

Identification of peptides that bind hepatitis C virus envelope protein E2 and inhibit viral cellular entry from a phage-display peptide library

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Abstract. Hepatitis C virus (HCV) envelope protein E2 is required for the entry of HCV into cells. Viral envelope proteins interact with cell receptors in a multistep process, which may be a promising target for the development of novel antiviral agents. In this study, a heptapeptide M13 phage-display library was screened for peptides that bind specifically to prokaryotically expressed, purified truncated HCV envelope protein E2. ELISA assay was used to quantify the binding of the peptides to HCV E2 protein. Flow cytometry, quantitative reverse-transcription PCR and western blotting were used to investigate the inhibition effect of one peptide on HCV infection in hepatoma cells (Huh7.5) *in vitro*. Four peptides capable of binding specifically to HCV E2 protein were obtained after three rounds of biopanning. Peptide C18 (WPWHNHR), with the highest affinity for binding HCV E2 protein, was synthesized. The results showed that peptide C18 inhibited the viral infectivity of both HCV pseudotype particles (HCVpp) harboring HCV envelope glycoproteins and cell-culture produced HCV (HCVcc). Thus, this study demonstrated that peptide C18 is a potential candidate for anti-HCV therapy as a novel viral entry inhibitor.

Introduction

Over 170 million individuals worldwide are infected with hepatitis C virus (HCV), which is a major causative agent of

liver disease. HCV infection persists after primary infection in the majority of infected individuals, often leading to fibrosis, cirrhosis or hepatocellular carcinoma (HCC). However, no effective prophylactic vaccines or drugs against HCV are currently available (1,2).

HCV is an enveloped virus that belongs to the genus *Hepacivirus* in the *Flaviviridae* family, with a positive single-stranded 9.5 kilobase (kb) RNA genome. HCV genomic RNA encodes a polyprotein precursor of ~3,000 amino acid (aa) residues, which is cleaved by a combination of host and viral proteases into at least 10 proteins: the core, envelope proteins (E1 and E2), p7, and non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B proteins (3). Envelope proteins are type 1 transmembrane proteins with N-terminal ectodomains and C-terminal hydrophobic anchors (4). The proteins E1 and E2, especially E2, mediate viral entry into target cells, which is an obligatory step in virus replication and the maintenance of infection (5,6). The frequency of HCV genomic mutation is relatively high, and the virus is divided into six major genotypes and multiple subtypes (7). In general, despite strong amino acid variability, almost all viruses enter cells via specific cell receptors, which are distinct for different genotypes of viruses (8-11). Drugs targeting the viral entry step may provide a potential candidate for anti-HCV therapy, with the HCV E2 protein serving as an ideal target.

Full-length E2 protein extends from amino acids 384 to 746 of the HCV polyprotein and contains regions of extreme variability. The C-terminal truncation of E2 at residue 661 (E2₆₆₁) or 715 (E2₇₁₅) leads to the secretion of E2, as this deletion has been suggested to remove the hydrophobic transmembrane anchor sequence (12,13). It has been shown that E2 truncated at residue 661 has a higher folding efficiency compared to E2 truncated at 715 (12,13). In a previous study, we constructed a recombinant prokaryotic plasmid expressing the truncated HCV E2 gene (E2₆₆₁), and expressed and purified the protein. We found that E2₆₆₁ retained the ability to react with the sera of HCV-infected individuals using western blotting (14). In the present study, we used the phage-display random peptide library, a useful tool for obtaining short peptides that specifically bind to a target ligand, to identify novel peptides that

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specifically bind to HCV E2. HCV-pseudotyped particles (HCVpp) harboring HCV envelope glycoproteins (15,16) and cell-culture-produced HCV (HCVcc) (17,18) were used as surrogate models to investigate the ability of selected identified peptides to inhibit the entry of HCV into cells *in vitro*.

Materials and methods

Cells and proteins. Huh7.5 cells (a kind gift from Professor Charles M. Rice, The Rockefeller University, New York, NY, USA) and 293T cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS; Gibco), 1% L-glutamine and 1% penicillin-streptomycin. The HCV proteins E2₆₆₁, NS3 (19) and NS5B were prokaryotically expressed with 6X His tags and purified using Ni-NTA affinity chromatography (Qiagen, Valencia, CA, USA).

Phage display peptide biopanning. The Ph.D.-7 phage display peptide library was obtained from New England Biolabs (Beverly, MA, USA). The original library revealed a wide diversity of sequences with no obvious positional biases. The *E. coli* host strain ER2738 provided in the library kit was used for M13 phage propagation. Phage panning procedures were performed according to the manufacturer's instructions.

Briefly, 35 mm-diameter polystyrene plates were coated with HCV E2₆₆₁ (100 µg) in 1.5 ml NaHCO₃ (pH 8.6, 0.1 mol/l) at 4°C overnight with gentle agitation in a humidified container. The coating solution was completely removed and each plate was filled with blocking buffer (PBS containing 5% BSA) for 2 h at 4°C. Approximately 2x10¹¹ phages were added to the blocked wells and mixed gently for 1 h at 37°C. The plates were washed 10 times with Tris buffer solution, pH 7.5, containing 0.1% (v/v) Tween-20 (TBST). The adherent phages were eluted with 1 ml 0.2 mol/l glycine-HCl (pH 2.2) containing 1 mg/ml bovine serum albumin (BSA) and neutralized with 150 µl 1 mol/l Tris-HCl (pH 9.1). The eluted phage titer was subsequently evaluated by a blue plaque-forming assay on agar plates containing tetracycline. The phages were amplified by mixing the eluate with 20 ml of *E. coli* ER2738 culture and then incubation at 37°C with vigorous agitation for 7 h. The titer of the amplified eluates was determined as described in the kit protocol, and the eluates were used for the following round of biopanning. To enrich for phages that displayed the target peptide on their surface, the phages were screened a total of three times, as described above. The panning stringency was gradually increased stepwise in each round, by decreasing the amount of input HCV E2₆₆₁ protein (100, 50, then 20 µg) and reducing the binding time (60, 30, then 20 min).

Binding assays. The reactivity of the third round peptide population for HCV E2₆₆₁ protein was estimated using an ELISA. Incubations were performed in a volume of 100 µl at 37°C. Microtiter wells were coated with HCV E2₆₆₁ protein overnight at 4°C, and then blocked with BSA (15 mg/ml) for 4 h. The third round peptide population was added [1x10¹¹ plaque-forming units (PFU)/well] and incubated for 1 h at room temperature with gentle agitation. The plate was washed 10 times with TBST containing 0.5% Tween-20. Subsequently,

horseradish peroxidase (HRP)-conjugated anti-M13 phage antibody (Pharmacia, Peapack, NJ, USA) diluted 1:5000 in phosphate-buffered saline (PBS, pH 7.4) was added, incubated for 1 h, and the wells were washed 10 times with TBST. Peroxidase activity was determined at 37°C using the substrate *o*-phenylenediamine-H₂O₂ in phosphoric acid buffer. The reaction was stopped using 25 ml of 2 mol/l H₂SO₄ for 15 min, and the absorbance values were measured at 490 nm (A490). HCV proteins NS3 and NS5B, and BSA and PBS were used as negative controls. Triplicate determinations were generated for each data point.

Selection of individual positive clones. Twenty-five clones were randomly selected from the eluates of the third round of biopanning and individually added to *E. coli* ER2738 cultures for amplification and titration. The relative binding affinities of the individual clones to HCV E2₆₆₁ protein were assayed using an ELISA, as described above. Triplicate determinations were generated for each data point.

DNA sequencing and peptide synthesis. The single-stranded DNA from the positive phages was purified using an M13 purification kit (Beijing Sunbiotech Co., Ltd., Beijing, China). DNA sequencing was performed by Beijing Aoke Biotechnology Ltd. (Beijing, China). The heptapeptide sequences were deduced from the DNA sequences and synthesized by Chinapeptides Co., Ltd. (Shanghai, China). Homologous analysis was performed using BLAST. The unrelated synthetic heptapeptide (VLRSDFK), the sequence deduced from the clone with the weakest affinity to HCV E2₆₆₁ protein of the 25 randomly selected positive clones, was used as the negative control.

Entry inhibition assay using HCV pseudotype particles. HCV pseudotype particles (HCVpp) were generated as previously described (15,16). Briefly, 2.25 µg of the HCV envelope glycoprotein expression plasmid PVRC-E1E2hebei (genotype 1b), 9 µg of the transfer plasmid pCS-CG expressing the reporter gene enhanced green fluorescent protein (EGFP), and 6.75 µg of the lentiviral packaging plasmid pHR'CMVΔ8.2 were co-transfected into 2x10⁶ 293T cells using Fugene HD transfection reagent (Roche, Penzberg, Germany) to produce infectious HCVpp. The three plasmids were a generous gift from Professor Wenjie Tan (National Institute for Viral Disease Control and Prevention, China CDC, Beijing, China). HCVpp-containing supernatant was collected 48 h after transfection, pooled, filtered and condensed using a Lenti-X™ Concentrator (Takara, Otsu, Japan).

Incorporation of HCV E1-E2 glycoproteins into the pseudoparticles was verified by western blotting using HCV-infected sera obtained from patients at Tangdu Hospital (Fourth Military Medical University, Xi'an, China) as a primary antibody. HCVpp titer was quantified by the p24 content of the samples using the HIV-1 p24 ELISA kit (PerkinElmer, Boston, MA, USA). Then, 1.2 ml HCVpp (1 ng/ml) was incubated with the peptides at final concentrations of 20, 50, 100 and 200 µg/ml for 1 h at room temperature, and added to the Huh7.5 cells. At 16 h post-infection, the media were removed, the cells were washed three times with PBS, incubated with fresh media for an additional 32 h, and then collected. EGFP activity, a reflection of the degree of entry of the pseudoparticles into the host

Table I. Primers used for quantitative real-time RT-PCR.

Target gene	Primer
HCV 5'-UTR	F: 5'-CGGGAGAGCCATAGTGGTC-3'
	R: 5'-AGTACCACAAGGCCTTTCG-3'
GAPDH	F: 5'-TGCACCACCAACTGCTTAG-3'
	R: 5'-GATGCAGGGATGATGTTC-3'

F, forward; R, reverse.

cells, was measured using flow cytometry. HCVpp bound to the unrelated peptide and naïve HCVpp were used as a negative and mock control, respectively.

Entry inhibition assay using HCVcc. The pFL-J6/JFH plasmid containing the full-length HCV chimeric genome (genotype 2a) was kindly provided by Professor Charles M. Rice. Cell-culture-produced HCV (HCVcc) was generated as previously described (17,18), without modifications. Full-length HCV RNA was transcribed *in vitro* from the pFL-J6/JFH plasmid, and electroporated into Huh7.5 cells. The copy number of HCVcc in the cell supernatants was determined by quantitative reverse-transcription (RT)-PCR. Then, 1×10^7 copies of HCVcc were incubated with the peptides (final concentration, $100 \mu\text{g/ml}$) for 1 h at room temperature and then added to Huh7.5 cells. HCVcc incubated with the unrelated peptide and naïve HCVcc were used as a negative and mock control, respectively.

For quantitative real-time RT-PCR, the media were discarded at 6 h post-infection. Subsequently, the cells were washed three times with PBS and collected. Total RNA was extracted using TRIzol (Gibco) according to the manufacturer's instructions and quantitative real-time RT-PCR was conducted using the SYBR-Green Real-time PCR Master Mix kit (Takara) with the primers listed in Table I.

For western blot analysis, the media were removed at 16 h post-infection, the cells were washed three times with PBS, incubated with fresh medium at 37°C for an additional 32 h and then collected. Mouse anti-HCV core antigen monoclonal antibody (1:20; Thermo Scientific Pierce Antibodies, Rockford, IL, USA) and mouse anti-HCV NS5A protein monoclonal antibody (1:100; generous gift of Professor Charles M. Rice) were used to detect the expression of HCV core protein and NS5A by western blotting, respectively.

Statistical analysis. Differences between groups were compared using the one-way ANOVA. Statistical significance was defined as $P < 0.05$.

Results

Specific enrichment of HCV E2₆₆₁ protein-bound phages. Phages specifically binding to HCV E2₆₆₁ protein were enriched through three rounds of panning. The output/input ratio of phages after each round of panning was used to determine the phage recovery efficiency. After three rounds of panning, the output/input ratio in the third round had increased by almost

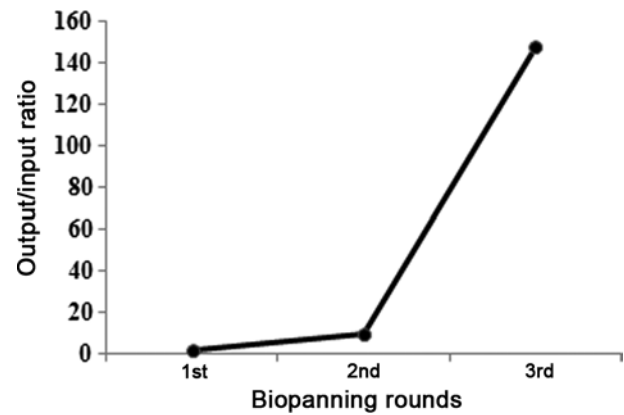


Figure 1. Phage output/input ratios in each biopanning round. The output/input ratios were 1.9×10^{-7} , 3.2×10^{-5} and 2.8×10^{-3} in the first, second and third rounds, respectively. The output/input ratio increased almost 150-fold from the first to the third round.

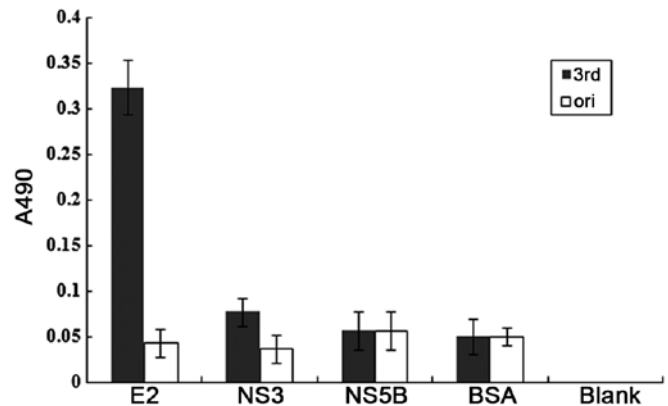


Figure 2. Binding of third round phages to HCV E2₆₆₁ and unrelated proteins, as determined by ELISA. The proteins coated to the ELISA wells were HCV E2₆₆₁, NS3, NS5B and BSA. HCV E2₆₆₁, NS3 and NS5B were prokaryotically expressed with a 6X His tag. The titers of the third round phages and original phages were both 1×10^{11} PFU/well.

150-fold, compared with the first round, demonstrating that effective biopanning of phages specifically binding to HCV E2₆₆₁ protein had occurred (Fig. 1).

Binding assays. As shown in Fig. 2, the binding reactivity of the third round phages was much higher in the HCV E2₆₆₁ protein as compared to other unrelated proteins. The original phages had similar reactivity in all the proteins.

Positive clones and their sequences. Twenty-five plaques were randomly selected from the third round phages, and their binding reactivity to the HCV E2₆₆₁ protein was determined individually using an ELISA. Clone no. 3 had the lowest affinity to E2₆₆₁ protein. Four clones with the highest affinities (nos. 7, 11, 17 and 18) were selected for subsequent analysis (Fig. 3).

Following DNA sequencing, the amino acid sequences of the selected clones were deduced and designated as C7, C11, C17 and C18, corresponding to the respective clone numbers (Fig. 4). The four peptides harbored the conserved motif WPWXXXR, where X is any residue, with a bias for H. This similarity suggested that the four peptides were

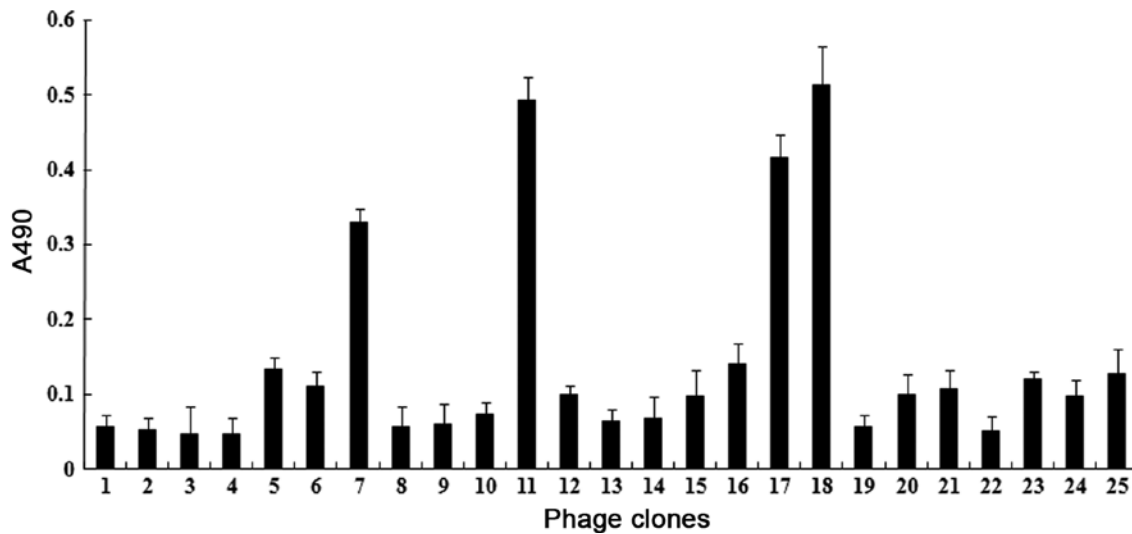


Figure 3. Binding of 25 randomly selected phages from the third round of biopanning to HCV E2₆₆₁. Binding to the HCV E2₆₆₁ protein was determined by an ELISA. Four clones (nos. 7, 11, 17 and 18) had higher affinities for binding HCV E2₆₆₁ protein compared to the other clones. Clone no. 3 had the weakest affinity for binding HCV E2₆₆₁ protein. The titers of the phage clones were 2×10^{10} PFU/well.

C07 H P W I P K R
 C11 W P W Q H H R
 C17 W P W H H V R
 C18 W P W H N H R

Figure 4. Sequences of positive phage clones from the third round of biopanning which bound HCV E2₆₆₁. Conserved amino acids are indicated by double underlines. The core epitope was WPWXXXR, where X is any residue, with a bias for H. Peptide C18 (WPWHNHR) derived from clone no. 18 had the highest affinity for HCV E2₆₆₁ protein of the four clones tested, and was synthesized.

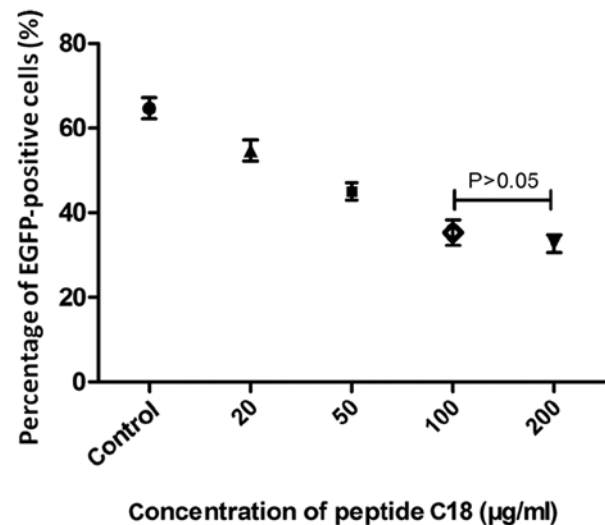


Figure 5. Determination of the concentration of peptide C18 used in inhibition experiments. Huh7.5 cells were infected with HCVpp in the presence of different concentrations of C18. The percentage of EGFP-positive Huh7.5 cells, reflecting the degree of HCVpp entry into host cells, was measured by flow cytometry.

capable of binding to the same site of the target protein E2₆₆₁. Furthermore, the four sequences were analyzed using BLAST; however, no homologous proteins were identified. The sequence VLRSDFK deduced from clone no. 3 was synthesized as an unrelated peptide.

Evaluation of the antiviral activity of peptide C18. Due to the high binding reactivity between clone no. 18 and HCV E2₆₆₁ protein, peptide C18 with the sequence WPWHNHR was synthesized. The most appropriate concentration of the peptide used in the inhibition assay was 100 µg/ml (Fig. 5). It is indicated that peptide C18 significantly inhibited HCVpp from entering Huh7.5 cells detected by flow cytometry. The HCVpp entry was notably inhibited in the presence of peptide C18, compared to the group of HCVpp and the group of the unrelated peptide (Fig. 6).

Furthermore, quantitative real-time RT-PCR demonstrated that the level of HCV RNA in HCVcc-infected Huh7.5 cells was significantly lower in the presence of peptide C18, compared to Huh7.5 cells incubated with HCVcc and the unrelated peptide (Fig. 7A). Western blotting revealed that the expression of the HCV core and NS5A proteins decreased in HCVcc-infected Huh7.5 cells incubated with peptide C18, compared to cells

incubated with HCVcc and the unrelated peptide (Fig. 7B). These results demonstrated that peptide C18 is able to block both HCVpp and HCVcc entering the host cells.

Discussion

Entry of hepatitis C virus (HCV) into cells may involve the cellular components CD81 tetraspanin (20,21), the scavenger receptor class B type I (SR-BI) (22,23), low-density lipoprotein receptor (LDLR) (24), the mannose-binding lectins DC-SIGN and L-SIGN (25), glycosaminoglycans (GAGs) (26), and the junction proteins claudin-1 (CLDN1) (27) and occluding (OCLN) (28). The HCV envelope glycoprotein E2 is also thought to play a major role in virus-cell attachment.

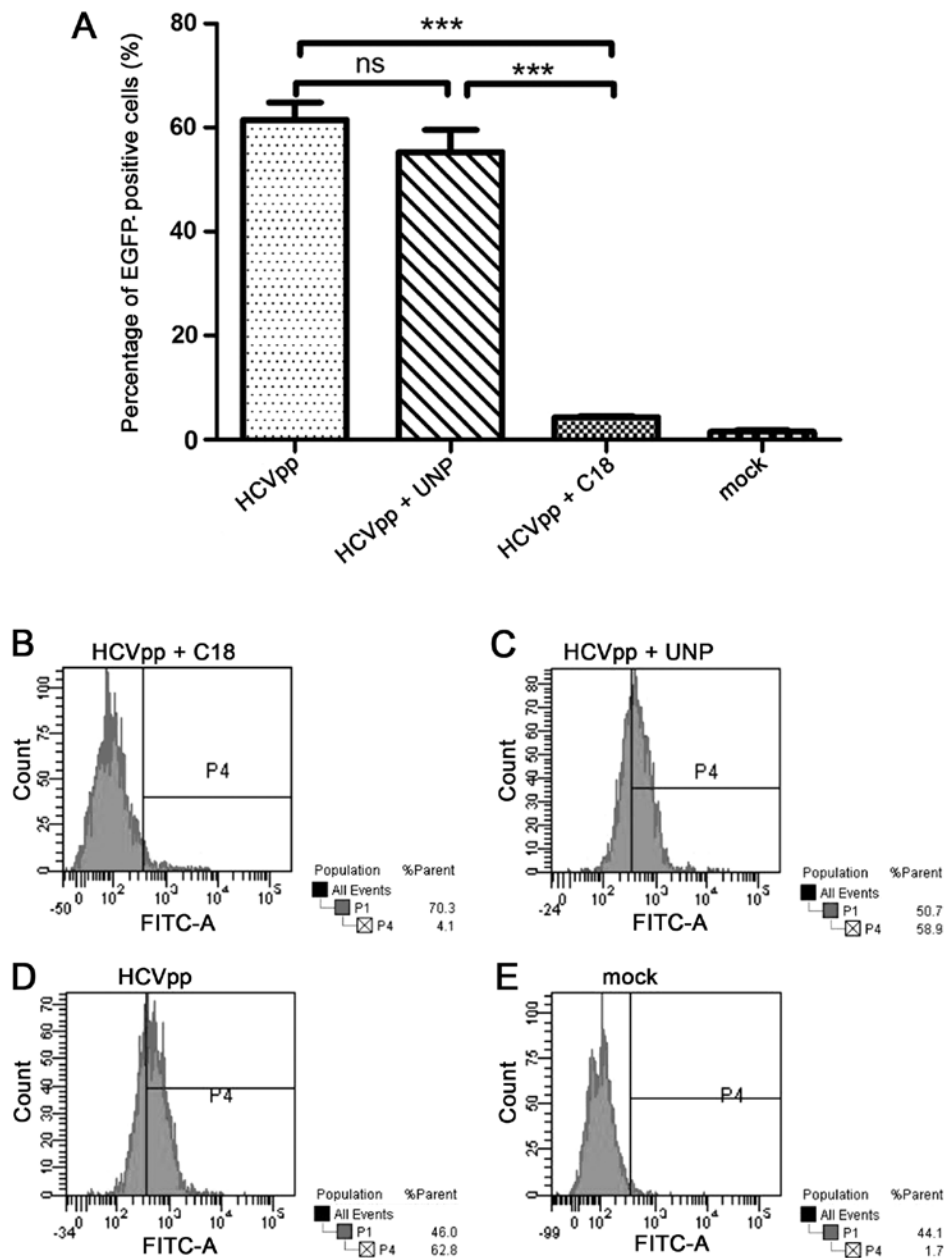


Figure 6. Peptide C18 inhibits the entry of HCVpp into Huh7.5 cells. (A) Huh7.5 cells were infected with HCVpp in the presence of C18 or an unrelated peptide (UNP). The percentage of EGFP-positive Huh7.5 cells was measured by flow cytometry. Data are presented as the mean \pm standard deviation of triplicate experiments (** $P < 0.001$; ns, 0.249). The results of a representative flow cytometric analysis of Huh7.5 cells infected with (B) HCVpp + C18, (C) HCVpp + UNP, (D) or HCVpp and (E) naïve Huh7.5 cells. Entry of HCVpp was significantly lower in the HCVpp + C18 group, compared with the HCVpp + UNP and HCVpp groups.

Inhibition of the process by which viruses bind to their target cells may provide an effective way to block viral entry and infection (10).

The emergence of entry inhibitors has derived from anti-HIV infection studies (29-31). Enfuvirtide, licensed in 2003, is the first drug in this class and provides significant clinical anti-HIV activity. Enfuvirtide is a 36 amino acid peptide that is based on the stem region of HIV gp41, a viral envelope protein that mediates the fusion of HIV with host cells (29). This new type of viral inhibitor may also provide a novel mechanism to treat other types of viral infection.

There have been some successful examples in the study of HCV entry inhibitors. Receptor-mimics or neutralizing anti-

bodies targeting HCV candidate cell receptors such as CD81, SR-BI and CLDN1 may be potential novel antiviral strategies (10,32). The CD81-like small peptide ATWVCGPCT, which aligns with 153-161 of the hCD81 sequence, is able to block the CD81 binding site of the HCV E2 protein (33). A CD81-binding peptide and mimotope of the HCV E2 protein, SPQYWTGPA, can competitively inhibit the binding of HCV E2 to native CD81-expressing MOLT-4 cells (34). However, cell receptor-based antagonists potentially affect the expression of these cell receptors and disrupt receptor associations with other cell surface proteins. Therefore, the identification of viral protein-based inhibitory compounds may be a superior and safer strategy.

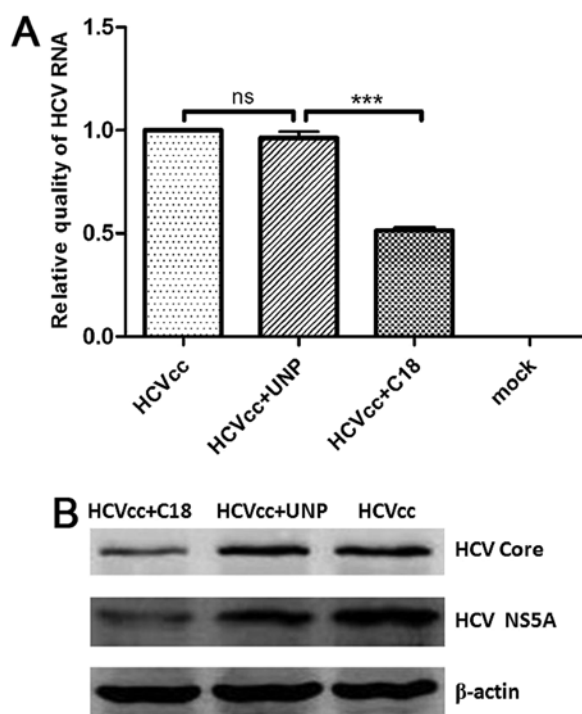


Figure 7. Peptide C18 inhibits the entry of HCVcc into Huh7.5 cells. (A) Huh7.5 cells were infected with HCVcc in the presence of C18 or an unrelated peptide (UNP). The entry of HCVcc into cells was measured by HCV-specific quantitative reverse-transcriptase RT-PCR and normalized to *GAPDH*. Results are expressed as the mean \pm standard deviation (copies/ml) of triplicate experiments. HCVcc entry was significantly lower in the HCVcc + C18 group, compared with the HCVcc + UNP and HCVcc groups, *** $P < 0.001$; ns, 0.171. (B) Entry of HCVcc into the cells was measured by western blotting using an anti-HCV core antibody and NS5A monoclonal antibody. β -actin was used as a loading control.

In the present study, we expressed truncated HCV E2₆₆₁ protein in *E. coli*, and selected the peptides which specifically bound to HCV E2₆₆₁ protein from a phage display peptide library. Phage display technology has been used in a number of studies for epitope mapping and the identification of peptides which bind to target proteins (33–36). The heptapeptide library used in this study contained a complexity of 2.7×10^9 individual clones, representing the entire obtainable repertoire of 7-mer peptide sequences ($20^7 = 1.28 \times 10^9$). As a result, effective panning was conducted after three rounds (Fig. 1). Although HCV E2₆₆₁ protein was prokaryotically expressed with a 6X His tag, the negative control HCV proteins NS3 and NS5B were also expressed with 6X His tags. Therefore, the third round phages did not bind to the 6X His tags, as they had a higher binding reactivity for E2₆₆₁ compared to the negative control proteins with the same tags (Fig. 2). Eventually, four peptides which were able to bind specifically to the HCV E2₆₆₁ protein were obtained. Of these peptides, C18, the candidate peptide with the greatest affinity for E2₆₆₁, was selected for subsequent analysis.

Investigation of virus-cell interactions were blocked until the development of HCVpp and HCVcc, which enabled efficient growth of HCV in cell culture and provided valuable tools to understand the viral life cycle and in the search for new antiviral compounds. Baldick *et al* screened a small molecule library and identified a potent HCV-specific

triazine inhibitor, EI-1, which blocked the cellular entry of a series of HCVpp and HCVcc with E1-E2 sequences prepared from various HCV isolates (genotype 1a, 1b and 2a) (37). Additionally, a 16-residue polypeptide containing a portion of the E2 transmembrane domain inhibited HCVpp infection at concentrations up to 50 mM (38). Hong *et al* prokaryotically expressed a 50 amino acid C-terminal region-truncated HCV E2 protein (genotype 2a) and performed biopanning using a phage display peptide against HCV-truncated E2. The peptide pep7-1 was found to notably decrease the infectivity of HCVcc in the cell culture (39).

Similarly, in the present study, it was shown that short peptides with a high affinity for E2₆₆₁ were capable of inhibiting the entry of HCV into cells, and serve as potential antiviral agents. The conserved motif WPWXXXR was identified from the HCV E2 protein with genotype 1a (Fig. 4). The representative synthetic peptide C18 demonstrated a good ability to inhibit the cell entry of HCVpp (with the E2 sequence from genotype 1b, Fig. 6) and HCVcc (with the E2 sequence from genotype 2a, Fig. 7). These results confirm the assumption that, viruses with different genotypes enter cells via a similar mechanism.

Notably, if the solution of peptide and HCVpp or HCVcc were not removed from the cells within 16 h, the entry of HCV (as indicated by the expression of the viral reporter EGFP, or HCV RNA and viral protein expression) was not significantly different in HCVpp- or HCVcc-infected cells incubated with C18 or the unrelated peptide (data not shown). This may be explained by the fact that if the HCVpp or HCVcc and peptide solution were not removed, the peptide may undergo degradation and/or endocytosis in the cells; thereby, weakening the inhibitory effect of peptide C18 on viral entry to the cells. Entry inhibitors block virus attachment and entry, and cannot exert an effect once the viruses have entered the cells.

Peptide C18 with the sequence WPWHNHR was identified in the present study and is a potential candidate for HCV therapeutic intervention. Entry inhibitors, a novel type of viral inhibitor, may be an effective anti-HCV strategy, regardless of the genotype of the virus, and may eventually provide a valuable component of the optimal therapy for HCV infection. A novel cocktail therapy, using a combination of entry inhibitors, HCV proteases and polymerase inhibitors, with a backbone of pegIFN/RBV, may have preferable, distinct modes of action and lead to resistance to HCV infection.

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