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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.

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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 tryznaei 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* DH5 α 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% CO₂ 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- μ m 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 μ g/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 μ g/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 μ g/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 Ca²⁺, 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 μ l 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 μ l 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 \times 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	TCATGACATCAAGAAGGTGGTGAAG
	Antisense	TCCTTGGAGGCCATGTGGCCAT

0.9% saline solution was used as a negative control, and 0.1% Triton X-100 was used as a positive control. The plate was centrifuged for 5 min at 1000 × g, and the absorbance of hemoglobin released in the supernatant was measured at 570 nm.

2.11. Statistical analysis

Data are expressed as the mean ± standard deviation from at least three separate experiments. Statistical significance was tested using a two-tailed Student's *t*-test; statistical significance was set at **P* < 0.05, and extreme significance was set at ***P* < 0.01.

3. Results

3.1. Screening of the anti-HCV peptides from the scorpion venom peptide library

The venom gland cDNA libraries of the scorpions *C. tricostatus* and *C. tryznai* were constructed and characterized by our group.

After sequence analysis, 13 peptides were classified as antimicrobial peptides, whose mature peptides mostly include 9 to 20 amino acid residues (Fig. 1A). The 13 mature peptides were chemically synthesized and screened for the ability of inhibiting HCV (JFH-1) infection in Huh7.5.1 cells (Fig. 1B) by RNA analysis. The peptide from the venom of scorpion *C. tryznai*, termed Ctry2459, was shown to inhibit HCV infection by >90% and was studied further.

3.2. Extracellular anti-HCV activity and viral inactivation of Ctry2459

The cytotoxicity of Ctry2459 on Huh7.5.1 cells was tested using the MTT assay. The concentration of Ctry2459 that inhibited 50% of cell growth (CC₅₀) was 79.8 μg/ml. At the concentration of 20 μg/ml, the viability of the peptide-treated cells was greater

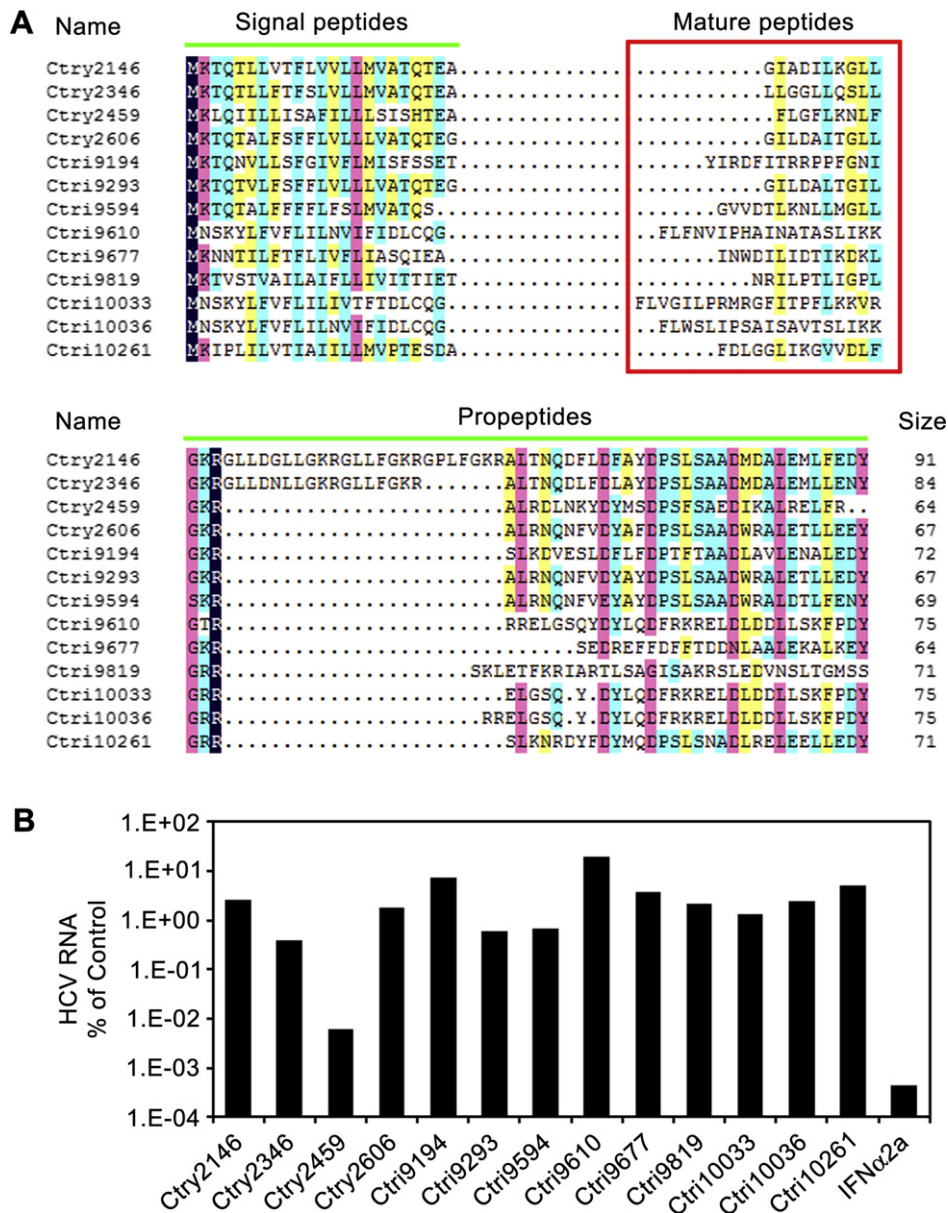


Fig. 1. Screen of anti-HCV peptides from the scorpion venom peptide library. (A) Thirteen antimicrobial peptides were identified, and the mature peptides are indicated by red boxes. (B) The 13 peptides (20 μg/ml) were screened for the ability to inhibit HCV infection (JFH-1) in Huh7.5.1 cells. HCV RNA levels were calculated as a percentage (%) of the negative control. IFNα2a (100 IU/ml) was used as a positive control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

than 95%, indicating that 20 $\mu\text{g/ml}$ or less was minimally cytotoxic to cells. The inhibitory activity against HCV infection was time-dependent at this concentration (Fig. 2A). When treated for 2 h, the inhibitory effect was the most effective. Under this condition, the inhibition of HCV infection was dose-dependent (Fig. 2B and C) and the 50% effective (EC_{50}) concentration was 1.84 $\mu\text{g/ml}$.

To investigate whether Ctry2459 inactivates HCV particles, viral supernatant was pretreated with Ctry2459 for 2 h, and then the HCV RNA content and infectivity were measured. As shown in Fig. 2E, the peptide reduced the HCV infectivity by 90% compared with the control, while the RNA content was hardly reduced. Moreover, a sedimentation method was used to determine the impact of Ctry2459 on the structural integrity of HCV particles. Briefly, concentrated HCV particles were incubated with or without peptide for 2 h at 37 °C and then were separated by ultracentrifugation. The viral structural proteins of the supernatant and sediment were determined by western blotting. As shown in Fig. 2D, the high-density sediment without treatment of Ctry2459 contained high levels of HCV E2, ApoE and HCV core proteins, whereas the supernatant contained only a small amount of these proteins. When treated with Ctry2459, the levels of HCV E2 and ApoE protein significantly increased in the supernatant and were reduced in the sediment. However, the HCV core protein content was hardly changed in the supernatant or the sediment. These data reveal that the peptide Ctry2459 can disrupt the membrane integrity of HCV particles; however, the viral capsid with core protein and RNA was hardly affected.

3.3. Time-dependent anti-HCV activities and cellular localization of Ctry2459

To determine the action time of Ctry2459, 20 $\mu\text{g/ml}$ of peptide was added to the virus or cells at different times relative to incubation. As shown in Fig. 3A and B, Ctry2459 displayed strong inhibitory activity when added to the virus before infection. By contrast, Ctry2459 was much less inhibitory when added to the cells together with the virus and entirely noninhibitory when added to the cells for 2 h and removed before the virus was added, as well as noninhibitory when added to the cells 4 h after infection. These results suggest that the peptide Ctry2459 only prevented initiation of HCV infection and had no intracellular anti-HCV activity. We speculated that there were some barriers in the infected cells that restricted the action of the peptide Ctry2459. To identify the exact barriers, the cellular localization of Ctry2459 was determined by confocal microscopy. As shown in Fig. 3C, labeled Ctry2459 peptide slightly entered the cells and accumulated in granular structures in the cytoplasm. These results suggest that the poor cellular uptake and aggregation of Ctry2459 in cytoplasmic endosomes restricted and silenced its intracellular antiviral activity.

3.4. Effect of lysosomotropic agents on the cellular uptake, intracellular distribution and anti-HCV activity of Ctry2459

To determine whether the granular structures of the labeled Ctry2459 peptide in the cytoplasm was generated by endosomes, lysosomotropic agents such as chloroquine and Ca^{2+} were added

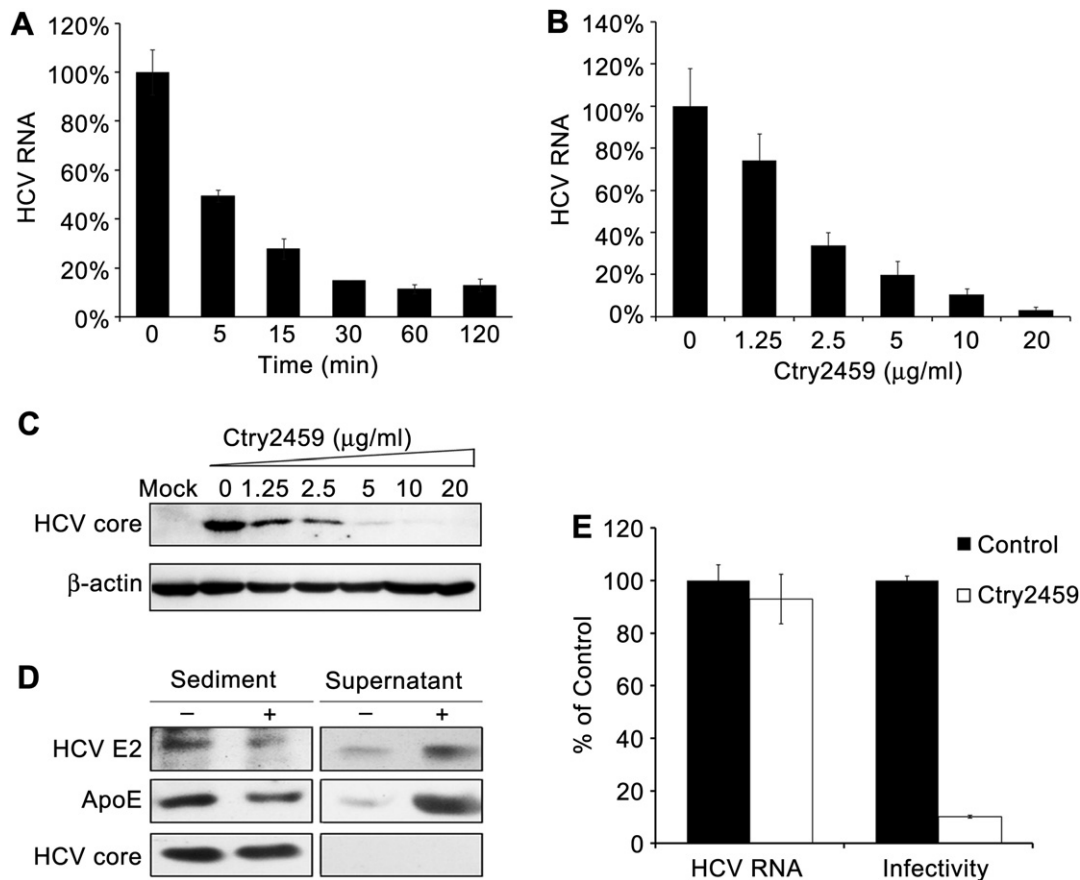


Fig. 2. Extracellular anti-HCV activity and viral inactivation of Ctry2459. (A, B, C) HCV RNA and core protein content in infected Huh7.5.1 cells decreased both time- and dose-dependently after treatment with indicated concentrations of the Ctry2459 peptide. (D, E) ApoE, HCV E2, HCV core protein, HCV RNA content and viral infectivity were determined by the virucidal assay.

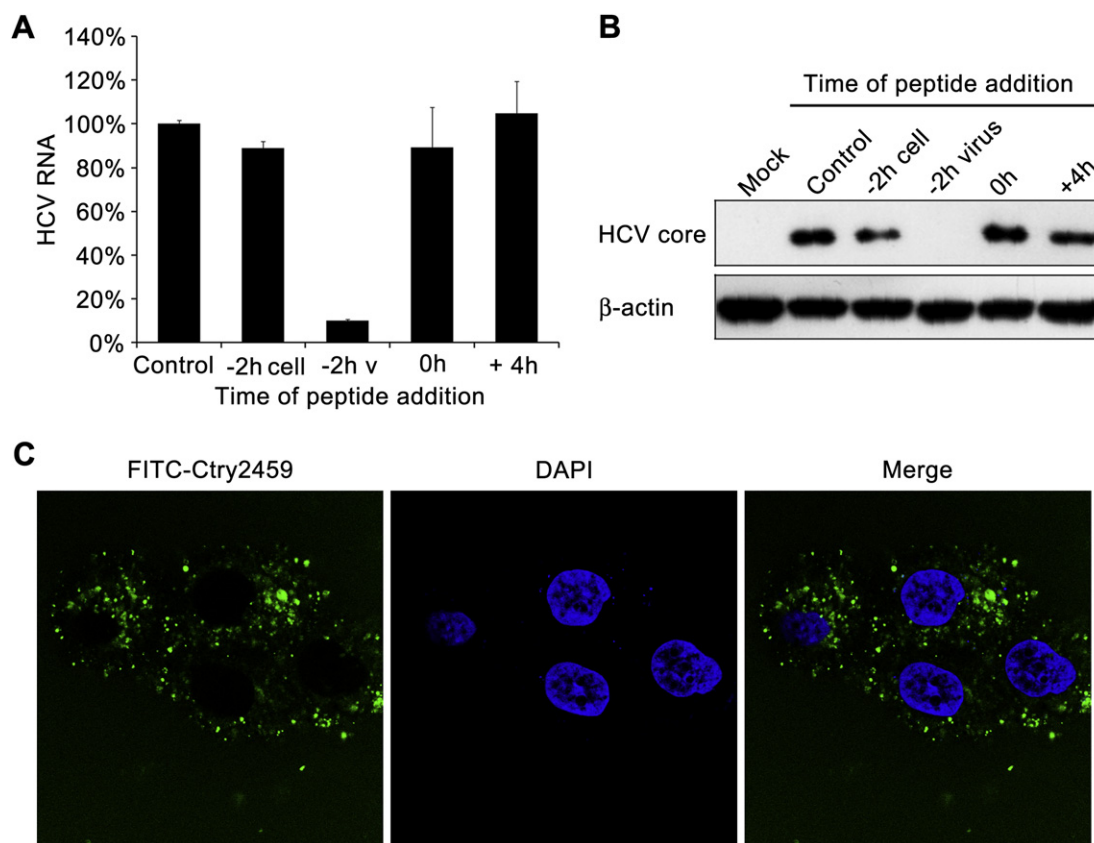


Fig. 3. Time-dependent anti-HCV activities and cellular localization of Ctry2459. (A, B) The Ctry2459 peptide was added at a final concentration of 20 μ g/ml under the following conditions: (i) preinoculation of cells: peptide was added to cells for 2 h at 37 $^{\circ}$ C followed by washing three times with growth medium before virus infection; (ii) preinoculation of virus: the peptide was added to the virus for 2 h at 37 $^{\circ}$ C, and then the mixture was used to infect cells for 4 h at 37 $^{\circ}$ C. The peptide–virus mixture was then removed, and then the cells were washed three times and replenished with growth medium without peptide. (iii) coinoculation: peptide was added to cells together with the virus for 4 h, at which time the cells were washed three times and replenished with growth medium without peptide; (iv) postinoculation: cells were infected for 4 h, at which point the virus was removed, and then the cells were washed as above and replenished with growth medium containing peptide throughout the experiment. After 48 h postinfection, HCV RNA and core protein levels were measured by real-time PCR and western blotting. (C) The cellular localization of the Ctry2459 peptide was determined by confocal microscopy after 48 h incubation.

with FITC-Ctry2459 before analysis by confocal microscopy. The cellular uptake of the Ctry2459 peptide was significantly promoted by treatment with 50 μ M chloroquine or 3 mM Ca^{2+} (Fig. 4A). The intracellular distribution of Ctry2459 was improved by dispersing the granular structures, causing the peptide to be equally distributed in the cytoplasm. These data clearly demonstrate that lysosomotropic agents can dramatically enhance the cellular uptake and improve the intracellular distribution of the peptide Ctry2459.

To further determine the intracellular anti-HCV effects of lysosomotropic agents, 50 μ M chloroquine or 3 mM Ca^{2+} was added with Ctry2459 after HCV infection. Ctry2459 showed no significant antiviral effect in HCV infection cells without chloroquine or Ca^{2+} (Fig. 4B). However, Ctry2459 produced a significant reduction (30% and 90%, respectively) in HCV RNA following treatment with chloroquine or Ca^{2+} compared with treatment with chloroquine or Ca^{2+} only. These results indicate that the intracellular anti-HCV activity of Ctry2459 is significantly promoted by activating the cellular uptake and endosomal escape pathways.

3.5. Design of histidine-rich peptides based on the molecular template of Ctry2459

The online prediction model showed that Ctry2459 adopts an α -helix structure. Circular dichroism (CD) spectroscopy revealed that Ctry2459 adopted a random coil structure in water but an

α -helix structure in 50% TFE (Fig. 5C). The helical wheel of Ctry2459 exhibited a strong amphipathic α -helix conformation with a hydrophobic face and a hydrophilic face (Fig. 5B). These results suggest that Ctry2459 is a good dipolar molecule that can form an amphipathic helical structure in the appropriate environment.

It has been reported that protonation of the imidazole groups of histidine residues plays an important role in endosomal escape, as the peptide LAH4 exhibits effective delivery functions [20]. Because of the similar secondary structure of the Ctry2459 and LAH4 peptides, we asked whether the addition of histidine can promote the endosomal escape of Ctry2459. Based on the design strategy of LAH4, two or three histidine residues were added, resulting in the derivation of the histidine-rich peptides Ctry2459-H2 (abbreviated as H2) and Ctry2459-H3 (abbreviated as H3), respectively (Fig. 5A). The secondary structures were predicted and determined to be the same as that of Ctry2459 (abbreviated as WT). As shown in Fig. 5B and C, the addition of histidine residues did not disrupt the stabilization of secondary structures but enhanced the amphipathic α -helix conformation.

3.6. Extracellular anti-HCV activities and viral inactivation of Ctry2459-H2 and Ctry2459-H3

The extracellular anti-HCV activities of Ctry2459-H2 and Ctry2459-H3 were determined to be the same as that of the

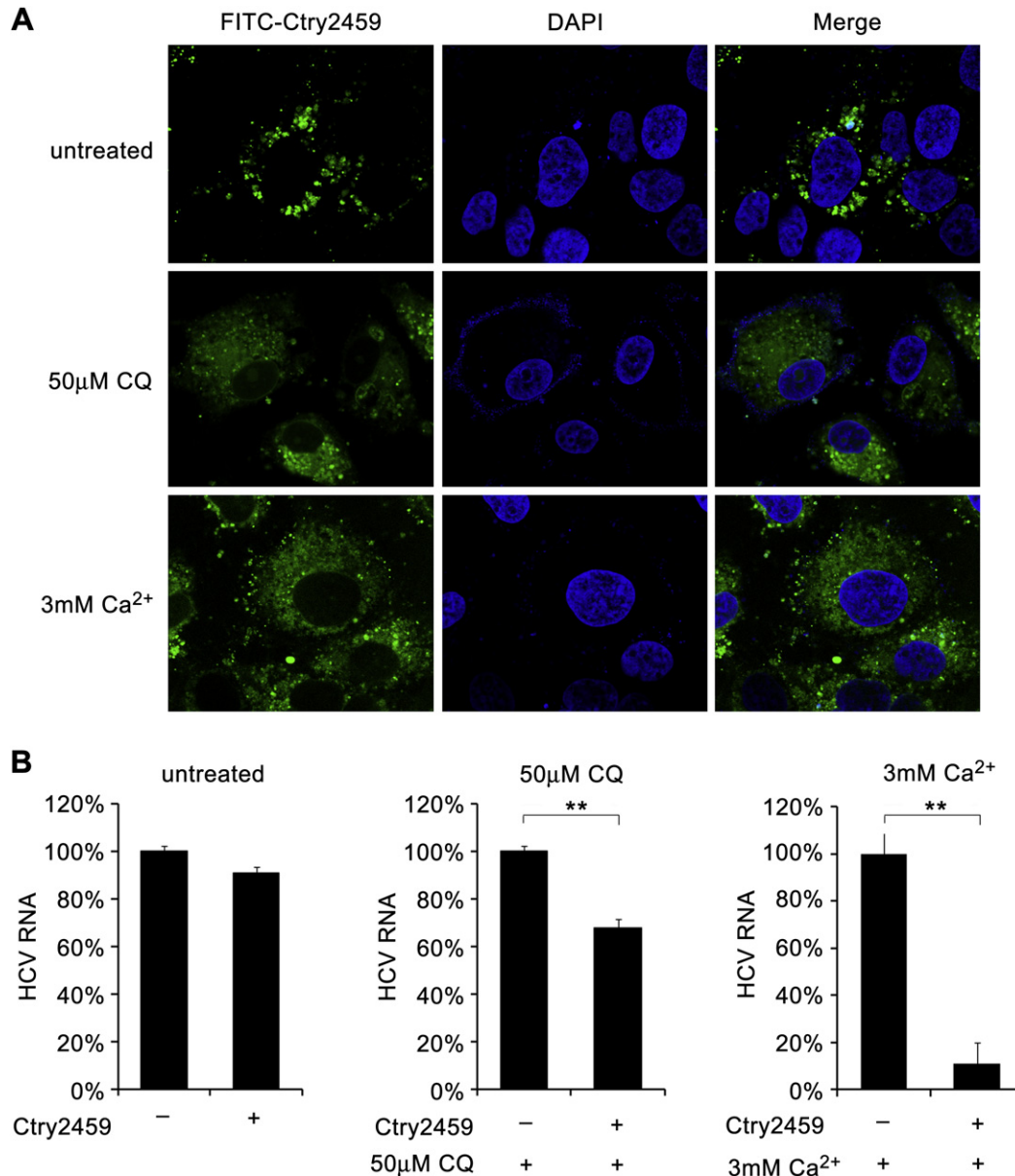


Fig. 4. Effect of lysosomotropic agents on the cellular localization and anti-HCV activities of Ctry2459. (A) Effect of chloroquine (50 μM) and Ca²⁺ (3 mM) on the cellular uptake and intracellular distribution of the Ctry2459 peptide by confocal microscopy after 48 h incubation. (B) Effect of chloroquine (50 μM) and Ca²⁺ (3 mM) on the anti-HCV activity of the Ctry2459 peptide by real-time RT-PCR analysis. ***P* < 0.01.

wild-type Ctry2459 peptide. Briefly, at the concentration of 20 μg/ml, the inhibitory activities of the two peptides against HCV infection were occurred in a time-dependent manner (Fig. 6A and B). The inhibitory activities of Ctry2459-H2 and Ctry2459-H3 against HCV infection both occurred in a dose-dependent manner (Fig. 6C and D). Additionally, the 50% effective (EC₅₀) concentrations were 1.08 μg/ml and 0.85 μg/ml, respectively. To determine whether Ctry2459-H2 and Ctry2459-H3 were virucidal for HCV, the viral supernatant was pretreated with peptide for 2 h, and the HCV RNA content and infectivity were measured. Unlike wild-type Ctry2459, Ctry2459-H2 and Ctry2459-H3 reduced the viral RNA by 40% and 70%, respectively, although the viral infectivities were both reduced by 90%, similar to that of the Ctry2459 peptide. These data suggest that the addition of histidine residues did not affect the mode of peptide action but significantly enhanced the disruption of viral integrity.

3.7. Intracellular anti-HCV activities and cellular localization of Ctry2459-H2 and Ctry2459-H3

To determine whether the internalization of Ctry2459-H2 and Ctry2459-H3 were promoted by the addition of histidine residues, FITC-labeled peptides were used and measured by confocal microscopy. As shown in Fig. 7A, Ctry2459-H2 and Ctry2459-H3 exhibited a higher amount of cellular uptake than wild-type Ctry2459. Specifically, Ctry2459-H2 and Ctry2459-H3 exhibited more dispersed distribution in the cytoplasm, indicating the property of endosomal escape.

Based on these improvements, the intracellular anti-HCV activities of Ctry2459-H2 and Ctry2459-H3 were determined. The three peptides were added 4 h, 24 h or 48 h postinfection. After incubation for another 48 h, the intracellular HCV RNA of samples was measured. As shown in Fig. 7B, all the three peptides exhibited

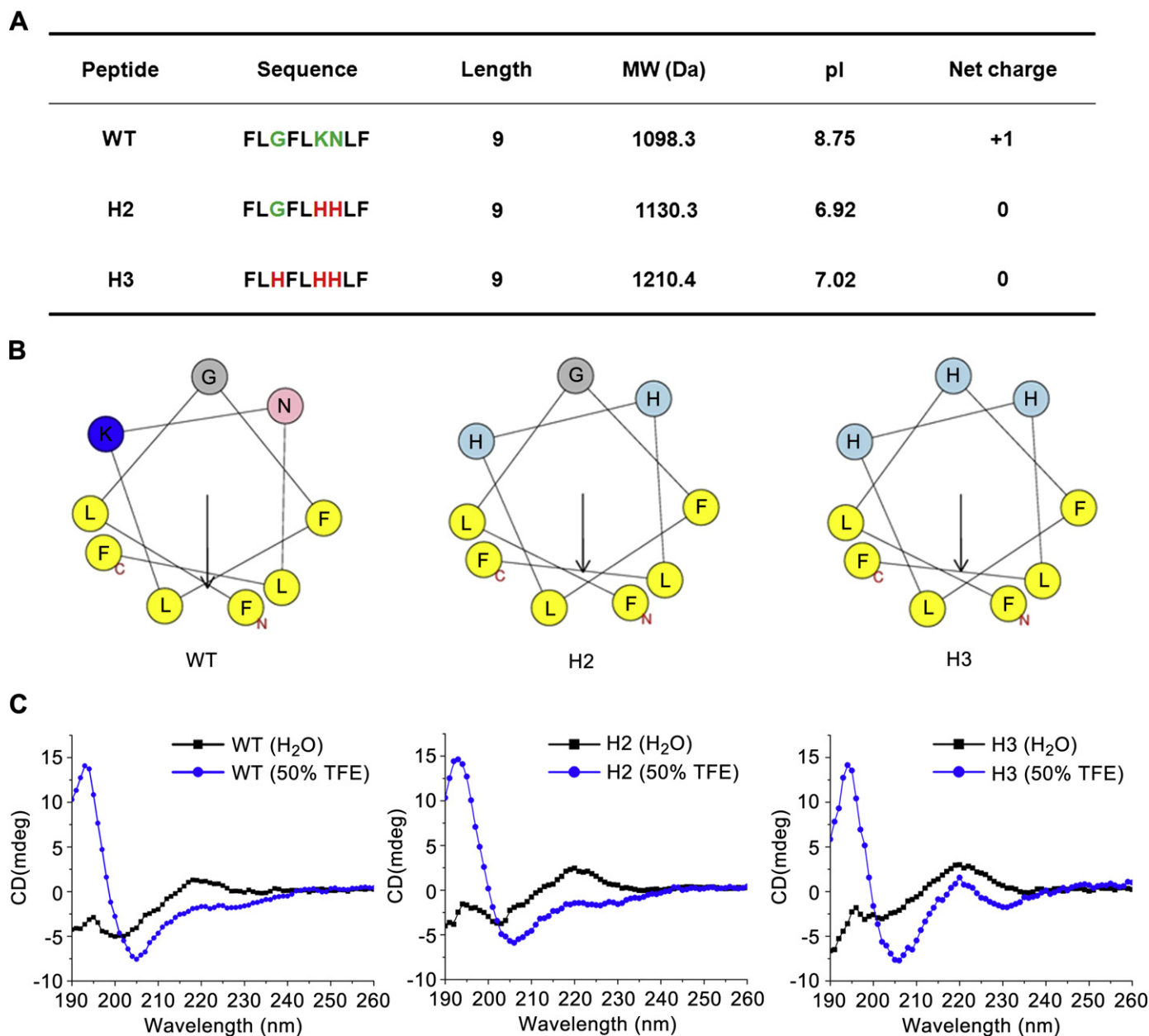


Fig. 5. Design of histidine-rich peptides based on the molecular template of Ctry2459. (A) Sequence alignments of the WT, H2 and H3 peptides. The mutation sites are shown in green, and the histidine residues are shown in red. (B) Helical wheels of the three peptides showed the hydrophilic and hydrophobic faces. (C) CD spectra of the three peptides. Measurements were performed in the UV range of 250–190 nm at 25 °C in water on a Jasco-810 spectropolarimeter. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a slightly inhibitory effect at the point of 4 h postinfection. However, Ctry2459-H2 and Ctry2459-H3 exhibited inhibitory activity when added 24 h postinfection and a stronger inhibitory effect (particularly Ctry2459-H3) when added 48 h postinfection. These results confirm that compared with the wild-type Ctry2459 peptide, Ctry2459-H2 and Ctry2459-H3 resulting in effective intracellular antiviral activity.

3.8. Inhibition of the acidification of endosomes

It has been shown that the acidification of endosomes can be significantly inhibited by H^+ -ATPase inhibitors such as bafilomycin A1. Protonation of the imidazole groups of histidine residues plays an important role in endosomal escape, as the peptide LAH4

exhibits effective delivery functions. However, inhibition of the acidification of endosomes significantly diminishes the delivery efficiency [20].

To confirm the endosomal escape pathway of Ctry2459-H2 and Ctry2459-H3, 150 nM bafilomycin A1 was added to Huh7.5.1 cells together with peptides followed by analysis by confocal microscopy. As shown in Fig. 8, the cellular uptake was slightly reduced, but the intracellular distribution was significantly affected in the presence of bafilomycin A1. Specifically, the peptides formed granular structures in the cytoplasm instead of being dispersed and equally distributed. These results demonstrated that the efficient action of such peptides requires acidification of the endosomes, further confirming the endosomal escape pathway of Ctry2459-H2 and Ctry2459-H3.

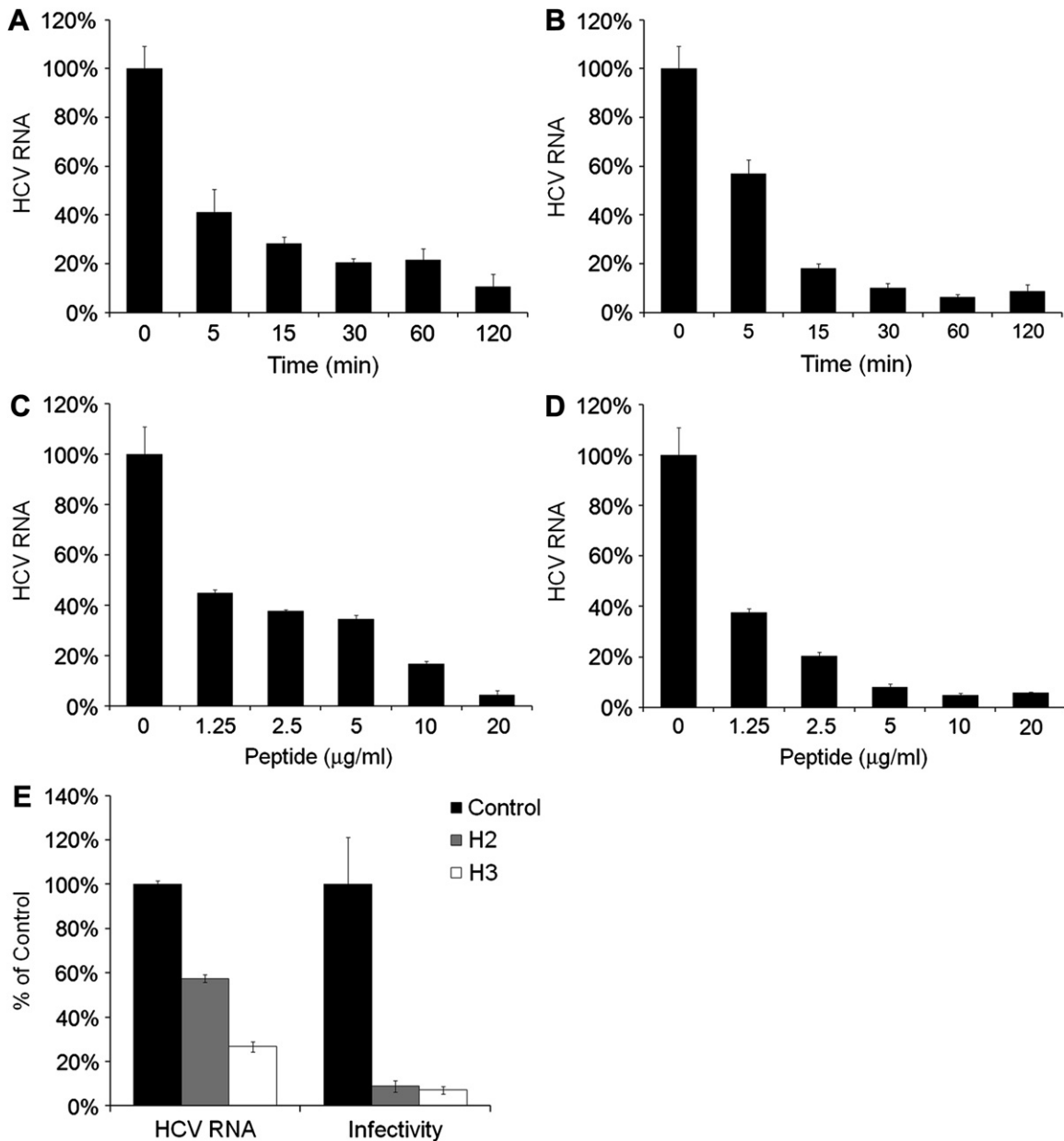


Fig. 6. Extracellular anti-HCV activities and viral inactivation of Ctry2459-H2 and Ctry2459-H3. The HCV RNA content in infected Huh7.5.1 cells decreased both time- and dose-dependently after treatment with the indicated concentrations of H2 (A, C) and H3 (B, D). (E) The HCV RNA content and viral infectivity were determined using a viral inactivating assay.

3.9. Cytotoxic and hemolytic activities of Ctry2459, Ctry2459-H2 and Ctry2459-H3

The cytotoxic activities of Ctry2459 and mutational peptides against Huh7.5.1 and L02 cells were tested using the MTT assay (Fig. 9A and B), and hemolysis activities were also tested (Fig. 9C). The 50% cytotoxicity concentration (CC_{50}) and the 50% hemolysis concentration (HC_{50}) were calculated. As shown in Table 2, the CC_{50} of Ctry2459-H2 and Ctry2459-H3 against Huh7.5.1 cells were both $>500 \mu\text{g/ml}$, which was more than 6-fold higher than the CC_{50} of Ctry2459. The HC_{50} of Ctry2459-H2 and Ctry2459-H3 were, respectively, $203.3 \mu\text{g/ml}$ and $416.4 \mu\text{g/ml}$, approximately 1.5-fold and 3-fold higher than the HC_{50} of the Ctry2459 peptide ($137.9 \mu\text{g/ml}$). These results indicated not only that the anti-HCV activities were improved by the addition of histidine residues but

also that the cytotoxic and hemolysis activities were significantly reduced. Although we do not understand the principle of toxicity improvement in detail, the designed histidine-rich peptides exhibited optimizing properties as peptide drugs, including bio-availability and toxicity (Fig. 10).

4. Discussion

In this study, we screened a new anti-HCV peptide from the peptide library of scorpion venom. The most potent peptide, Ctry2459, whose IC_{50} ($1.84 \mu\text{g/ml}$) is over 40-fold lower than the CC_{50} ($79.8 \mu\text{g/ml}$) observed in Huh7.5.1 cells (Table 2), was selected for further analysis. Ctry2459 displays an entirely noteworthy antiviral mechanism that potently destabilizes the viral structural integrity, thus reducing the initiation of HCV infection. However,

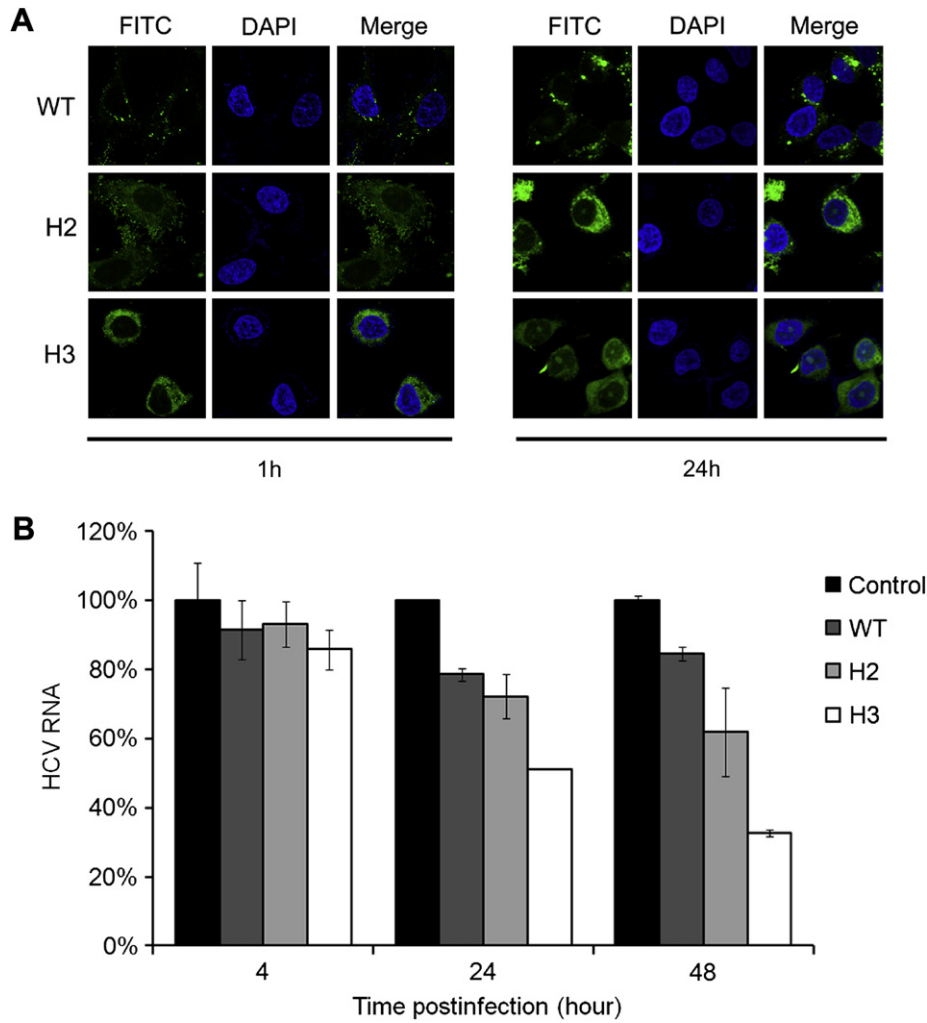


Fig. 7. Intracellular anti-HCV activities and cellular localization of Ctry2459-H2 and Ctry2459-H3. (A, B) The cellular localization of Ctry2459-H2 and Ctry2459-H3 was determined by confocal microscopy after incubation for 1 h and 24 h (C) Ctry2459-H2 or Ctry2459-H3 was added 4 h, 24 h and 48 h postinfection, and then were incubated for another 48 h. At the indicated times, the HCV RNA content was determined by real-time RT-PCR.

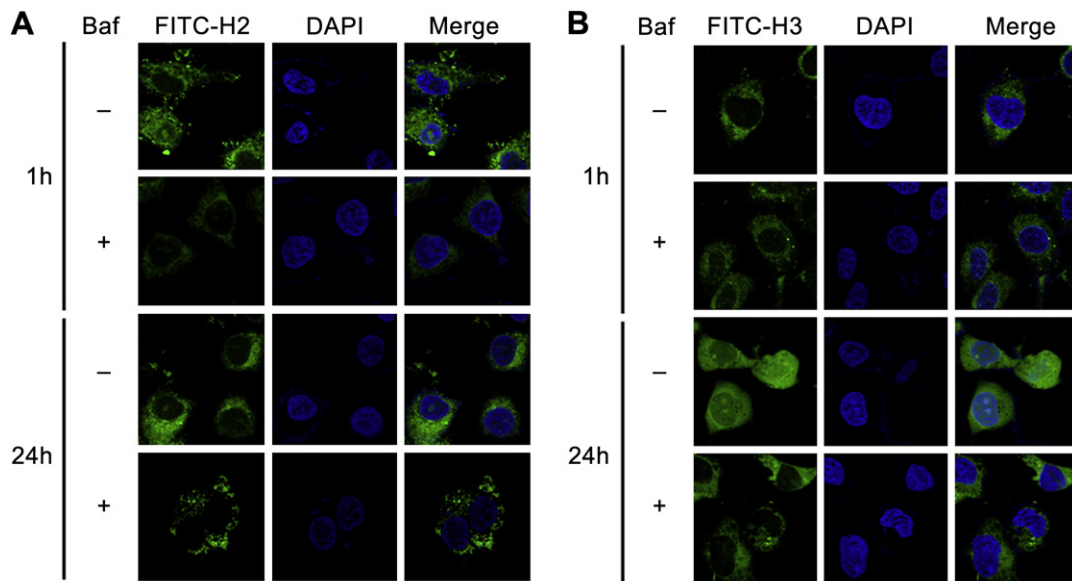


Fig. 8. Effect of H⁺-ATPase inhibition on the intracellular distribution of Ctry2459-H2 and Ctry2459-H3. Bafilomycin A1 (150 nM) was added to cells together with Ctry2459-H2 and Ctry2459-H3, and then the cells were incubated for 1 h and 24 h. At the indicated time, the cellular localization was determined by confocal microscopy.

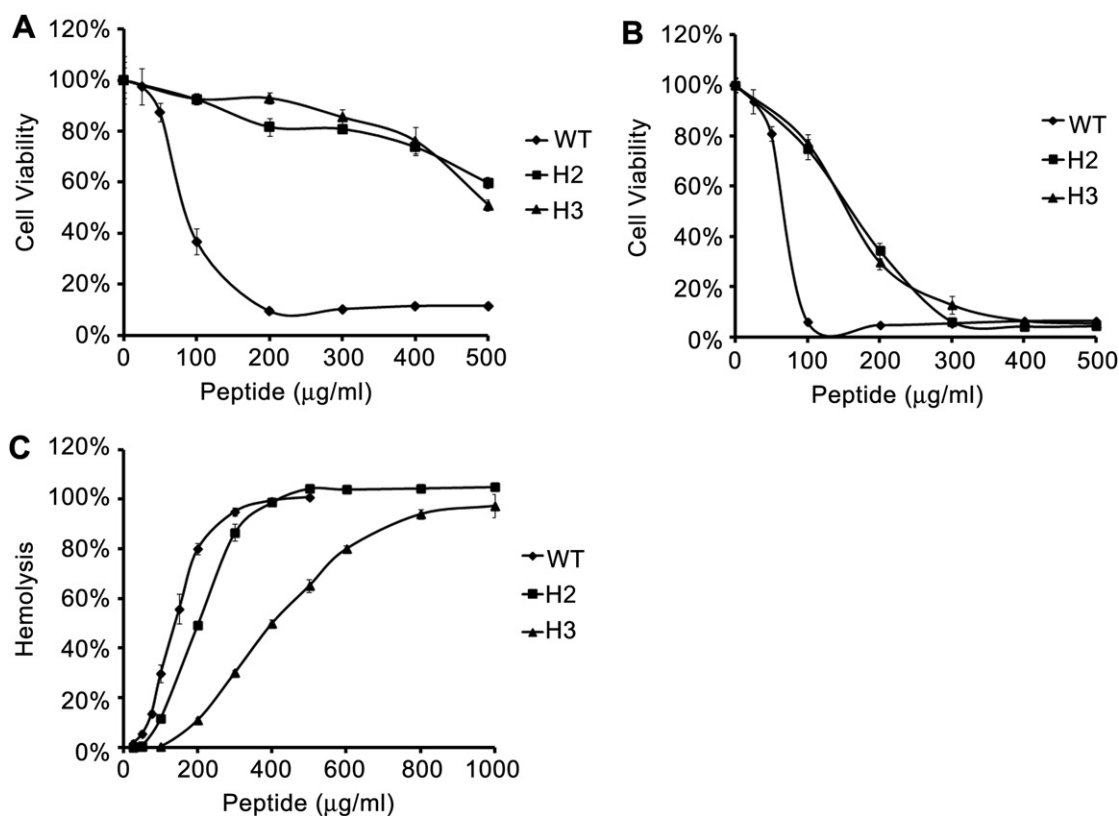


Fig. 9. Cytotoxic and hemolytic activities of Ctry2459, Ctry2459-H2 and Ctry2459-H3. Cytotoxic activities of Ctry2459, Ctry2459-H2 and Ctry2459-H3 against the Huh7.5.1 (A) and L02 (B) cell lines. The concentrations ranged from 0 to 500 μg/ml. (C) Hemolytic activity against human erythrocytes. The concentrations ranged from 0 to 1000 μg/ml.

Ctry2459 lacks suppression of acquired infection because of its poor cellular uptake and aggregation in cytoplasmic endosomes. Therefore, a potent peptide design strategy is needed for this challenge.

Recently, lysosomotropic agents such as chloroquine and Ca^{2+} have been used to enhance the transfection efficiency of peptide nucleic acid (PNA) peptide conjugates. Ca^{2+} could increase the rate of endocytosis or facilitate endosomal release [21]. Specifically, it increases the interaction between the conjugates and cellular membrane that may in turn increase internalization and induce endosomal escape [22]. Chloroquine is a well-known endosome-disrupting agent that can promote endosomal escape and enhance transfection efficacy [23]. In the present study, we also demonstrated that either 50 μM chloroquine or 3 mM Ca^{2+} can significantly enhance the cellular uptake and endosomal escape of Ctry2459, which promoted its intracellular antiviral activity. These results indicated that a large amount of peptide remains entrapped in endosomes, and the strategy to improve the low bioavailability is likely to involve the cellular uptake and endosomal escape pathways. Peptide drug administration

accompanied by the addition of lysosomotropic agents is not so effective and clinically practical.

During the past two decades, many peptides have been identified as having the property of causing the cellular import of cargo molecules [24]. These peptides are collectively called cell-penetrating peptides (CPPs), and they have attracted increasing attention. Sharing many similarities with antimicrobial peptides, they can interact with, disorder and cross cell membranes [25]. CPPs can trigger the endocytotic uptake of various biomolecules into the cell [26], such as the HIV-1 Tat-derived peptide [27], the influenza-virus hemagglutinin protein HA2, and elastin-like polypeptide (ELP) [28]. An artificially designed peptide, LAH4, which is rich in pH-responsive histidine residues, was shown to promote endosomal escape [29]. Histidine-rich peptides have been shown to exert a PEI-like proton sponge effect and mediate effective gene transfection. These activities have also been shown for another histidine-rich peptide, Endoportor [30].

According to this theory, we designed two histidine-rich peptide derivatives of Ctry2459 to improve the peptide bioavailability (Fig. 5). The designed peptides, Ctry2459-H2 and Ctry2459-H3, exhibited the same α -helix structure as the wild-type Ctry2459 peptide (Fig. 5), indicating that the addition of histidines did not affect the peptide structure but enhanced the helicity. Based on the structure consistency, Ctry2459-H2 and Ctry2459-H3 exhibited more effective virucidal activities *in vitro* than that of Ctry2459 by displaying stronger disruption of the viral integrity (Fig. 6). The cellular uptake of Ctry2459-H2 and Ctry2459-H3 significantly increased, and their intracellular distributions were completely different from the intracellular distribution of Ctry2459. In particular, Ctry2459-H2 and Ctry2459-H3 exhibited dispersed distribution in the cytoplasm, indicating the property of endosomal escape. When used as treatments for established HCV infection, Ctry2459-H2 and Ctry2459-H3

Table 2
Pharmacological profiles of Ctry2459 and its mutational peptides.

Peptide	EC ₅₀ (μg/ml) ^a	HC ₅₀ (μg/ml) ^b	CC ₅₀ (μg/ml) ^c	SI ^d
WT	1.84	137.9	79.8	43.4
H2	1.08	203.3	>500	>500
H3	0.85	416.4	>500	>500

^a The EC₅₀ (50% effective concentration) value of HCV (moi 0.1) was determined in Huh7.5.1 cells.

^b The HC₅₀ (50% hemolysis concentration) value was determined as described above.

^c The CC₅₀ (50% cytotoxic concentration) value of Huh7.5.1 cells was determined using the MTT assay.

^d The SI (select index) was calculated using the ratio CC₅₀/EC₅₀.

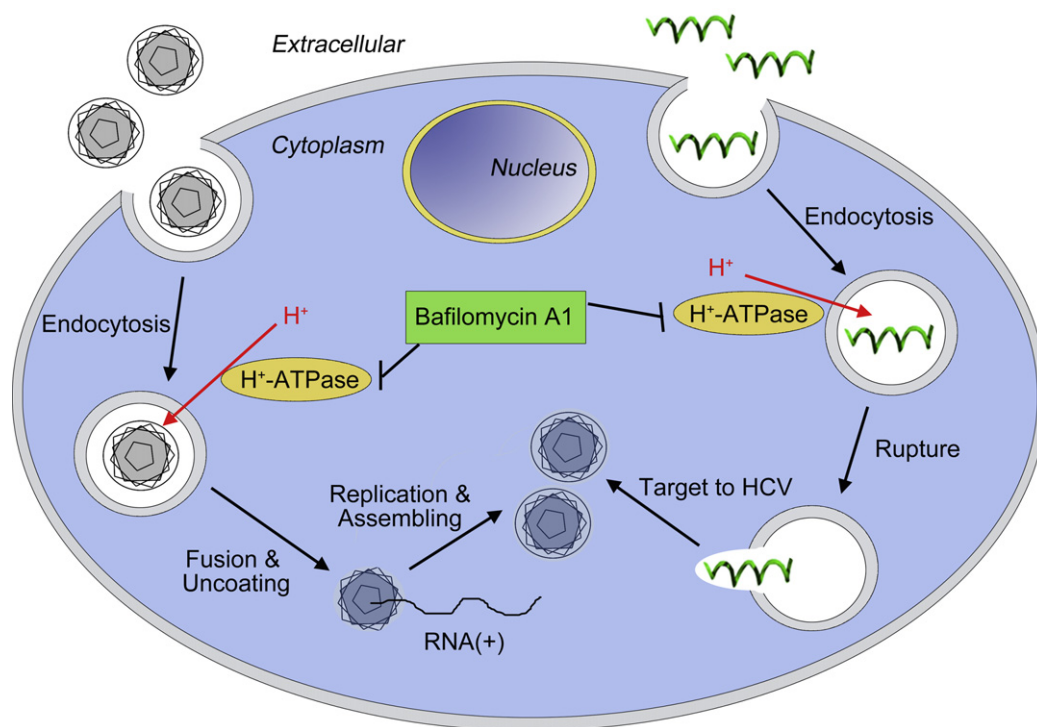


Fig. 10. Schematic diagram illustrating a mechanism of the histidine-rich peptide action via the cellular uptake and endosomal escape pathways. The peptides were taken up by the cells via endocytosis and were restricted in endosomes. With the acidification of endosomes by H^+ -ATPase, common peptides would be digested by the enzyme, whereas Ctry2459-H2 and Ctry2459-H3 with endosomal escape properties can easily break through the endosomal barrier. The released peptides in the cytoplasm would be targeted to intracellular HCV particles and act as an antiviral compound.

exhibited a slightly inhibitory effect when added 4 h postinfection, much less inhibition when added 24 h postinfection but stronger inhibition when added 48 h postinfection. The cause may be that at the early state of infection, there were very few mature viral particles in the cytoplasm so that the virucidal effect could not be significantly reflected. However, HCV replicated to high levels 24 h–48 h postinfection, and intracellular mature viral particles rapidly accumulated [31]. Therefore, the mutational peptides Ctry2459-H2 and Ctry2459-H3 effectively enter the cells, break through the endosomes, interact with the mature viral particles, and exhibit significant antiviral activities.

The mechanism of enhancement of cellular uptake most likely involves the addition of histidine residues, which enhance modification with stearic acid and increase fusion with the endosomal membrane because of the high membrane affinity of stearic acid [23]. The mechanisms of endosomal escape mostly include the interaction of the peptide with the endosomal membrane and the proton sponge effect: (i) Interaction with the endosomal membrane. The histidine residues are uncharged at neutral pH; however, when the pH of the endosomal lumen drops, the imidazole rings become protonated and can adopt an in-plane orientation at the membrane surface that induces membrane disorder. Hence, pH-induced changes in the peptide charge during endosomal acidification enhance the ability of the escape from the endosome and the ability to reach the cell cytosol and/or, eventually, the nucleus [25,32]. (ii) Proton sponge effect. Protonation induces an extensive inflow of ions and water into the endosomal environment that subsequently leads to osmotic swelling, endosome rupture and release of the entrapped components [33].

The histidine residue-mediated endosomal escape was dependent on the acidification of endosomes, which can be inhibited by H^+ -ATPase inhibitors such as bafilomycin A1 [20]. To determine the rationality of the action mode, 150 nM bafilomycin A1 was used in

the confocal analysis of Ctry2459-H2 and Ctry2459-H3. After inhibition of the acidification of endosomes, the peptides accumulated in granular structures (particularly Ctry2459-H2) in the cytoplasm, similar to Ctry2459, as shown in Fig. 8. This result suggests that Ctry2459-H2 and Ctry2459-H3 interact with the intracellular viral particles via the endosomal escape pathway.

Interestingly, Ctry2459-H2 and Ctry2459-H3 both exhibit significantly reduced cytotoxic activities in the carcinoma cell Huh7.5.1 (>6-fold) and an immortalized nonmalignant cell line L02. Moreover, the hemolytic activities were also reduced by 1.5-fold and 3-fold, respectively. This reduction was most likely related to the net charge of the peptides. At neutral pH, the net charge of peptide Ctry2459 is +1, whereas Ctry2459-H2 and Ctry2459-H3 are uncharged. The positive charge may enhance the interaction between the peptide and plasma membrane, causing a higher disruption of the plasma membrane. Thus, uncharged Ctry2459-H2 and Ctry2459-H3 exhibit lower cytotoxic and hemolytic activities. However, the specific mechanism of the reduced pharmacological toxicity is not fully understood. Further studies are needed to determine the mechanism, and more optimized peptides could be designed and tested on multiple viruses.

5. Conclusions

In summary, we screened the natural anti-HCV peptide Ctry2459 from the venom peptide library of the scorpion *C. tryznaei*. Ctry2459 inactivated HCV particles and reduced viral infectivity; however, Ctry2459 cannot suppress established infection. The addition of lysosomotropic agents such as chloroquine and Ca^{2+} significantly improves the cellular uptake and intracellular distribution of Ctry2459, thus promoting the antiviral activity against established HCV infection. Therefore, we designed two histidine-rich peptides to overcome the cellular uptake and endosome barriers. The designed

peptides, Ctry2459-H2 and Ctry2459-H3, exhibited effective cellular uptake and endosomal escape properties, thus significantly reducing the intracellular viral levels. They also displayed lower pharmacological toxicities than that of wild-type Ctry2459. Thus, in the present study, two histidine-rich peptides were designed to possess intracellular antiviral activities. This is the first report to show optimization of bioactive peptides via the cellular uptake and endosomal escape pathways. Our study defined a new optimizing strategy to enhance the bioavailability of peptide drugs.

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