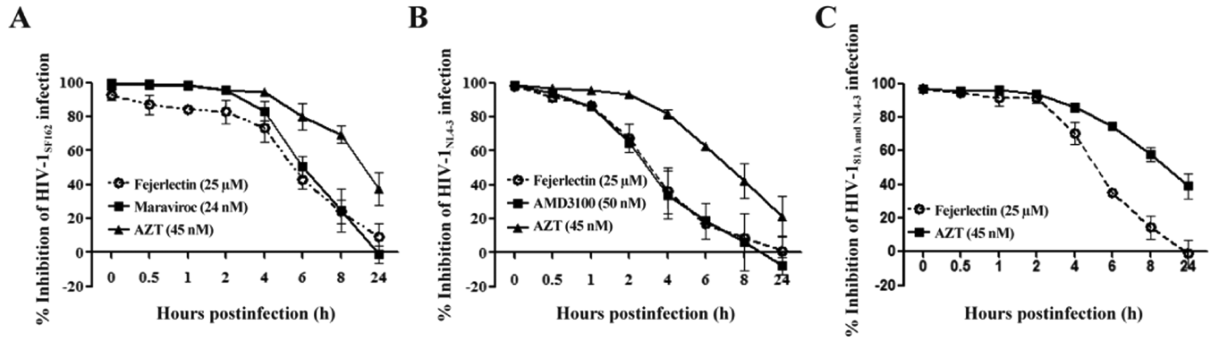

Figure 4. Time-of-addition assay with fejerlectin: (A) R5-monotropic HIV-1_{SF162}, (B) X4-monotropic HIV-1_{NL4-3}, and (C) X4R5 dual-tropic HIV-$1_{81A\ and\ NL4-3}$. Experimental data are expressed as mean ± SD (n = 3).

G pseudovirus infection. These results suggest that fejerlectin might be an HIV-1 entry inhibitor that specifically targets HIV-1 Env membrane protein.

Inhibition of HIV-1 Env-Mediated Cell−Cell Fusion. Env-mediated membrane fusion is pivotal for HIV-1's early entry into a target cell. The above results suggest that the anti-HIV-1 activity of fejerlectin could be attributable to targeting HIV-1 Env. Therefore, we further examined the inhibitory activity of fejerlectin on HIV-1 Env-mediated cell−cell fusion with CHO-WT cells as the effector cells and MT-2 cells as the target cells.[21] As shown in Figure 5D, fejerlectin dose-dependently inhibited cell−cell fusion with an IC_{50} value of 6.57 ± 0.25 μM, which was comparable to that of the ADS-J1-positive control. This result further suggests that fejerlectin might inhibit HIV-1 entry by blocking HIV-1 Env-mediated membrane fusion.

Inhibition of HIV-1 Gp41 6-HB Formation. Gp41 exerts a crucial role during fusion of the viral and cellular membranes.[22] The HIV-1 gp41 6-HB is composed of N- and C-terminal heptad repeat sequences (NHR and CHR), a key structure enhancing HIV-1 fusion and entry into target cells. Gp41 core formation *in vitro* was mimicked with the N- and C-peptides based on an effective model system established in our laboratory.[23,24] Both fejerlectin and ADS-J1 significantly inhibited gp41 6-HB formation in a dose-dependent manner with IC_{50} values of 2.61 ± 0.17 and 1.53 ± 0.48 μM, respectively (Figure 6A). In agreement, as shown by circular dichroism (CD) spectroscopy, the conformational formation of α-helicity between the N36 and C34 peptide was inhibited by incubating various concentrations of fejerlectin with N36 before addition of C34 peptides (Figure 6B). Therefore, fejerlectin inhibited the formation of α-helical complexes and the 6-HB structure by blocking the interaction between the viral gp41 NHR and CHR regions. To further ensure that fejerlectin bound N36, the kinetic reaction between fejerlectin and N36 was analyzed by SPRi. Fejerlectin strongly bound to N36 peptide with a K_D of 4.66×10^{-4} M (Figure 6C). Taken together, these results suggest that fejerlectin blocks the

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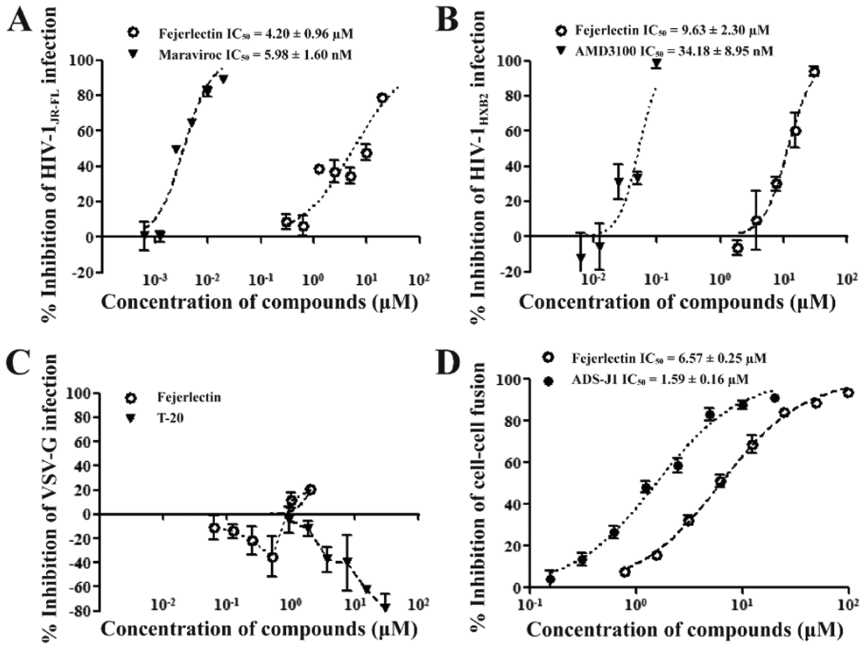

Figure 5. Effect of fejerlectin on the HIV-1 infection cycle. Inhibitory activities of fejerlectin against HIV-1_{JR-FL} (A), HIV-1_{HXB2} (B), and VSV-G (C) pseudotyped viruses. Maraviroc, AMD3100, and T-20 were used as positive controls. (D) Effects of fejerlectin on the formation of syncytia between CHO-WT and MT-2 cells. ADS-J1 was chosen as a positive control. Experimental data are expressed as mean ± SD (n = 3).

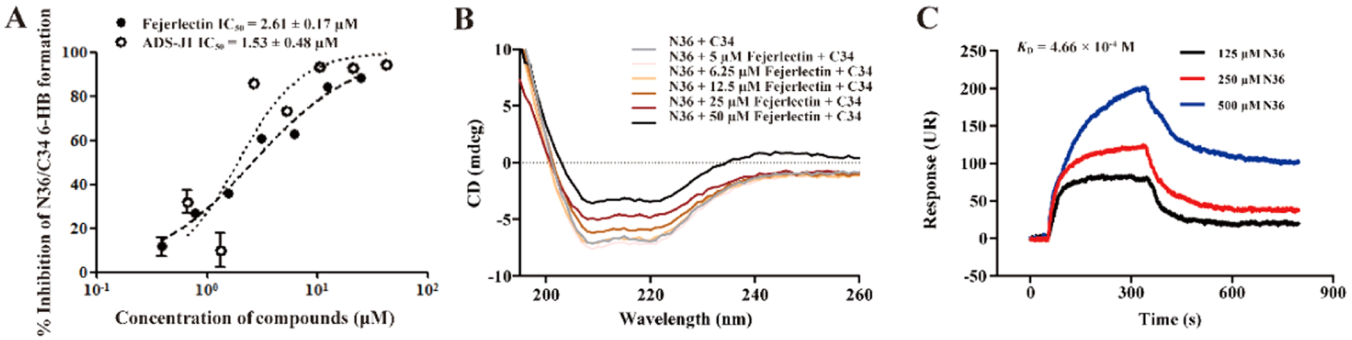

Figure 6. Effect of fejerlectin on the formation of 6-HB. The inhibitory effect of fejerlectin on the formation of 6-HB was analyzed by enzyme-linked immunosorbent assay (ELISA) (A) and CD spectra (B). ADS-J1 was chosen as a positive control. Experimental data are expressed as mean ± SD (n = 3). (C) Binding affinity between fejerlectin and N36 peptide derived from HIV-1_{JR-FL} gp41 analyzed by SPRi.

formation of gp41 6-HB and inhibits entry of HIV-1 into target cells.

DISCUSSION

Amphibians produce a variety of defense molecules, such as antimicrobial peptides and lectins, in their skin to protect them from invading microbes and predators.[16] Although a common amphibian species in Asia, only a few studies have examined the activity of skin secretions from *F. limnocharis*, and reports on its defense peptides are limited.[25,26] Here, for the first time, we identify and describe a novel lectin-like peptide called fejerlectin from *F. limnocharis* skin. Although its precursor shares some structural similarities to other amphibian defense peptides and its mature peptide contains a disulfide-bridged cyclic region comprising seven amino acid residues present in some known antimicrobial peptides (Figure 1A), fejerlectin has no antimicrobial activity (Figure 1S). However, the 10 amino acid fejerlectin peptide shows significant hemagglutination and D-(+)-galacturonic acid-binding activities. Moreover, fejerlectin has significant anti-HIV-1 activities by blocking the formation of gp41 6-HB and inhibiting HIV-1 entry into target cells. Therefore, fejerlectin is the smallest lectin-like peptide reported to date and represents a new family of lectins.

Lectins are a class of non-immune-origin proteins that can specifically bind to carbohydrates and that are involved in development, infectious diseases, and immunological functions, making them suitable as drugs and drug carriers.[27,28] However, most lectins are large and cannot be used as drugs due to their immunogenicity and toxicity. Smaller peptides or even organic molecules that can mimic the function of lectins are ideal drug candidates.[29] However, only a limited number of small peptide lectins have shown anti-HIV activity. Moreover, the reported natural peptide lectin with anti-HIV activity contains more than 10 residues and more complex in structure.[7] For example, retrocyclin-1 is a circular octadecapeptide with an internal tridisulfide ladder.[13] Thus, it is overburdened and expensive for current production systems if they are used as anti-HIV drugs. As the smallest lectin with a single disulfide bridge in its structure, fejerlectin should be stable and easy to synthesize. Consistent with this, fejerlectin is

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easily synthesized (Figure 1B,C) and a small range of temperatures, pH changes, and serum incubation conditions do not alter its HA activity (Figure 2 and Table 1). Furthermore, fejerlectin has low toxicity and can suppress early viral entry into host cells at the sites of infection, making it desirable for development into a microbicide like most naturally occurring anti-HIV peptides (Figure 3). Mutations in the envelope proteins of HIV-1 can easily result in resistance to many drugs targeting gp120 but not the molecules binding to carbohydrates.[30] Therefore, fejerlectin, with its lectin-like activity, can easily retain broad-spectrum anti-HIV activity. In addition, several protein lectins have reduced HIV transmission in organotypic models, suggesting that naturally occurring lectins are promising anti-HIV drugs.[31,32] It is worth noting that such a small peptide can be so powerful to agglutinate bacterial cells and erythrocytes. The *N* value measured in ITC is 0.415, indicating that one D-(+)-galacturonic acid may bind to multiple fejerlectin molecules (Table S2). Furtherly, the size distribution of fejerlectin is obviously different in number and intensity (Figure S2), indicating that fejerlectin agglomerates in the solution and may exist in the form of a larger complex.[33] Considering that more than one target sugar molecule is on cell surface, fejerlectin serves as a bridge and its cross-link in solution plays an important role during cell agglutination. Overall, the small size, stable properties, low toxicity, and significant antiviral activities make fejerlectin an intriguing prototype for the development of an ideal anti-HIV drug.

HIV-1 entry into host cells mainly depends on Env-mediated membrane fusion. Consequently, HIV-1 Env, with its large number of glycosyl sites, is a target for anti-HIV-1 lectins.[12,34] As a lectin-like peptide, fejerlectin inhibits HIV entry by suppressing Env-mediated membrane fusion during HIV infection, and indeed our compelling results support this hypothesis (Figure 5). By contrast, almost all anti-HIV peptides from amphibian skin exert their functions through direct inactivation involving their oligomerization in bilayers and subsequent disruption of membrane integrity, as demonstrated by caerins and dermaseptins.[18,19,35,36] Thus, fejerlectin is the first amphibian-derived peptide to directly interfere with HIV entry. Surprisingly, fejerlectin can bind directly nonglycosylated N36 peptide and inhibit the formation of 6-HB (Figure 6). Furthermore, to the best of our knowledge, the presence of D-(+)-galacturonic acid in Env has yet to be reported. Therefore, the anti-HIV activity of fejerlectin seems to be unrelated to its carbohydrate-binding activity. In recent years, distinct classes of HIV-1 entry inhibitors targeting 6-HB of gp41 have been discovered. For example, our previously studies confirmed that a glycosylated dihydrochalcone derived from natural products (trilobatin) and a N-substituted pyrrole derivative 12 m (NSPD-12 m) inhibited HIV-1 entry/fusion by binding the gp41 NHR region and blocking the formation of fusion-active 6-HB.[24,37] In addition, there are also a large number of literature reports on peptide-based HIV entry/fusion inhibitor targeting gp41. The first peptide-based HIV entry inhibitor, enfuvirtide (T20), was approved by Food and Drug Administration (FDA) to treat HIV/AIDS patients in 2003.[38] The second-generation CHR-derived peptides in a hope to replace T20 have potent anti-HIV-1 activity by inhibiting 6-HB formation, such as sifuvirtide and albuvirtide.[39,40] The third-generation CHR peptides are CHR-derived peptides containing MT-hook or IDL anchor, and lipopeptides with 50-fold more potent anti-HIV-1 activity than T20 including CP32, HP23, and CP-IDL.[41,42] However, our data support it is possible that fejerlectin can bind both carbohydrates and the gp41 NHR.

Previous studies have shown that mannose, the target of anti-HIV lectins, is mainly present in gp120.[43−45] However, fetuin-specific retrocyclin-1 can protect cells from HIV-1 infection by binding with high affinity to gp120, CD4, and galactosylceramide rather than nonglycosylated gp120.[13] Furthermore, gp41 contains a large amount of complex-type glycans other than mannose and can be bound by the *β*-galactose-specific lectin CVL, suggesting that other monosaccharides or glycoproteins in HIV-1 Env may also serve as targets for molecules inhibiting HIV-1 entry.[9,46,47] Considering that we only investigated a limited number of monosaccharides and fejerlectin inhibits Env-mediated fusion, we cannot exclude that other sugar molecules or glycoproteins in Env are fejerlectin targets and related to its anti-HIV activity.

In summary, this is the first report identifying a lectin-like peptide from the skin of *F. limnocharis*. The primary sequence of fejerlectin contains 10 amino acid residues including a disulfide-bridged heptapeptide ring. Fejerlectin has significant hemagglutination and D-(+)-galacturonic acid-binding activities. Thus, it is the smallest lectin-like peptide reported to date. Furthermore, fejerlectin can prevent the formation of 6-HB by binding to gp41 NHR, thus inhibiting Env-mediated fusion and HIV-1 entry. These characteristics make fejerlectin a promising new template for future anti-HIV drug development.

MATERIALS AND METHODS

Animals and Ethics Statement. Adult *F. limnocharis* frogs of both genders weighing 3−6 g (n = 4), which are not an endangered or protected species, were captured in the countryside of Guangzhou, Guangdong Province, China. The frogs were humanely euthanized by CO_2 inhalation, washed with deionized water, and their skin was separated immediately for RNA extraction. All protocols and procedures involving live animals were approved by the Animal Care and Use Ethics of Southern Medical University and carried out in full compliance with the guidelines approved for Animal Care and Use at Southern Medical University.

Reagents. HEK-293T, TZM-bl, CHO-WT, and MT-2 cells were obtained from the American Type Culture Collection. *p*NL4-3E-R-Luc plasmid, HIV-1 and vesicular stomatitis virus-G (VSV-G) Env-encoding plasmids, maraviroc (MVC, a CCR5 antagonist), AMD3100 (a CXCR4 inhibitor), and zidovudine (AZT, a nucleoside reverse transcriptase inhibitor) were purchased from the National Institutes of Health AIDS Research and Reference Reagent Program. Plasmids encoding HIV-1_{JR-FL} (R5 strain), HIV-1_{HXB2} (X4 strain), HIV-1_{NL4-3} (CXCR4-tropic), HIV-1_{SF162} (CCR5-tropic), and dual-tropic HIV-$1_{81A\ and\ NL4-3}$ infectious clones were kind gifts from Jan Munch of Ulm University. All virus stocks were produced by transfecting HEK-293T cells. The well-characterized peptides N36 and C34 derived from the N- and C-terminal core structure of HIV-1 gp41 were synthesized by Scilight Biotechnology LLC (Beijing, China).[23,48] ADS-J1, a small-molecule HIV-1 entry inhibitor that blocks gp41 six-helix bundle (6-HB) formation, was purchased from ComGenex. Mouse monoclonal antibodies targeting NC-1 and T-20 specific for the gp41 6-HB were prepared using a previously described method.[49−51]

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Molecular Cloning and Characterization of cDNA Encoding Fejerlectin. Total RNA from *F. limnocharis* skin was extracted using TRIzol reagent (Life Technologies, Inc.), purified by oligo (dT) cellulose chromatography (Life Technologies, Inc.), quantified with a Merinton SMA1000 (Merinton), and used for cDNA synthesis with a SMART cDNA library construction kit (Takara Biotechnology) according to the manufacturer's instructions. cDNA encoding fejerlectin was obtained by PCR amplifications with synthesized skin total cDNA as template and HG (5′-AGATGTT(G/C)ACC(T/A)TGAAGAAATC-3′) in the sense direction plus 3′ PCR primer CDS III (5′-ATTCTAGAGGCC-GAGGCGGCCGACATG-3′) in the antisense direction as primers.[52] PCR amplifications were carried out in a reaction mixture containing Gene Taq polymerase (TianGen) in a thermal cycler. The PCR program was as follows: 4 min at 95 °C; 30 cycles of 20 s at 95 °C, 30 s at 48 °C, and 30 s at 72 °C, and finally 10 min at 72 °C for extension. The PCR product (~300 bp) was purified by gel electrophoresis and cloned into the pMD18-T vector (Takara Biotechnology) for DNA sequencing. The physical and chemical parameters of fejerlectin and its precursor were predicted and analyzed using the ExPASy Bioinformatics Resource Portal (http://www.expasy.org/tools/) and the blastp suite of the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/blast.cgi).

Peptide Synthesis. Fejerlectin and FITC-labeled fejerlectin (FITC conjugated to the N terminus of fejerlectin) were synthesized by GL Biochem Ltd. The crude synthetic peptide was purified with an Inertsil ODS-SP (C-18) RP-HPLC column (Shimadzu) to >95% purity. The high-purity peptide was pooled, lyophilized, and further characterized by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry.

Hemagglutination Activity Test. Mice erythrocytes were washed with Alsever's solution and diluted into a 2% suspension (v/v) with phosphate-buffered saline (PBS, pH 7.5). HA activity tests were carried out in V-bottom microtiter plates by the twofold dilution method. After erythrocytes in the blank had fully subsided, the results were recorded with PBS as a negative control. The HA titer, defined as the reciprocal of the lectin displaying HA activity at the lowest concentration, was considered as one HA unit.[53] To determine the effects of temperature, pH, serum, metal ions, and carbohydrates on its HA activity, fejerlectin was incubated at three different temperatures (25, 37, and 50 °C); in selected buffers (100 mM sodium citrate, pH 6.5; 100 mM Tris−HCl, pH 7.5; 100 mM glycine, pH 8.5); with PBS with 10 mM EDTA, 10 mM $CaCl_2$, or 10 mM $MgCl_2$; in human serum at 37 °C for 0−4 h; or with PBS containing 25 different sugars including D-(+)-galacturonic acid for 1 h before HA titers were measured. All experiments were repeated at least three times.

Bacterial Agglutination Assay. The agglutination tests against *S. aureus* and *E. coli* were performed to further verify the lectin-like activity of fejerlectin. *S. aureus* and *E. coli* were diluted to a density of 2×10^8 cells/mL and incubated at room temperature for 1 h with BSA, 5 μM fejerlectin, or 5 μM fejerlectin premixed with 4 mM D-(+)-galacturonic acid for 30 min, respectively. Then, the mixtures were individually stained with Gram dye and observed under an inverted microscope.

Isothermal Titration Calorimetry. The interaction between limnolectin and D-(+)-galacturonic acid was measured with a VP-ITC microcalorimeter (Malvern, U.K.) as previously reported by us.[52] In short, limnolectin and D-(+)-galacturonic acid were dissolved in 50 mM PBS, pH 7.2, and degassed under vacuum before use. D-(+)-Galacturonic acid (10 mM) was added in 1.0 μL aliquots every 2 min intervals into 50 μM limnolectin in the sample cell under constant stirring at 25 °C for 21 injections. PBS solution was used as a blank control to subtract the dilution heat. High feedback mode of the instrument was selected to analyze the data. The enthalpy change (ΔH) and the equilibrium dissociation constant (K_D) were calculated by fitting to a single-site binding model with the MicroCal Origin software. The basic thermodynamic equations were used to calculate the entropy change (ΔS) and Gibb's free-energy change (ΔG). The experiment was repeated at least three times.

Surface Plasmon Resonance Imaging Assay. The affinity between fejerlectin and D-(+)-galacturonic acid was investigated with the PlexArray HT system. Briefly, fejerlectin (2 mM) was fixed on the chip surface and then incubated at 4 °C overnight in a humid box. Before SPRi, the chip was washed and blocked with 5% (m/v) nonfat milk overnight. After the baseline was stabilized with PBS, different concentrations of D-(+)-galacturonic acid (10, 20, and 40 mM) or N36 peptide (125, 250, and 500 μM) were flowed over the chip at 2 μL/s. H_3PO_4 (0.5% (v/v)) in deionized water was used for chip surface regeneration. Standard deviations were plotted using the average results of 10 different points, and the average kinetics of the complexes were obtained from the 1:1 Langmuir kinetic model of three different concentrations. The K_D value was calculated according to the kinetic constants derived from curve-fitting association and dissociation rates to the real-time binding and washing data. All data analysis was performed with ORIGIN-Lab software. The experiment was repeated at least three times.

Anti-HIV-1 Infection Assay. The anti-HIV-1 infection activity of fejerlectin was measured with three different HIV-1 infectious clones (CXCR4-tropic HIV-1_{NL4-3}, CCR5-tropic HIV-1_{SF162}, and dual-tropic HIV-$1_{81A\ and\ NL4-3}$) and HIV-1_{NL4-3}-*Luc* virions. First, TZM-bl cells (1×10^5/mL) were seeded into 96-well plates and incubated at 37 °C overnight. Then, 100-fold 50% tissue culture infective dose ($TCID_{50}$) HIV-1 or VSV-G viruses were incubated with fejerlectin at various concentrations at 37 °C for 30 min. Then, the mixture was added to cells and further incubated for 48 h. Finally, a Dual-Luciferase Reporter Assay Kit was used to determine the luciferase activity of lysed TZM-bl cells. Maraviroc, AMD3100, and AZT were used as positive controls, respectively. For the single-round HIV-1 infection assay, TZM-bl cells were challenged with HIV-1 Env-pseudotyped HIV-1_{JR-FL} (R5 strain) and HIV-1_{HXB2} (X4 strain) viruses, which were preincubated with fejerlectin (0.5−50 μM) at 37 °C for 30 min. Fresh Dulbecco's modified Eagle's medium (DMEM) was used to replace the culture supernatant at 24 h after viral infection, and the cells were collected 72 h post-infection for luciferase activity measurement.

Cytotoxicity *In Vitro*. The *in vitro* TZM-bl cell cytotoxicity of fejerlectin was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, TZM-bl cells (2×10^4/well) were inoculated into 96-well culture plates containing DMEM with 1% penicillin/streptomycin and 10% fetal bovine serum. After 24 h, graded concentrations of fejerlectin from 1 to 1000 μM were added to the medium prior

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to another 48 h of incubation. The medium was removed, and 100 μL of MTT solution (0.5 mg/mL) was applied to each well. After 4 h, the MTT solution was replaced with 150 μL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. Absorbance at 570 nm was determined with an ELISA reader. The 50% cytotoxicity concentrations (CC_{50}) were computed with CALCUSYN software.

Time-of-Addition Assay. To investigate the *in vitro* antiviral activity of fejerlectin at different time points after virus infection, the time-of-addition assay was carried out as previously reported.[54] In short, 50 μL of HIV-1_{NL4-3}, HIV-1_{SF162}, and HIV-$1_{81A\ and\ NL4-3}$ at 100 $TCID_{50}$ were used to infect 100 μL of 5×10^5/mL TZM-bl cells for 0, 0.5, 1, 2, 4, 6, 8, and 24 h at 37 °C before adding 50 μL of fejerlectin (25 μM), AZT (45 nM), maraviroc (24 nM), or AMD3100 (50 nM). The culture supernatants were replaced with fresh medium 24 h post-infection, and the culture supernatants were collected for measuring p24 antigen levels, as described in the above anti-HIV-1 infection assay after 48 h of infection.

HIV-1 Env-Mediated Cell−Cell Fusion Assay. The syncytium formation assay was performed using our previous method to directly evaluate the inhibition of fusion between MT-2 cells (expressing CD4 and CXCR4) and CHO-WT cells (expressing HIV-1 Env).[21] Briefly, 2×10^5/mL CHO-WT cells were incubated with graded concentrations of fejerlectin for 30 min and then incubated with 4×10^5/mL MT-2 cells for 48 h at 37 °C. ADS-J1 was used as a positive control. The number of positive syncytia formed by MT-2 and CHO-WT cells in three fields per well was recorded using an inverted microscope. The percentage inhibition of cell fusion was computed according to the following formula: %inhibition = (1 − syncytia number of inhibitor/syncytia number of positive control) × 100. The IC_{50} values were counted with CALCUSYN software.

Circular Dichroism Spectroscopy Assay. N36 at 10 μM was incubated with fejerlectin at a final concentration of 5, 6.25, 12.5, 25, or 50 μM in PBS at 37 °C for 30 min before incubation with 10 μM C34 for another 30 min. The CD spectra of all samples from 190 to 260 nm were measured with a Chirascan CD spectrometer (Applied Photophysics Ltd., U.K.) using a 5 nm bandwidth, 0.1 cm path length, 0.1 nm resolution, 4 s response time, and 50 nm/min scanning speed. The data were corrected by subtraction of the blank corresponding to the solvent and are presented as the mean residue ellipticity (θ) in deg cm^2/dmol.[55]

ELISA for Inhibiting Gp41 6-HB Formation. To determine whether fejerlectin interfered with gp41 6-HB formation, a sandwich ELISA was established as described previously.[56] First, 2 μM N36 peptide was incubated with a graded concentration of fejerlectin (5−50 μM) at 37 °C for 30 min before further incubation with 2 μM C34 at 37 °C for another 30 min. Then, the mixture was added to a 96-well polystyrene plate precoated with 2 μg/mL rabbit anti-6-HB IgG. Then, 1 μg/mL NC-1 monoclonal antibody, biotin-labeled anti-mouse IgG (Sigma-Aldrich; 1:5000 diluted in 0.01 M PBS with 2% dry nonfat milk), streptavidin-labeled horseradish peroxidase (HRP) (Zymed, South San Francisco, CA; 1:10 000 diluted in PBS with 10% goat serum), 3,3′,5,5′-tetramethylbenzidine, and 1 M H_2SO_4 were sequentially added. Finally, the absorbance value at 450 nm was measured with an ELISA reader.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00033.

List of various carbohydrates tested (Table S1); thermodynamic parameters of ITC (Table S2); antibacterial activity of fejerlectin (Figure S1); and size of fejerlectin in H_2O (Figure S2) (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Lin Li − *Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China;* orcid.org/0000-0002-2443-3121; Phone: 86-20-61648655; Email: li75lin@126.com

Xueqing Xu − *Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China;* orcid.org/0000-0002-4525-5803; Phone: +86-20-61648537; Email: xu2003@smu.edu.cn; Fax: +86-20-61648655

Authors

Weichen Xiong − *Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China*

Chenliang Zhou − *Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China*

Shuwen Yin − *Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China; Department of Pharmacy, Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou 510060, China*

Jinwei Chai − *Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China*

Baishuang Zeng − *Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China*

Jiena Wu − *Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China*

Yibin Li − *Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c00033

Author Contributions

W.X. and C.Z. contributed equally to this work. W.X., C.Z., S.Y, J.C., B.Z., J.W., and Y.L. performed the experiments and analyzed the data. L.L. and X.X. designed the experiments, supervised the study, evaluated the data, and revised the manuscript for publication. All authors contributed to the manuscript and approved the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Riddell, J.; Amico, K. R.; Mayer, K. H. HIV Preexposure Prophylaxis. *JAMA* **2018**, *319*, 1261.
(2) Checkley, M. A.; Luttge, B. G.; Freed, E. O. HIV-1 Envelope Glycoprotein Biosynthesis, Trafficking, and Incorporation. *J. Mol. Biol.* **2011**, *410*, 582−608.
(3) Zhang, C.; Zhu, R.; Cao, Q.; Yang, X.; Huang, Z.; An, J. Discoveries and Developments of CXCR4-Targeted HIV-1 Entry Inhibitors. *Exp. Biol. Med.* **2020**, *245*, 477−485.
(4) Jurado, S.; Cano-Muñoz, M.; Morel, B.; Standoli, S.; Santarossa, E.; Moog, C.; Schmidt, S.; Laumond, G.; Cámara-Artigas, A.; Conejero-Lara, F. Structural and Thermodynamic Analysis of HIV-1 Fusion Inhibition Using Small Gp41 Mimetic Proteins. *J. Mol. Biol.* **2019**, *431*, 3091−3106.
(5) Li, W.; Lu, L.; Li, W.; Jiang, S. Small-Molecule HIV-1 Entry Inhibitors Targeting Gp120 and Gp41: A Patent Review (2010-2015). *Expert Opin. Ther. Pat.* **2017**, *27*, 707−719.
(6) Singh, S.; Devi, S.; Ng, T. Banana Lectin: A Brief Review. *Molecules* **2014**, *19*, 18817−18827.
(7) Akkouh, O.; Ng, T.; Singh, S.; Yin, C.; Dan, X.; Chan, Y.; Pan, W.; Cheung, R. Lectins with Anti-HIV Activity: A Review. *Molecules* **2015**, *20*, 648−668.
(8) Swanson, M. D.; Winter, H. C.; Goldstein, I. J.; Markovitz, D. M. A Lectin Isolated from Bananas Is a Potent Inhibitor of HIV Replication. *J. Biol. Chem.* **2010**, *285*, 8646−8655.
(9) Wang, J. H.; Kong, J.; Li, W.; Molchanova, V.; Chikalovets, I.; Belogortseva, N.; Luk'yanov, P.; Zheng, Y. T. A *β*-Galactose-Specific Lectin Isolated from the Marine Worm *Chaetopterus variopedatus* Possesses Anti-HIV-1 Activity. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2006**, *142*, 111−117.
(10) Reynoso-Camacho, R.; González De Mejía, E.; Loarca-Piña, G. Purification and Acute Toxicity of a Lectin Extracted from Tepary Bean (*Phaseolus acutifolius*). *Food Chem. Toxicol.* **2003**, *41*, 21−27.
(11) Vineethkumar, T. V.; Shyla, G.; George, S. Smallest Lectin-like Peptide Identified from the Skin Secretion of an Endemic Frog, Hydrophylax Bahuvistara. *Acta Biol. Hung.* **2018**, *69*, 110−113.
(12) Mitchell, C. A.; Ramessar, K.; O'Keefe, B. R. Antiviral Lectins: Selective Inhibitors of Viral Entry. *Antiviral Res.* **2017**, *142*, 37−54.
(13) Wang, W.; Cole, A. M.; Hong, T.; Waring, A. J.; Lehrer, R. I. Retrocyclin, an Antiretroviral *θ*-Defensin, Is a Lectin. *J. Immunol.* **2003**, *170*, 4708−4716.
(14) Lü, S.; Liang, S.; Gu, X. Three-Dimensional Structure of *Selenocosmia huwena* Lectin-I (SHL-I) from the Venom of the Spider *Selenocosmia huwena* by 2D-NMR. *J. Protein Chem.* **1999**, *18*, 609−617.
(15) Li, J.; Wu, H.; Hong, J.; Xu, X.; Yang, H.; Wu, B.; Wang, Y.; Zhu, J.; Lai, R.; Jiang, X.; Lin, D.; Prescott, M. C.; Rees, H. H. Odorranalectin Is a Small Peptide Lectin with Potential for Drug Delivery and Targeting. *PLoS One* **2008**, *3*, No. e2381.
(16) Xu, X.; Lai, R. The Chemistry and Biological Activities of Peptides from Amphibian Skin Secretions. *Chem. Rev.* **2015**, *115*, 1760−1846.
(17) Lai, R.; Zheng, Y.-T.; Shen, J.-H.; Liu, G.-J.; Liu, H.; Lee, W.-H.; Tang, S.-Z.; Zhang, Y. Antimicrobial Peptides from Skin Secretions of Chinese Red Belly Toad Bombina Maxima. *Peptides* **2002**, *23*, 427−435.
(18) Lorin, C.; Saidi, H.; Belaid, A.; Zairi, A.; Baleux, F.; Hocini, H.; Bélec, L.; Hani, K.; Tangy, F. The Antimicrobial Peptide Dermaseptin S4 Inhibits HIV-1 Infectivity in Vitro. *Virology* **2005**, *334*, 264−275.
(19) VanCompernolle, S. E.; Taylor, R. J.; Oswald-Richter, K.; Jiang, J.; Youree, B. E.; Bowie, J. H.; Tyler, M. J.; Conlon, J. M.; Wade, D.; Aiken, C.; Dermody, T. S.; KewalRamani, V. N.; Rollins-Smith, L. A.; Unutmaz, D. Antimicrobial Peptides from Amphibian Skin Potently Inhibit Human Immunodeficiency Virus Infection and Transfer of Virus from Dendritic Cells to T Cells. *J. Virol.* **2005**, *79*, 11598−11606.
(20) Dong, Z.; Luo, W.; Zhong, H.; Wang, M.; Song, Y.; Deng, S.; Zhang, Y. Molecular Cloning and Characterization of Antimicrobial Peptides from Skin of Hylarana Guentheri. *Acta Biochim. Biophys. Sin.* **2017**, *49*, 450−457.
(21) Li, M.; Li, L.; Jiang, S.; Zhang, J. A Non-Infectious Assay for Detecting HIV Env-Induced Cell-Cell Fusion. *J. Jinan Univ. (Med. Ed.)* **2007**, *6*, 9.
(22) Barreto Vasconcelos, A. L.; Monteiro-Cunha, J. P. HIVfird: A Tool for Detection of Resistance to Fusion Inhibitor Drugs in HIV-1 Sequences. *AIDS Res. Hum. Retroviruses* **2019**, *35*, 941−947.
(23) Liu, S.; Lu, H.; Niu, J.; Xu, Y.; Wu, S.; Jiang, S. Different from the HIV Fusion Inhibitor C34, the Anti-HIV Drug Fuzeon (T-20) Inhibits HIV-1 Entry by Targeting Multiple Sites in Gp41 and Gp120. *J. Biol. Chem.* **2005**, *280*, 11259−11273.
(24) Yin, S.; Zhang, X.; Lai, F.; Liang, T.; Wen, J.; Lin, W.; Qiu, J.; Liu, S.; Li, L. Trilobatin as an HIV-1 Entry Inhibitor Targeting the HIV-1 Gp41 Envelope. *FEBS Lett.* **2018**, *592*, 2361−2377.
(25) Suhyana, J.; Artika, I. M.; Safari, D. Activity of Skin Secretions of Frog *Fejervarya limnocharis* and *Limnonectes macrodon* against *Streptococcus pneumoniae* Multidrug Resistant and Molecular Analysis of Species *F. limnocharis*. *Curr. Biochem.* **2016**, *2*, 99−112.
(26) Yoshimura, Y.; Kasuya, E. Odorous and Non-Fatal Skin Secretion of Adult Wrinkled Frog (Rana Rugosa) Is Effective in Avoiding Predation by Snakes. *PLoS One* **2013**, *8*, No. e81280.
(27) Cavada, B. S.; Pinto-Junior, V. R.; Osterne, V. J. S.; Oliveira, M. V.; Lossio, C. F.; Silva, M. T. L.; Bari, A. U.; Lima, L. D.; Souza-Filho, C. H. D.; Nascimento, K. S. Comprehensive Review on Caelsalpinioideae Lectins: From Purification to Biological Activities. *Int. J. Biol. Macromol.* **2020**, *162*, 333−348.
(28) Lagarda-Diaz, I.; Guzman-Partida, A.; Vazquez-Moreno, L. Legume Lectins: Proteins with Diverse Applications. *Int. J. Mol. Sci.* **2017**, *18*, No. 1242.
(29) Jaffé, W. G.; Seidl, D. S. Toxicology of Plant Lectins. *Food Poisoning*; Routledge, 2019; Vol. 7, pp 263−290.
(30) Eade, C. R.; Wood, M. P.; Cole, A. M. Mechanisms and Modifications of Naturally Occurring Host Defense Peptides for Anti-HIV Microbicide Development. *Curr. HIV Res.* **2012**, *10*, 61−72.
(31) Balzarini, J. Inhibition of HIV Entry by Carbohydrate-Binding Proteins. *Antiviral Res.* **2006**, *71*, 237−247.
(32) Mori, T.; O'Keefe, B. R.; Sowder, R. C.; Bringans, S.; Gardella, R.; Berg, S.; Cochran, P.; Turpin, J. A.; Buckheit, R. W.; McMahon, J. B.; Boyd, M. R. Isolation and Characterization of Griffithsin, a Novel HIV-Inactivating Protein, from the Red Alga Griffithsia Sp. *J. Biol. Chem.* **2005**, *280*, 9345−9353.
(33) Yeap, S. P.; Lim, J.; Ngang, H. P.; Ooi, B. S.; Ahmad, A. L. Role of Particle−Particle Interaction Towards Effective Interpretation of Z -Average and Particle Size Distributions from Dynamic Light Scattering (DLS) Analysis. *J. Nanosci. Nanotechnol.* **2018**, *18*, 6957−6964.
(34) Tan, S.; Li, W.; Li, Z.; Li, Y.; Luo, J.; Yu, L.; Yang, J.; Qiu, M.; Cheng, H.; Xu, W.; Jiang, S.; Lu, L.; Liu, S.; Ma, W. A Novel CXCR4 Targeting Protein SDF-1/S4 as an HIV-1 Entry Inhibitor. *Viruses* **2019**, *11*, No. 874.
(35) Marcotte, I.; Wegener, K. L.; Lam, Y.-H.; Chia, B. C. S.; de Planque, M. R. R.; Bowie, J. H.; Auger, M.; Separovic, F. Interaction

https://dx.doi.org/10.1021/acsomega.1c00033
ACS Omega 2021, 6, 6414−6423

of Antimicrobial Peptides from Australian Amphibians with Lipid Membranes. *Chem. Phys. Lipids* **2003**, *122*, 107−120.
(36) Zairi, A.; Tangy, F.; Bouassida, K.; Hani, K. Dermaseptins and Magainins: Antimicrobial Peptides from Frogs' Skin—New Sources for a Promising Spermicides Microbicides—A Mini Review. *J. Biomed. Biotechnol.* **2009**, *2009*, No. 452567.
(37) Qiu, J.; Liang, T.; Wu, J.; Yu, F.; He, X.; Tian, Y.; Xie, L.; Jiang, S.; Liu, S.; Li, L. N-Substituted Pyrrole Derivative 12M Inhibits HIV-1 Entry by Targeting Gp41 of HIV-1 Envelope Glycoprotein. *Front. Pharmacol.* **2019**, *10*, No. 859.
(38) Zhu, Y.; Chong, H.; Yu, D.; Guo, Y.; Zhou, Y.; He, Y. Design and Characterization of Cholesterylated Peptide HIV-1/2 Fusion Inhibitors with Extremely Potent and Long-Lasting Antiviral Activity. *J. Virol.* **2019**, *93*, No. e02312-18.
(39) Yu, D.; Ding, X.; Liu, Z.; Wu, X.; Zhu, Y.; Wei, H.; Chong, H.; Cui, S.; He, Y. Molecular Mechanism of HIV-1 Resistance to Sifuvirtide, a Clinical Trial−Approved Membrane Fusion Inhibitor. *J. Biol. Chem.* **2018**, *293*, 12703−12718.
(40) Chong, H.; Yao, X.; Zhang, C.; Cai, L.; Cui, S.; Wang, Y.; He, Y. Biophysical Property and Broad Anti-HIV Activity of Albuvirtide, a 3-Maleimimidopropionic Acid-Modified Peptide Fusion Inhibitor. *PLoS One* **2012**, *7*, No. e32599.
(41) Pu, J.; Wang, Q.; Xu, W.; Lu, L.; Jiang, S. Development of Protein- and Peptide-Based HIV Entry Inhibitors Targeting Gp120 or Gp41. *Viruses* **2019**, *11*, No. 705.
(42) Chong, H.; Xue, J.; Xiong, S.; Cong, Z.; Ding, X.; Zhu, Y.; Liu, Z.; Chen, T.; Feng, Y.; He, L.; Guo, Y.; Wei, Q.; Zhou, Y.; Qin, C.; He, Y. A Lipopeptide HIV-1/2 Fusion Inhibitor with Highly Potent In Vitro, Ex Vivo, and In Vivo Antiviral Activity. *J. Virol.* **2017**, *91*, No. e00288-17.
(43) Go, E. P.; Hewawasam, G.; Liao, H.-X.; Chen, H.; Ping, L.-H.; Anderson, J. A.; Hua, D. C.; Haynes, B. F.; Desaire, H. Characterization of Glycosylation Profiles of HIV-1 Transmitted/Founder Envelopes by Mass Spectrometry. *J. Virol.* **2011**, *85*, 8270−8284.
(44) Bonomelli, C.; Doores, K. J.; Dunlop, D. C.; Thaney, V.; Dwek, R. A.; Burton, D. R.; Crispin, M.; Scanlan, C. N. The Glycan Shield of HIV Is Predominantly Oligomannose Independently of Production System or Viral Clade. *PLoS One* **2011**, *6*, No. e23521.
(45) Raska, M.; Takahashi, K.; Czernekova, L.; Zachova, K.; Hall, S.; Moldoveanu, Z.; Elliott, M. C.; Wilson, L.; Brown, R.; Jancova, D.; Barnes, S.; Vrbkova, J.; Tomana, M.; Smith, P. D.; Mestecky, J.; Renfrow, M. B.; Novak, J. Glycosylation Patterns of HIV-1 Gp120 Depend on the Type of Expressing Cells and Affect Antibody Recognition. *J. Biol. Chem.* **2010**, *285*, 20860−20869.
(46) Gallo, S. A.; Wang, W.; Rawat, S. S.; Jung, G.; Waring, A. J.; Cole, A. M.; Lu, H.; Yan, X.; Daly, N. L.; Craik, D. J.; Jiang, S.; Lehrer, R. I.; Blumenthal, R. θ-Defensins Prevent HIV-1 Env-Mediated Fusion by Binding Gp41 and Blocking 6-Helix Bundle Formation. *J. Biol. Chem.* **2006**, *281*, 18787−18792.
(47) Pritchard, L. K.; Vasiljevic, S.; Ozorowski, G.; Seabright, G. E.; Cupo, A.; Ringe, R.; Kim, H. J.; Sanders, R. W.; Doores, K. J.; Burton, D. R.; Wilson, I. A.; Ward, A. B.; Moore, J. P.; Crispin, M. Structural Constraints Determine the Glycosylation of HIV-1 Envelope Trimers. *Cell Rep.* **2015**, *11*, 1604−1613.
(48) Li, J.; Chen, X.; Huang, J.; Jiang, S.; Chen, Y. H. Identification of Critical Antibody-Binding Sites in the HIV-1 Gp41 Six-Helix Bundle Core as Potential Targets for HIV-1 Fusion Inhibitors. *Immunobiology* **2009**, *214*, 51−60.
(49) Neurath, A. R.; Debnath, A. K.; Strick, N.; Li, Y. -Y.; Lin, K.; Jiang, S. Blocking of CD4 Cell Receptors for the Human Immunodeficiency Virus Type 1 (HIV-1) by Chemically Modified Bovine Milk Proteins: Potential for AIDS Prophylaxis. *J. Mol. Recognit.* **1995**, *8*, 304−316.
(50) Jiang, S.; Lin, K.; Lu, M. A Conformation-Specific Monoclonal Antibody Reacting with Fusion-Active Gp41 from the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein. *J. Virol.* **1998**, *72*, 10213−10217.
(51) Neurath, A. R.; Jiang, S.; Strick, N.; Lin, K.; Li, Y. Y.; Debnath, A. K. Bovine β-Lactoglobulin Modified by 3-Hydroxyphthalic Anhydride Blocks the CD4 Cell Receptor for HIV. *Nat. Med.* **1996**, *2*, 230−234.
(52) Zeng, B.; Chai, J.; Deng, Z.; Ye, T.; Chen, W.; Li, D.; Chen, X.; Chen, M.; Xu, X. Functional Characterization of a Novel Lipopolysaccharide-Binding Antimicrobial and Anti-Inflammatory Peptide in Vitro and in Vivo. *J. Med. Chem.* **2018**, *61*, 10709−10723.
(53) Zhang, W.; Tian, G.; Geng, X.; Zhao, Y.; Ng, T. B.; Zhao, L.; Wang, H. Isolation and Characterization of a Novel Lectin from the Edible Mushroom Stropharia Rugosoannulata. *Molecules* **2014**, *19*, 19880−19891.
(54) Zhang, X.; Chen, J.; Yu, F.; Wang, C.; Ren, R.; Wang, Q.; Tan, S.; Jiang, S.; Liu, S.; Li, L. 3-Hydroxyphthalic Anhydride- Modified Rabbit Anti-PAP IgG as a Potential Bifunctional HIV-1 Entry Inhibitor. *Front. Microbiol.* **2018**, *9*, No. 1330.
(55) Su, Y.; Chong, H.; Xiong, S.; Qiao, Y.; Qiu, Z.; He, Y. Genetic Pathway of HIV-1 Resistance to Novel Fusion Inhibitors Targeting the Gp41 Pocket. *J. Virol.* **2015**, *89*, 12467−12479.
(56) Su, S.; Zhu, Y.; Ye, S.; Qi, Q.; Xia, S.; Ma, Z.; Yu, F.; Wang, Q.; Zhang, R.; Jiang, S.; Lu, L. Creating an Artificial Tail Anchor as a Novel Strategy To Enhance the Potency of Peptide-Based HIV Fusion Inhibitors. *J. Virol.* **2017**, *91*, No. e01445-16.

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