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A new natural α-helical peptide from the venom of the scorpion *Heterometrus petersii* kills HCV

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ABSTRACT

Hepatitis C virus (HCV) is a major cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma. There is no vaccine available for HCV, and almost half of patients cannot be cured using standard combination therapy. Thus, new anti-HCV strategies and drugs are urgently needed. Here, the gene encoding a new α-helical peptide, Hp1090, was screened from the venomous gland cDNA library of the scorpion *Heterometrus petersii*. Structural analysis showed that Hp1090 is an amphipathic α-helical peptide. *in vitro* HCV RNA inhibitory assays indicated that Hp1090 peptide inhibited HCV infection with an IC_{50} of 7.62 μg/ml (5.0 μM), whereas Hp1035 peptide, showing high homology to Hp1090, exhibited no anti-HCV activity. Hp1090 acted as a viricide against HCV particles *in vitro* and prevented the initiation of HCV infection. Furthermore, this peptide interacted with HCV particles directly and rapidly permeabilized phospholipid membranes. Collectively, it seems that Hp1090 is virocidal for HCV *in vitro*, directly interacting with the viral membrane and decreasing the virus infectivity. These results suggest that Hp1090 could be considered an anti-HCV lead compound with virocidal mechanism that offers a potential therapeutic approach to HCV infection. Our work opens a new avenue for antiviral drug discovery in natural scorpion venom.

1. Introduction

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus classified in the *Flaviviridae* family [24]. Since its discovery in 1989, HCV has been recognized as the primary cause of chronic hepatitis, hepatic steatosis, cirrhosis and hepatocellular carcinoma (HCC) worldwide. Chronic hepatitis C is particularly grave, representing the most common indication for liver transplantation [36]. Currently, more than 200 million people are estimated to be infected by HCV globally, posing a major public health problem [38]. However, a protective vaccine is not yet available, and therapeutic options remain limited. The current standard therapy, which consists of a combination of pegylated interferon α (Peg-IFN-α) and ribavirin, results in a sustained viral response in only 50% of patients, and HCV treatment efficacy is markedly dependent upon the viral genotype [28]. Thus, new classes of antiviral compounds with improved efficacy are needed.

Previous studies have demonstrated that a wide variety of living organisms produce a large repertoire of antimicrobial peptides (AMPs) that play an important role in innate immunity to microbial invasion [9–11,13–15,31]. These peptides can be divided into at least four structural classes: amphipathic α-helices, amphiphilic peptides with two to four β-strands stabilized by disulfide bridges, loop structures, and extended structures [2,10,16]. They possess a broad spectrum of antimicrobial activity, killing or neutralizing Gram-negative and Gram-positive bacteria, fungi (including yeasts), parasites (including planaria and nematodes), cancer cells, and even enveloped viruses [12]. Some AMPs have been reported to display antiviral activity as well. For example, dermaseptin S4 inhibits human immunodeficiency virus 1 (HIV-1) [26], caerin 1.1 inhibits HIV-1 and viral transfer from dendritic cells to T cells [35], and recombinant scorpine prevents replication of the dengue-2 virus [3].

Scorpion venom is a rich resource of AMPs and has been used as a traditional drug in China for thousands of years. In the past, scorpion venomous peptides have been reported to possess activity against Gram-positive and Gram-negative bacteria [6,39], but were never tested for anti-HCV activity. In this investigation, Hp1090, a new antimicrobial peptide, shared high homology in amino acid sequences with the known AMP IsCT, was screened and characterized from the cDNA library of the venomous gland of *Heterometrus petersii*. The structure analysis showed that Hp1090 had the amphipathic α-helical feature which is beneficial for peptide's anti-HCV activity reported previously [5]. Furthermore, the real-time RT-PCR

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assays showed that Hp1090 had a significant inhibitory effect on HCV infection with an IC_{50} (the 50% inhibitory concentration) of 7.62 μg/ml, which is well-below the LC_{50} (the 50% lethal concentration) of 126.82 μg/ml for cells. We also found that Hp1090 potently inhibited HCV before viral entry into cells and killed HCV rapidly *in vitro*. These results reveal that Hp1090 is a potential anti-HCV lead peptide.

2. Materials and methods

cDNA library construction. H. petersii scorpions were collected in the Guangxi Province of China. Their glands were then isolated 2 days after electrical extraction of their venom. Total RNA was prepared from these glands using TRIzol reagent (Invitrogen). Poly(A) mRNA was purified with a Poly(A) Tract mRNA isolation system (Promega). A cDNA library was constructed using a 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.

cDNA library screening by PCR. A specific primer was designed and synthesized to screen for Hp1090 gene, which is homologous to IsCT, an antimicrobial peptide derived from the scorpion *Opisthacanthus madagascariensis*. Specifically, the cDNA library of the venomous gland of *H. petersii* was screened using a PCR-based method as described previously [39]. The general forward primer and specific reverse primer were 5′-TCGACCCACGCGTCCG-3′ and 5′-TCCTCTCTTTCCGAA-3′, corresponding to the *Sal*I adapter region of the recombinant vector and the conserved processing region of the propeptide, respectively.

cDNA sequencing and computer analysis. The plasmids identified as positive clones were characterized using an ABI Prism 377XL DNA sequencer with a universal T7 promoter primer. Sequence analysis was carried out with BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences obtained for Hp1090 and Hp1035 homologs were retrieved from GenBank by the BlastP method (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple-sequence alignments of Hp1090 proteins were also carried out using CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw/).

Peptide synthesis, purification, and characterization. Hp1090 (IFKAIWSGIKSLF) and Hp1035 (IFSAIGGFLKSIF) were synthesized on an Abimed AMS 422 synthesizer by Fmoc solid-phase peptide synthesis. Both peptides were C-terminally amidated. The peptides were then deprotected and released from the resin by trifluoroacetic acid (TFA) treatment. After precipitation using cold diethyl ether, the peptides were filtered (Millex-HV, 0.45 μm; Millipore) and further purified on a C18 column (EliteHPLC, 10 mm × 250 mm, 5 μm, 300 A). A linear gradient from 30% to 95% water with 0.1% TFA was used for 40 min at a constant flow rate of 5 ml/min. Peaks were detected at a wavelength of 220 nm and were collected manually. The peptides were then lyophilized and their purity was assessed by HPLC (Elite-HPLC) and mass spectrometry (Voyager-DESTR; Applied Biosystems). The secondary structures of the Hp1090 and Hp1035 peptides were measured by circular dichroism (CD) spectroscopy. Measurements were carried out in the UV range of 250–190 nm at 25 °C in water, 30% TFE and 70% TFE using a Jasco-810 spectropolarimeter, at a concentration of 0.1 mg/ml. For each peptide, spectra were collected from three separate recordings and averaged after subtracting the blank spectrum of pure water.

Cell culture and reagents. Huh7.5.1 were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco), as described previously [40].

MTT assay. Huh7.5.1 cells were plated in 96-well plates (7000 cells/100 μl/well). The cells were incubated for 24 h at 37 °C, followed by the addition of serial 2-fold dilutions of peptides. After 48 h at 37 °C, 20 μl of 5 mg/ml MTT solution in phosphate buffered saline (PBS; Invitrogen) was added to each well, and the plates were returned to the incubator. Four hours later, the medium was removed, 100 μl dimethyl sulfoxide (DMSO) was added, and the plates were shaken for 20 min at room temperature to completely dissolve the purple formazan precipitate. The absorbance was then measured at 570 nm on a Multiskan MK3 (Thermo).

Amplification of HCV viral stocks. HCV (JFH-1) was prepared as described previously [37]. 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 (Corning). Infected cells were trypsinized and replated in a T75 flask before confluence on day 2–3 postinfection (p.i.). The supernatant from infected cells was then harvested 6-7 days p.i. and aliquoted for storage at −80 °C.

in vitro HCV RNA inhibitory assay. To quantify the inhibitory effect of peptides on HCV infection, the HCV RNA contents of the supernatants derived from peptide-treated and untreated cells were quantitated by real-time RT-PCR. The peptide stock solution was mixed with viral supernatant, resulting in a final peptide concentration of 20 μg/ml. The virus-peptide mixture was then incubated at 37 °C for 2 h, after which it was used to infect Huh7.5.1 cells at an MOI of 0.1. The cells were washed after 4 h of incubation and incubated in fresh medium containing peptides. As the positive control, the virus and infected cells were treated with 100 units/ml of recombinant human IFN-α as the same time of peptides added as above. After 48 h of infection, the supernatant was collected and the HCV RNA levels were measured by real-time RT-PCR according to the manufacturer's instructions (KHB, China).

Dose-dependent effect assay. Huh7.5.1 cells were plated in a 6-well plate at 4.5×10^5 cells per well for 24 h. Different concentrations (0, 6, 9, 12, 15, and 20 μg/ml) of Hp1090 peptide were added to the virus (MOI of 0.1) for 2 h at 37 °C, and then the mixture were added to the cells respectively for 4 h, at which time the cells were washed three times and replenished with complete medium. Two days p.i., supernatant and intracellular HCV RNA levels were measured as described above.

Time-dependent effect assay. Huh7.5.1 cells were seeded at 4.5×10^5 cells per well in a 6-well plate and infected with HCV (MOI of 0.1) one day later. Hp1090 peptide was added to the cultured cells to a final concentration of 20 μg/ml under the following conditions: (i) preinoculation of cells: Hp1090 peptide was added to the cells for 2 h at 37 °C followed by three washes with medium before viral infection; (ii) preinoculation of virus: Hp1090 peptide was added to the virus for 2 h at 37 °C, and this mixture was added to the cells for 4 h and then removed by washing and replaced with virus/Hp1090-free medium; (iii) co-inoculation: Hp1090 peptide was added to the cells together with virus for 4 h, after which the cells were washed and replenished with virus/Hp1090-free medium; (iv) postinoculation: the cells were infected for 4 h, and then the virus was removed by washing and the cells were treated with Hp1090 for different time intervals (0–48 h, 12–48 h, 24–48 h, or 36–48 h p.i.). After 48 h, the supernatant and intracellular HCV RNA levels were measured as described above. The relative HCV RNA content in infected cells was determined after normalization to cellular GAPDH mRNA levels.

Virocidal HCV assay. Hp1090 peptide was added to HCV to a final concentration of 20 μg/ml for 2 h at 37 °C. The peptide was then separated from the peptide-virus mixture using the Amicon Ultra Centrifugal Filter Units (100,000 NMWL, Millipore). Both the separated ultrafiltrate contained peptide in the centrifuge tube (down) and the concentrate with virus (up) were used to infect Huh7.5.1 cells, respectively, as above. The supernatant and intracellular HCV RNA levels were measured after 48 h. The virus without Hp1090 treated with or without ultrafiltration served as controls.

Coupling of the Hp1090 peptide to the CM5 biosensor chip. All interactions between HCV and peptides were examined at 25 °C