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Design of histidine-rich peptides with enhanced bioavailability and inhibitory activity against hepatitis C virus

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ABSTRACT

Recently, peptide drugs have evolved into mainstream therapeutics, representing a significant portion of the pharmaceutical market. However, their bioavailability remains to be improved compared with that of chemical drugs. Here, we screened and identified a new peptide, Ctry2459, from a scorpion venom peptide library that was proven to inhibit hepatitis C virus (HCV) infection via inactivating infectious viral particles. However, Ctry2459 cannot suppress established infection of HCV because of the poor cellular uptake and restriction of endosomes. Based on the molecular template of the Ctry2459 peptide, we designed two histidine-rich peptides (Ctry2459-H2 and Ctry2459-H3) with significantly enhanced cellular uptake and improved intracellular distribution. Moreover, the two mutated peptides, as well as the wild-type peptide Ctry2459, exhibited virucidal activities against HCV. In distinct contrast to the Ctry2459 peptide, the mutated peptides significantly suppressed the established HCV infection at the cellular level but demonstrated lower cytotoxic and hemolytic activities. Our work presents an effective design strategy for optimizing natural antiviral peptides and opens a new avenue for enhancing the bioavailability of peptide drugs.

1. Introduction

Recently, peptide drugs have evolved into mainstream therapeutics, representing a significant portion of the pharmaceutical market. Peptide drugs are advantageous because they are amenable to rational design and exhibit highly diverse structures and broad biological activities. Owing to this superiority, peptide drugs play significant roles as both therapeutics and biomarkers [1–3]. However, many obstacles must be overcome before peptide drugs can be applied clinically. Peptide drugs have short half-lives in systemic circulation, are easily degraded by proteases in plasma and target cells, are often easily cleared by the reticuloendothelial system and can be immunogenic [4]. New synthetic strategies for limiting metabolism and alternative routes of administration have emerged recently and have resulted in numerous peptide-based drugs that are now being marketed [5]. Nevertheless, new strategies are still urgently needed to solve problems such as nonspecific targeting, poor uptake, low effective biological activity and bioavailability.

As a family of peptide drugs, antiviral peptides have attracted much attention, and some of these molecules have displayed effective bioactivities. A series of synthetic antiviral peptides have been reported. For example, T-20, a 36-mer peptide derived from the HIV-1 transmembrane glycoprotein (gp41), showed potent inhibition of HIV-1 membrane fusion and virus entry and is now in clinical use in HIV-1-infected patients [6]. Despite the therapeutic potency of T-20, resistant strains have emerged. To achieve fusion inhibition without this setback, a similar peptide (T-1249) was designed and synthesized. The peptide T-1249 was more effective than T-20 even with a single daily administration instead of the two administrations used for T-20 and retained activity against most T-20-resistant strains [7,8]. Another synthetically derived peptide, C5A, prevented HCV infection by inactivating both extra- and intracellular infectious particles. As a potent anti-HCV peptide, C5A destabilizes the viral membrane based on its lipid composition, offering a unique therapeutic approach to HCV and other types of viral infection [9]. However, improving C5A for clinical use would be challenging. These antiviral peptides still face problems such as drug resistance and bioavailability.

Natural antimicrobial peptides (AMPs) provide plentiful raw materials for developing peptide drugs. AMPs are key effectors of the innate immune response of animals to combat microbial challenge [10,11]. Thus far, approximately 1000 eukaryotic AMPs have been isolated; these molecules are expected to be a rich source of antibiotics [12,13]. These small peptides are multifunctional as

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effectors of innate immunity on the skin and mucosal surfaces and have demonstrated direct antimicrobial activity against various bacteria, viruses, fungi, and parasites [14]. Natural AMPs from scorpion venoms have attracted much attention due to their antiviral bioactivities. The mutational peptide mucroporin-M1 was shown to be virucidal against the measles, SARS–CoV and influenza H5N1 viruses [15], and inhibited HBV replication *in vitro* and *in vivo* [16]. A natural α-helical peptide, Hp1090, was proven to have the property of killing HCV [17]. Another mutational peptide, Kn2-7, was also effective in inhibiting HIV-1 infection [18]. These studies indicated that scorpion venom is a rich source of antiviral peptides.

Here, we present a new scorpion peptide, Ctry2459, that efficiently inhibits initial HCV infection by inactivating the viral particles. However, Ctry2459 cannot suppress an established infection because of its low bioavailability, resulting from the poor cellular uptake and restriction of intracellular endosomes. Since the addition of histidine residues can enhance the helicity, amphiphilicity and endosomal escape of peptides, we try to design histidine-rich peptides from the template of Ctry2459 to improve antiviral activities, cellular uptake and intracellular distribution. The helicity and amphiphilicity of peptides were characterized by circular dichroism (CD). The antiviral activities were studied using real-time RT-QPCR. Confocal microscopy assay was used to investigate the bioavailability. The potential biotoxicity of peptides was characterized by measuring their cytotoxic effect on various cell lines and hemolytic effect on human red blood cells. The overall objective of this study is to enhance the bioavailability and bioactivity of peptide drugs by overcoming cellular barrier.

2. Materials and methods

2.1. cDNA library construction and sequence analysis

Chaerilus tryznai and *Chaerilus tricostatus* scorpions were collected in the Xizang Province of China. Their glands were collected 2 days after the electrical extraction of their venom. Total RNA was prepared from the glands using TRIzol reagent (Invitrogen). Poly(A) mRNA was purified with a Poly(A) Tract mRNA isolation system (Promega). The cDNA libraries were constructed with the Superscript plasmid system cDNA library construction kit (Gibco/BRL). cDNAs were cloned into the pSPORT1 plasmid (Gibco/BRL) and transformed into *Escherichia coli* DH5α cells. Randomly chosen cDNA clones were sequenced to obtain a reliable representation of the toxin content in the venom gland. Sequence analysis was performed with the BLASTX program, and peptides were classified.

2.2. Peptide synthesis and purification

Peptides were synthesized using the solid-phase synthesis method and amidated at the C-terminus with a purity of >95% (ChinaPeptides Co., Ltd., China). The synthetic peptides were assessed by HPLC (Elite-HPLC) and mass spectrometry (Voyager-DESTR; Applied Biosystems).

2.3. Circular dichroism analysis

The secondary structure was measured by circular dichroism (CD) spectroscopy. Measurements were performed in the UV range of 250–190 nm at 25 °C in water and 50% TFE using a Jasco-810 spectropolarimeter, at a concentration of 0.1 mg/ml. Spectra were collected from three separate recordings and averaged after subtracting the blank spectrum of pure water.

2.4. Cell culture and virus

Huh7.5.1 and L02 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin, and 1% streptomycin at 37 °C in a 5% CO_2 incubator. HCV (JFH-1) was prepared as described previously [19]. Briefly, the viral stocks were diluted in complete DMEM and used to inoculate naive 50% confluent Huh7.5.1 cells at a multiplicity of infection (moi) of 0.1 in a T25 flask (NEST Biotechnology Co. Ltd. China). Infected cells were trypsinized and replated in a T75 flask before confluence on day 2–3 postinfection (p.i.). Stock virus was made by collecting and filtering the supernatant, and the viral titer was measured by real-time RT-PCR according to the manufacturer's instructions (KHB, China). Aliquots were stored at −80 °C prior to use.

2.5. In vitro HCV RNA analysis

Huh7.5.1 cells were seeded in six-well plates with 5×10^5 cells per well. The next day, cells were infected with HCV (moi 0.1) and incubated for 4 h. Cells were washed three times and replenished with growth medium. After 48 h, cells were harvested for detection. Total RNA was extracted using TRIzol reagent and was transcribed into cDNA using the First-Strand Synthesis Supermix (Invitrogen, CA, USA). Real-time PCR was performed using the SYBR Green PCR assay and an ABI 7500 system according to the manufacturer's instructions. The HCV and GAPDH primer sets used for mRNA detection are summarized in Table 1.

2.6. Western blot analysis

Cells were lysed in a radioimmunoprecipitation assay buffer (50 mM Tris–HCl [pH 7.5], 150 mM sodium chloride, 1% Nonidet P40, and 0.5% sodium deoxycholate) with phenylmethylsulfonyl fluoride. The concentration of total protein was determined using a BCA protein quantification kit. Equal amounts of protein were electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel, which was then transferred onto a nitrocellulose membrane. The membrane was blocked by incubation with 5% skim milk. The primary antibodies used were as follows: mouse monoclonal anti-ApoE (E6D7) (Calbiochem, GER), goat polyclonal anti-HCV E2 (Abcam, Cambridge, UK), mouse monoclonal anti-Hep C cAg (C7-50) and mouse polyclonal anti-β-actin (Santa Cruz Biotechnology, CA, USA).

2.7. HCV inactivating assay

Infected Huh7.5.1 cells were grown in T75 cell culture flasks. For viral protein analysis, a large quantity of cell culture medium was prepared and cleared by differential centrifugation and then passed through a 0.22-μm filter unit. The HCV particles in the medium were concentrated by ultracentrifugation at 40,000 rpm at 4 °C for 2 h in a Beckman SW41 rotor. The obtained HCV pellets were dissolved in PBS and were treated with the peptides at a final concentration of 20 μg/ml. The mixtures were then incubated at 37 °C for 2 h, at which time they were separated by ultracentrifugation. The supernatant was collected and lyophilized. Proteins of HCV particles from both the separated HCV precipitate and supernatant component were measured by western blot analysis. For viral RNA analysis, the peptides were diluted in complete growth medium at a final concentration of 20 μg/ml and incubated with virus (moi 0.1) at 37 °C for 2 h, at which point the virus–peptide mixture was analyzed for HCV RNA content and infectivity compared with the control. The HCV RNA content was measured by real-time RT-QPCR, and the viral infectivity was determined by infecting Huh7.5.1 cells and then measuring 2 days later.

2.8. Confocal microscopy

N-terminus FITC-labeled peptide was added to the cells at a final concentration of 20 μg/ml, and then was incubated at 37 °C. After incubating for the indicated time, cells were washed with PBS, fixed with 4% paraformaldehyde, and washed twice. Cell nuclei were stained with DAPI (diluted 1:500 in PBS). Cells were washed three times with PBS. The cellular localization of the peptide was analyzed by confocal microscopy. For the Ca^{2+}, CQ and bafilomycin A1 supplementation studies, the listed chemicals were added to the medium together with the labeled peptide and then analyzed as above.

2.9. MTT assay

Cells were seeded in a 96-well plate (7000–10000 cells per well) and cultured at 37 °C for 24 h. A series of concentrations of peptides were added into the medium, and the plate was incubated for at 37 °C for 48 h, at which time 20 μl of MTT solution (5 mg/ml in PBS buffer; Invitrogen) was added to each well, and the plate was incubated at 37 °C for 4 h. The medium was removed, 100 μl DMSO was added, and then the plate was shaken for 20 min at room temperature to completely dissolve the crystal purple formazan. The absorbance was measured at 570 nm.

2.10. Hemolysis

Freshly obtained human red blood cells were washed three times with HEPES buffer (pH 7.2) by centrifugation for 10 min at 1200 × g. The cells were then resuspended in 0.9% saline and seeded in a 96-well plate with 10^7–10^8 cells per well. A series of concentrations of peptides were added and incubated at 37 °C for 1 h. A

Table 1
Primers used in real-time RT-PCR.

Gene name	Direction	Sequence (5′–3′)
HCV (JFH-1)	Sense	TCGTATGATACCCGATGCT
	Antisense	GTTTGACCCTTGCTGTTGA
GAPDH	Sense	TGATGACATCAAGAAGGTGGTGAAG
	Antisense	TCCTTGGAGGCCATGTGGGCCAT