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Short Communication

A cyclic peptide mimic of an RNA recognition motif of human La protein is a potent inhibitor of hepatitis C virus

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ABSTRACT

Due to limited available therapeutic options, developing new lead compounds against hepatitis C virus is an urgent need. Human La protein stimulates hepatitis C virus translation through interaction with the hepatitis C viral RNA. A cyclic peptide mimicking the β-turn of the human La protein that interacts with the viral RNA was synthesized. It inhibits hepatitis C viral RNA translation significantly better than the corresponding linear peptide at longer post-treatment times. The cyclic peptide also inhibited replication as measured by replicon RNA levels using real time RT-PCR. The cyclic peptide emerges as a promising lead compound against hepatitis C.

1. Introduction

Hepatitis C has emerged as a major challenge to the medical community with limited therapeutic options. The NS3 protease of the virus is a major drug target (Lamarre et al., 2003). Due to potential resistance and genotype specificity of molecules targeting the NS3 protease, development of new drug leads and validation of new targets is of high priority in this disease area (Pawlotsky, 2011). The mechanism of initiation of internal translation of hepatitis C virus (HCV) RNA is unique and fundamentally different from the cap-dependent translation of the host cell mRNAs. Human La protein plays an important role in the IRES mediated translation of HCV (Ali et al., 2000). Previously it was shown that a heptapeptide corresponding to an RNA recognition motif [RRM (112-184)] of the human La protein inhibits IRES mediated translation of HCV by competing with the human La protein for the HCV RNA. In this article, a cyclic peptide mimic of this motif is shown to inhibit the HCV translation and replication, more potently and at longer time scales, making it a promising lead compound for further development.

2. Materials and methods

2.1. Peptide synthesis and purification

Peptide synthesis methodology is given in Mondal et al., 2008.

2.2. NMR spectroscopy

The peptides were dissolved in 20 mM (*D11*)-Tris–HCl buffer, pH 7 containing 0.1 M NaCl, and 10% D_2O using WATERGATE water suppression. All spectra were recorded at 10 °C on a Bruker Avance 600-MHz NMR spectrometer fitted with TCI cryoprobe (Bruker Biospin, Switzerland). Total correlation spectroscopy (TOCSY; mixing time, 80 ms) and nuclear overhauser effect spectroscopy (NOESY) (mixing time, 400 ms) spectra were run in the water suppression mode. Structure was determined as described before Mondal et al., 2008.

2.3. Plasmid

The bicistronic plasmid construct containing HCV IRES in between renilla luciferase and firefly luciferase gene (RLuc-HCV-IRES-FLuc) was cloned into the pCDNA3.1 vector (Mondal et al., 2008).

2.4. Transient transfection and peptide transduction

Cell culture and other details are as per Mondal et al. (2008); Ray and Das (2011).

2.5. Real time RT-PCR

The monolayer Huh7 cells harboring the HCV monocistronic replicon (Frese et al., 2003) was overlaid with arginine tagged peptides (linear or cyclic or non-specific) at different concentrations.

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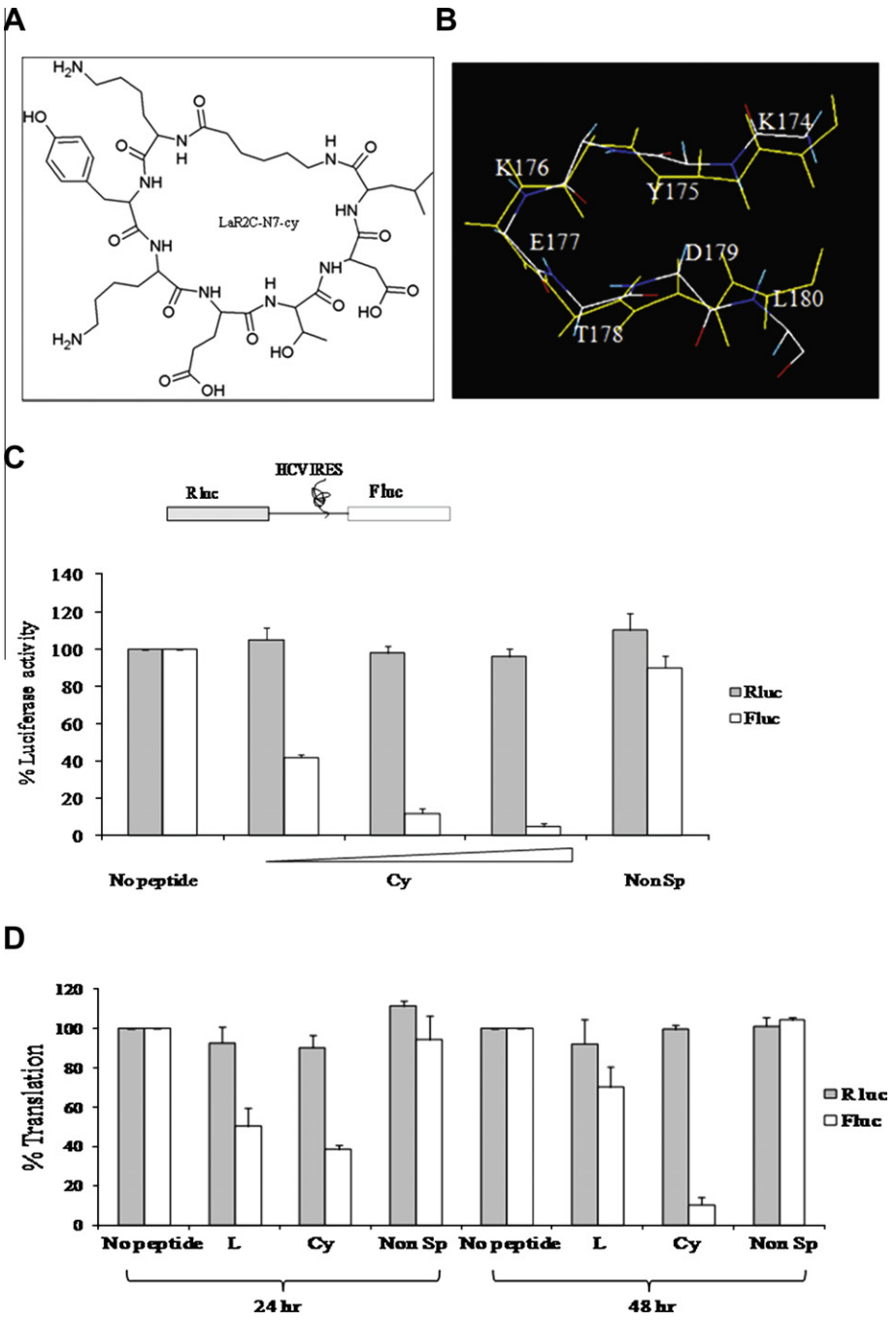

Fig. 1. (A) The chemical structure of LaR2C-N7-cy. (B) Structural alignment of LaR2C-N7-cy (CPK, only the KYKETDL part is shown) and the corresponding region of human La protein, PDB ID-1S79, 174-180AA) (Yellow). The alignments were done by online structure alignment server "SuperPose Version 1.0"15 and then the color was changed by Swiss-PDB (Maiti et al., 2004). (C) Huh7 cells were transfected with 2 μg of HCV bicistronic construct followed by peptide transduction in the absence or presence of increasing concentrations (2, 4, 8 μM) of DR_6-LaR2C-N7-cy (indicated as Cy). Twenty-four hours post transduction luciferase assay was performed and relative RLuc and FLuc activities were plotted. Grey bar indicates R luc activity and white bar indicates F luc activity. (D) Huh7 cells were transfected with 2 μg of HCV bicistronic construct followed by peptide transduction (2.5 μM). Twenty-four and 48 h post transduction, cells were lysed and luciferase assay was performed. Percentage translation was plotted on *Y* axis taking different time points on *X* axis. L refers to DR_6-LaR2C-N7 and Cy refers to DR_6-LaR2C-N7-cy. A DR_6 non-specific peptide (2.5 μM) was used as a negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

After 24 h, total RNA was isolated using TRI reagent (Sigma) and reverse transcribed using HCV 5′ primer and GAPDH 3′ primer by RevertAid™ M-MuLV RT (Fermentas) to detect the level of HCV negative strand RNA. Resulting cDNA was used for PCR amplification corresponding to HCV IRES using a real time assay mixture (Finnzymes) as per manufacturer's instructions. The data were analyzed by using ABI-Prism's real time PCR machine. GAPDH was used as an internal control.

2.6. Western blotting

Concentrations of cell lysates were assayed using Bradford reagent (Bio-Rad) and equal amounts of extracts were separated by SDS–PAGE and transferred to nitrocellulose membrane (PALL Life Sciences). Analysis was done using rabbit polyclonal anti-NS5B antibody (ab35586; Abcam, Cambridge, MA) followed by secondary antibody (horseradish peroxidise conjugated anti-rabbit IgG;

Sigma). Rabbit monoclonal anti-actin peroxidase antibody (Sigma) was used as an internal control for equal loading.

2.7. MTT assay

MTT assay was used to measure the toxicity of peptides using standard protocol (Loske et al., 1998). Briefly, Huh7 cells were seeded in 96-well plate at a density of 5000 cells per well in DMEM at 37 °C with 5% CO_2. Peptides were added to final concentrations of 2, 4, 8, 20, 40, 80, 100, 200 μM. The plate was incubated for further 48 h under the same conditions. 20 μl MTT (Sigma) solution (5 mg/ml in phosphate buffered saline) was added to each well and incubated at 37 °C for 4 h. MTT solution was decanted off and formazan was extracted from the cells using 100 μl of DMSO per well. Color intensity was measured using ELISA plate reader at 550 nm.

3. Results and discussion

3.1. Design of the cyclic peptide

Previously we have reported that a heptapeptide from human La protein, LaR2C-N7, has inhibitory activity against hepatitis C viral translation (Mondal et al., 2008). This peptide sequence (174-KYKETDL-180; La numbering) encompassed a β-turn in the human La protein structure which is known to interact with the HCV RNA (Alfano et al., 2004). Previously we have shown that the RNA bound structure of this peptide is similar to the conformation of the corresponding peptide in the free La protein (Mondal et al., 2008). The conformational properties of this heptapeptide in the free state was first investigated by NMR with an aim to understand the difference between the free and the RNA bound state of the peptide and hence, develop better lead molecules that would inhibit viral translation more effectively. Fig. S1A shows the structure of the free heptameric peptide (LaR2CN7) as obtained from NMR spectroscopy. The peptide has an extended structure but a turn-like structure is preserved around the residue E177. The substantial conformational difference between the free and the RNA bound conformation of the linear unmodified peptide suggested that imposing a conformational constraint may create a better La-HCV RNA interaction inhibitor.

3.2. A cyclic peptide mimicking the RNA bound β-turn has better inhibitory activity

Cyclization is often used to stabilize peptides for *in vivo* applications (Alfano et al., 2004), provided the restricted conformation of the cyclic peptide is compatible with target recognition. Since the RNA bound conformation of LaR2C-N7 is a β-turn which has C1α-C5α within a distance of 7 Å, possibility existed to design a cyclic peptide that preserves this β-turn. The synthetic scheme of this designed cyclic peptide is shown in Figs. S2A and B. The cyclic peptide (Fig. 1A) and the DR_6 counterpart were synthesized and purified to homogeneity as described in Mondal et al., 2008 and Fig. S2. The conformation of the cyclic peptide was analyzed using

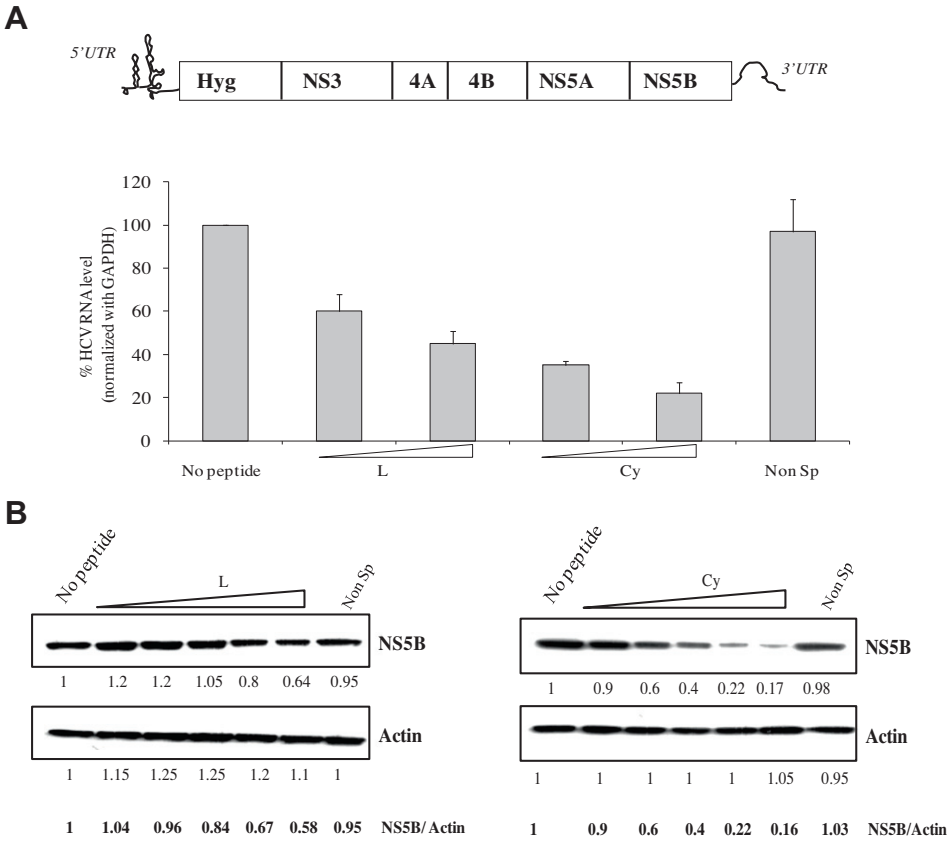

Fig. 2. (A) Schematic representation of HCV monocistronic replicon (adapted from Frese et al., 2003).The monolayer of Huh7 cells harboring the monocistronic replicon was transduced with linear DR_6-LaR2C-N7 (L) or DR_6-LaR2C-N7-cy (Cy) at increasing concentrations (2 and 4 μM). A DR_6 non-specific peptide (8 μM) was used as negative control. Total RNA was isolated at 24 h post transduction and subjected to real time RT-PCR for negative strand synthesis. GAPDH was used as an internal control. (B) A monolayer of Huh7 cells harboring the monocistronic replicon was transduced with linear DR_6-LaR2C-N7 (L) or DR_6-LaR2C-N7-cy (Cy) at increasing concentrations (0.5, 1, 2, 4 and 6 μM). Cells were lysed at 48 h post transduction and western blot was performed using anti-NS5B antibody. Actin was used as an internal control. A DR_6 non-specific peptide (6 μM) was used as negative control.

two-dimensional proton NMR spectroscopy. All the hydrogen atoms were assigned using standard NOESY/TOCSY walk. The constraints were derived from the NOE distances and the J-coupling. The derived structure using DYANA (Herrmann et al., 2002) was then structurally aligned with the corresponding part of the NMR structures of the RRM (112-184) (Fig. 1B) or the RNA bound structure (Fig. S1B). The β-turn part of the cyclic peptide is remarkably similar to the RNA bound structure. To test the potency of the cyclic peptide vis-à-vis the linear peptide, we have attached a cell penetration tag (hexa-D-arginine) to each of the peptides and transduced it to Huh7 cells carrying a reporter construct (luciferase expression system). The concentration dependence of the translation inhibition at 24 h is shown in Fig. 1C. The inhibition by the cyclic peptide at this time point was somewhat more potent than the corresponding linear peptide. To compare the effect of cyclization at longer time scales, expression of the luciferase gene was measured at 24 and 48 h post peptide treatment. At the shorter time points, the cyclic peptide shows modestly higher inhibitory potency than the linear peptide (Fig. 1D). However, at longer time points, this difference in potency becomes much more pronounced. The likely cause of the time-dependence of the difference in potency may be due to faster degradation of the linear peptide. Furthermore, the effect of cyclic peptide on HCV replication was examined using Huh7 cells harboring HCV monocistronic replicon. These cells were transduced with increasing concentrations of cyclic La peptide (2 and 4 μM). Twenty-four hours post transduction, cells were harvested and level of negative strand of HCV RNA was measured by real time (Fig. 2A) and semi quantitative RT PCR (Fig. S3A) using primers listed in Fig. S3B. Further, to clearly differentiate the effect of linear and cyclic peptide activities on viral protein expression, western blot analysis was performed at 48th hr using anti-NS5B antibody (Fig. 2B). Although both peptides showed significant reduction in HCV RNA and protein level without affecting cell viability (Fig. S4), the cyclic peptide showed greater inhibition compared to the linear one. Our study suggests that the cyclic peptide is more effective and preserves the potency of the translation inhibition over a much longer time scale than the linear peptide. Thus the cyclic peptide could be an effective lead compound for anti-virals against hepatitis C.

4. Glossary

IRES, Internal Ribosome Entry Site; NOESY, Nuclear Overhauser Effect spectroscopy; TOCSY, Total Correlation Spectroscopy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.12.026.

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