Cyclophilin A: a possible host modulator in Chandipura virus infection

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Abstract
Chandipura virus (CHPV), belonging to the genus Vesiculovirus of the family Rhabdoviridae, has been identified as one of the causes of pediatric encephalitis in India. Currently, neither vaccines nor therapeutic drugs are available against this agent. Considering that the disease progresses very fast with a high mortality rate, working towards the development of potential therapeutics against it will have a public health impact. Although the use of viral inhibitors as antiviral agents is the most common way to curb virus replication, the mutation-prone nature of viruses results in the development of resistance to antiviral agents. The recent development of proteomic platforms for analysis of purified viral agents has allowed certain upregulated host proteins that are involved in the morphogenesis and replication of viruses to be identified. Thus, the alternative approach of inhibition of host proteins involved in the regulation of virus replication could be explored for their therapeutic effectiveness. In the current study, we have evaluated the effect of inhibition of cyclophilin A (CypA), an immunophilin with peptidyl-prolyl cis/trans-isomerase activity, on the replication of CHPV. Treatment with cyclosporin A, used in vitro for the inhibition of CypA, resulted in a 3-log reduction in CHPV titer and an undetectable level of CypA in comparison to an untreated control. An in silico analysis of the interaction of the CHPV nucleoprotein with the human CypA protein showed stable interaction in molecular docking and molecular dynamics simulations. Overall, the results of this study suggest a possible role of CypA in facilitating CHPV replication, thus making it one of the potential host factors to be explored in future antiviral studies.

Introduction
Chandipura virus (CHPV), a negative-sense RNA virus, belongs to the genus Vesiculovirus of the family Rhabdoviridae [1]. The virus was first isolated in the village of Chandipur in the Nagpur district of Maharashtra state, India, in 1965 [2]. Later, in the year 2003, large encephalitis outbreaks were reported in the states of Andhra Pradesh, Telangana, and Maharashtra [3]. The disease was clinically characterized by a high-grade fever of short duration, vomiting, altered sensorium, generalized convulsions, and decerebrate posture, leading to grade IV coma and death within 48 h of hospitalization [3]. The CHPV genome encodes five proteins, namely, nucleocapsid protein N, phosphoprotein P, matrix protein M, glycoprotein G, and large protein L. These proteins interact with host cell machinery to enable the various stages of the viral life cycle, beginning with entry and uncoating of the virus, followed by transcription and replication of its genome, and finally the assembly and release of progeny virions [4].

The role of different host proteins in the virus life cycle has been studied for different viral infections. Recently, with the help of computational approaches based on structure and sequence similarity, 121 putative host interactors were identified for CHPV [5]. One of the host proteins, cyclophilin A (CypA), which has been demonstrated to be associated with the life cycle of several viruses, including VSV, the prototype virus of the genus Vesiculovirus [6]. CypA is an immunophilin with peptidyl-prolyl cis/trans isomerase (PPIase) activity that plays a key role in protein folding [7]. It is also associated with cellular functions such as cell signaling, transcriptional regulation, protein folding, and trafficking [8, 9] besides playing an important role as a receptor for the immunosuppressive drug cyclosporin A (CsA) [10]. Dawar et al. [11] reviewed its diverse roles in infections with viruses including hepatitis B virus (HBV), vaccinia virus (VV), human immunodeficiency virus type 1 (HIV-1),...
hepatitis C virus (HCV), coronaviruses (CoVs), vesicular stomatitis virus (VSV), human cytomegalovirus (HCMV), flaviviruses, and enterovirus 71. An antiviral role of CypA has been demonstrated against vesicular stomatitis virus (VSV) [6], a related virus belonging to the genus Vesiculovirus of the family Rhabdoviridae. The recent observations by Zhang et al. [12] on proteomic profiling of purified rabies virus particles have also documented the association of CypA with virus particles, emphasizing its possible role in virus replication. The cyclophilins are known for their high binding affinity for CsA [10]. Thus, inhibition of CypA using known inhibitors such as CsA and its synthetic analogs (NIM811, alisporivir) [11] may pave the way for exploring the role of CypA in viral infections. The availability of cyclosporin A and its analogs for clinical use makes it easier to target this particular host protein to curtail viral infection, thus making it one of the attractive targets for antiviral therapy [13]. Owing to the diverse role of CypA in viral infections, the aim of the current study was to explore the role of CypA in CHPV infection using in silico as well in vitro approaches.

Materials and methods

Study design

Chandipura virus strain 034627 (CIN0327) was used for the study. To investigate the involvement of CypA in CHPV infection, CsA was used as an inhibitor of CypA. The cell cytotoxicity of CsA in RD cells was determined using an MTT assay. Concentrations at which > 90% cell viability was observed were selected for further analysis. To assess the effect of CypA inhibition on CHPV replication, a plaque reduction assay was carried out. The growth kinetics of CHPV in RD cells in the presence or absence of CypA inhibitor were compared. The presence of extracellular CypA during CHPV infection in the presence or absence of CsA was determined using a commercially available CypA ELISA. The experimental work was further supported by in silico studies. The 3D structure of the N protein of CHPV 034627 was predicted and validated. The validated structure was then analyzed for its interaction with human cyclophilin A by in silico docking. The stability of the docked complex was analyzed by molecular dynamics simulation.

Virus and cells

A CHPV strain isolated during the 2003 outbreak, CHPV-034627 [3], maintained at the lowest passage level, was used for all in vitro experiments, including plaque reduction assays and growth kinetics assays carried out in this study. The RD cell line (a human rhabdomyosarcoma cell line) was procured from ATCC (ATCC CCL-136, USA). It was maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) and 100 U of penicillin and 100 µg of streptomycin (Invitrogen, USA) per ml at 37 °C in a 5% CO₂ incubator for propagation of the virus and growth kinetics experiments.

Virus stock preparation and quantitation

RD cells were infected with CHPV 034627 at a multiplicity of infection (moi) of 0.01 for 1 h at 37 °C. The unabsorbed virus was removed, and DMEM with 2% FBS was added. The culture supernatant was harvested when 80-90% of cells showed a cytopathic effect (CPE). This material was further clarified by centrifugation and stored at – 80 °C for further use.

The quantitation of the reference virus stock in plaque-forming units was carried out in RD cells using a plaque assay as described earlier [14]. The assay was terminated at 24 h postinfection, and plaques were counted manually. The virus titer was expressed as plaque-forming units/ml (PFU/ml) and was used to determine the moi in further infections and experimental growth kinetics assays.

Inhibition of CypA

To determine the inhibitory concentrations of CsA, the 50% cytotoxic concentration (CC₅₀) of commercially available CsA (CypA inhibitor) was first determined using an MTT assay carried out in RD cells with CsA concentrations in the range of 5-500 μM in quadruplicate as described earlier [15].

Effect of inhibition of cyclophilin A (CypA) with cyclosporin A (CsA) on CHPV replication

Based on the results of the MTT assay, non-toxic concentrations (> 90% cell viability) of CsA were chosen for studying the effect of inhibition of CypA on CHPV replication. In a preliminary experiment to assess the effect of CypA inhibition on CHPV replication, a plaque reduction assay in RD cells was carried out using 100 PFU of CHPV and 5 μM and 10 μM concentrations of CsA in 1.8% CMC overlay medium after virus adsorption. Wells overlaid with CMC overlay after virus infection served as a positive control, and uninfected wells overlaid with CMC overlay served as a negative control.

The effect of CypA inhibition on CHPV replication was assessed by comparing the growth kinetics of CHPV in RD cells in the presence or absence of CsA. In these experiments, cells were infected with CHPV at an moi of 0.01 and 5 μM or 10 μM concentrations of CsA were used. Uninfected cells suspended in CsA-containing medium and in medium
Without CsA served as a drug control and a negative control, respectively, whereas cells infected with the virus and suspended in medium without CsA served as a virus control. The culture supernatant was harvested at 6, 12, 18, and 24 h postinfection and stored at -80°C for further virus titer determination by plaque assay.

In order to investigate the association of CypA inhibition with CHPV replication kinetics, we measured extracellular and intracellular levels of CypA in uninfected and infected cells in the presence and absence of CsA.

For extracellular detection of CypA, culture supernatant harvested at different time points was tested by ELISA. For intracellular detection of CypA, cells were collected from each well in RIPA lysis buffer (Cell Signalling Technology, USA) containing protease inhibitor cocktail. Cell lysis was carried out for 10 min on ice, followed by centrifugation at 14,000 rpm for 10 min. The clarified cell lysate was then used for measurement of CypA levels by ELISA. The level of CypA was analyzed in triplicate using a commercially available ELISA kit (Human Cyclophilin A ELISA, Sigma, USA) following the manufacturer’s instructions.

### 3D structure prediction and molecular docking

The 3D structure of the N protein of CHPV strain 034627 (GenBank Protein ID ADO63665) was predicted using a Swiss-Model online workstation [16] using the vesicular stomatitis virus (VSV) N protein as a template (PDB ID: 2GIC) [17]. The predicted structure was analyzed for the quality of modeling using the PROCHECK tool available at http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html [18]. The 3D structure of the human CypA protein was retrieved from the Protein Data Bank (PDB ID: 1CWA).

Visualizations of protein structure and images were generated using Discovery Studio Visualizer.

The 3D structure of human CypA (1CWA.pdb) retrieved from the RCSB Protein Data Bank (PDB) was used for docking with the predicted structure of the N protein of CHPV using the PATCHDOCK server (Molecular Docking Algorithm Based on Shape Complementarity Principles) (https://bioinfo3d.cs.tau.ac.il/PatchDock/https://bioinfo3d.cs.tau.ac.il/PatchDock/) [19]. The top 10 docking complexes generated by PATCHDOCK were refined using the FireDock tool (Fast Interaction Refinement in molecular DOCKing) available on the PATCHDOCK server. The refined complex with the lowest binding energy was selected for studying the stability of interaction using molecular dynamics simulation. The binding affinity of the selected complex was then calculated using PRODIGY (PROtein binDIng enerGY prediction) tool available online at https://bianca.science.uu.nl/prodigy/ [20].

### Molecular dynamics simulation

The stability of the docked complex of the CHPV N protein with the human CypA protein was evaluated by molecular dynamics simulation using the GROMACS-2020.2 molecular dynamics package [21]. The bonded and non-bonded interactions between the protein molecules were analyzed using the OPLS-AA/L all-atom force field [22]. The system was equilibrated, and charge neutralization was carried out by adding suitable ions as per established protocols. The system pressure and temperature were equilibrated at 1 bar and 300°K for an equilibration period of 100 ps. The MD simulations were set for 10 ns, and the RMSD was determined to obtain a stable protein-protein complex [23].
Results

Virus titration

Using a plaque assay, the titer of the virus was found to be $1 \times 10^7$ PFU/ml. This reference stock was used to calculate the moi for the growth kinetics assay (Supplementary Fig. S1).

Inhibition of CypA

Using an MTT assay to analyze the toxic effects of the CypA inhibitor CsA on RD cells, the $CC_{50}$ was found to be 40 µM. The non-toxic concentrations of CsA (5 µM and 10 µM) with > 90% cell viability were used in the inhibition study. A plaque reduction assay showed a fourfold reduction in the number of plaques (mean plaque number ± SD: 24 ± 5) in cells treated with 10 µM CsA (SD: 100 ± 8) compared to those treated with 5 µM CsA (100 ± 7) or untreated (Supplementary Fig. S2).

The growth kinetics of CHPV in the presence of CsA showed a dose-dependent effect on virus replication (Fig. 1). The infection of RD cells reached a titer of $1 \times 10^7$ PFU/ml (mean ± 1SE, log_{10} PFU/ml) in the untreated virus control cells and cells treated with 5 µM CsA, but it reached only $1 \times 10^5$ PFU/ml (mean ± 1SE, log_{10} PFU/ml) in the presence of 10 µM CsA. Figure 1 shows the effect of inhibition of CypA on CHPV replication. A reduction in virus titer by $> \log 3$ (mean ± 1SE, log_{10} PFU/ml; $2 \times 10^4$ PFU/ml vs. $1 \times 10^7$ PFU/ml in the virus control and $3 \times 10^6$ PFU/ml in 5-µM-CsA-treated cells) was observed at 18 h postinfection in cells treated with 10 µM CsA. The impairment of replication of CHPV observed after inhibition of CypA indicated a possible role of this protein in virus replication.
Modulation of Chandipura virus infection by cyclophilin A

Furthermore, measurement of CypA levels in the culture supernatants harvested for the viral growth kinetics experiment revealed the association of the extracellular level of CypA with that of viral egress. The cells treated with CypA inhibitor after virus infection showed lower levels (decrease of 3 logs) of both the virus and CypA in the culture supernatant at different time points, whereas the untreated virus-infected cells showed a substantial increase in CypA concentration at 18 and 24 h postinfection. In the case of the uninfected drug control cells treated with 5 µM and 10 µM CsA, the CypA was not detectable, whereas a slight increase in CypA was detected in control cells (Fig. 2a).

The assays for intracellular detection of CypA revealed an approximately fourfold reduction in the CypA level in the presence of 10 µM CsA in both the CHPV-infected and uninfected cells (Fig. 2b).

Analysis of the virus multiplication rate as a function of time using the Mann-Whitney test showed a significant difference in growth kinetics of CHPV in CsA-treated (10 µM) vs. untreated RD cells ($p < 0.01$).

3D structure prediction and molecular docking

The 3D structure of the N protein of CHPV-034627 was predicted using the VSV N protein as a template (PDB ID: 2GIC). Assessment of the Ramachandran plot revealed that the overall percentage of residues in favored and allowed regions was 99.5%, indicating the reliability of the modeled structure. The in silico molecular docking of the predicted N protein structure with Human CypA (PDB ID: 1CWA) was retrieved from the RCSB Protein Data Bank (PDB). The top-ten-ranking complexes generated by PatchDock were subjected to refinement using the FireDock tool. The docked complex refined by FireDock with a binding energy in solution of -3.19 kcal/mol formed one hydrogen bond between Ala20 of the N protein and Glu120 of cyclophilin A. The van der Waals energy component was the principal stabilizing energy for their interactions, as calculated by FireDock. The best solution is shown in Figure 3. As determined using the online PRODIGY tool, the binding energy of the selected complex was $\Delta G = -8.5$ kcal/mol. After FireDock refinement, the selected docked complex with the lowest binding energy was used to analyze the stability of the interaction in a molecular dynamics simulation.

Molecular dynamics

To evaluate the stability of the CHPV N protein complexed with human CypA, a molecular dynamics simulation was performed using GROMACS-2020.2. The temperature and pressure were equilibrated by applying the NVT and NPT ensemble, respectively, with a period of 100 ps (Fig. 4a and b). The minimized potential energy was estimated to be $-3.717 \times 10^6$ kJ/mol for the docked protein complex.

The RMSD plot of the complex is shown in Figure 4c. The RMSD gradually increased from 0.2 nm to 0.6 nm after 6 ns and then stabilized further to ~0.7 nm after 7 ns. The RMSF plot shown in Figure 4d indicated fluctuations in the CHPV N protein, covering the amino acid stretch from residues 9 to 26. Fluctuation in the human CypA protein was observed in the amino acid stretch from residues 100 to 125. It should be noted that these regions of maximum fluctuation form the binding interface in the complex with H-bonds formed between Ala 20 of the N protein and Glu 120 of CypA. These findings...
demonstrate the flexibility and stability of the docked protein complex.

**Discussion**

Like many other viruses, CHPV encodes few proteins of its own. Consequently, concerted interactions between host and viral proteins are essential for efficient virus replication [24]. Earlier studies carried out on vesicular stomatitis virus demonstrated the role of CypA as a chaperone protein and one of the cellular factors required for VSV replication. Bose et al. concluded that the interaction of CypA with the VSV N protein enables the folding of nucleocapsids into a transcriptionally competent conformation required for optimal transcription efficiency of the VSV-NJ genome [6].

In the present study, we evaluated the role of CypA in CHPV replication. We first analyzed the *in silico* interaction of the CHPV N protein with the human CypA protein. In the absence of a known structure of the CHPV N protein, the 3D structure was predicted using the VSV N protein as a template. The predicted structure was judged to be stable and reliable by PROCHECK analysis (Ramachandran plot). Molecular docking of the predicted structure of the CHPV N protein with human CypA showed stable binding. MD simulations indicate that the complex is highly stable.

*In vitro* experiments indicated elevated extracellular levels of CypA in CHPV-infected cells at 18-24 h postinfection (Fig. 2a), and the virus titer was $1 \times 10^7$ PFU/ml at 18 h postinfection (Fig. 1). These findings indicate that an increased level of extracellular CypA may be associated with cytopathic effects. The effect of CsA-induced inhibition of CypA was observed in viral growth kinetics experiments. A greater than three-log reduction in virus titer was observed at 18 h postinfection when infected cells were treated with 10 µM CsA.

![Fig. 4](attachment:image.png)
We have also conducted laboratory experiments for the determination of intracellular levels of CypA. It was observed that treatment of both uninfected cells and cells infected with CHPV with 10 µM CsA (a CypA inhibitor) resulted in a fourfold reduction in CypA levels.

Drawing an analogy from reports on the N protein of VSV, a virus related to CHPV, it could be concluded that the host CypA may play a similar role in the replication of CHPV. In parallel, in silico studies indicated stable binding of the CHPV N protein with the host CypA, thus supporting our in vitro observations. However, experimental work to study the interaction of CypA with the CHPV N protein needs to be performed to test the accuracy of the in silico predictions.

For the first time, we have obtained evidence for a possible role of the host CypA protein in the inhibition of CHPV replication. The information obtained in the present study can be further evaluated using different non-immunosuppressive analogs of CsA with conserved CypA inhibition potential.

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Declarations Conflict of interest The authors declare that they have no conflict of interest.

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