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A peptide targeted against phosphoprotein and leader RNA interaction inhibits growth of Chandipura virus – An emerging rhabdovirus

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ABSTRACT

The fatal illness caused by Chandipura virus (CHPV), an emerging pathogen, presently lacks any therapeutic option. Previous research suggested that interaction between the virally encoded phosphoprotein (P) and the positive sense leader RNA (le-RNA) may play an important role in the viral lifecycle. In this report, we have identified a β-sheet/loop motif in the C-terminal domain of the CHPV P protein as essential for this interaction. A synthetic peptide encompassing this motif and spanning a continuous stretch of 36 amino acids ($Pep_{208-243}$) was found to bind the le-RNA *in vitro* and inhibit CHPV growth in infected cells. Furthermore, a stretch of three amino acid residues at position 217–219 was identified as essential for this interaction, both *in vitro* and in infected cells. siRNA knockdown-rescue experiments demonstrated that these three amino acid residues are crucial for the leader RNA binding function of P protein in the CHPV life cycle. Mutations of these three amino acid residues render the peptide completely ineffective against CHPV. Effect of inhibition of phosphoprotein–leader RNA interaction on viral replication was assayed. Peptide $Pep_{208-243}$ tagged with a cell penetrating peptide was found to inhibit CHPV replication as ascertained by real time RT-PCR. The specific inhibition of viral growth observed using this peptide suggests a new possibility for designing of anti-viral agents against *Mononegavirale* group of human viruses.

1. Introduction

Chandipura virus (CHPV) has been repeatedly associated with several fatal outbreaks of acute encephalitis among children in different parts of India, and thus has been identified as a novel virus causing febrile illness in human (Bhatt and Rodrigues, 1967; Gurav et al., 2010; Rao et al., 2004; Rodrigues et al., 1983; Van Ranst, 2004). Consistently high case fatality rates (CFR), >53% and 78.3% during the 2003 and 2005 epidemics respectively, and mortality within 48 h of onset of symptoms, have prompted its recognition as an important emerging human pathogen in the Indian subcontinent (Chadha et al., 2005; Rao et al., 2004; Van Ranst, 2004). Manifestation of CHPV infection starts with flu like symptoms and includes abdominal pain, vomiting, altered consciousness, and impaired neurologic functions (Menghani et al., 2012). Eventually, encephalitis sets in causing diffused or focal inflammation of the brain parenchyma associated with brain dysfunction(Menghani et al., 2012; Narasimha Rao et al., 2008). Being a prototype member of the *Rhabdoviridae* family, CHPV belongs to the *Mononegavirales* order which includes other significant human pathogens like Ebola virus, human respiratory syncytial virus, measles virus, Nipah virus, and rabies virus. Apart from India, it has also been isolated in Nigeria from hedgehogs (Kemp, 1975) and in Sri Lanka from macaques (Peiris et al., 1993). Though the CHPV closely resembles the prototype Vesiculovirus, Vesicular Stomatitis Virus (VSV), it can be readily distinguished by its ability to infect humans (Basak et al., 2007). As no real therapeutic option exists against this virus, the demands of developing novel antiviral strategies are high (Joshi et al., 2012).

Abbreviations: CHPV, Chandipura virus; le-RNA, Chandipura virus leader (+) RNA; RdRp, RNA dependent RNA polymerase; NMR, nuclear magnetic resonance; TOCSY, total correlation spectroscopy; HPLC, high performance liquid chromatography; ESI-MS, electrospray ionization mass spectroscopy; MALDI TOF, matrix assisted laser disruption ionization time of flight; Fmoc, fluorenylmethyloxycarbonyl chloride.

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The 11.1 Kb long, encapsidated genome of the Chandipura virus comprises of a 49 nt transcribed but untranslated leader gene (l), five transcriptional units coding for the Nucleocapsid protein (N), Phosphoprotein (P), Matrix Protein (M), Glycoprotein (G) and the Large protein (L) and another short non-translated trailer sequence (t) arranged in the order 3′ l-N-P-M-G-L-t 5′ (Basak et al., 2007; Menghani et al., 2012). Upon infection, CHPV RNA-dependent RNA polymerase (RdRp), composed of the L and P proteins, transcribes its genes to produce the leader RNA (le-RNA) and five capped and polyadenylated mRNAs (Basak et al., 2007). At a certain point after infection, replication of the whole negative stranded genome is initiated, thereby producing a polycistronic, positive strand RNA intermediate which subsequently serves as the template for the synthesis of negative sense genomic RNA needed for mature virion particles (Basak et al., 2003).

The interaction between the P protein and the le-RNAhas been previously established to play an important role in the CHPV life cycle (Basak et al., 2004; Roy et al., 2013). *In vitro* experiments demonstrated that unphosphorylated P protein (P_0) binds specifically to the positive sense le-RNA to form two stoichiometrically different complexes, I and II, depending on its oligomerization status (Basak et al., 2004). Recent studies on CHPV infected Vero-76 cells confirmed the occurrence of this interaction in the intercellular milieu too (Roy et al., 2013). However, both *in vitro* and *in vivo* studies showed that upon phosphorylation (P_1), this le-RNA binding ability of P is lost (Basak et al., 2004; Roy et al., 2013). Basak et al. further demonstrated that the accumulation of a le-RNA binding, phosphorylation null mutant of P in cells results in enhanced genome RNA replication with concurrent increase in the viral yield (Basak et al., 2004). RNA immunoprecipitation experiments demonstrated that the le-RNA/P protein interaction is augmented during the replication phase of the viral cycle (Roy et al., 2013). Thus, this RNA–protein interaction is vital for the replication of CHPV. Similar phosphoprotein–leader RNA interactions have also been reported in other negative strand RNA viruses, such as VSV (Keene et al., 1981), Rabies (Yang et al., 1999) and Rinderpest virus (Raha et al., 2004). Based on these evidences we hypothesized that the P protein–le-RNA interaction provides an attractive antiviraltarget against this class of viruses.

Till date, there is no specific antiviral agent or human vaccine available against CHPV. Recently, a tissue culture based inactivated vaccine candidate was reported (Jadi et al., 2011). In another study, Balakrishnan et al. suggested that inhibition of specific cytokines like TNFα or overall inhibition of proinflammatory cytokines might help to reduce CHPV pathogenesis (Balakrishnan and Mishra, 2008). Kumar et al. demonstrated that intracranial administration of P gene silencing siRNA in mice protected them from CHPV encephalitis (Kumar and Arankalle, 2010). However, at present, symptomatic treatment is the only option against CHPV encephalitis (Menghani et al., 2012). Peptide antivirals are proving to be promising in recent times. Peptides mimicking amino terminal end of Rabies virus P protein have been shown to possess antiviral activity (Castel et al., 2009) and recently Zhang et al. have reported that porcine Mx1 protein when fused to the Protein transduction domain (PTD) derived from HIV Tat protein efficiently inhibits VSV replication (Zhang et al., 2013). A peptide derived from the signal sequence of fibroblast growth factor 4 was shown to exhibit broad-spectrum antiviral activity against influenza viruses *in vitro* (Jones et al., 2006).

In the present work, a β-strand/turn in the C-terminal domain of the P-protein was identified as the le-RNA interacting motif. A synthetic peptide of 36 amino acids constituting the β-turn was found to retain the le-RNA binding activity, albeit with reduced affinity. Using NMR spectroscopy, we have identified three amino acid residues within the 36-mer peptide which are the most likely interacting points with the le-RNA. Mutational analysis and siRNA mediated knockdown-rescue experiments confirmed the role of these three amino acids in binding to the le-RNA. Subsequently, an N-terminal hexa D-arginine tagged version of the peptide was found to be cell permeable, non-cytotoxic which effectively impedes CHPV growth in Vero-76 cells. Real time RT-PCR analysis confirmed that this peptide efficiently inhibited viral replication. Thus, this peptide could be a promising lead towards a new class of antiviral agents against CHPV.

2. Materials and methods

2.1. Cell culture and viral infection

Vero-76 cells were cultured in DMEM supplemented with 10% FBS. CHPV (Strain 1653514) was purified from BHK-21 cells. All CHPV infections were performed in serum free DMEM for 1 h, after which cells were washed twice with D-PBS, and supplemented with 10% FBS containing DMEM.

2.2. Site directed mutagenesis, expression and purification of P protein

PCR based overlap extension method of site directed mutagenesis was used in creating the mutated full-length P proteins discussed in this study (Ho et al., 1989). P protein was expressed in protease deficient BL21(DE3) strain of *Escherichia coli* and purified using Q-Sepharose Fast Flow resin (Amersham Biosciences) as mentioned previously (Basak et al., 2004). Purity of all proteins was confirmed on SDS–PAGE. A representative figure showing purity of wild type P is included in Fig. S4. All the purified proteins were dialyzed against the Binding Buffer (10 mM Tris–HCl buffer, pH 8.0 containing 100 mM NaCl, 40 mM KCl, 5 mM $MgCl_2$, 0.1% Triton ×100 and 10% Glycerol) and centrifuged at 1,00,000*g* in a Beckman LE-80K ultracentrifuge (Beckman, CA, USA) for 2 h at 4 °C prior to each experiment (Basak et al., 2004). Concentrations of all proteins were measured using BioRad RC-DC Protein Assay Kit and by measuring absorbance at 280 nm assuming $\varepsilon = 25{,}200\ M^{-1}\ cm^{-1}$ unless otherwise mentioned.

2.3. Synthesis of RNA probes

CHP-le II RNA was chemically synthesized (IDT, USA). Full length le-RNA was *in vitro* transcribedas mentioned previously (Basak et al., 2004). Positive sense le-RNA was synthesized *in vitro* from CHP-le/pGEM-3Z clone linearized with Hind III using T7 RNA polymerase as discussed previously (Basak et al., 2004). This RNA is 65 nt long, bearing 16 nt vector derived sequence at its 5′ end. To obtain radiolabeled RNA, α ^{32}P-UTP (5000 Ci/mmol, BRIT, India) was included in the transcription reaction. *In vitro* synthesized RNA was eluted from the urea-polyacrylamide gel, precipitated twice with ethanol and suspended in RNase free water. RNAs used in this study were quantified spectrophotometrically by their absorbance at 260 nm. Extinction coefficients for different RNA probes were calculated to be $\varepsilon = 672{,}000\ M^{-1}\ cm^{-1}$ for le-RNA, and $280{,}400\ M^{-1}\ cm^{-1}$ for CHP-le II.

2.4. Gel electrophoretic mobility shift assay (EMSA)

Gel electrophoretic mobility shift assays were performed as mentioned previously (Basak et al., 2004). Briefly, le-RNA probe (60,000 cpm) was incubated with unphosphorylated P protein in binding buffer (10 mM Tris–HCl buffer, pH 8.0 containing 100 mM NaCl, 40 mM KCl, 5 mM $MgCl_2$, 0.1% Triton ×100 and 10% Glycerol) supplemented with 0.01 mg/ml bovine serum albumin (BSA) and 1 mg yeast tRNA (serving as excess non-specific RNA) for 15 min at 25 °C in a total volume of 15 μl. Native gel

loading dye was added to the reaction mixture (final 3% v/v, containing Ficoll, 0.1 mM EDTA, 0.025% w/v bromophenol blue and 0.025% w/v xylene cyanol) after incubation. Complexes were finally resolved on a native 6% w/v polyacrylamide gel containing 5% glycerol, run at 4 °C in 1× TAE. The gel was dried and developed using a Phosphorimager (Typhoon, GE Healthcare).

2.5. Synthesis and purification of labeled and unlabeled peptides

The $Pep_{208-243}$ and $Pep_{208-243AAA}$ along with their hexa-arginine tagged variety were synthesized on a PS3 Protein Technologies peptide synthesizer at 0.1 mmol scale by using a solid-phasepeptide synthesis strategy using 9-fluorenylmethoxy carbonyl chemistry and Rink amide MBHA resin (Banerjee et al., 2002). The crude peptides were purified by reversed-phase high-performance liquidchromatography (Waters Inc.) with a C_{18} column (Hypersil gold, Thermo Fisher) employing linear gradients of water/acetonitrile containing 0.1% trifluoroacetic acid. Peptide mass and purity (>95%) were checked by ESI (Waters Inc.) and MALDI TOF (Applied Bio Systems) Mass spectrometry (Fig. S2). Peptides were labeled with 5,6-carboxy fluorescein in solid phase and then cleaved and purified using similar methodology as in case of unlabeled peptides.

2.6. Circular Dichroism spectroscopy

Circular Dichroism (CD) spectra were measured in a JASCO spectropolarimeter. The measurements were conducted in 20 mM potassium phosphate buffer, pH 7.0 containing 100 mM KCl for the peptide and 10 mM Tris–HCl buffer, pH 8.0 containing 200 mM NaF for the proteins at 25 °C. The measurements were performed in a 1 mm path length cuvette with a band width of 1.0 nm and a scan speed of 50 nm/min. Ten spectra were averaged to improve the signal-to-noise ratio. All protein and peptide concentrations were 5 μM. Secondary structure contents were estimated by a neural network based model.

2.7. Fluorescence spectroscopy

Fluorescence was measured using a Quantamaster 6 (PTI) T-geometry and Perkin Elmer L-geometry fluorometers. The fluorescence experiments were carried out at 25 °C. The experiments were carried out in a 1 cm path length cuvette.Values were corrected for buffer fluorescence value, volume and inner filter effect. Curve fittings and calculation of K_d value were carried out with Kyplot software using single site binding equation.

2.8. NMR spectroscopy

NMR experiments were conducted on an Avance 600-MHz spectrometer (Bruker) equipped with TXI cryoprobe. The buffer for peptide alone and peptide:RNA complex experiments was 20 mM potassium phosphate buffer, pH 7 containing 100 mM KCl. The 1D proton spectra were recorded using the pulse program *zgesgp* and water suppression was achieved by excitation sculpting. TOCSY spectra for the peptide and peptide:RNA complex with a mixing time 60 ms, were recorded at 298 K wherein solvent signal was suppressed by WATERGATE and spinlock was attained by MLEV sequence.

2.9. Confocal microscopy

Hexa-D-arginine tagged peptide (DR_6-$Pep_{208-243}$) was labeled with carboxyfluorescein for assessing the intracellular permeability of the peptide. DR_6-$Pep_{208-243}$ was treated at 20 μM onto monolayers of Vero-76 cells, and at different time post-addition, cells were washed in PBS (X2) and fixed with 2% paraformaldehyde for 40 min at RT. FITC fluorescence was monitored under a laser scanning confocal microscope (Carl Zeiss) using appropriate settings.

2.10. Cyto-toxicity assay (MTT)

Cellular toxicity of DR_6-$Pep_{208-243}$ was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Briefly, monolayers of Vero-76 cells in 96 well tissue culture plates were treated with different concentrations of $Pep_{208-243}$ in complete DMEM for 24 h. Following which, MTT was added to a final concentration of 1 mg/ml, and incubated for 4 h at 37 °C. DMSO was used to dissolve the resulting formazan crystals. Absorbance was recorded in a microplate reader (Molecular Devices) at 590 nm with a reference filter of 620 nm. Data for three independent experiments were plotted.

2.11. [^{3}H]thymidine incorporationassay

Twenty hours after DR_6-$Pep_{208-243}$ treatment, Vero-76 cells in 96 well tissue culture plates were treated with 0.2 μCi of methyl[^{3}H]thymidine, and then incubated for an additional 24–96 h at 37 °C. Plates were harvested onto filter paper using a cell harvester. Scintillation Cocktail was added to dried filters and radioactivity was estimated in a liquid scintillation counter.

2.12. Determination of viral titer reduction potential of DR_6-$Pep_{208-243}$ and DR_6-$Pep_{208-243AAA}$

Vero-76 cells were cultured in 35 mm tissue culture dish (BD Falcon) for 24 h at 37 °C in DMEM supplemented with 10% FBS. Cells were pre-incubated with indicated concentrations of the corresponding peptide for 1 h, following which they were washed twice with D-PBS, and infected with CHPV at an MOI of 0.1. 1 h post infection, cells were again washed twice with D-PBS, and were treated with different concentrations of peptides along with untreated controls, in 10% FBS supplemented DMEM medium. Treated and untreated cells were then incubated for 24 h at 37 °C in the presence of 5% CO_2. Virus particles were released from the infected cells by freeze–thaw cycle method and virus yield was measured by plaque assay. The percent of inhibition of viral replication in cells treated with DR_6-$Pep_{208-243}$ or DR_6-$Pep_{208-243AAA}$ was expressed as percentage of untreated control and was calculated as follows: [(number of plaques form peptide treated cells/number of plaques form control cells) × 100]. The data from three independent experiments were plotted and standard deviations shown.

2.13. Real time RT-PCR

Total RNA was isolated from those cells using TRI Reagent (Ambion) with subsequent RNase-free DNase (Fermentas) treatment following manufacturer's instructions. 1 μg of total cellular RNA was reverse transcribed using random hexamer primers according to the manufacturer's protocol (Epicenter). cDNAs were used as template and real time PCR was carried out using Maxima® SYBR Green qPCR Master Mix (2X) (Fermentas) in a real time cycler (Applied Biosystems StepOnePlus™), using ROX as passive reference dye for internal reference for normalization of the SYBR® Green fluorescence signal. The following primers were used: Upstream (5′-TGGAAAGGGTAGGAGATATTCGA-3′) and downstream (5′-GAGAGTGTCCTGAAGCTTTGG-3′). These primers are specific for a 150 bps amplicon of the N–P gene junction to facilitate viral genome replication assessment. The housekeeping gene, GAPDH, was used as an internal control (Upstream primer, 5′-GTCTTCACCACCATGGAGAA-3′, and downstream primer, 5′-AGGCATTGCT-

GATGATCTTGA-3′). The $2^{(-\Delta\Delta CT)}$ method was used to analyze the relative changes in real-time quantitative PCR experiments (Livak and Schmittgen, 2001). Normalized Relative replication to untreated sample was plotted as a function of time post infection.

2.14. siRNA knockdown-rescue experiment

Knockdown of the CHPV P gene was performed using P-2 siRNA, that has beenpreviously described by Kumar and Arankalle (2010). P-2 siRNA targets the sequence 5′-ACCGAATCACCTGGCTCCAAA-3′. For knockdown-rescue experiments, cells were first transfected with siRNA-resistant P protein clones using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. Resistance to siRNA was attained by introduction of seven silent point mutations in the siRNA target sequence of the P constructs: 5′-ACCGAAAGCCCGGGCAGTAAA-3′. 24 h post transfection; cells were infected with CHPV at MOI of 0.1. One hour post infection, cells were transfected with 100 pmol of P-2 siRNA, or its scrambled form, PS-2 siRNA (Qiagen, India) using Lipofectamine 2000 (Invitrogen). Cell supernatants were harvested 24 h post infection and viral titers assessed by plaque assay. To determine siRNA transfection efficiency, P-2 siRNA tagged with Alexa Fluor-647 (Qiagen, India) was used.

2.15. Immunoblotting

Whole cell lysate from siRNA and/or plasmid DNA transfected cells were resolved on a 12.5% polyacrylamide gel. Western blot analyses were performed according to standard protocols, using polyclonal antibodies against the CHPV P protein and β-actin (Cell Signaling Technology).

2.16. Statistics

All data were presented as means ± standard deviation (S.D.) from at least three separate experiments. A two tailed unpaired Student's *t*-test was used to determine the significance of differences between treated and control groups. A *P* value <0.05 was considered statistically significant. GraphPad PRISM software was used for statistical analysis.

3. Results

3.1. Identification of a β-strand/loop motif as important in binding le-RNA

To investigate the importance of le-RNA/P-protein interaction in the life cycle of CHPV, we undertook a chemical genetic approach. The first task was to identify the le-RNA binding region of the P-protein. Homology modeling of the residues 201–257 of the C-terminal domain of Chandipura virus P protein (Fig. 1A) was performed using the NMR structure of VSV P_{CTD} (Green and Luo, 2009) as a template (PDB ID: 2K47). The C-terminal domain of Chandipura virus P-protein shows appreciable similarity with that of VSV and other *vesiculoviruses* (Fig. S1). The modeled structure shows that the residues of the C-terminal domain form several small α-helices and an anti-parallel β-sheet connected by flexible loop (Fig. 1A). β-Sheet/loop structures have often been found to be binding sites for cognate macromolecules in the past (Del Angel et al., 1989; Dildine and Semler, 1992). We tested the possibility of the β-sheet/loop structure encompassing residues 208–243 in the C-terminal domain of the P protein to be involved in the le-RNA binding. To this end, a peptide corresponding to amino acids 208–243 of CHPV P protein (to be called $Pep_{208-243}$ henceforth), was synthesized and purified by reversed phase HPLC; purity was checked using ESI-MS (Fig. S2A). Far UV CD spectra (Fig. 1B) of this peptide revealed the predominant secondary structural element to be β-sheet/turn (43.2% β-sheet, 8.1% turn and 48.7% random coil).

The le-RNA binding ability of this 36 residue peptide was tested by fluorescence anisotropy. For this purpose, the N-terminal end of the peptide was labeled with fluorescein. The labeled peptide was then titrated with increasing concentrations of the C-terminal half of the CHPV le-RNA (CHP-le II; Fig. S3) and the anisotropy were measured. CHP-le II was used because previous studies showed that the P protein binds tightly to this region (Basak et al., 2004). Anisotropy of the fluoresceinated $Pep_{208-243}$ (open circles in Fig. 2A) was found to increase as a function of CHP-le II concentration and was largely saturated at or above 4 μM of the RNA. The binding isotherm was fitted to a single site binding equation to estimate the value of the dissociation constant (K_d), which was found to be 1.43 ± 0.77 μM.

After confirming the binding of CHP-le II RNA with $Pep_{208-243}$, we attempted to identify the residues on the peptide that are

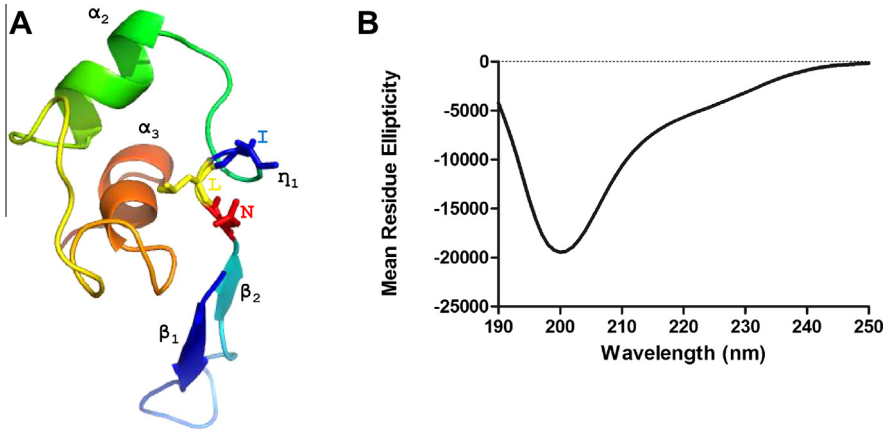

Fig. 1. Leader RNA recognition motif of CHPV P protein is located in its C-terminal domain (CTD). (A) Homology model of CHPV P_{CTD} (residues 201–257), using VSV P_{CTD} as template (PDB ID: 2K47). The predicted secondary structure elements are shown in different colors. Stick representation of residues N217, L218 and I219 in the β-sheet/turn are shown in red, yellow and blue respectively. Modeling was performed using the SwissModel server. The image was prepared with PyMOL Molecular Graphics System (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC). (B) Far-UV CD spectra of $Pep_{208-243}$ at 5 μM concentration in 20 mM potassium phosphate buffer, pH 7.0 containing 100 mM KCl at 25 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

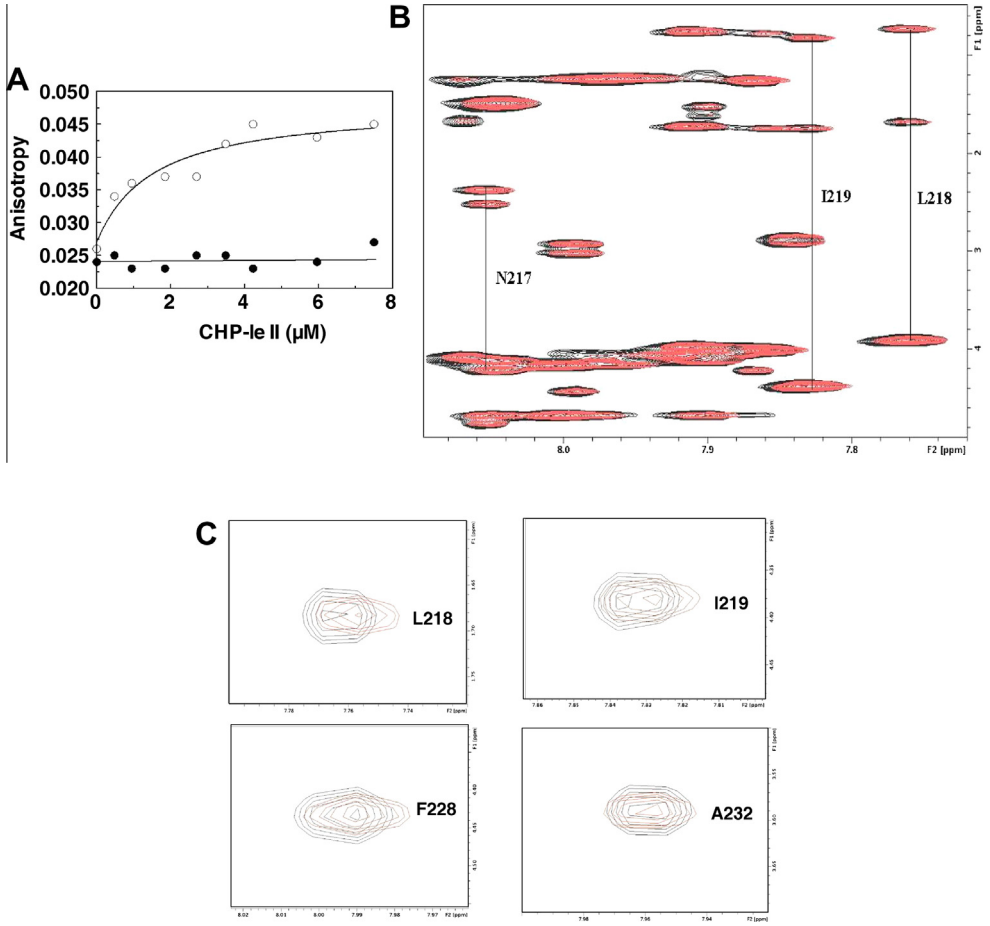

Fig. 2. (A) Binding isotherm of $Pep_{208-243}$ (open circles) and $Pep_{208-243AAA}$ (solid circles) with CHP-le II RNA. 5′ Fluorescein labeled wt and mutant peptides were titrated with increasing concentrations of CHP-le II RNA in 20 mM potassium phosphate buffer, pH 7.0 containing 100 mM KCl at 298 K. Binding isotherms were fitted to single site binding equation using KyPlot software.The representative isotherms are average of ten independent measurements. (B) 2D homonuclear TOCSY overlap spectra of $Pep_{208-243}$:CHP-le II complex. Concentration of the peptide and RNA was kept at 500 μM and 50 μM respectively. Contours in black indicate peaks of the peptide alone and those in red represent peaks of the peptide in presence of CHP-le II. Residues undergoing substantial shift upon binding to the RNA are labeled. (C) An enlarged view of the above overlaid 2D ^{1}H–^{1}H TOCSY spectrashowing distinct chemical shift perturbation tentatively assigned to leucine 218(L218) and isoleucine 219 (I219) (top panel), and another part of the overlaid spectra assigned to phenylalanine 228 (F228) and alanine 232 (A232) that show no chemical shift perturbation (bottom panel). The assignments are tentatively based on through-bond connectivity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

crucial in interacting with the RNA by chemical shift perturbation NMR. TOCSY spectra of the peptide, in absence or presence of CHP-le II RNA (peptide:RNA of 1:10), were overlaid to identify the RNA interacting residues (Fig. 2B). Residues that undergo chemical shift changes upon complex formation were tentatively assigned by through-bond connectivity. Asparagine (N217), leucine (L218) and isoleucine (I219) are the residues whose chemical shifts in this region of the spectra have been most significantly perturbed compared to other residues in the peptide. Fig. 2C (top panel) represents an enlarged portion of this overlaid spectra (Fig. 2B) showing distinct chemical shift perturbation tentatively assigned to leucine (L218) and isoleucine (I219), while the bottom panel shows another part of the overlaid spectra assigned to phenylalanine (F228) and alanine (A232) where no chemical shift perturbation was observed. A triple mutant version of $Pep_{208-243}$ where all three residues (N217, L218 and I219) have been mutated to alanine ($Pep_{208-243AAA}$) was synthesized and purified (Fig. S2B). Using similar methods of synthesis, labeling and purification described above, the fluoresceinated $Pep_{208-243AAA}$ peptide was prepared and used in the anisotropy assay to quantitatively determine its association with the CHP-le II RNA. It is apparent from Fig. 2A (solid circles) that CHP-le II RNA failed to cause any increase in anisotropy value of the labeled $Pep_{208-243AAA}$, indicating loss of CHP-le II RNA binding ability of $Pep_{208-243AAA}$ *in vitro*.

3.2. Mutation of residues 217–219 in CHPV P protein perturbs its RNA binding ability in vitro

A triple alanine mutant of the P protein, $P_{NLI217,\ 218,219AAA}$ (henceforth called P_{AAA}), was created by site-directed mutagenesis and purified from *E. coli* using similar methodology like the wt full-length protein (Fig. S4). The secondary structure of the protein was judged to be similar to the wild-type protein by circular dichroism (Fig. S5 and Table S1). P_{AAA} was subjected to electrophoretic mobility shift assay (EMSA) using ^{32}P-labeled full-length le-RNA as the probe (Fig. 3A). It is clear from the result that while the wild-type P protein forms both complex I and II at two different concentrations of the protein (Basak et al., 2004), P_{AAA} does not form any of those complexes in the concentration range tested in this assay (lanes 4 and 5). To further substantiate our claim that $Pep_{208-243AAA}$ is deficient in le-RNA binding, we devised a chase experiment in which ^{32}P-labeled le-RNA was allowed to form complex with wild type P protein in binding buffer in the presence of yeast tRNA, for

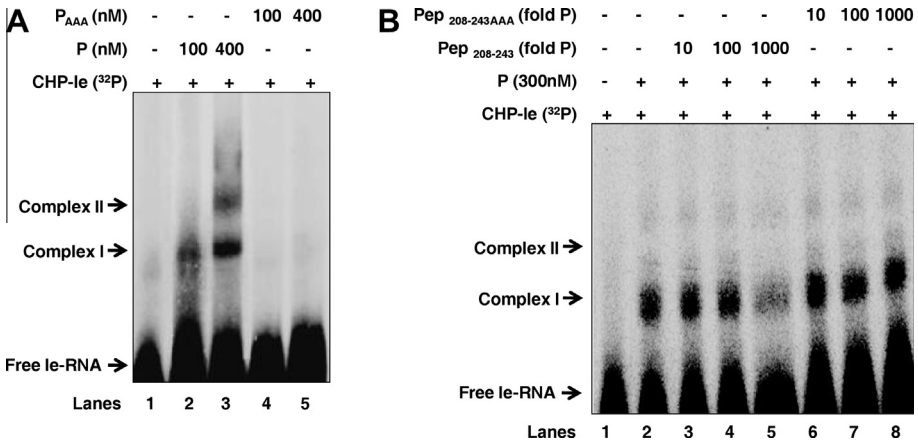

Fig. 3. Residues 217, 218 and 219 of P protein are instrumental in binding to le-RNA. (A)Interaction of P_{AAA} (residues 217, 218 and 219 mutated to alanine) with le-RNA. ^{32}P labeled full length le-RNA was incubated with 100 and 400 nM of wt P protein (lanes 2, 3) or P_{AAA} (lanes 4, 5). (B) Competition assay of wt P protein and le-RNA complex with $Pep_{208-243}$ and $Pep_{208-243AAA}$. ^{32}P labeled le-RNA was allowed of form complex with wild type P protein in binding buffer in the presence of yeast tRNA for 15 min. 300 nM P protein was used for the assay to facilitate formation of complex I only (lane 2). This complex was chased with 10, 100 and 1000 fold molar excess of $Pep_{208-243}$ (lanes 3, 4 and 5) and $Pep_{208-243AAA}$ (lanes 6, 7 and 8). Lane 1 represents no protein control.

15 min. 300 nM P protein was used in the assay to facilitate formation of complex I only. This complex was chased with 10, 100 and 1000 fold molar excess of $Pep_{208-243}$ and $Pep_{208-243AAA}$. It is evident from the result (Fig. 3B) that, $Pep_{208-243}$ efficiently chased out labeled CHP-le RNA (compare lanes 3, 4, 5 with lane 2) at 1000 fold excess, whereas $Pep_{208-243AAA}$ failed to exhibit considerable chase under the same conditions (compare lanes 6, 7, 8 with lane 2). A K_d of 1.43×10^{-6} M for $Pep_{208-243}$ compared to 4.07×10^{-8} M for full length P protein (Basak et al., 2004) explains the high molar requirement of the peptide to chase out the full length P protein. These observations clearly indicate that residues 217–219 of the P protein are involved in le-RNA binding *in vitro*.

3.3. siRNA knockdown-rescue experiment suggests importance of P protein/le-RNA interaction in viral life cycle

To understand the importance of P protein/le-RNA interaction in the viral lifecycle and to reinstate the importance of residues 217–219 *in vivo*, a siRNA knockdown-rescue experiment was performed. P gene targeted siRNA (P-2) which was previously reported by Kumar et al. (Kumar and Arankalle, 2010) was used to knock down the viral P mRNA in infected cells.PS-2, a scrambled version of P-2 siRNA was used as a negative control. In order to rescue the P protein knockdown, a siRNA immune construct of the P gene (Psi_{wt}) was generated by introducing seven silent point mutations in the siRNA target sequence (see materials and methods). Another similar siRNA immune P gene construct was generated but with residues 217–219 mutated to alanine (Psi_{AAA}). Fig. 4A shows the P-2 siRNA mediated silencing of wild typeP protein (P_{wt}) as well as the resistance of Psi_{wt} and Psi_{AAA} to silencingby immunoblotting with anti-P antibody. It is seen that both Psi_{wt} and Psi_{AAA} are expressed at equivalent levels in the presence of P-2 siRNA. PS-2 siRNA did not have any effect on the expression of the P protein.

For the knockdown-rescue experiment, Vero-76 cells were first transfected with either Psi_{wt} or Psi_{AAA} or an empty vector. 24 h post transfection, the cells were infected with CHPV (MOI of 0.1), followed by siRNA transfection (P-2 siRNA or PS-2 siRNA) after 1 h. After 24 h, the cell supernatants were collected and assayed for viral titer by plaque assay (Fig. 4B and Fig. S6).The logic behind the experiment was that, CHPV knocked down with P-2 siRNA could be rescued with the extraneous expression of P protein from siRNA-resistant constructs. Rescue with Psi_{wt} and not with Psi_{AAA} would imply the importance of the three amino acids Asparagine (N), Leucine (L) and Isoleucine (I) at positions 217, 218 and 219 respectively, in viral propagation. Four sets of experiments were performed simultaneously. Set 1, served as a control where Vero-76 cells were first transfected with an empty vector and then infected with CHPV followed by treatment with scrambled siRNA (PS-2). In set 2, cells were transfected with an empty vector and infected with CHPV but treated with P-2 siRNA. In set 3, cells were transfected with Psi_{wt} and infected with CHPV followed by treatment with P-2, whereas, in set 4, cells were transfected with Psi_{AAA} instead of Psi_{wt}. As observed from the results (Fig. 4B and Fig. Fig. S6), there has been a significant reduction ($P < 0.05$) in viral titer (close to 1 log) in set 2 compared to set 1 control. This confirms the reduction in CHPV titer by P-2 siRNA. Rescue of this knockdown by Psi_{wt} is observed in set 3, where the viral titer has increased significantly as compared to set 2. However, the difference in titer between set 2 and set 4 is non-significant. This signifies that Psi_{AAA} failed to exhibit a similar rescuing ability. Taken together, these data indicate that introduction of Psi_{wt} and not Psi_{AAA} could rescue P-2 siRNA knocked down CHPV. So, these three amino acids N, L and I at positions 217–219 are important for the function of P protein in infected cells. Since these three amino acids have shown to be crucial for binding le-RNA, we can conclude that P protein/le-RNA interaction is essential for propagation of CHPV.

3.4. Growth characteristics of DR6-$Pep_{208-243}$ treated Vero-76 cells

The importance of P-protein/le-RNA interaction on the viral growth suggests that this could be a potential drug target. To evaluate this we conjugated 6 D-arginine residues to the N-terminus of $Pep_{208-243}$ (henceforth called DR_6-$Pep_{208-243}$) for facilitating cell entry (Balhorn et al., 2009). To confirm the cell permeability of this hexa-arginine tagged peptide we labeled it with fluorescein and found that the tagged peptide was indeed permeable to the cell membrane and after 3 h of incubation it was almost homogeneously distributed within the cell (Fig. 5A). Assessment of the cytotoxicity of DR_6-$Pep_{208-243}$ by MTT assay revealed no significant cellular toxicity (Fig. 5B).

In order to further address the concern that DR_6-$Pep_{208-243}$ may exhibit non-specific, pleotropic effect on normal cellular processes, we performed a [^{3}H] thymidine incorporation assay. The ^{3}H-thymidine is incorporated into dividing cells and the level of this incorporation measured using a liquid scintillation counter is

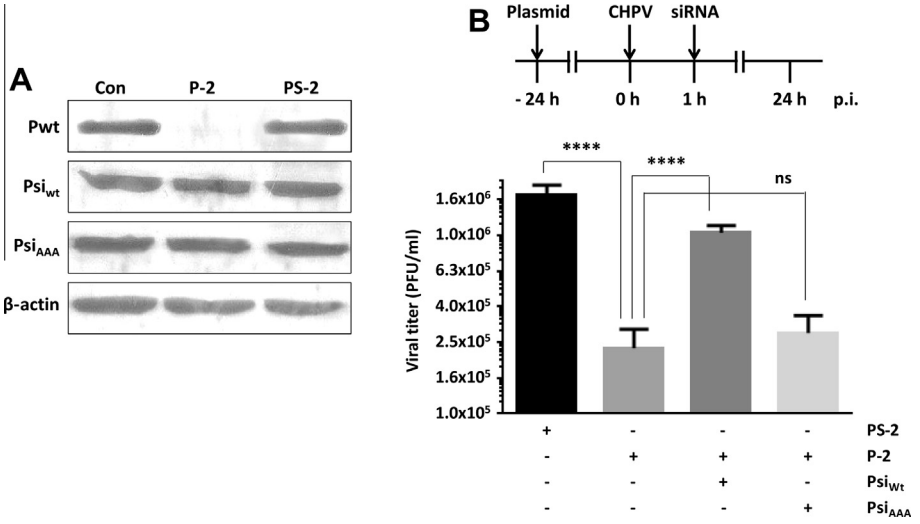

Fig. 4. siRNA knockdown-rescue experiment. (A) Whole-cell lysates from Vero-76 cells transfected with P_{wt} (siRNA sensitive), Psi_{wt} orPsi_{AAA}(siRNA resistant) plasmids followed by treatment with either P-2 siRNA or scramble siRNA (PS-2) immunobloted with anti-P antibody. β-Actin was used as a loading control. Control represents no siRNA treatment. (B, C) siRNA resistant wild typeP protein (Psi_{wt}) rescues P-2 siRNA knocked down CHPV, whereas, Psi_{AAA} fails to do so. Viral titers were assessed by plaque assay 24 h post siRNA treatment as shown. Four sets of experiments were performed simultaneously on Vero-76 cells infected with CHPV. Differential treatment in each set has been shown. All experiments were performed in triplicates. Results are expressed as mean ± standard deviation. A two tailed unpaired Student's *t*-test was used to determine the significance of differences at *P* value <0.05. Non-significant, ns. An uninfected control has been shown.

proportional to the amount of cell proliferation. Monolayers of Vero-76 cells were treated with 0.2 μCi of methyl-[^{3}H]-thymidine, 20 h after DR_6-$Pep_{208–243}$ treatment. Growth was monitored as percentage [^{3}H]thymidine incorporation with respect to control (no peptide) at 24, 48, 72 and 96 h post treatment. Fig. 5C clearly demonstrates that DR_6-$Pep_{208–243}$ has no effect on growth of Vero-76 cells even when incubated up to 96 h.

3.5. Peptide DR_6-$Pep_{208–243}$ inhibits virus propagation

To assess the potential of DR_6-$Pep_{208–243}$ to reduce CHPV yield, overnight monolayer cultures of Vero-76 werepre-treated with varying amounts ofDR_6-$Pep_{208–243}$ (or with PBS control) for 1 h, following which, they were infected with CHPV at an MOI of 0.1. Post infection (1 h), cells were again treated or mock treated with

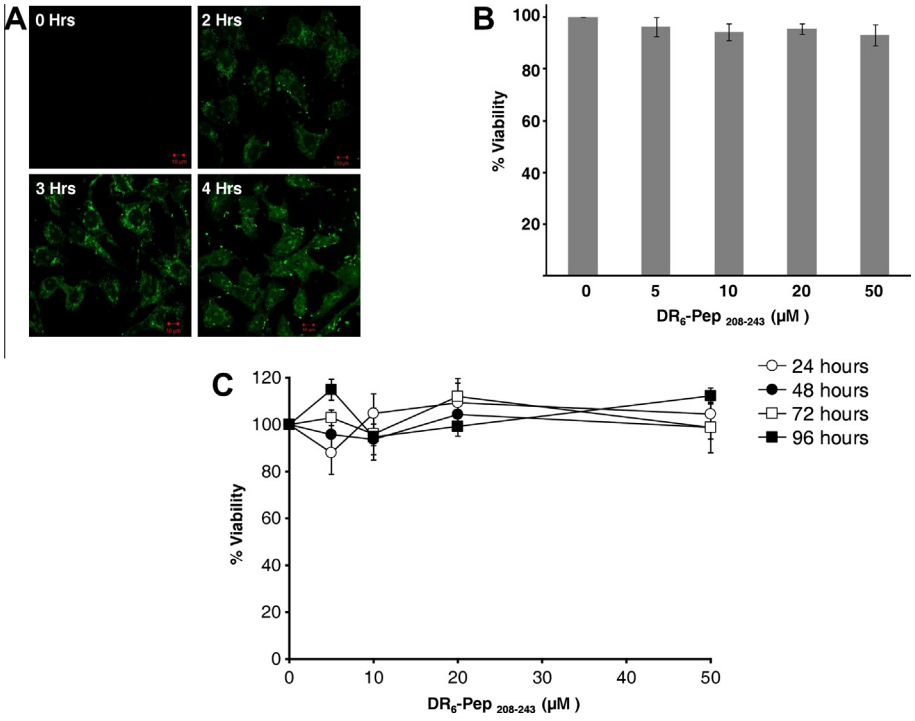

Fig. 5. Cellular permeability and non-toxicity of peptide DR6-$Pep_{208–243}$. (A) Hexa-D-arginine tagged peptide ($Pep_{208–243}$) was labeled with carboxyfluorescein and treated (20 μM) onto monolayers of Vero-76 cells, and FITC fluorescence monitored at different time points post addition under a laser scanning confocal microscope (Carl Zeiss). Time in hours is represented on the top left hand side of the figures. The bar represents 10 μm. (B) MTT assay was performed on Vero-76 cells treated for 24 h with increasing concentrations of DR6-$Pep_{208–243}$. The experiment was performed thrice and data points are shown as mean ± standard deviation. (C) [^{3}H] thymidine incorporation in DR_6-$Pep_{208–243}$ treated Vero-76 cells at different time intervals. Four different concentrations of DR_6-$Pep_{208–243}$ were used (5, 10, 20, 50 μM). The data is plotted as percentage cell viability with respect to a control not treated with DR_6-$Pep_{208–243}$ (% viability). Results are expressed as mean ± standard deviation. Standard deviation was estimated from triplicate wells.

DR_6-$Pep_{208-243}$and incubated for another 24 h. Titer and thus the yield of the released viral particles from three independent experiments were quantified by viral plaque assay (Fig. S7) after collecting the virus containing media from the plates.

The result of plaque assay (circles in Fig. 6) shows that the viral titer decreases in a dose dependent manner with a 50% effective inhibitory concentration (EC_{50}) value of 17.41 ± 4.2 μM for the cell line tested. As a control, a cell permeable version of the mutant peptide (DR_6-$Pep_{208-243AAA}$) was tested for its effect on virus yield in exactly the same manner as described above for the wt peptide. The mutant peptide has very little effect, if any, on the virus yield (squares in Fig. 6). This observation confirms that the specific inhibitory effect of DR_6-$Pep_{208-243}$ on virus production is due to its ability to interact with the le-RNA, and mutation of the interacting residues abolished this activity.

3.6. DR_6-$Pep_{208-243}$ inhibits CHPV RNA replication

The P protein of *Mononegavirales* has been implicated to be an essential component of the viral RNA polymerase machinery (Banerjee, 1987a,b; Basak et al., 2007). Moreover, according to our previous observations, the P protein/le-RNA interaction possibly plays a decisive role during viral RNA replication (Basak et al., 2004, 2003). Hence, we decided to investigate if peptide DR_6-$Pep_{208-243}$ interferes with viral RNA replication. A time dependent Real Time RT-PCR assay was developed for this purpose. Total RNA was isolated from DR_6-$Pep_{208-243}$ and DR_6-$Pep_{208-243AAA}$ (20 μM) treated cells at 4and 8 hpi (MOI of 10). For assessment of relative viral RNA replication, cDNAs were synthesized using random hexamers, and then PCR was performed with primers specific for a 150 bps amplicon in the N–P gene junction (Roy et al., 2013). A 150 bps amplicon of the GAPDH gene was used as the reference housekeeping gene. The results show that (Fig. 7) DR_6-$Pep_{208-243}$ inhibits viral RNA replication by 78% at 8 hpi as compared to untreated control. It has been shown previously that in CHPV, when infected at an MOI of 10, viral RNA replication is initiated at the late phase of the viral cycle, i.e., between 6 and 8 hpi (Roy et al., 2013). On the other hand, DR_6-$Pep_{208-243AAA}$ fails to inhibit the viral RNA replication (Fig. 7). Thus, $Pep_{208-243}$ selectively interferes with the viral RNA replication machinery, whereas, mutation of residues 217–219 abrogated this potential.

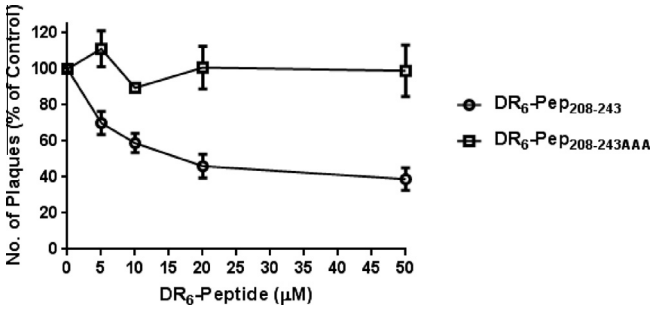

Fig. 6. Effect of peptide DR6-$Pep_{208-243}$ and DR6-$Pep_{208-243AAA}$ on CHPV growth. Plaque assay showing the effect of DR6-$Pep_{208-243}$ (50 μM) on CHPV 24 h post-infection of Vero-76 cells in presence or absence of peptides, as described. Four different concentrations of both peptides were used (5, 10, 20, 50 μM). Viral growth inhibition has been represented as percentage of plaques with respect to that untreated with peptide (% viability). The experiment was performed thrice and data points are shown as mean ± standard deviation. EC_{50} (50% effective inhibition concentration) of DR6-$Pep_{208-243}$ was calculated to be 17.41 ± 4.2 μM. A two tailed unpaired Student's *t*-test with Welch's correction was used to determine the significance of differencein titers between DR_6-$Pep_{208-243}$ and DR_6-$Pep_{208-243AAA}$ and the calculated *P* value was 0.0225. Being less than 0.05, this difference is significant.

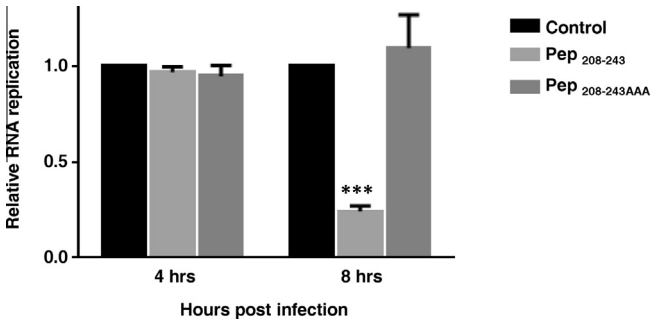

Fig. 7. Real Time RT-PCR analysis of CHPV RNA replication inhibition with peptides DR_6-$Pep_{208-243}$ and DR_6-$Pep_{208-243AAA}$ at two different time points post-infection. Total RNA was isolated from DR_6-$Pep_{208-243}$, or DR_6-$Pep_{208-243AAA}$ (20 μM) treated, or untreated cells (control) at 4 and 8 hpi. Real Time RT-PCR was performed with the primers designed to assess CHPV RNA replication as described in the materials and methods. This experiment was performed in 3 biological replicates, and standard deviations are indicated. Test for significance was performed with a two tailed unpaired Student's *t*-test. *P* value <0.0001 (***) for DR_6-$Pep_{208-243}$ at 8 hpi compared to control is significant.

4. Discussion

CHPV infection is a major concern in the Indian subcontinent. In recent times, CHPV has emerged as an important pediatric encephalitis-causing pathogen with very high case fatality rate in central India (Jadi et al., 2011; Venkateswarlu and Arankalle, 2009). Statistical analysis of the epidemiology data shows an increase in cases from 2003 to 2010 (Menghani et al., 2012). Till date, two attempts have been made to develop vaccines against CHPV – one, a recombinant glycoprotein based vaccine and the other, a cell based inactivated vaccine (Jadi et al., 2011; Venkateswarlu and Arankalle, 2009). However, no clinical trials have been reported with any of the above vaccine.

Previous contributions have outlined the critical role of the interaction between the viral P protein and the le-RNA in CHPV replication (Basak et al., 2003, 2004, 2007; Roy et al., 2013). Therefore, this interaction may prove to be a novel target for suppression of CHPV replication by peptidomimetic approaches. In recent times, peptide drugs and anti-viralshave evolved into mainstream therapeutics and are finding clinical applications (Alhoot et al., 2013; Hong et al., 2013; Palu and Loregian, 2013). A variety of synthetic antiviral peptides have been reported till date.For example, a 36-mer peptide named T-20derived from the HIV-1 transmembrane glycoprotein (gp41), exhibited effective inhibition of HIV-1 membrane fusion and virus entry and is now in clinical use in HIV-1-infected patients (Imai et al., 2000). Another synthetic peptide, C5A, was found to be active against HCV infection by inactivating both extra- and intracellular infectious particles (Cheng et al., 2008).

In the current study, we have identified a stretch of three residues, 217–219 N, L, I between residues 208–243 in the C-terminal domain of the P protein as essential in binding the le-RNA, both *in vitro* and in infected cells. Though the interaction between the le-RNA and the P protein has been reported previously (Basak et al., 2003, 2004; Roy et al., 2013), this is the first report of the interaction site. While, the existence of other points of interaction cannot be ruled out, data presented in this work confirms that these three residues are vital for the interaction. Subsequently, the siRNA knock-down rescue experiment confirmed the importance of this interaction in the viral life cycle (Fig. 4). Next, we hypothesized that if a small peptide can be designed so as to competitively inhibit the P/le-RNA interaction in CHPV infected cells, then, effective viral control can be achieved. With this aim, a synthetic, cell permeable peptide encompassing the three interacting

residues (DR_6-$Pep_{208–243}$) was tested for potential anti-viral activity. As evident from the data (Fig. 6), DR_6-$Pep_{208–243}$ showed appreciable antiviral potential, with an EC50 value of 17.41 ± 4.2 μM. The role of residues, 217–219 were further ascertained when the mutant peptide, DR_6-$Pep_{208–243AAA}$ failed to cause any significant change in the viral titer. Finally, it was tested whether inhibition of this P/le-RNA interaction has any effect on viral replication. In agreement with previous reports, DR_6-$Pep_{208–243}$ was found to inhibit CHPV replication at 8 hpi, the time point at which viral genome RNA synthesis culminates (Roy et al., 2013). Therefore, these *in vitro* findings indicate that use of this peptide or a more developed derivative may be an effective antiviral strategy against CHPV infection. At this stage, the peptide, when compared to the full length P protein, has a lower affinity towards the le-RNA. In addition the peptide is linear and unmodified. Linear unmodified peptides are generally unstable *in vitro* and not suitable as therapeutic agents. Thus, to develop the peptide further for therapeutic purposes, we may have to stabilize it against *in vivo* degradation by constraining the active conformation. This can be done by structure-guided incorporation of non-natural amino acids and modifications, such as, PEGylation.

Given widespread need of new antivirals in the *mononegavirales* group of viruses, this report may have wider implication for designing of antivirals against this important group of human pathogens. Also, as similar le-RNA/P protein interactions has been reported in other viruses (Horikami and Moyer, 1991; Keene et al., 1981; Raha et al., 2004; Yang et al., 1999), it will be interesting to investigate if a similar strategy is successful in these systems too. Moreover, evaluating the efficacy of this peptide in a suitable animal model will be required before commenting on its therapeutic possibilities. However, we propose it as a good lead for future generation of peptidomimetic anti-virals against the *mononegavirale* group of viruses.

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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.2013.09.003.

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