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Short communication

N-protein-RNA interaction is a drug target in a negative strand RNA virus

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ABSTRACT

The negative strand RNA virus family contains many human pathogens. Finding new antiviral drug targets against this class of human pathogens is one of the significant healthcare needs. Nucleocapsid proteins of negative strand RNA viruses wrap the viral genomic RNA and play essential roles in gene transcription and genome replication. Chandipura virus, a member of the *Rhabdoviridae* family, has a negative strand RNA genome. In addition to wrapping the genomic RNA, its nucleocapsid protein interacts with the positive strand leader RNA and plays a vital role in the virus life-cycle. We have designed a peptide, based on prior knowledge and demonstrated that the peptide is capable of binding specifically to the positive strand leader RNA. When the peptide was transported inside the cell, it inhibited viral growth with IC_{50} values in the low micromolar range. Given the widespread occurrence of leader RNAs in negative strand RNA viruses and its interaction with the nucleocapsid protein, it is likely that this interaction could be a valid drug target for other negative strand RNA viruses.

RNA viruses form a deadly group of human pathogens that cause Ebola, SARS, COVID-19, rabies, influenza, hepatitis C, polio, and HIV/AIDS. In most cases, available therapeutic interventions are insufficient, and there is a crying need for new drug targets. This is now highlighted with the COVID-19 pandemic. Genomes of many RNA viruses are encapsidated with a nucleocapsid protein (Albertini et al., 2006; Green et al., 2006), usually abbreviated as N. It plays an essential role in many aspects of the viral life-cycle (Green et al., 2000; Zhang et al., 2008). Chandipura virus is a putative human pathogen and proposed to have caused several outbreaks in the Indian sub-continent (Chadha et al., 2005; Gurav et al., 2010; Menghani et al., 2012; Van, 2004). It is a negative-strand RNA virus belonging to the *Rhabdoviridae* family (Basak et al., 2007; Dragunova and Závada, 1979; Rao et al., 2004). In this article, we have explored and validated the interaction of the nucleocapsid protein and the positive-strand leader RNA as a drug target. Using a peptide derived from the N-protein sequence, we have demonstrated that disruption of this interaction leads to the abrogation of viral growth in more than one assay system.

Proteins of the Chandipura virus have strong homologies to those of the Vesicular Stomatitis Virus (VSV). Due to this similarity and the known genomic RNA binding site of the VSV N-protein, we predict that the C-terminal domain of the N protein is a major RNA-binding region (Blumberg et al., 1983; Green et al., 2006). Apart from general encapsidation of the single-stranded RNA, the N protein is known to specifically interact with positive sense leader RNA in CHPV (Mondal et al., 2010). In the rabies virus, the genomic RNA is unable to compete with the leader RNA, implying some differences in the mode of binding of the leader RNA and the genomic RNA (Albertini et al., 2006). The genomic RNA binds in the central cavity, between the two domains (Fig. 1A) (Yang et al., 1998). RNABINDR predicted two major RNA binding regions within the C-terminal domain, amino acid residues: 351–374 & 404–422, as shown in (Fig. S1). The 351–374 segment is an unstructured loop projecting outward, making this an unlikely candidate for the leader RNA binding. The 391–422 segment, encompassing the 404–422 segment, is a helix-turn-helix motif (Fig. 1A). A peptide was, thus, synthesized comprising the C-terminal 32 amino acid residues, named

Abbreviations: CHPV, Chandipura virus; RdRp, RNA dependent RNA polymerase; HPLC, high performance liquid chromatography; ESI-MS, electrospray ionization mass spectroscopy; MALDI TOF, matrix assisted laser desorption ionization time of flight; Fmoc, 9-fluorenylmethyloxycarbonyl chloride; VSV, Vesicular stomatitis virus; MTT, (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide); MOI, Multiplicity of Infection; PBS, Phosphate buffered saline; IC_{50}, 50% inhibitory concentration.

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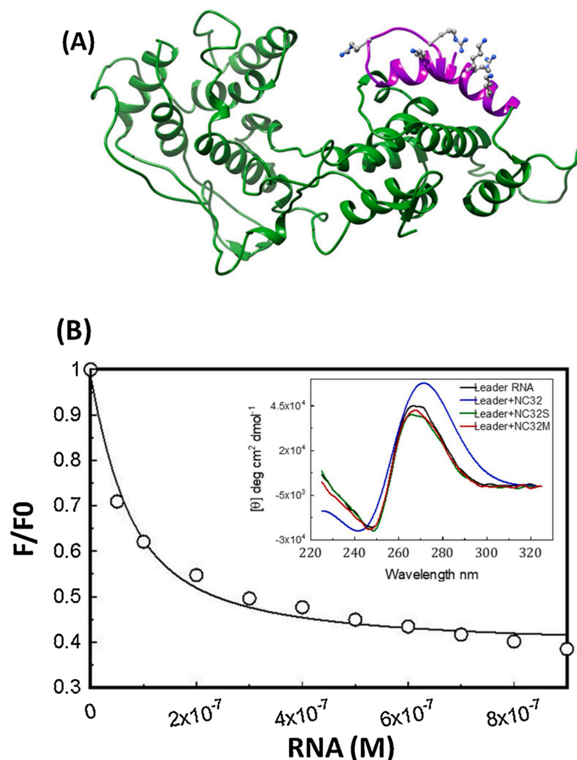

Fig. 1. The peptide NC32 specifically interacts with the leader RNA CHP-le 1. (A) Homology-modeled structure of N-protein from Chandipura. The violet-colored part is the segment from which NC32 is derived. The ball and stick side-chains are lysine and arginine residues present in that segment. Homology model was generated by swiss model (https://swissmodel.expasy.org/) using vsv N-protein as template (PDB2gic). (B) Binding isotherm of NC32 with CHP-le 1. Inset, CD spectrum of CHP-le 1 in the absence (black) and presence of NC32 (blue), NC32M (green), NC32S (red). The buffer conditions were 50 mM Tris buffer, pH 8 at 25 °C. The data shows an average of ten spectra.

NC32. Mass and CD spectra of NC32 are shown in Figs. S2 and S3.

The leader RNA of the Chandipura virus contains two distinct halves, a stem-loop half, and an unstructured half, having different structural features. The N protein is predicted to interact with the unstructured part (Bhattacharya et al., 2006). NC32 interaction with the unstructured 5' end of CHPV leader RNA, CHP-le 1, was analyzed by Circular Dichroism (CD) and fluorescence spectroscopy. In order to confirm the specificity of the interaction, two mutant varieties of NC32 were designed and synthesized. One of the mutant peptides is a scrambled form of NC32, hereafter referred to as NC32S. Another mutant peptide named NC32M, which contains a mutation of the arginine residue to alanine at position 408 (R408A) of the full-length nucleocapsid protein of CHPV. Among other residues in the C-terminal domain, R408 forms a critical contact with the RNA genome of VSV (Rainsford et al., 2010). The corresponding residue is the 19th residue in the construct marked in red in Table S1. The molecular weight and purity of the mutant peptides were characterized by MALDI-TOF. Near-UV CD spectra, which reflects only the signal from the nucleic acid bases, of 5 μM CHP-le 1 was recorded in the absence and presence of an equimolar concentration of NC32, NC32S, and NC32M. There is an approximately 1.5-fold increase in CD signal of CHP-le 1 in the near-UV region upon the addition of NC32, indicating that the peptide NC32 interacts with the leader RNA CHP-le 1 (Fig. 1B, inset). The enhancement of the CD signal at these wavelengths is likely due to some changes in base-stacking in the peptide-RNA complex. No significant change in CD signal was observed upon the addition of NC32S or NC32M. The lack of change of CD signal upon the addition of mutant peptides shows that NC32 interacts specifically with CHP-le 1. Fluorescence intensity of 5(6)-carboxyfluorescein labeled NC32 decreased on titrating with increasing concentrations of CHP-le 1 and was mostly saturated at or above 600 nM (Fig. 1B). The binding isotherm, when fitted to a single site binding equation gives a K_d value of 133 nM. The observed quenching of the labeled peptide by CHP-le 1 confirms that the 5′-half of CHPV leader RNA is binding to the C-terminal end of the viral Nucleocapsid protein, specifically in a concentration-dependent manner.

Although the peptide is significantly disordered in solution, it binds to the leader RNA specifically. Many examples of specific peptide binding to nucleic acids are known (Kjems et al., 1992; Talanian et al., 1990). In order to test the antiviral effect of NC32, six D-arginine residues were attached to its amino-terminal end. Hexa D-arginine residues act as a cell-penetrating peptide tag (Balhorn et al., 2009). Hexa-D-arginine tagged NC32 (DR-NC32) was labeled with 5(6)-carboxyfluorescein for assessing the intracellular permeability of the peptide. 5(6)-carboxyfluorescein-labeled DR-NC32 was added to monolayers of CV-1 cells and incubated for 3 h. Live cells were then visualized under an Andor spinning disk live-cell confocal microscope. After 3 h of incubation, DR-NC32 was almost homogeneously distributed within the cellular milieu. Cytotoxicity of DR-NC32 was measured by MTT assay. The peptide showed no significant cellular toxicity in concentrations up to 20 μM at 24 h (Fig. 2B).

To assess the potential of DR-NC32 for inhibiting CHPV growth, overnight monolayer cultures of Vero-76 cells were infected with CHPV at a Multiplicity of Infection (MOI) of 0.1. At 1 h post-infection, CHPV infected cells were treated with varying amounts of DR-NC32 or with Phosphate buffered saline (PBS) as control, and further incubated for 24 h. Titer and thus, the yield of the released viral particles in the cell culture supernatant was quantified by plaque assay (Fig. S4). The result shows that DR-NC32 decreases viral titer in a dose-dependent manner, with a 50% inhibitory concentration (IC_{50}) value of 3.4 ± 0.7 μM (Fig. 3A). Thus, DR-NC32 is effective in suppressing the growth of CHPV. Interaction of N protein with leader RNA has been shown to play a role in the initiation of encapsidation that is essential for the genomic RNA replication process (Bhattacharya et al., 2006). To evaluate whether DR-NC32 interferes with CHPV genome replication, we set up a minigenome assay based on plasmids under control of the T7 promotor in CV-1 cells infected by recombinant vaccinia virus expressing bacteriophage T7 polymerase (vTF7-3). This system reconstitutes a functional CHPV ribonucleocapsid in the cytoplasm upon transfection of four plasmids, one plasmid encoding a minigenome composed of the leader/trailer genomic regions flanking a luciferase reporter gene under the control of CHPV transcription signals (MGCL) (Chakraborty, 2017), and three separate plasmids encoding the viral N, P, and L proteins. The production of luciferase in this system combines the encapsidation of the minigenome successively into ribonucleocapsid by the N protein and its transcription by the L/P complex (Chakraborty, 2017). To investigate the inhibitory potential of DR-NC32 on the transcription/replication process, we have measured the luciferase expression at 24 h after co-transfection of plasmids constituting the minireplicon system. At 5 h post-transfection, CV-1 cells encoding the minigenome were treated

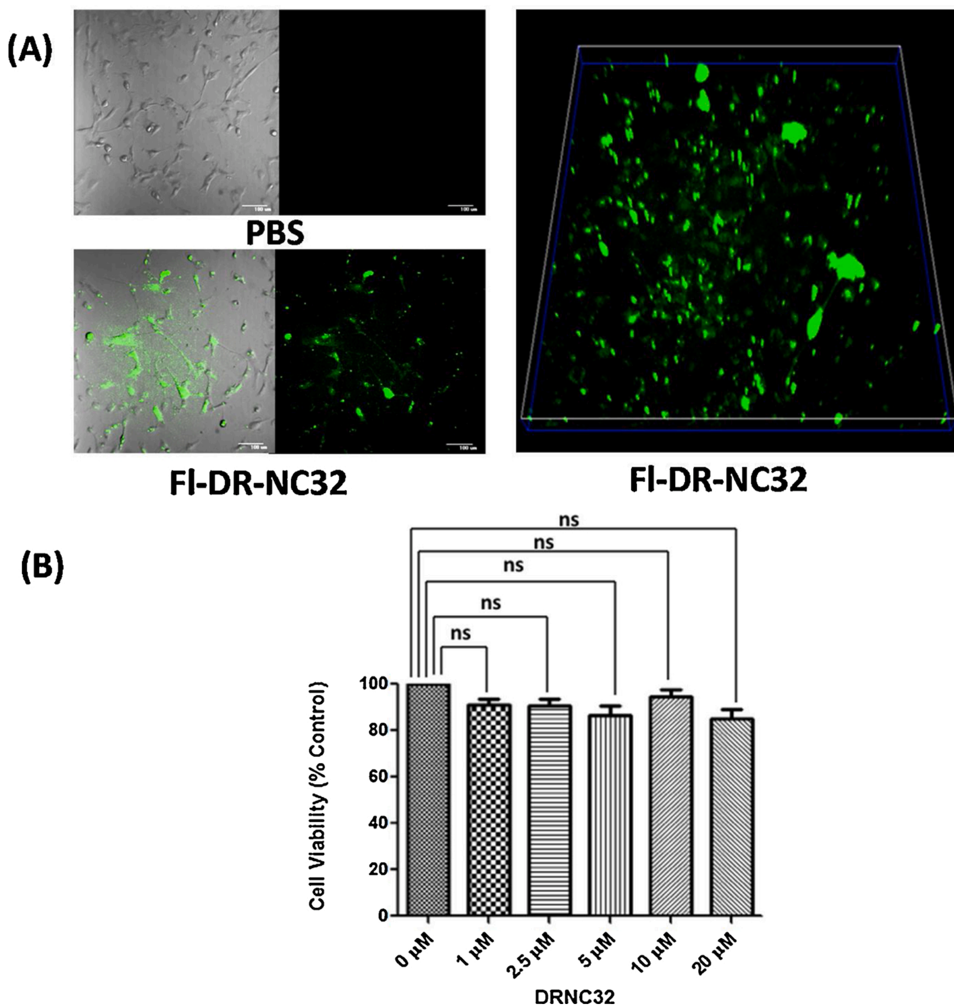

Fig. 2. Determination of cellular permeability and cytotoxicity of the peptide DR-NC32. (A) CV-1 cells were treated with PBS (Blank), or 5 (6)-carboxyfluorescein labeled DR-NC32 at a final concentration of 2.5 μM for 3 h. Fluorescence of live cells was monitored by excitation at 488 nm under Andor spinning disk live-cell confocal microscope. A Z-stack image of CV-1 cells treated with 5(6)-carboxyfluorescein labeled DR-NC32 at a final concentration of 2.5 μM for 3 h is also shown. The bar on the bottom right-hand side of the figures represents 10 μm. (B) MTT assay was performed on CV-1 cells treated for 24 h with increasing concentrations of DR-NC32. Cells treated with PBS were used as control.

with varying amounts of DR-NC32 or with PBS control (Fig. 3B). The data indicated that DR-NC32 inhibited the expression of luciferase from the CHPV minigenome system. The IC_{50} value was 5.0 ± 0.7 μM. This value is close to the one obtained with the live virus. N protein is a multifunctional protein facilitating many important roles in the life cycle of CHPV. As a monomer it recognizes and binds specific sequences of the viral RNA in complex with the P protein. Subsequent N-N oligomerization generates an RNA binding interface capable of encapsidating the entire virus genome in coordination with P protein and positive sense leader RNA. Given the affinity of the peptide DR-NC32 towards CHPV leader RNA, it is likely that the observed antiviral activity is due to the disruption of the N protein-leader (+) RNA interaction. DR-NC32 corresponds to a highly integral part of the N protein C-terminal domain. So, another plausible explanation for the inhibition of viral growth could be the misfolding of N protein caused by the peptide. More research is needed in order to accurately determine the mechanism of action of the peptide DR-NC32 and improve upon its antiviral activity. Recently, a study has appeared that suggests that the nucleocapsid protein of the SARS-COV2-RNA interaction could be a potential drug target against COVID-19 (Kang et al., 2020). Given the general importance of nucleocapsid proteins in life-cycles of pathogenic RNA viruses, peptides or peptidomimetics could be successfully used to disrupt virus life-cycles. Even if the peptides prove unsuitable, ultimately, validation of N-RNA interaction provides a path for developing small molecules that may interfere with this interaction.

Author statement

Prasenjit Chakaraborty carried out the experiments, partially designed the experiments, and wrote the initial manuscript.

Dhrubajyoti Chattopadhyay analyzed the data, plan the experiments, and edited the manuscript.

Siddhartha Roy planned the overall study, partially analyzed the data, and edited the final manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

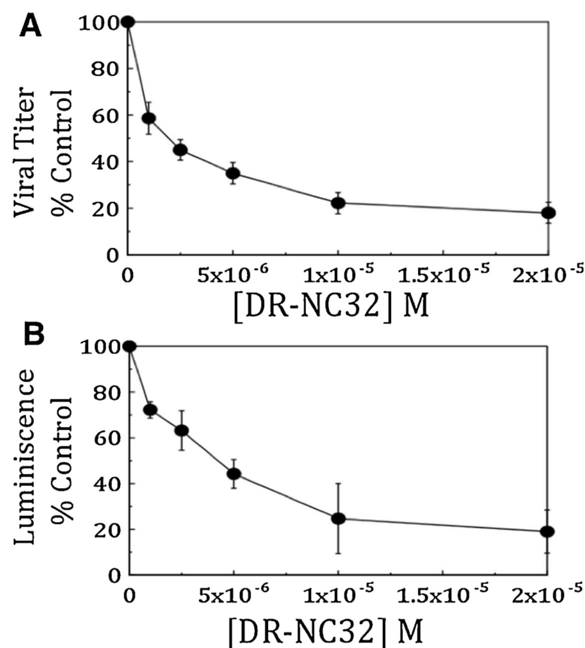

Fig. 3. Inhibition of CHPV growth by the peptide DR-NC32. (A) The graph showing the effect of DR-NC32 on CHPV titer 24 h post-infection of Vero-76 cells in the presence or absence of peptides, as described. Five different concentrations of the peptide were used (1, 2.5, 5, 10, and 20 μM). Viral growth inhibition measurement is reported as the percentage of plaques in peptide-treated samples with respect to that obtained with the untreated samples. (B) The CHPV minireplicon expressing luciferase was treated with five different concentrations of DR-NC32 (1, 2.5, 5, 10, 20 μM).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2021.198298.

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