A p7 Ion Channel-derived Peptide Inhibits Hepatitis C Virus Infection in Vitro*

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Viral infection is an early stage of its life cycle and represents a promising target for antiviral drug development. Here we designed and characterized three peptide inhibitors of hepatitis C virus (HCV) infection based on the structural features of the membrane-associated p7 polypeptide of HCV. The three peptides exhibited low toxicity and high stability while potently inhibiting initial HCV infection and suppressed established HCV infection at non-cytotoxic concentrations in vitro. The most efficient peptide (designated H2-3), which is derived from the H2 helical region of HCV p7 ion channel, inhibited HCV infection by inactivating both intracellular and extracellular viral particles. The H2-3 peptide inactivated free HCV with an EC50 (50% effective concentration) of 82.11 nM, which is >1000-fold lower than the CC50 (50% cytotoxic concentration) of Huh7.5.1 cells. H2-3 peptide also bound to cell membrane and protected host cells from viral infection. The peptide H2-3 did not alter the normal electrophysiological profile of the p7 ion channel or block viral release from Huh7.5.1 cells. Our work highlights a new anti-viral peptide design strategy based on ion channel, giving the possibility that ion channels are potential resources to generate antiviral peptides.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1, 2). More than 200 million people are infected with HCV worldwide (3). In recent decades the standard of care for patients with chronic hepatitis C depended on combination therapy with pegylated interferon (IFNα) and ribavirin (4, 5). An optimized therapy consists of the HCV NS3/4A protease inhibitors telaprevir and boceprevir (6–8). However, these therapies are unsatisfactory clinically because of widespread side effects, drug resistance, and low sustained virological responses. Furthermore, there is no effective vaccine against HCV infection. Therefore, the development of potential novel antiviral agents is urgently needed.

Viral entry is the first step of the viral life cycle and is a promising target for inhibiting viral infection (9). Short polypeptides derived from viral envelope sequences have been used to probe protein-protein interactions involved in viral entry (10), and peptides containing membrane-transiting motifs have the ability to inhibit virus entry to cells (11). For example, a peptide mimetic of an essential region of gp41, which is involved in fusion, has been successfully developed as an antiviral agent against HIV infection (12). Another peptide containing a portion of the HCV E2 transmembrane domain inhibits HCV pseudoparticle infection (10). The amphipathic α-helical peptide C5A, which was derived from the membrane anchor domain of the HCV NS5A protein, showed significant inhibitory effects against HCV, HIV, and HSV infection in vitro (13–15). Some of these peptides selectively and reversibly blocked viral infection without inactivating virions, whereas the effects of other peptides were not readily distinguishable from virus inactivation. Nevertheless, the strategy of developing antiviral peptides based on viral membrane proteins is promising.

The p7 is an HCV-encoded protein and plays an essential role in the assembly and release of infectious viral particles throughout the HCV life cycle (16–18). p7 contains a high proportion of hydrophobic residues and is defined as a membrane protein because it located in the endoplasmic reticulum membrane where it forms a homohexamer transmembrane ion channel (19–21). Previous studies have determined that each p7 monomer forms three highly hydrophobic membrane-associated α-helical segments (19, 22). Thus, referring to the peptide design strategy presented above, we sought to design and identify p7-derived peptides that may prevent HCV infection. In this study we analyzed the sequence and structural characteristics of the HCV p7 polypeptide, designed and screened peptides that inhibit HCV infection in a cell culture infection model, and characterized the functional antiviral mechanism of a typical peptide. Overall, our work opens a new avenue for screening anti-HCV peptides and uncovers potential anti-HCV agents.

**Background:** Membrane-associated viral proteins are potential resources to generate antiviral peptides.

**Results:** A 19-aa peptide, named H2-3, derived from HCV p7 ion channel, inhibits HCV infection.

**Conclusion:** H2-3 inhibits initial and established HCV infection by inactivating virions and protecting host cells from viral entry.

**Significance:** The discovery of H2-3 peptide supports the feasibility of developing novel antiviral peptides from ion channels.

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3 The abbreviations used are: HCV, hepatitis C virus; m.o.i., multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; aa, amino acids.
Experimental Procedures

**Peptides**—Peptides were synthesized using the solid-phase synthesis method and amidated at the C terminus (ChinaPeptides Co., Ltd., Hangzhou, China). The purity of the synthetic peptides was assessed by analytical high pressure liquid chromatography (HPLC), and the molecular weight was determined by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Voyager-DESTR; Applied Biosystems). All peptides were initially dissolved in dimethyl sulfoxide (DMSO; Sigma) at a storage concentration of 2 mM.

**Cell Culture**—Huh7.5.1 cells were kindly provided by Chisari and co-workers (23). Bel7402 cells, HeLa cells, and HEK293T cells were obtained from China Center for Type Culture Collection. The above three cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin in a humidified 5% CO2 incubator at 37 °C.

**Virus**—HCV genotype 2a strain JFH-1 was kindly provided by Takaji Wakita. The production of HCV cell culture virus was performed as previously described (24). In brief, Huh7.5.1 cells were infected with JFH-1 at a multiplicity of infection (m.o.i.) of between 0.1 and 5. HCV was propagated for 6 days before collection. Virus stock was obtained after filtering the cell supernatant. Viral titers were quantified using a commercial kit (VOLUME 290 • NUMBER 38)

**RNA Analysis**—Total intracellular RNA was isolated using TRIzol reagent (Invitrogen, CA) and was transcribed into cDNA using First-Strand Synthesis Supermix (Invitrogen). Real-time PCR was performed using the SYBR Green PCR assay and an ABI 7500 system according to the manufacturer’s instructions. The relative HCV RNA content in infected cells was determined after normalization to cellular GAPDH mRNA levels. The primer pair 5’-TCTGCGGATCCCATGAAAGTCGCA-3’ (sense) and 5’-TCAGGCAGTACCACAAGGC-3’ (antisense) was used to quantify the HCV RNA. The primer pair 5’-CAAGAAGGTTGAGTGAAC-3’ (sense) and 5’-AGGTGGAGGAGTGAGGTG-3’ (antisense) was used to quantify the GAPDH mRNA.

**MTT Assay**—Cells were seeded in a 96-well plate (7,000–10,000 cells per well) and cultured at 37 °C for 24 h. A series of concentrations of peptides was added to the media, and the plate was incubated 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 the plate was shaken for 20 min at room temperature to completely dissolve the crystal purple formazan. The absorbance was measured at 570 nm.

**Hemolysis**—Freshly obtained human red blood cells were washed 3 times with HEPES buffer (pH 7.2) by centrifugation for 10 min at 1200 × g. The cells were then suspended in 0.9% saline and seeded in a 96-well plate with 102–104 cells per well. A series of concentrations of peptides was added and incubated at 37 °C for 1 h. A 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.

**Serum Stability**—Peptides were incubated in DMEM medium containing 10% FBS at 37 °C for 1, 2, 4 and 24 h. At the indicated times, the pretreated peptides were added to infected Huh7.5.1 cells. The inhibitory effect of viral proliferation in cell culture was determined by RT-PCR 72 h post-infection. The serum stability of peptides was evaluated by the antiviral effect after incubation.

**Time-of-addition Assay**—Huh7.5.1 cells were seeded at 5 × 105 cells/well in 6-well plates. The next day virus inoculum was added to the plates and then was then incubated at 37 °C for 4 h. Peptides were added to the cells for 4 h and removed before inoculation (−4 h) together with virus at the time of inoculation (0 h) or 4 h post-inoculation after virus had been removed by washing (+4 h). The −4 h and 0-h cells were washed after 4 h of incubation and replaced with virus/peptide-free medium, whereas the peptide remained in the +4 h cultures throughout the experiment. Seventy-two hours post-infection the cells were lysed for HCV RNA quantitation, and the inhibitory rates were calculated.

**Viral Inactivation**—Viral supernatants were treated with peptides for the indicated times before inoculation. Four hours post-infection the cells were washed and replenished with culture medium. To determine viral infectivity, intracellular and supernatant HCV RNA contents were measured by real-time quantitative PCR 72 h post-infection. For direct virucidal evaluation, viral supernatants were diluted in PBS or serum-free medium and detected using the HCV RNA quantitative PCR diagnostic kit as described above.

**Confocal Microscopy**—An N-terminally FITC-labeled peptide was added to the cells at a final concentration of 10 μM and was then incubated at 37 °C. After incubating for the indicated times, the cells were washed with PBS, fixed with 4% paraformaldehyde, and washed twice. The cell nuclei were stained with DAPI (diluted 1:500 in PBS). The cells were washed three times with PBS. The cellular localization of the peptide was analyzed by confocal microscopy.

**Electrophysiological Analysis**—The construct pIRES2-eGFP-p7 was transfected into HEK293T cells using the FuGene Transfection Reagent (Roche Diagnostics) following the manufacturer’s instructions 24–48 h before electrophysiological experiments. Currents from HEK293T cells expressing the p7 channel were measured using the whole-cell patch clamp technique at room temperature. Current measurements and data acquisition were performed with an EPC 10 patch-clamp amplifier (HEKA Elektronik, Lambrecht (Pfalz), Germany)
Novel anti-HCV Peptide Derived from p7 Ion Channel

FIGURE 1. Design and identification of p7-derived peptides as inhibitors of HCV infection. A, structure of p7 (JFH-1 2a) monomer. Three helix domains are labeled in dotted line boxes: H1 (5–15), H2 (19–39), and H3 (48–57). B, inhibitory effects of p7-derived peptides on HCV infection. Peptides (10 μM) were added to the cells together with the virus at the time of inoculation. The cells were washed after 4 h of incubation and replaced with culture medium. 72 h post-infection, and the cells were lysed for HCV RNA quantitation.

controlled by PULSE software (HEKA Elektronik). For measuring p7 currents, the internal pipette solution contained 140 mM KCl, 1 mM MgCl2, 1 mM EGTA, and 5 mM HEPES (pH 7.2 with KOH) and the external solution containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). Currents were generated by applying the voltage protocol from −100 to +100 mV in 10-mV increments with a holding voltage of −60 mV. Peptides were dissolved in external solution containing 0.01% BSA for application in electrophysiological experiments. A multichannel microperfusion system, MPS-2 (INBIO Inc., Wuhan, China), was used to exchange the external recording bath solution. Patch clamp data were analyzed with Sigmaplot 9 (SPSS Inc.).

Viral Release—Huh7.5.1 cells were seeded in a 12-well plate at a density of 2 × 10^5/well the day before infection. The next day the cells were infected with virus (m.o.i. 0.1) for 4 h, after which they were washed and replenished with culture medium containing CD81 antibody (0.5 μg/ml). To investigate the effect on viral release, peptides (10 μM) were added together with the CD81 antibody 4 h post-infection, and infected cells were subsequently cultured for 24 and 48 h before HCV supernatant RNA copies were measured. The effect of peptides on HCV release was evaluated by the supernatant RNA quantitative assay normalized to the DMSO control. Mouse anti-human CD81 (JS-81) antibody was purchased from BD (BD Biosciences).

CD Spectroscopy—Circular dichroism (CD) measurements were recorded on a Jasco J-810 spectropolarimeter. Spectra were recorded in a 1-mm-path length cell with a peptide concentration of 0.2 mg/ml in 50% tetrafluoroethylene, averaged over three scans, and corrected with a buffer blank. Measurements were performed in the UV range of 250–190 nm at 25 °C.

Statistical Analyses—All assays were performed in triplicate. Data from repeated experiments are expressed as the means ± S.D.

Results

Design and Identification of Antiviral Peptides from p7—The structure of the HCV p7 (JFH-1 2a) monomeric peptide was generated through homology modeling based on p7 (5a) template (22). Each monomer consists of an N-terminal helix (H1) from residues 5–15, a mid-region helix (H2) from residues 19–39, and a C-terminal helix (H3) from residues 48–57 (Fig. 1A). Based on the strategy described above, 14 peptides spanning different membrane motifs of the p7 monomer were generated. These peptides were associated with the three α-helical segments, including the H1, H2, and H3 peptides and different forms of residue extensions or truncations (Table 1). Six peptides inhibited >50% HCV infection when added to cell cultures at the same time as the viral inoculation (Fig. 1B). The most efficient inhibitory peptides, H2-2, H2-3, and H3, were selected for further study.

p7-derived Peptides Inhibit Initial HCV Infection at Non-cytotoxicity Concentrations—To provide a comprehensive pharmacological profile of p7-derived peptide H2-2, H2-3, and H3, we tested their cytotoxicity on three different human cell lines. By MTT assay, we found that the three p7-derived peptides have very low cytotoxicity against the three cell lines tested (Fig. 2, A–C). For Huh7.5.1 cells, their 50% cytotoxicity concentrations (CC50) were calculated to be 83.64 ± 6.95, 115.99 ± 16.68, and 47.22 ± 5.29 μM, respectively (Fig. 2A). For Bel7402 and HeLa cells, the CC50 were all calculated to be ≈200 μM (Fig. 2, B and C).

The MTT assay suggested that the viabilities of peptide-treated cells were all >95% at 10 μM (Fig. 2A), indicating this concentration or less was minimally cytotoxic to cells. Below the concentration of 10 μM, the inhibitory activities of these three peptides against HCV infection were dose-dependent (Fig. 2D). The 50% effective concentrations (EC50) of H2-2, H2-3, and H3 were 1.60 ± 0.11, 0.54 ± 0.08, and 0.52 ± 0.07 μM (Table 2), yielding selective indexes of 52.28, 214.80, and 90.81, respectively.

p7-derived Peptides Inhibit Established HCV Infection with Low Hemolytic Activity and High Stability—A hemolytic assay revealed that three p7-derived peptides displayed minor hemolytic activity, with <10% hemolysis even at 400 μM (Fig. 3A). In addition, these three peptides exhibited very high serum stability, with an ~70% reduction of HCV proliferation in cell culture even with a 24-h preincubation under serum conditions (Fig. 3B). These results suggest that the p7-derived peptides displayed very advanced pharmacological profiles, which makes them strong candidates as therapeutic antiviral agents.
To evaluate the therapeutic functions of three p7-derived peptides against HCV infection, a time- and dose-dependent treatment with DMSO and peptides were carried out in Huh7.5.1 cells. As shown in Fig. 4, A and B, intracellular and supernatant HCV RNA content of the peptide-treated cultures decreased significantly with a time-dependent manner, revealing an efficient suppression of viral proliferation in cell cultures.

### TABLE 1

| Peptide  | Amino acid sequence | Position | Length | Description |
|----------|---------------------|----------|--------|-------------|
| H1       | LVVLHAASAAN         | 5–15     | 11 aa  |             |
| H1-1     | KLVVLHAASAAN        | 4–15     | 12 aa  | Extend N-, 1 aa |
| H1-2     | KLKVVLHAASAAN       | 3–15     | 13 aa  | Extend N-, 2 aa |
| H1-3     | LVVLHAASAANC        | 5–16     | 12 aa  | Extend C-, 1 aa |
| H1-4     | LVVLHAASAANCH       | 5–17     | 13 aa  | Extend C-, 2 aa |
| H2       | LLYFAIFFVAWHIRGRVVP | 19–39    | 21 aa  |             |
| H2-1     | LLYFAIFFVAWHIRGR    | 19–35    | 17 aa  |             |
| H2-2     | GLLYFAIFFVAWHIRGR   | 18–35    | 18 aa  |             |
| H2-3     | HGLLYFAIFFVAWHIRGR  | 17–35    | 19 aa  |             |
| H3       | WPFCILLLMAL         | 48–57    | 10 aa  |             |
| H3-1     | LGFLWPFCILLLMAL     | 44–57    | 14 aa  | Extend N-, 4 aa |
| H3-2     | TFFCILGWPFCILLMAL   | 40–57    | 18 aa  | Extend N-, 8 aa |
| H3-3     | WPFCILLMALPR        | 48–59    | 12 aa  | Extend C-, 2 aa |
| H3-4     | WPFCILLMALPRQ       | 48–60    | 13 aa  | Extend C-, 3 aa |

### TABLE 2

| Pharmacological profiles of p7-derived peptides against HCV |
|---------------|
| Peptide | Infection | Post-infection | EC$_{50}$$^a$ | CC$_{50}$$^b$ |
|----------|-----------|----------------|---------------|---------------|
| H2-2     | 1.60 ± 0.11 | 2.19 ± 0.16 | 63.64 ± 6.95 |
| H2-3     | 0.54 ± 0.08 | 1.09 ± 0.11 | 115.99 ± 16.68 |
| H3       | 0.52 ± 0.07 | 1.54 ± 0.73 | 47.22 ± 5.29 |

$^a$ EC$_{50}$ (50% effective concentration) value of HCV (moi 0.1) was determined on Huh7.5.1 cells;

$^b$ CC$_{50}$ (50% cytotoxic concentration) value of Huh7.5.1 cells was determined by MTT assay.

### FIGURE 2

p7-derived peptides inhibited HCV infection at non-cytotoxicity concentrations. Cytotoxicity of H2-2, H2-3, and H3 peptides against Huh7.5.1 cells (A), Bel7402 cells (B), and HeLa cells (C) is shown. The concentrations ranged from 0 to 400 μM. D, dose-dependent inhibition of HCV infection by H2-2, H2-3, and H3 peptides. A series of concentrations of peptides were added to the cells together with virus at the time of inoculation. The cells were washed after 40 h incubation and replaced with culture medium. 72 h post-infection, the cells were lysed for HCV RNA quantitation.

### FIGURE 3

p7-derived peptides had low hemolytic activity and high stability. A, hemolytic activity of H2-2, H2-3, and H3 peptides against human erythrocytes. The concentrations ranged from 0 to 400 μM. B, serum stability of H2-2, H2-3, and H3 peptides. Peptides (10 μM) were incubated in medium containing 10% FBS for 0, 1, 4, and 24 h at 37°C. At the indicated times, preincubated peptides were used to clear the established HCV infection.
displayed a dose-dependent behavior (Fig. 4, C and D). For instance, after a 72-h post-infection treatment with H2-2, H2-3, and H3 peptides, the supernatant HCV RNA levels were reduced with EC50 of 2.19 ± 0.16, 1.09 ± 0.13, and 1.45 ± 0.13 μM, respectively (Table 2). These results indicate that H2-3 was the most efficient anti-HCV peptide from p7, and it was selected for further study.

**H2-3 Peptide Inhibits Multiple Steps of the HCV Life Cycle**—A time-of-addition assay was employed to determine the antiviral mechanism of the peptide H2-3 (Fig. 5A). H2-3 peptide had a strong inhibitory effect when added to the cells 4 h before the virus was added, added to the cells together with virus, or added to the cells 4 h after infection, with inhibitory rates of 8-, 14-, and 10-fold, respectively (Fig. 5B). These results suggest that H2-3 most likely not only interacts with host cells but also affects virus directly or interferes with virus-host interaction. In addition, H2-3 peptide reduced HCV infectivity by ~200-fold in the viral inactivation assay, whereas the viral RNA content was not affected by H2-3 (Fig. 5C), revealing a viral inactivation mechanism but not a virucidal mechanism. These results suggest that H2-3 most likely interacts with both virus and host cells, thereby blocking the viral entry step, which generates a comprehensive inhibitory effect on an established viral infection.

**H2-3 Peptide Potently Inactivates HCV**—Because the H2-3 peptide has the strongest inhibitory effect when added to virus before infection, we determined the precise activity with time- and dose-dependent experiments. As shown in Fig. 5D, H2-3 peptide reduced HCV infectivity by ~100-fold after a 5-min treatment and ~200-fold after a 60-min treatment, revealing a very rapid viral inactivation function. Simultaneously, H2-3 peptide reduced HCV infectivity in a dose-dependent manner (Fig. 5E), with an EC50 of 82.11 nM, which is ~1000-fold lower than its CC50. These results suggest that H2-3 inactivates HCV in a highly effective manner.

**H2-3 Peptide Adsorbs to the Cells and Blocks Initial HCV Infection**—The time of peptide addition experiment suggested that H2-3 peptide most likely interacts with host cells (Fig. 5B). To confirm this hypothesis, a fluorescent H2-3 peptide containing a FITC group at its N-terminal was produced, and its cellular localization was tested by confocal microscopy. As shown in Fig. 6A, H2-3 peptide efficiently adsorbed to Huh7.5.1 cells after 4 h of incubation and the initially internalized virus was reduced by 74% (Fig. 6B), whereas after 24 h of incubation, H2-3 peptide entered Huh7.5.1 cells and located in the cytoplasm. At this time of pretreatment, initially internalized virus was still reduced by 56% (Fig. 6B). These results suggest that H2-3 peptide potently inhibits initial HCV infection by binding to cell surface, and the inhibitory effect slightly decreased when the cell-bound peptide was gradually internalized.

**H2-3 Peptide Enters the Cell and Reduces Viral Infectivity**—Using confocal microscopy, we observed that H2-3 peptide ultimately entered Huh7.5.1 cells after a 24-h incubation. As shown in Fig. 6A, H2-3 peptide is distributed throughout most regions of the cell cytoplasm, revealing a high cellular uptake rate. Because H2-3 peptide inactivates cell-free virus and reduces its infectivity, we investigated whether the peptide H2-3 inactivates intracellular viruses. To test this possibility, infected cells were lysed, intracellular virus was collected, and its infectivity was detected according to a secondary infection
step. As shown in Fig. 6, intracellular HCV RNA content and extracellular HCV RNA copies were slightly reduced after a 24-h treatment with H2-3 peptide, whereas intracellular and extracellular HCV infectivity were significantly reduced (Fig. 6D). These results indicate that H2-3 peptide enters the cell and inactivates intracellular viruses.

**H2-3 Peptide Has No Effect on the Electrophysiological Profile of HCV p7 Ion Channel**—Because the peptide H2-3 was derived from a monomer of p7, it is necessary to determine whether the derived peptide interfered with the p7 ion channel. Therefore, we established an assay that uses the patch clamp technique to record p7-mediated currents in HEK293T cells. Transfection of p7 (2a) plasmid induced a distinct current in HEK293T cells, and this current was activated by voltage (Fig. 7A). Previous results indicated that H2-3 peptide efficiently adsorbed to the cell membrane and entered the cells after 24 h of incubation (Fig. 6A). Thus, a comprehensive incubation experiment was carried out to determine the influence of H2-3 peptide on the channel conductance of p7. However, the peptide H2-3 had no effect on the channel conductance of p7 even though it acted 24 h before transfection and persistently acted throughout the p7 expression and assembly process (Fig. 7A). These results show that although H2-3 is derived from p7, it does not affect the electrophysiological profile of HCV p7 ion channel.

**H2-3 Peptide Has No Effect on Viral Release from Host Cells**—It has been reported that the p7 ion channel plays an essential role in HCV assembly and secretion (17, 18). In the above experiment we confirmed that the peptide H2-3 does not impair the electrophysiological profile of the p7 ion channel through a comprehensive treatment (Fig. 7B). Here, we established a simple viral release model to test whether H2-3 peptide has any influence on HCV release. Huh7.5.1 cells were infected...
with virus and replenished with medium containing CD81 antibody, which blocks the re-entry of released virus. Using this system, we observed that H2-3 peptide reduced the supernatant HCV level significantly in the absence of CD81 antibody (Fig. 7B), consistent with the intracellular HCV level (Fig. 4A). In contrast, in the presence of CD81 antibody, there is no reduction of supernatant HCV levels by treatment with H2-3 peptide 24 h post-infection and a minor reduction at 48 h post-infection. These results indicate that H2-3 peptide does not block HCV release from host cells, corresponding to the functional deficiency of the p7 channel.

### Structural Basis for the Antiviral Activities of H2-3 Peptide

Although H2-3 peptide is derived from the helical region of p7 polypeptide, it might be entirely different as a single molecule. To illustrate the structural characteristic of H2-3 peptide, CD spectroscopy was employed. As shown in Fig. 8A, H2-3 peptide adopts an α-helix conformation in the approximate membrane environment. As a result, this peptide adopts the same conformation as a synthetic molecule and the helical region (Fig. 8B).

Charged residues are commonly key elements of bioactive peptides, and there were two charged residues (Arg-33 and Arg-35) in H2-3 under physiological condition. Therefore, we investigated whether these residues were necessary for the antiviral function. We generated mutant peptides in which Arg-33 and Arg-35 were partially or completely replaced with different types of residues (Table 3). Compared with H2-3, mutant peptides displayed greatly reduced antiviral activities when two arginines were replaced with non-charged (H2-3M1) or negatively charged residues (H2-3M2, H2-3M3, and H2-3M4). Mutant peptides with one histidine (H2-3M5 and H2-3M6) possess half-inhibitory activity, whereas the peptide with two histidines (H2-3M7) entirely lost antiviral function. Furthermore, mutant peptides with one or two lysines (H2-3M8, H2-3M9, and H2-3M10) exhibited similar or even stronger antiviral activities than H2-3 (Fig. 8C).

We further measured the structural characteristics of different types of mutant peptides by CD spectroscopy. As shown in Fig. 8A, all peptides adopt an α-helix conformation in approximate membrane environment. Even though replacement of two arginines with glutamic acids (H2-3M4) severely reduced the helicity, replacement with asparagine/glutamine (H2-3M1)
or two lysines (H2-3M10) barely affect the helicity, and replacement with two histidines (H2-3M7) enhanced the helicity. The helicity of these peptides are not consistent with their antiviral activities (Fig. 8C), suggesting that net charge but not helical conformation or polarity is most functionally linked to the ability of the peptide to inhibit HCV infection.

Discussion

Recent NMR studies have shown that the p7 ion channel adopts an unusual funnel-like structure with three helical segments (22, 25), which provides a beneficial basis for the development of a p7-related antiviral strategy. To design new antiviral peptides from p7, we generated the monomeric structure of p7 (2a) based on the sequence and structure of p7 from a genotype 5a virus strain using a homology modeling method. Each monomer of p7 (2a) consists of three α helical segments, H1 (5–15), H2 (19–39), and H3 (48–57) (Fig. 1A), which differ from p7 (5a) because of the sequence differences between viral strains. The α helix is the most prevalent secondary structure in proteins (reflecting ∼30% of each protein across the entire Protein Data Bank) and is characterized by a right-handed coil stabilized by hydrogen bonds between the backbone residues of the polypeptide chain (26, 27). Peptides with a helical conformation are likely to be more stabilized than those with a random coil conformation. Therefore, in this study, peptides derived from p7 were designed with three α helical segments rather than a random coil. As shown in Table 1, the three α helical segments formed the initial three peptides, which were named H1, H2, and H3. Because the entire p7 peptide is composed predominantly of hydrophobic residues, the three peptides also display highly hydrophobic properties, and two of them (H1 and H3) are even non-charged under normal conditions, which is not advantageous for antiviral peptides. To make sure the derived peptides have a rational polarity and net charge, we designed a series of peptides with different forms of residue extensions or truncations either at the N or C termini (Table 1). The inhibitory effects of these peptides on HCV infection were evaluated through a co-inoculation assay. Six peptides, H2-1, H2-2, and H2-3 from the H2 helix and H3, H3-3, and H3-4 from the H3 helix, displayed 50% inhibition at 10 μM. We evaluated the comprehensive pharmacological profiles of three p7-derived peptides: H2-2, H2-3, and H3 (Table 2). All these peptides not only inhibit initial HCV infection (Fig. 2D) but also suppress an established HCV infection (Fig. 4). In addition, all these peptides display low cytotoxicity against several cell lines, with a CC50 of ∼200 μM (Fig. 2, A—C) and weak hemolytic activity even at a concentration of 400 μM (Fig. 3A). In addition, these three peptides exhibit high serum stability (Fig. 3B). There is no apparent explanation for the low toxicity and high stability features, but they most likely depend on the original template of these peptides. Because p7 is a virally
encoded membrane protein, it is likely that p7-derived peptides adopt the same properties as p7 in the presence of host cells, including low toxicity and high stability.

The most efficient peptide in this study, H2-3, which consists of residues from His-17 to Arg-35 of the H2 helix, was chosen for detailed characterization. Mutational experiments revealed that the antiviral activity of the H2-3 peptide is net charge-dependent, which is supported by the two positively charged residues, Arg-33 and Arg-35 (Table 3 and Fig. 8C). For instance, the peptide H2-3M1 with Asn-33/Gln-35, the H2-3M2 peptide with Glu-35, the H2-3M3 peptide with Glu-33, and the H2-3M4 peptide with Glu-35/Glu-33 entirely lost their antiviral activities. In addition, the H2-3M5 peptide with His-33 and the H2-3M6 peptide with His-35 partially lost their antiviral activities, and the H2-3M7 peptide with both His-33 and His-35 entirely lost its antiviral activity. The peptides H2-3M8 and H2-3M9 have almost the same antiviral activities because they contain the most conservative changes with Lys-33 and Lys-35, respectively. These results revealed that the positive net charge, but not polarity, plays a predominant role in the antiviral activity of the H2-3 peptide. Although histidine can be easily charged in an acidic environment, the pH value of the cell culture-based viral infection system is insufficient, which is consistent with the antiviral activities of the three peptides with a histidine mutation.

In the structure-activity analysis, we found that the p7-derived H2-3 peptide adopts the similar conformation as the helical region of p7 (Fig. 8A). Unlike previously reported C5A (14) and GBVA10-9 peptide (9), H2-3 peptide consists of approximately 80% of hydrophobic amino acids, which makes it impossible to form a typical amphipathic α-helical conformation. Besides, the hydrophilic amino acids only locate at the two terminal regions but not the mid-region of H2-3, which is an unusual composition of a derived antiviral peptide. Thus, we hypothesize that the special sequence composition of H2-3 peptide is the most crucial basis for its antiviral activity, whereas the helical conformation might be only essential but not sufficient, because there is no mutant peptide that has antiviral activity but adopt non-helical conformations.

The comprehensive antiviral activity of H2-3 peptide most likely correlates with multiple modes of action, as the time of peptide addition experiment revealed that the H2-3 peptide displayed a strong inhibitory effect at multiple steps of the HCV life cycle. Because the most efficient antiviral effect occurs in the preincubation group, the predominant mode of the H2-3 peptide is most likely viral inactivation (Fig. 5). To confirm this possibility, we estimated in detail the viral inactivating effect of the H2-3 peptide. Interestingly, the H2-3 peptide inactivated HCV with an EC50 of 82.11 nM (Fig. 5E), which was 7-fold and 14-fold lower than the concentrations in the co-inoculation and post-incubation assays (Table 2). Previous studies have reported that the antiviral effect of some viral inactivating peptides depends on the disruption of the whole viral particle (14, 28), whereas others only disturb viral envelopes without any significant influence on the viral genome (29, 30). In this study, although the H2-3 peptide reduced HCV infectivity by ~100-fold after pretreatment for 5 min, the viral RNA was not affected (Fig. 5, C and D), suggesting an apparent inactivating mechanism of action. Because p7 is a highly hydrophobic membrane-associated protein, it is reasonable to assume that the H2-3 peptide has a strong membrane affinity. Through confocal microscopy, we observed that a FITC-labeled H2-3 peptide efficiently bound to the cell surface (Fig. 6A), which blocked the initial HCV infection to host cells (Fig. 6B). Furthermore, labeled H2-3 peptide gradually entered cells after 24 h, which resulted in the inactivation of both intracellular and extracellular progeny viral particles (Fig. 6, C and D). Because of the multiple modes of action of the H2-3 peptide, the eventual antiviral effect is reflected in the viral proliferation curve at least 3 days post-infection (Fig. 4, A and B).

As reported previously, the virus replicated to high levels after 24 h post-infection, and intracellular mature viral particles rapidly accumulated from that time in the JFH-1 and Huh7.5.1 systems (23). In the present study, we observed that the H2-3 peptide entered cells and accumulated in the cytoplasm by 24 h post incubation (Fig. 6A). As a result, it is likely that the inhibitory effect would only appear at 24 h post-infection if the peptide H2-3 were used as a therapeutic antiviral agent. In fact, this prediction was eventually confirmed in the post-infection assay; there is no significant difference between the proliferation curve of the DMSO control and the H2-3 peptide-treated group before 16 h post-infection (data not shown), whereas the growth trend differed after 24 h post-infection, and the difference became stronger thereafter (Fig. 4, A and B), which correlated with the timing of both the peptide uptake and viral maturation.

Although the H2-3 peptide is derived from p7, it does not affect the electrophysiological profile of the p7 ion channel (Fig. 7A), which might correlate with its membrane affinity. As we described above, the H2-3 peptide can interact with both the cell and the virus by its positive charge, which improves the electrostatic interactions with the negatively charged membrane. Structural analysis revealed that the H2 helix appears to associate with the H3 helix by intertwining with it, thus playing an essential role in the assembly of the p7 channel. In comparison, the electrostatic interaction between the H2-3 peptide and the membrane is likely stronger. Therefore, the H2-3 peptide most likely binds cellular and viral membranes but does not assemble into the p7 channel or associate with full-length p7 monomers even when treatment begins 24 h before channel expression to ensure the uptake of sufficient peptide. Because the p7 ion channel is essential for viral assembly and release, further antibody blockage experiments were performed and revealed that H2-3 had no effect on viral release (Fig. 8B). We conclude that the H2-3 peptide acts as a pre-infection blocker of HCV and is also a potential therapeutic inhibitor after viral infection, which differs from many previously reported viral entry inhibitors and virucidal agents (9, 31).

In this study, we successfully developed an efficient anti-HCV peptide (H2-3) from p7 ion channel. However, the precise antiviral mechanism and the direct target are still unclear. Besides, this peptide is derived from a viral encoded protein and has the similar conformation as part of it. Therefore, it is possible to induce a similar host immune reaction once used as a therapeutic antiviral drug, a reminder that there is a risk to be cleared by the host immune system. The approach to optimize this anti-
novel peptide discovery strategy includes 1) finding the antiviral target and mechanism, 2) modifying a derived peptide to mimic similar immunoreaction with full-length protein, and 3) generating multiple peptides for combination therapy.

In conclusion, we designed, tested, and characterized three new p7-derived peptides that inhibit HCV infection and suppress HCV proliferation in cell culture. These peptides exhibit low toxicity and high stability, which make them strong candidates for further antiviral drug development. In addition, our work provides a new peptide design strategy that is a useful reference for peptide drug discovery.

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