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A peptide derived from hepatitis C virus E2 envelope protein inhibits a post-binding step in HCV entry

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ABSTRACT

The HCV envelope proteins E1 and E2 are required for virus binding to cellular receptors and pH-dependent fusion with endosomal membranes. Envelope protein interactions within this multistep process may provide novel targets for development of antiviral agents. To identify E1 and E2 regions involved in critical steps of HCV entry, we screened an E1E2 overlapping peptide library for inhibition of infection using a lentiviral reporter vector pseudotyped with E1E2 envelope proteins. A 16-residue polypeptide containing a portion of the E2 transmembrane domain (Peptide 75) inhibited HCV pseudoparticle infection with an IC50 of approximately 0.3 μM and did not inhibit infection by VSV-g pseudoparticles at concentrations up to 50 μM. Structure–activity analysis of Peptide 75 showed that antiviral activity was dependent upon L-configuration and hydrophobic character, and that the native sequence was required for maximal activity. Peptide 75 did not show virocidal activity against HCV pseudoparticles or other viruses. Temperature-shift experiments showed that the peptide acted at a post-binding step and that inhibition was further increased when used in combination with an anti-CD81 antibody previously shown to inhibit pseudoparticle entry at a post-binding step. These data suggest that interactions involving the C terminal region of E2 may play an important role in the HCV entry process.

1. Introduction

Chronic infection of hepatitis C virus (HCV) affects 170 million people world wide. HCV shows a remarkable tendency to establish persistent infections and chronic liver disease, this can ultimately lead to cirrhosis and hepatocellular carcinoma (Chevaliez and Pawlotsky, 2007). The efficacy of the current standard therapy, peg-interferon and ribavirin, is less than satisfactory and is associated with side effects (Di Bisceglie et al., 2002), and no treatment is available to prevent new infection. Thus, more effective and better tolerated antiviral therapies are needed to treat chronic hepatitis C.

HCV is an enveloped virus and has a positive stranded RNA genome of ~9.6 kb (Reed and Rice, 2000). Its genome encodes a 3000 amino acid polyprotein, which is processed co- or post-translationally by host and viral proteases into at least 11 proteins including two envelope proteins E1 and E2 (Reed and Rice, 2000). HCV E1 and E2 are type I membrane proteins which consist of an N-terminal ectodomain and a C-terminal transmembrane domain. The transmembrane domains contain herterodimerization signals that allow E1 and E2 to form non-covalent heterodimers that are required for virus entry to the target cells (Cocquerel et al., 2002).

Host factors, including CD81, Scavenger Receptor Class BI (SRBI), Claudin-1, and occludin (Liu et al., 2009; Ploss et al., 2009) and most likely others, have been shown to play a critical role in HCV entry (Barth et al., 2006; Bartosch and Cosset, 2006; Cocquerel et al., 2006; Evans et al., 2007). Although direct interactions between HCV E2 and host factors including CD81 (Pileri et al., 1998) and SRBI (Scarselli et al., 2002) have been shown, the exact role of each receptor in HCV entry remains to be determined. HCV particles attach to the target cells through their interactions to cell surface receptors and are endocytosed in a clathrin-dependent manner (Blanchard et al., 2006). HCV entry also requires a low pH environment which is thought to trigger E1E2 protein conformational changes needed for fusion between viral and endosomal membranes (Hsu et al., 2003; Tscherne et al., 2006).

HCV is a member of the flaviviridae, which includes yellow fever virus (YFV), dengue virus (DENV), tick borne encephalitis virus (TBEV) and west Nile virus (WNV). The flaviviridae are a subgroup of the arboviridae, a large virus family which also includes the alphaviruses, such as Semliki Forest virus (SFV). After binding to cellular receptors, these viruses are taken up in endosomes. Endosome acidification activates structural rearrangements of the viral

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envelope proteins, exposing fusion polypeptides which then insert into endosomal membranes to mediate fusion of the viral membrane and release of the nucleocapsid into the cytoplasm. Based on sequence analysis and comparison with related viruses, the HCV E2 protein is likely to contain a class II fusion polypeptide (Yagnik et al., 2000), similar to those found in TBEV (Rey et al., 1995), DENV (Kuhn et al., 2002) and SFV (Lescar et al., 2001).

Short polypeptides derived from viral envelope sequences have been used to probe protein–protein interactions involved in viral entry. In the case HIV, a peptide mimetic of an essential region of gp41 involved in fusion has been successfully developed as an antiviral agent (Greenberg and Cammack, 2004). Peptide inhibitors that mimic regions of the DENV and WNV envelope proteins also have shown to inhibit viral entry in a sequence specific manner (Bai et al., 2007; Hrobowski et al., 2005).

In this study we used HCV pseudoparticles (HCVpp) as a surrogate model to screen for HCV E1E2 peptides that inhibit HCV entry. HCVpp contain full length HCV E1E2 envelope proteins assembled onto retroviral core particles containing a retrovirus genome harboring the luciferase gene (Bartosch et al., 2003; Hsu et al., 2003). These HCVpp were previously validated as useful tools to study HCV entry as they have tropism to liver cells and can be neutralized by HCV patient serum and E2 antibodies (Hsu et al., 2003). The recently developed HCV cell culture system (HCVcc) most closely resembles HCV infection and allows confirmation of results obtained from HCVpp system (Lindenbach et al., 2005; Yi et al., 2006). We identified one peptide that mimics the sequence in HCV E2 C-terminal domain and inhibited HCV entry. This peptide did not inhibit control pseudoparticles that have glycoproteins from vesicular stomatitis virus (VSVpp). HCVpp and VSVpp share the same retroviral particle core and differ in viral envelope proteins. Our results showed that this peptide inhibited HCV entry at a post-binding step and suggest that E2 C-terminus may play a role in HCV fusion into target cells.

2. Materials and methods

2.1. Plasmids and antibodies

The fragment encompassing aa 171–746 was PCR amplified from pFKl389/core3′ plasmid (Pietschmann et al., 2002) with forward primer GATCAAGCTTATGGGTTGCTCCTTTTCTATCTTC and reverse primer GATCAGATCTAGTGATAATCCGGAGTCGAACTCGATAGTC. The amplified product was digested with HindIII and XbaI and ligated into the plasmid pCMV-hygro (Invitrogen Corp., Carlsbad, CA). This plasmid was transfected into 293T cells and the expression of the envelope proteins was confirmed by Western blot. The pNL43E$^-$R$^-$luc plasmid (Chen et al., 1994) was obtained from NIH AIDS Reference and Reagent Program.

Anti-CD81 antibody, JS81, was obtained from BD Pharmingen (San Jose, CA). The anti-core antibody, C7-50, was obtained from Affinity BioReagents (Golden, CO).

2.2. Cells

293T cells were obtained from ATCC. Cells were propagated in DMEM with 10% FBS, 1% P/S, 4 mM L-glutamine and 1.5 g/L sodium bicarbonate and not allowed to grow to more than 50% confluency. The HuH7 cells were routinely maintained in DMEM medium supplemented with 4 mM L-glutamine, 1.8 mM sodium bicarbonate, 1× nonessential amino acids and 1 mM sodium pyruvate (Mediatech, VA). Primary hepatocytes were obtained from Cambrex (Walkersvill, MD) and were used within 1 week of their arrival.

2.3. Generation of HCV pseudoparticles and determination of infectivity

Single cycle HCV pseudoparticles were generated by transfecting 2 million 293T cells with 5 μg of each of pNL43E$^-$R$^-$luc and the HCV envelope expression plasmid, pCMV/E1-2, using Profection transfection kit from Promega (Madison, WI). The culture medium was refreshed 12–24 h after transfection and pseudoparticle-containing supernatant was collected 48 h after transfection. As a control, the pseudoparticles lacking viral envelope proteins were generated by transfection of the pNL43E$^-$R$^-$luc plasmid alone. These no envelope pseudoparticles were used as a control for non-specific entry events in infection assays.

To determine the amount of particle-associated p24 in supernatant, 1 ml of viral supernatant was pelleted through 100 μl of 20% sucrose cushion and the amount of p24 antigen in pellet was measured using a HIV-1 p24 ELISA kit (PerkinElmer, Boston, MA).

To determine the infectivity of HCVpp, HuH7 cells were seeded at 8000 cells in 90 μl media in 96-well plate. 50 μl of viral supernatant of two-fold titration was added to the cells 24 h after seeding. The cells were incubated at 37 °C with 5% CO_2 for an additional 72 h and were harvested for luciferase assay using the Bright-glo luciferase reagent from Promega. Pseudoparticle infectivity was determined by normalizing luciferase activity to particle-associated p24 (RLU/ng p24).

2.4. Tissue culture infectious HCV production and infection assay

HCV RNA was in vitro transcribed from plasmid pH77-S and electroporated into cells as previously described (Yi et al., 2006). Virus was concentrated 50–100-fold by addition of one-fourth volume of steril-filtered 40% (w/v) polyethylene glycol-8000 in PBS and overnight incubation at 4 °C. Virus precipitates were collected by centrifugation (8000 × *g*, 15 min) and resuspend in PBS.

HuH7 cells were seeded at 10,000 cell/well on 8-chamber slides (Nalge Nunc, Rochester, NY) 24 h before inoculation with 100 μl of concentrated virus. Cells were analyzed 96 h later for HCV core antigen expression by immunofluorescence as described in reference (Yi et al., 2006). The number of foci staining positive for core antigen in each well was counted.

2.5. Peptide library preparation

The peptide library was synthetically assembled on MBHA Rink amide or NovaSyn TG Sieber resin (at 5 μmol per peptide) in 96-well microtiter plate using a MultiPep synthesizer (Intavis AG, Germany) with standard Fmoc chemistry. After successful completion of the synthesis, the resins were washed with dichloromethane five times and completely dried under vacuum in the MultiPep. The microtiter plate was removed from the MultiPep and placed on top of a 96-well collection plate (2 ml volume per well). 200 μl of concentrated (80–90%) TFA with proper scavengers such as water and triisopropylsilane were added to the microtiter wells containing the assembled peptide resin. This cleavage process was repeated three additional times with 10 min intervals. The collected cleavage solution was let to stand at room temperature for 2 h to effect deprotection. Each peptide was then transferred to a 15-ml polypropylene tube containing 10 ml anhydrous ethyl ether. The tubes were spun down in a clinical centrifuge. The ether phase was carefully decanted. The precipitated peptides were taken up in water and lyophilized over night. LCMS analysis was conducted for each peptide to verify the molecular weight and assess purity. The peptides were finally dissolved in 100% DMSO and used in assays without further purification unless otherwise noted for selected bioactive peptides. The bioactive peptides were synthesized at larger scale (250 μmol) by an ABI model 431A peptide

synthesizer (Applied Biosystems, Foster City, CA) using the same chemistry as mentioned above. Peptide 75 and derivatives were highly hydrophobic and batches with ≥70% purity were used.

2.6. Antiviral activity assay

HuH7 cells were seeded at 8000 cells/well in 90 μl medium in a 96-well plate. Peptides were incubated with cells for 1 h at 37 °C and then 50 μl viral supernatant was added to the cells 24 h after seeding. The HCVpp and VSVpp stocks were pre-titered and dilutions of the stocks were used in infection assays to yield approximately 10,000–20,000 RLU. Cells were incubated at 37 °C with 5% CO_2 for additional 72 h and were harvested for luciferase assay (Promega, Madison, WI). Peptide stocks were prepared in 100% DMSO at 10 mg/ml and were initially screened at 100 μg/ml. Antiviral activity was measured comparing to vehicle control (0.5% DMSO).

3. Results

3.1. A peptide from HCV E2 protein specifically inhibits HCVpp entry

HCV pseudoparticle (HCVpp) infection of target cells requires HCV envelope proteins E1 and E2, which make specific interactions with target cell receptors and with each other during viral attachment and fusion process. We hypothesized that these interactions involve discrete polypeptide regions in E1 and E2 and that it might be possible to compete these interactions using free peptides derived from E1 and E2. To test this hypothesis, we designed a library of 16 residue peptides with 9 residue overlaps from the HCV genotype 1b con1 strain E1 and E2 envelope sequences. This library was then screened for inhibition using luciferase HCV pseudoparticles (HCVpp-luc) assays (Lohmann et al., 1999). In the initial screen, one peptide (Peptide 75) from a region which partially overlaps the E2 transmembrane domain inhibited HCVpp entry by more than 70% at 50 μM (Fig. 1A). Dose response studies subsequently showed that Peptide 75 inhibited HCVpp infection of Huh-7 human hepatoma cells with EC_{50} and EC_{90} values of 0.3 ± 0.4 μM (95% CI, $n = 10$) and 14 ± 12 μM (95% CI, $n = 8$), respectively.

To determine whether the inhibition of infection was specific for pseudoparticles carrying HCV E1E2, we tested the inhibitory activity of Peptide 75 against control pseudoparticles produced using the vesicular stomatitis virus G protein (VSVpp). Like HCVpp, infection of cells by VSVpp requires clathrin-dependent endocytosis pathway, and both pseudoparticles contain the same lentiviral vector core and RNA, therefore VSVpp serve as post-entry and cytotoxicity control for HCVpp. Peptide 75 did not show significant inhibition of control VSVpp infection of Huh-7 cells at concentrations up to 50 μM. In addition, Peptide 75 did not inhibit infection of U87 cells by a second control virus, HIV, at concentrations up to 50 μM (Fig. 1B). To determine whether the ability of Peptide 75 to block infection by HCVpp was restricted to HuH7 cells, we also tested its inhibitory activity using human primary hepatocytes. Peptide 75 inhibited HCVpp infection of primary human hepatocytes by 50% at 0.3 μM ($n = 1$) (Fig. 1C). These experiments showed that Peptide 75 inhibited infection by HCVpp but not VSVpp or HIV, suggesting that its inhibitory effect may be specific to HCV.

3.2. Peptide 75 inhibits infection by cell culture adapted HCV

Our initial series of experiments demonstrated that Peptide 75 inhibited infection of Huh-7 cells and primary human hepatocytes by HCV pseudoparticles. To further characterize the ability of Peptide 75 to inhibit HCV entry, we tested its ability to inhibit infection by cell culture adapted HCV (HCVcc). HCVcc particles were generated using strain H77S, a cell culture adapted replication competent virus derived from genotype 1a strain H77 (Yi et al., 2006). The amino acid sequences of E1 and E2 from H77S clone and con1 are 81% identical overall and have three amino acid differences within the portion of E2 corresponding to Peptide 75 (Fig. 1A). H77S grows less efficiently than the genotype 2a virus JFH-1, yielding viral titers that are about 1/100 those produced by JFH-1 (Yi et al., 2006). Because H77S titers were limited, we tested the inhibitory effect of Peptide 75 in two independent experiments at 5 μM (approximately 17 x IC_{50}). Infection of Huh-7 cells with H77S HCVcc was followed by counting foci immunostained with anti-HCV core antibody. As shown in Table 1, treating Huh-7 cells with 5 μM Peptide 75 before and during incubation with HCVcc reduced the number of core-positive foci by 80–90% (Table 1). Together with the previous experiments, these results showed that Peptide 75 is capable of inhibiting genotype 1 HCV infection by both pseudoparticles and by cell culture adapted replication competent HCVcc-H77S.

Table 1
Peptide 75 inhibits H77S HCVcc infection in cell culture.

	ffu/well	
	Experiment 1	Experiment 2
DMSO	20, 20	8, 10
Peptide 75 5 μM	4, 0	2, 2

Peptide 75 was also tested with HCVcc particles made from genotype 2a JFH isolate (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The amino acid sequences of E1 and E2 from JFH clone and con1 are 64% identical overall and have 8 amino acid differences within the 16 amino acids of E2 corresponding to Peptide 75 (Fig. 1A). Treating Huh-7 cells with 5 μM Peptide 75 before and during incubation with JFH HCVcc did not have an effect on infection comparing to vehicle control (data not shown).

3.3. Amino acid sequence requirement for peptide activity

To determine the essential amino acid residues that are required to inhibit HCVpp entry, sequential deletions were generated at the N- and C-termini (Table 2). Peptides with the first six amino acids deleted from the N-terminus retained 80–90% of inhibitory activity; however, deletion of the five amino acids from the C-terminus resulted in loss of up to 80% of the activity. The sensitivity of this peptide to the C-terminus deletions indicates the importance of the residues from the transmembrane domain for its activity. The 13-mer peptide, A-I-K-W-E-Y-V-L-L-L-F-L-L (missing the first three amino acids from the N-terminus), retained about 90% activity compared with the original 16-mer Peptide 75, and was used for further studies.

A scrambled peptide of the 13 amino acids, L-K-L-F-E-V-Y-L-I-L-W-L-A, was 10-fold less active compared to the wild-type sequence in the entry assay (Table 3). The low level activity observed for

Table 2
Deletion analysis of Peptide 75.

Peptide sequence	HCVpp %inhibition[a]
S-F-A-I-K-W-E-Y-V-L-L-L-F-L-L	91
F-A-I-K-W-E-Y-V-L-L-L-F-L-L	95
A-I-K-W-E-Y-V-L-L-L-F-L-L	92
I-K-W-E-Y-V-L-L-L-F-L-L	88
K-W-E-Y-V-L-L-L-F-L-L	78
W-E-Y-V-L-L-L-F-L-L	82
V-S-F-A-I-K-W-E-Y-V-L-L-L-F-L	63
V-S-F-A-I-K-W-E-Y-V-L-L-L-F	49
V-S-F-A-I-K-W-E-Y-V-L-L-L	49
V-S-F-A-I-K-W-E-Y-V-L-L	44
V-S-F-A-I-K-W-E-Y-V-L	17

[a] All peptides were tested at 50 μM.

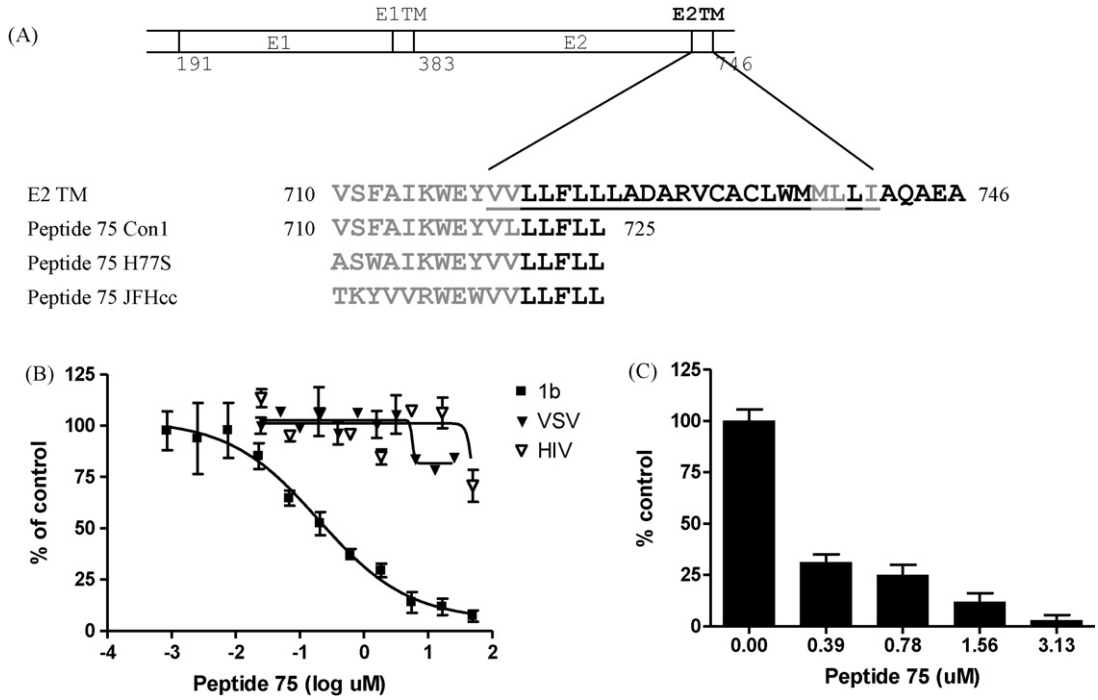

Fig. 1. Peptide 75 from E2 specifically inhibited HCVpp entry. (A) Schematic representation of HCV glycoprotein E1 and E2 region. The transmembrane (TM) domains in E1 and E2, and the number for the last amino acid residue in core, E1 and E2 are indicated. The sequence of the E2 TM domain and vicinity is shown and the amino acid residues in TM are underlined (Cocquerel et al., 2002). Alignment of Peptide 75 with the E2 sequence is shown. The residues in darker color are more than 80% conserved among genotypes 1–6 (Ping et al., 2002). (B) Peptide 75 (aa 710–725) inhibited HCVpp-con1 in a dose-dependent manner. Cells and Peptide 75 were pre-incubated for 1 h, then HCVpp-con1 or VSVpp were added and incubated for 72 h before cells were harvested and analyzed for luciferase activity. (C) Fresh human hepatocytes in 96-well plate was purchased from Lonza Walkersville, Inc. (Walkersville, MD) and was treated with Peptide 75 in duplicate wells and infected with HCVpp as described in (B).

this scrambled peptide could be due to the overall hydrophobicity retained in the scrambled peptide, and/or the preeminent importance of perhaps as few as two or three of the residues or the accumulative small contributions from residues in the right places. To address the question of whether the overall hydrophobicity of the peptide was the key feature, the equally hydrophobic retro (reverse of sequence), inverso (replacement of levo- with dextro-configuration), and retro-inverso versions of the 13-mer peptide were synthesized and tested. The inverso peptide was 20-fold weaker (IC_{50} = 7 μM, n = 3) than the wild-type sequence, suggesting the importance of L-configuration for the activity. The retro and retro-inverso peptides were not active when tested up to 30 μM. This suggested that the native amino acid sequence was important and that the overall hydrophobic nature of the peptides was not sufficient for activity.

The contribution of each amino acid to the activity of the 13 amino acid peptide was examined by Gly, Ala, Pro and Lys scanning analysis. The results are summarized in Table 4. Glycine substitutions eliminated the side chains, and therefore were expected to greatly increase local backbone conformational flexibility. This enhanced conformational freedom can effectively dilute out the presence of bioactive conformations, leading to weaker activities. Most glycine mutations did not dramatically (<5-fold) increase the IC50, with the exception of W716G which increased the IC50 by 9.4-fold. In contrast to the Gly scan, Ala substitutions serve to maintain a similar backbone conformational rigidity. The Ala mutants all exhibited potencies similar to the wild-type peptide with the exception of L722A. These data suggest that the inhibitor activity may be contributed collectively by many residues. Introducing proline into the peptide has the potential to create local contortions in the peptide backbone and thus allows determination if a less aggregating and more water soluble peptide remains active. Mutations were tolerated in the N-terminal half but changes at C-terminal end significantly reduced activity. These data are consistent with the results of the deletion analysis which suggested that the C terminal residues were critical for activity. The Lysine residue is positively charged under the assay condition and thus reverses the local hydrophobic character of the peptide. Introduction of lysine at N-terminal (713–719) were tolerated, however, substitutions in the C-terminus (720–725) significantly reduced activity.

Table 3
Amino acid sequence requirement for peptide activity.

Sequence	Name	IC_{50} (μM)
A-I-K-W-E-Y-V-L-L-L-F-L-L	Wild-type sequence	0.3 ± 0.1 (n = 6)
a-i-k-w-e-y-v-l-l-l-f-l-l	Inverso (D isomers) sequence	6.7 ± 2.8 (n = 3)
L-L-F-L-L-L-V-Y-E-W-K-I-A	Retro sequence	>30 (n = 3)
l-l-f-l-l-l-v-y-e-w-k-i-a	Retro-inverso sequence	>30 (n = 3)
L-K-L-F-E-V-Y-L-I-L-W-L-A.	Scramble sequence	>3 (n = 3)

3.4. Peptide 75 is not virocidal to HCVpp

To determine the mechanism of action of Peptide 75, Peptide 75 was added to HCVpp infection at different time points relative to the inoculation. Peptide 75 was active when added together with HCVpp to HuH7 cells, and was ineffective when added 4 or 8 h after inoculation (Fig. 2A). This suggested that Peptide 75 either inhibited an early step in HCVpp infection, i.e. entry, or it was virocidal to HCVpp particles.

To determine if Peptide 75 could inactivate HCVpp particles, viral supernatant was incubated with Peptide 75 and HCVpp was purified with centrifugation through sucrose cushion to remove excess amount of peptide. The slight infectivity decrease in peptide-treated HCVpp was likely caused by residue peptide precipitates after incubation at 4 °C as shown by that addition of centrifuged peptide to untreated HCVpp also caused a similar reduction in

Table 4
Gly, Ala, Pro and Lys scanning analysis.

Fold increase in IC50	A713	I714	K715	W716	E717	Y718	V719	L720	L721	L722	F723	L724	L725
G	+	+	NT	++	+	+	NT	NT	+	+	+	+	+
A	+	+	+	+	+	+	+	+	+	++	+	+	+
P	+	+	+	+	+	+	+	+++	+++	+++	+++	+++	+++
K	+	+	+	+	+	+	+	+++	++	+++	+++	+++	+++

NT: not tested. Peptide was not available.
+, >0.4-fold, <5-fold; ++, >5-fold, <10-fold; +++, >10-fold.

infection (Fig. 2B). Therefore, the infectivity of HCVpp was not significantly affected by incubation with Peptide 75.

3.5. The peptide inhibits a post-binding step in HCVpp entry

During the entry process of HCVpp, the peptide may disrupt the binding of pseudoparticles to cells or it may interfere with post-binding steps, e.g. membrane-membrane fusion. To address this, cells and HCVpp were co-incubated with the peptide at 4 °C for 30 min to allow HCVpp to bind to the cells. The cells were then washed and incubated at 37 °C to allow post-binding events to occur. When Peptide 75 was incubated with cells and HCVpp at 4 °C for 30 min, it inhibited HCVpp infection by 60–80% at concentrations up to 50 μM (Fig. 3A). This inhibition was not seen when cells and the peptide were incubated with VSVpp. It is possible that the peptide disrupted HCVpp binding to cells. Alternatively, due to the highly hydrophobic nature of the peptide, the peptide may have remained associated with cell membrane despite washing and interfered with post-binding steps that occurred at 37 °C.

To differentiate these two possibilities and to further examine the peptide's effect(s) on viral entry, cells were exposed to HCVpp at 4 °C for 30 min in the absence of the peptide, and then washed to remove excess HCVpp. Cells were then moved to 37 °C, and various concentrations of Peptide 75 were added immediately (referred to as "0 hour" in Fig. 3B and blow) or after 4 h of incubation at 37 °C ("4 hour"). Peptide 75 remained effective in inhibiting HCVpp entry when added at 0 h after HCVpp had been allowed to bind to cells at 4 °C for 30 min ("0 hour" in Fig. 3B). However, when addition of Peptide 75 was delayed until 4 h after HCVpp-exposed cells had been moved to 37 °C, no inhibition of entry was observed ("4 hour" in Fig. 3B). The EC_{50} of Peptide 75 in this experiment was 0.2 μM, comparable to the EC_{50} observed when the Peptide 75 was present during the entire infection process (Fig. 1B). This experiment showed that antiviral activity of Peptide 75 was not reduced when it was added after virus and cells had been incubated together at 0° for 30 min.

3.6. Combination study using Peptide75 and an anti-CD81 antibody, JS81

CD81 is a member of the tetraspanin membrane protein superfamily whose members function to organize signaling complexes on the cell surface (Levy et al., 1998). CD81 was shown to bind to truncated soluble E2 protein (Pileri et al., 1998). In HCVpp entry assay, CD81 is required but not sufficient for infection. An antibody to CD81, JS81, was previously shown to block HCVpp entry; reportedly at a post-binding step (Cormier et al., 2004). We tested the combined effect of JS81 and Peptide 75 in blocking HCVpp entry. When JS81 was titrated by itself, it inhibited HCVpp entry with EC_{50} and EC_{90} values of 0.5 nM (0.08 μg/ml) and 1.7 nM (0.3 μg/ml), respectively. These values are consistent with previous reports. Combination experiments were performed using 10 concentrations of Peptide 75 and 8 concentrations of JS81 in a matrix format. A decrease in JS81 EC_{90} value in the presence of increasing amount of Peptide 75 was observed and *vice versa* (Fig. 4). The experiment was repeated by mixing JS81 and Peptide 75 at their respective IC50 concentrations and testing titrations of the two reagent mixture as suggested by the combination index method (Chou and Rideout, 1991; Johnson et al., 2004). The combination index, which was calculated based on the IC_{50}s for each agent alone and in combination, was 1.1 ± 0.4 (95% CI, $n = 4$), this value also suggested an additive interaction between these two agents.

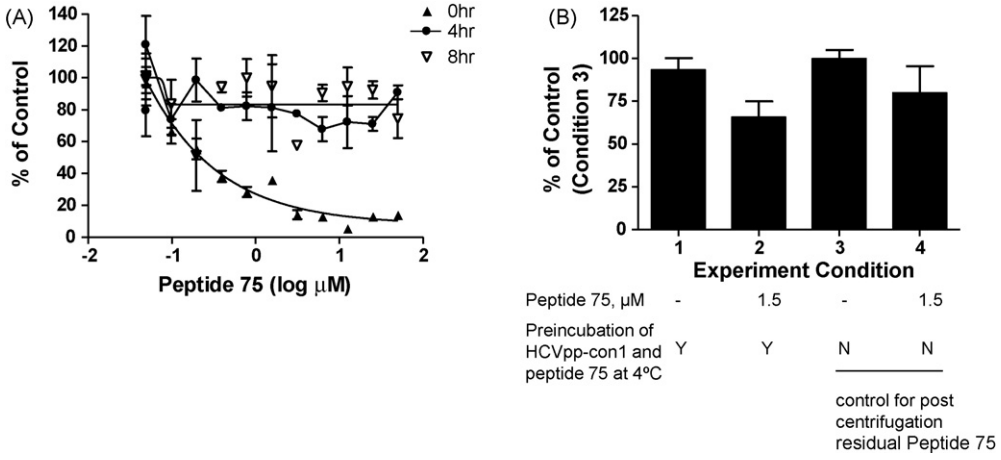

Fig. 2. Peptide 75 inhibited HCVpp at an early step and was not virocidal to HCVpp. (A) Peptide 75 inhibited HCVpp at an early step. Peptide 75 was added at the start (filled triangle), after 4 h (filled circle) or after 8 h (inverse hollow triangle) of infection. (B) Peptide 75 was not virocidal to HCVpp. Viral supernatant containing HCVpp-con1 particles were incubated with (experiment condition 2) or without (condition 1) 1.5 μM of Peptide 75 at 4 °C for 30 min followed by centrifugation as described (Hsu et al., 2003). The pellets were resuspended in HuH7 medium and used to infect HuH7 cells. To monitor the effects of Peptide 75 precipitates that might have formed during incubation at 4 °C on infection, Peptide 75 alone was incubated at 4 °C for 30 min and centrifuged. Untreated HCVpp viral supernatant alone (condition 3) or mixed with pellet from centrifuged Peptide 75 (condition 4) were used to infect Huh7 cells.

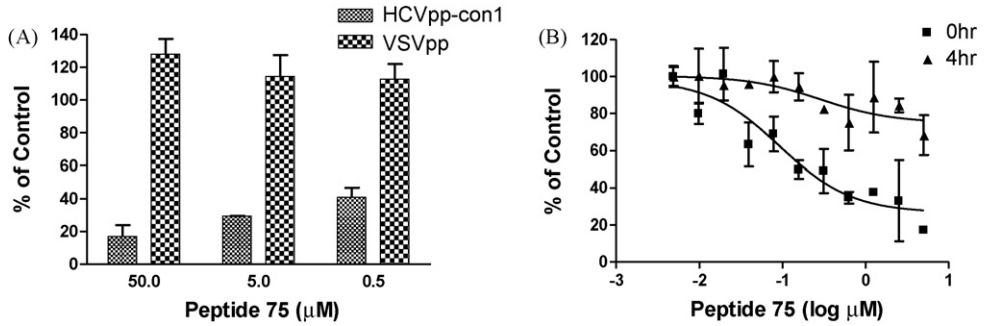

Fig. 3. Peptide 75 inhibited HCVpp at a post-binding step. (A) Peptide 75 appeared to inhibit HCVpp binding to cells. Cells and HCVpp or VSVpp were incubated with increasing amount of Peptide 75 at 4 °C, washed, and moved to 37C for 3 days before collected for luciferase assay. (B) Peptide 75 inhibited HCVpp at a post-binding step. Cells and HCVpp were incubated at 4 °C for 30 min, washed, and moved to 37 °C. Peptide 75 was added at 0 h (square) or 4 h (triangle) after cells were moved to 37 °C and incubated for 3 days before cells were harvested for luciferase assay.

4. Discussion

Inhibition of viruses at the stage of viral entry provides a target for therapeutic intervention, as evidenced by the recent development of HIV entry inhibitors (Rusconi et al., 2007). It is possible that a HCV entry inhibitor used in combination with protease and/or polymerase inhibitors would, by targeting multiple steps of viral infection cycle, have advantages over single drug therapy in reducing viral titers as well as in suppressing the emergence of viral resistance.

The best characterized peptide inhibitor of virus entry is Enfuvirtide (T20), which mimics the N terminal sequence in HIV fusion protein, gp41. This protein together with gp120 mediates viral attachment and fusion with host cells. The binding of gp120 to CD4 and CCR5 or CXCR4 leads gp41 to extend and interact with the host cell membrane. Subsequently, gp41 re-folds to form a trimer of hairpin structure to bring the viral and host membranes together. Enfuvirtide targets the extended state of gp41 and prevents refolding of gp41 from occurring and therefore inhibits HIV entry into host cells (Poveda et al., 2005; Root and Steger, 2004). HIV gp41 is a class I fusion protein, which contains two prominent alpha helices (Weissenhorn et al., 2007). Peptide inhibitors were also reported for dengue virus and West Nile virus, which belongs to the flavivirus family. The envelope glycoprotein (E) of flaviviruses, such as dengue virus (DENV) (Kuhn et al., 2002) and tick-borne encephalitis virus (TBEV) (Rey et al., 1995) are class II fusion proteins which comprise predominantly of beta sheets (Weissenhorn et al., 2007). Peptides from DENV and West Nile virus envelope sequences that are predicated for protein–protein and protein–lipid interactions were tested in infection assays. Peptides that inhibit infection in a sequence specific manner at non-cytotoxic concentrations were identified. However, it was not clear if these peptides inactivate free virion or inhibit at subsequent steps in viral infection cycle.

When this manuscript was in preparation, a virocidal peptide that inhibited HCV JFH infection in cell culture was identified by Cheng et al. (2008). In the study by Cheng et al., overlapping peptides that encompass the entire HCV genome (H77 strain) were

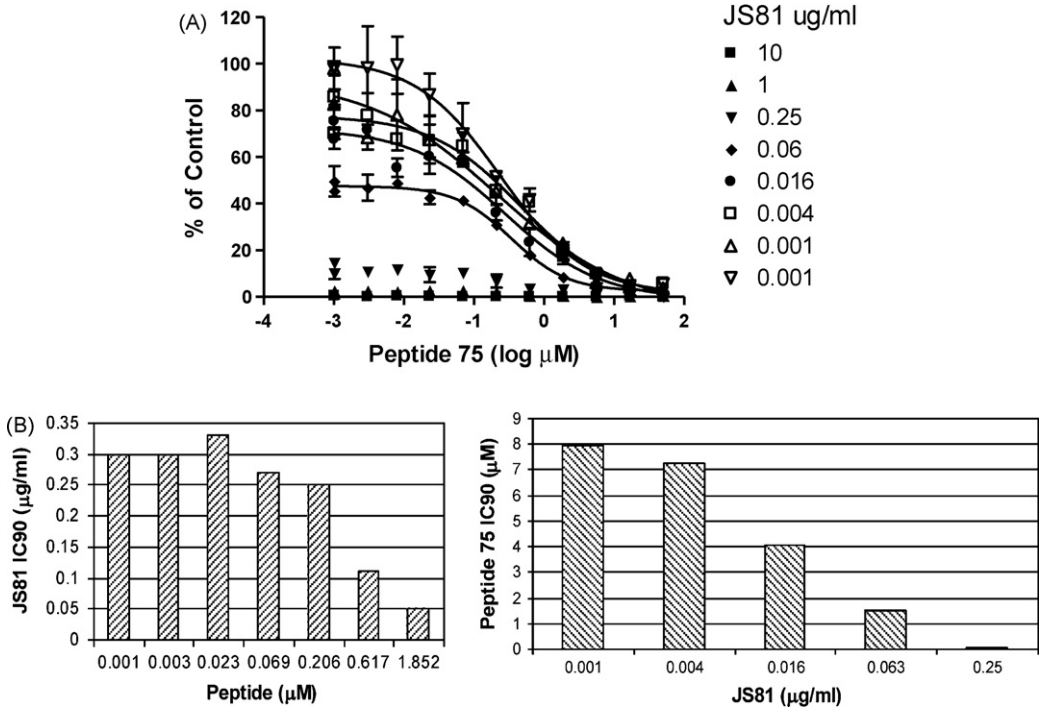

Fig. 4. Combination studies of Peptide 75 and JS81 antibody. (A) The dose response curves of Peptide 75 in the presence of increasing amount of JS81. (B) Decrease in EC_{90} of JS81 and Peptide 75 in the presence of Peptide 75 and JS81, respectively.

screened with the recently published HCV JFH infection system in a focus reduction assay. The most active peptide identified in the assay, peptide C5A, was found to inhibit viral expansion by greater than 100,000 fold. This peptide, which was derived from the NS5A N terminus, also inactivated intracellular and extracellular HCV particles. C5A activity was dependent on its hydrophobicity, α-helical structure and amino acid composition, but not its primary amino acid sequence. C5A was shown to permeabilize membranes of HCV and other viruses from flavivirus family. A second peptide described by Cheng et al., $E2_{701}$, which partially overlaps the N-terminus of Peptide 75 was 10,000 fold less active than C5A against JFH in the viral expansion assay. Its effect on virus particles was not described. Peptide 75 was not active against JFH HCVcc in our assay. This suggests that either the 9 amino acids shared with $E2_{701}$ are not sufficient to confer JFH-inhibitory activity in the context of Peptide 75 or that the dynamic range of our assay is not sufficient to detect the low level inhibitory activity of $E2_{701}$.

Development of HCVpp and HCVcc systems has had a major impact on the study of HCV entry. Like flaviruses, HCV enters cells through interaction with receptors on host cell surface followed by clathrin-dependent endocytosis (Diedrich, 2006). HCV entry requires a low pH environment (Hsu et al., 2003; Tscherne et al., 2006), which most likely triggers rearrangement of the envelope proteins and exposure of the fusion peptide, allowing it to interact with the target membrane to initiate the fusion process, as seen in dengue (Kuhn et al., 2002) and tick-borne encephalitis viruses (Rey et al., 1995). Using a similar strategy to Cheng et al., we identified a peptide that specifically inhibits HCVpp entry into target cells. Our data suggests that Peptide 75 was not virocidal and inhibited a post-binding step in HCV entry. Deletion and scanning analysis demonstrated that the hydrophobic nature in the C terminus of the peptide is required but not sufficient for its activity. Use of the native E2 peptide sequence and L-amino acids were required for activity of Peptide 75. Our study suggests that the E2 C-terminus may play a role in HCV fusion with target cells and that key interactions may be competed by a homologous peptide. Consistent with this hypothesis, Ciczora et al. identified mutations in the E2 transmembrane domain that altered the fusion properties of HCVpp (Ciczora et al., 2005). These mutations had no effects on formation of E1E2 heterodimers or incorporation of E2 into viral particles.

HCV entry is a multiple-step process and several host proteins have been identified as receptors, including CD81, SRBI, Claudin I and occludin (Barth et al., 2006; Bartosch and Cosset, 2006; Cocquerel et al., 2006; Evans et al., 2007). It is clear from recent work that more than one receptor is required for HCV entry. It is possible that engagement of one receptor causes conformational changes in the HCV envelope proteins leading to interaction with additional receptors and endocytosis. The anti-CD81 antibody, JS81, inhibits HCVpp and HCVcc infection at a post-binding step (Cormier et al., 2004), possibly by affecting conformational changes in HCV envelope proteins and their interaction with additional receptors. Our data suggests that Peptide 75 inhibits fusion triggered by exposure to low pH. Consistent with the hypothesis that Peptide 75 and JS81 act at different steps in the entry process, our results showed that Peptide 75 and JS81 had additive inhibitory effects on HCVpp infection.

Our study demonstrates that the steps after HCV particle binding to the cell surface can be inhibited with a peptide from the E2C terminus, suggesting that this region plays an important role in viral entry. Insights into the HCV entry process will help to identify inhibitors that can be potentially used to prevent new infection of cells and in combination therapy with HCV protease or polymerase inhibitors to suppress emergence of resistance.

Transparency declarations

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