Virology 526 (2019) 91–98

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Glycan-dependent chikungunya viral infection divulged by antiviral activity of NAG specific chi-like lectin

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ARTICLE INFO

Keywords:
Alphavirus
Chikungunya virus
Chitinase (chi)-like lectin
N-glycans
Lectin therapeutics

ABSTRACT

Highly pathogenic alphaviruses display complex glycans on their surface. These glycans play a crucial role in viral pathogenesis by facilitating glycan-host interaction during viral entry which can be targeted. Various studies have reported antiviral activity of lectins that bind to the glycans present on the surface of enveloped viruses. This study evaluates the antiviral potential of a chitinase (chi)-like lectin from Tamarind (TCLL) having specificity for *N*-acetylglucosamine (NAG). Thus, TCLL might bind to N-glycan rich surface of alphavirus and inhibit the entry of virus into the host cells. The direct treatment of TCLL with virus reduced the virus infection. Remarkably, the addition of NAG to TCLL abolished antiviral activity confirming that NAG binding property of TCLL is accountable for its antiviral activity. Further, an ELISA assay confirmed the binding of TCLL to alphaviruses. Taken together, this study will prove to be beneficial in developing lectin therapeutics targeting alphavirus glycan.

1. Introduction

The presence of glycans on the surface of enveloped viruses is one of the very important features that are vital to viral infectivity (Merry and Astrautsova, 2010; Raman et al., 2016). These glycans play a key role in virus pathogenesis by enabling the entry of virus into its target cells. Many antiviral therapeutics can be developed by targeting these glycosylation sites present on the surface of the virus (Balzarini, 2007; Merry and Astrautsova, 2010; Swanson et al., 2010; Van der Meer et al., 2007; Wang et al., 2015; Yamazaki and Tagaya, 1980).

Alphaviruses cause mild to severe viral diseases in human and animals such as encephalitis, arthritis, fever and rash. These viruses are transmitted by mosquitoes and replicate in both mosquito cells and vertebrate hosts. Members of the alphavirus genus such as Sindbis virus (SINV), Aura virus (AV), Ross River virus (RRV), chikungunya virus (CHIKV), Semliki Forest virus (SFV), O'nyong'nyong virus (ONNV), Mayaro virus (MAYV), Eastern, Western and Venezuelan equine encephalitis virus (EEEV, WEEV and VEEV) are distributed worldwide (Ryman and Klimstra, 2008; Strauss and Strauss, 1994). The CHIKV reemergence in 2005 brings the need to study these alphaviruses and their mechanisms that pose a potential threat to human health (Muniaraj, 2014; Roth et al., 2014; Sergon et al., 2007; Simon et al., 2006). There is currently no vaccine or antiviral therapy commercially available. Prevention of mosquito bites is the only method to save the public from the possible alphavirus epidemics.

Alphaviruses are arthropod-borne, single-stranded, positive-sense, enveloped viruses and they use the mechanism of membrane fusion for the introduction of their viral RNA genomes into the host cell. These viruses possess the membrane-anchored surface glycoproteins for recognizing the receptor and for their entry into the host cells through membrane fusion. The receptor-mediated endocytosis is used as an entry route by alphaviruses (Helenius et al., 1980; Marsh and Helenius, 1989). The viral entry is carried out by two viral glycoproteins, E1 and E2 (Schlesinger and Schlesinger, 1986; Simons and Warren, 1984). The membrane proteins, E1 and p62 (precursor to the E2 and E3 proteins before the furin cleavage of p62) are the result of precursor structural polyprotein processing by host proteases. p62 after the furin cleavage results in E2 and E3 glycoproteins. The glycoprotein, E1 helps in the membrane fusion, whereas, E2 helps in receptor binding (Garoff and Simons, 1974; Li et al., 2010; Lobigs et al., 1990; Metsikkö and Garoff, 1990; Omar and Koblet, 1988; Voss et al., 2010).

Blocking of viral surface glycoprotein epitopes that are used by viruses to interact with the host cell receptor for entry is one of the methods for the prevention and inhibition of viral infections (Balzarini, 2007). Recently, these carbohydrate or sugar moieties on viral surfaces are being exploited for drug development to control virus infections (Alen et al., 2009; Balzarini, 2007; Bertaux et al., 2007; François et al., 2008; Mounce et al., 2017). Sugar binding proteins/lectins or

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https://doi.org/10.1016/j.virol.2018.10.009
Received 7 July 2018; Received in revised form 25 September 2018; Accepted 10 October 2018
Available online 25 October 2018

neutralizing monoclonal antibodies that specifically bind epitopes on the virus surface are being investigated for various pathogenic viruses for development of antiviral therapy (Alen et al., 2009; An et al., 2006; Balzarini, 2007; Balzarini et al., 1992; Crill and Roehrig, 2001; Li et al., 2010; Ooi et al., 2010; Swanson et al., 2010; Wang et al., 2015). A banana lectin (BanLec) has been shown to inhibit the replication of HIV through its interaction with glycosylated envelope protein, gp120 (Swanson et al., 2010).

Alphaviruses attach to the receptor on the host cell membrane using their surface glycoprotein and enter into the target host cell (Jose et al., 2009; Smith et al., 1995). The cell membrane then encloses and seals the virus particles in the endocytic vesicles. The virus, via these vesicles, is transported to the successive cell compartments and delivered to the pre-lysosomal endosomes. The acidic pH of these endosomes results in conformational changes in the viral spike proteins and leads to the activation of E1 (Gibbons et al., 2003; Hammar et al., 2003; Lescar et al., 2001; Qin et al., 2009). The E1 protein then triggers the fusion of endosomal and viral membranes, as a result of which the virus releases its nucleocapsid into the host cell cytoplasm and initiates the virus replication process (Justman et al., 1993; Kielian, 1995). Disassembly of nucleocapsid results in the release of viral RNA genome. The genome is directly translated into viral replication proteins that replicate the genome (Strauss et al., 1984). After the replication of the viral RNA genome, the viral structural proteins form new infectious virus particles that bud out of the host cellular membrane. These new progeny virus released from infected cells interact with the neighboring host cell receptors through the mature glycoproteins on the surface of virions and spread infection. Any chemical compound or molecule that blocks the interaction of alphaviruses with the host cell can be developed as potential therapeutics for alphavirus treatment. The presence of N-glycans has been shown in the crystal structures of E3-E2-E1 glycoprotein complexes (mature) and p62–E1 glycoprotein (immature) of CHIKV (PDB IDs: 3N40, 3N42) (Voss et al., 2010). The crystal structure of E2-E1 heterodimer of SINV been reported which shows the presence of the N-glycan sites (PDB IDs: 3MUU) (Li et al., 2010).

The chitinase (chi)-like lectin from tamarind (*Tamarindus indica*) (TCLL) belongs to glycosyl hydrolase 18 (GH18) families of chitinases. The members of this family possess $(\beta/\alpha)_8$ barrel topology with an active site (DxDxE) of two aspartic acid and one glutamic acid residues separated by isoleucine and phenylalanine (Kesari et al., 2015). It lacks chitinase activity as the glutamic acid an active site residue is substituted making it an inactive chitinase. It has evolved to adopt an *N*-acetylglucosamine (NAG) binding property (Patil et al., 2013). The lectin TCLL isolated from the seeds of tamarind is a chitinase (chi)-like lectin, exists as a monomer having a molecular mass ~33 kDa (Patil et al., 2009, 2013). As TCLL has an ability to bind to the glycans/sugar molecules, so we sought to find out whether it binds to the N-glycan rich surface of alphavirus/chikungunya and further block the alphaviral infection.

In the present study, TCLL has been purified from tamarind seeds to near homogeneity. The binding of TCLL to the alphavirus envelope associated glycans was confirmed by an enzyme-linked immunosorbent assay (ELISA). Here we report that TCLL possesses an inhibitory and/or antiviral activity investigated using luciferase based assay and plaque reduction assay. The decreased levels of viral RNA were seen in the presence of TCLL confirming the antiviral effect of TCLL. The reversal of the antiviral effect of TCLL in the presence of NAG revealed that the inhibitory and/or antiviral activity of TCLL is due to its association with the N-glycan rich envelope of alphaviruses. Thus, TCLL has potential to be used as an antiviral therapeutics against the members of the alphavirus genus such as CHIKV and SINV. This study for the first time evaluated the antiviral use of chitinase (chi)-like lectin having specificity for NAG and assessed its ability to bind alphaviruses and block the entry step of the virus.

2. Materials and methods

2.1. TCLL purification

Seeds of tamarind were obtained from the fruit of locally growing plant. TCLL was purified as described previously with slight modifications (Patil et al., 2009). Briefly, the pulp from the fruit was removed manually and seeds were soaked overnight in buffer I (50 mM Tris-HCl buffer pH 7.4). The seed coat was removed manually using a pair of forceps and a razor. Using a blender the soaked seeds were homogenized to obtain the crude extract. The crude extract was centrifuged at 12,000 $\times g$ for 20 min at 4 °C. The collected supernatant was loaded onto the Affi-Gel Blue matrix (Bio-Rad, USA). Elution of the protein was done by using a stepwise gradient of NaCl (0.1, 0.2, 0.3, 0.4, 0.5, 0.8 and 1 M) in buffer I. Fractions containing the purified protein were dialyzed in buffer I at 4 °C. The sample was then subjected to an anion-exchange matrix (MacroPrep DEAE (diethylaminoethyl), Bio-Rad, USA). The matrix was pre-equilibrated with buffer I. Elution of the protein was done by using a stepwise gradient of NaCl (0.05, 0.1, 0.2, 0.3 and 0.5 M). The eluted fractions were run on 15% SDS-PAGE and staining was done with Coomassie brilliant blue. Amicon Ultra-15 (10 kDa cutoff) microconcentrator (Millipore, USA) was used to concentrate the protein. The protein sample was further purified using pre-equilibrated Superdex 75 prep grade (HiLoad 16/600) size exclusion chromatography column on ÄKTA purifier (GE Healthcare). The purity of the protein was checked by running the sample on 15% SDS PAGE and staining was done with silver stain. The extinction coefficient method was used to measure the concentration of protein with UV absorbance spectroscopy at 280 nm (extinction coefficient of TCLL is 33,725 M^{-1} cm^{-1}).

2.2. Cells

BHK-21 (Baby hamster kidney) and Vero cells were obtained from the National Center for Cell Science, Pune. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Himedia) containing 1% penicillin-streptomycin solution (PenStrep; Invitrogen, 5000 units/ml of penicillin and 5000 µg/ml of streptomycin) and 10% fetal bovine serum (FBS; Invitrogen), at 37 °C and 5% CO_2 in a humidified incubator by regular passaging at periodic intervals of 3–4 days.

2.3. Viruses

SINV was derived from the full-length SINV cDNA clone, pTOTO64. It has a firefly luciferase reporter gene (FLuc) driven by an internal ribosome entry site (IRES) inserted 3′ to the E1 gene The FLuc is followed by 3′ untranslated region (UTR). Luciferase is expressed off of an internal ribosome entry site (IRES) so that infectivity of the virus can be monitored directly as the amount of luciferase produced in cells with time (Snyder et al., 2013). The SINV with luciferase reporter gene was a kind gift from Prof. Richard J. Kuhn, Purdue University, USA.

Capped RNA transcripts were made by in vitro transcription of the full-length SINV cDNA clone, pTOTO64. The plasmid was linearized with a restriction enzyme, *Sac*I and the linear DNA was transcribed using SP6 RNA polymerase. Transfection into BHK-21 cells was done using electroporation. Briefly, confluent monolayers of BHK-21 cells were collected after trypsinization. Washing of cells was done twice using phosphate buffered saline (PBS) and resuspended in 400 µl PBS along with 10 µg of in vitro transcribed RNA. RNA was electroporated into cells using a Gene Pulser Xcell™ electroporator (Bio-Rad) at 25 µF, 1.5 kV and 200 Ω. Then the MEM with 2% FBS was added to the cells. The media was collected at 48 h post-electroporation and the virus titer was determined by standard plaque assay.

CHIKV was propagated in Vero cells using standard virus adsorption techniques. The titer of virus was determined using a standard plaque assay. Viral stocks were filtered and stored at −80 °C until use (Singh

et al., 2018).

2.4. Cell cytotoxicity assay

The cell cytotoxicity was evaluated by performing MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl tetrazolium bromide)] assay. Approximately 1×10^4 BHK-21 cells were seeded in 100 µl media per well in a 96-well plate and incubated at 37 °C. At ~80% cell confluency, the different concentrations of TCLL protein (5, 10, 25, 50, 100, 250 and 500 µM) were added to the cells. In another set, different concentrations of NAG (1, 5, 10, 15, 25 mM) were taken and incubated for 36 h at 37 °C. The MTT (0.5 mg/ml) reagent was added to the cells and further, incubated at 37 °C for 4 h in dark. Finally, dimethyl sulfoxide (DMSO) was added and the absorbance at 570 nm was recorded using multi-mode plate reader Cytation 3 (BioTek Instruments, Inc.). In case of control, cells were treated with medium without the compound and were considered to have 100% viability. The data are shown as a mean percentage of the viable cells versus control values.

2.5. TCLL and alphavirus interaction using ELISA

50 µl of each concentration of 0, 5, 10, 25, 50, 100 and 150 µM of TCLL protein were used to coat the wells of a 96-well ELISA micro-plate (MICROLON®; Greiner bio-one, Germany) for 2 h at 25 °C in duplicates. The blocking was done by adding an equal volume of 5 mM EDTA and 2% SDS and washed with TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20). For virus interaction, SINV virus was diluted in PBS for a multiplicity of infection (MOI) of 0.1. In another set, the same procedure was followed using the TCLL protein that was pre-incubated with 1 mM of NAG for 15 min before its incubation with virus and incubated at 25 °C for 1.5 h. The blocking was done with 3% skimmed milk in TBST for 1 h followed by incubation with the mouse anti-E2 alphavirus glycoprotein monoclonal antibody (Santa Cruz Biotechnolgy, Inc.) at a dilution of 1:3000 in TBST for 2 h at 25 °C. Subsequently, incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 secondary antibody (Affymetrix eBioscience) at a dilution of 1:7000 for 1 h at 25 °C. The interaction of TCLL protein with SINV was detected using TMB (tetramethylbenzidine)/H_2O_2 substrate (GeNei). After sufficient color development, 100 µl of 1 N H_2SO_4 was added to stop the reaction and the absorbance of resulting yellow color was taken at 450 nm with the help of a multi-mode plate reader Cytation 3 (BioTek Instruments, Inc.).

For a negative control reaction, Bovine serum albumin (BSA) was used in place of TCLL protein. In another control, only TCLL was taken without the virus. Also, the TCLL was pre-incubated with BSA before its incubation with virus and used as a control. Further, to determine the interaction of TCLL with CHIKV, a silimlar procedure of ELISA was followed using CHIKV at MOI of 0.1.

2.6. Luciferase based assay for SINV

The antiviral activity of TCLL protein was investigated by performing luciferase based assay using SINV with firefly luciferase. BHK-21 cells were seeded at a cell density of approximately 1×10^5 in 500 µl media per well in 24-well plate and incubated at 37 °C. The virus at MOI of 0.1 was incubated with 100 µM TCLL for 1 h at room temperature. This pre-incubated mixture was added to the cells and incubated at 37 °C. The cells were lysed after 0, 4, 6, 8 and 12 h using lysis buffer provided with firefly luciferase assay system (Promega, USA). The relative luminescence units (RLU) were recorded using a multi-mode plate reader Cytation 3 (BioTek Instruments, Inc.). In a separate experiment, TCLL protein was pre-incubated with 1 mM NAG for 15 min before its incubation with virus. Further to investigate whether TCLL inhibits virus infection by interacting with the cells, BHK-21 cells were pre-incubated with 100 µM TCLL by direct addition of diluted compound to the media for 1 h prior to infection with virus. The cell lysates were collected at different interval of time at 0, 4, 6, 8 and 12 h. As a control, only virus was taken without the compound.

2.7. Antiviral assay

To check the antiviral activity of TCLL protein against SINV and CHIKV, plaque assays were performed using BHK-21 and Vero cells, respectively. BHK-21 cells were seeded in a 12-well plate. 100 µM TCLL was incubated with SINV at an MOI of 0.1 for 1 h at room temperature and serial 10-fold dilutions of this pre-incubated mixture were made. In another set, TCLL protein was pre-incubated with 1 mM NAG for 15 min before its incubation with the virus. As a control SINV was taken without TCLL or NAG. At confluency of ~90%, the medium was removed from the cells and the cell monolayer was infected with viral dilutions along with the control and incubated at 37 °C for 1.5 h. Afterward, the viral inoculums were removed and 2 ml of overlay medium was added i.e. 1:1 mixture of 2 × media and 2% carboxymethyl cellulose (CMC). Further incubation was done at 37 °C for 3 days without any disturbance. The overlay medium was discarded and cells were fixed with 10% formaldehyde solution and stained with a minimal amount of 1% crystal violet. The reduction in viral titer (PFU/ml) was calculated as compared to control. A similar procedure of antiviral assay was followed for CHIKV using Vero cells. To explore the time-dependence of phenotype, SINV or CHIKV was incubated with 100 µM TCLL at different time intervals of 0.5, 1, 4 and 8 h. This incubated mixture was added to cells and virus titers were determined and compared with the virus control.

2.8. Viral titer reduction assay

The antiviral effect of TCLL protein on SINV and CHIKV was checked using viral titer reduction assays. Approximately 1×10^5 BHK-21 cells in 500 µl medium were seeded per well in a 24-well plate. SINV (MOI of 0.1) was pre-incubated with increasing concentrations of TCLL protein (1, 10, 50, 100 and 250 µM) for 1 h at room temperature. As a control, SINV was taken without TCLL. Media was removed from 24-well plate and the pre-incubated mixture was added to the cells and incubated at 37 °C. The supernatant was collected after 24 h post-infection (hpi) and subjected to plaque assays on BHK-21 cells. The viral titers (PFU/ml) were calculated and compared with the virus control without compound. A similar procedure of plaque assay for CHIKV (MOI of 0.1) using Vero cells was performed.

2.9. Viral RNA isolation and cDNA synthesis

The compound treatment was same as above. For quantification of viral genomes after compound treatment, Trizol (Invitrogen, USA) was directly added to the cells and RNA from virus infected host cell at 24 hpi was extracted using Trizol (Invitrogen, USA) using manufacturer's instructions. AccuScript high fidelity Ist strand cDNA synthesis kit (Agilent) was used to synthesize the cDNA from the isolated RNA using the manufacturer's protocol. cDNA was stored at −20 °C until processed.

2.10. Quantitative real time PCR (qPCR)

The amplification of SINV and CHIKV E1 genes along with GAPDH as internal control were done by using the KAPA SYBR® FAST qPCR kit master mix (2 ×) universal (Kapa Biosystems, USA) according to manufacturer's protocol in a Real-Time PCR StepOnePlus™ System (Applied Biosystems, USA). Briefly, samples were assayed in a 10 µl reaction mixture containing 5 µl of KAPA SYBR FAST, 0.4 µl of ROX high, 1 µl of cDNA, 2.6 µl of H_2O and a final concentration of either SINV E1 or CHIKV E1 or GAPDH forward and reverse primers: 10 pmol/µl each. The thermal profile was: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and

extension at 60 °C for 1 min. The oligonucleotide primers 5′-ACAGCC AGATGAGTGAGGCGTA-3′ (forward) and 5′-CGATGCTGAAATTGGTCC AGCTATG-3′ (reverse) were used for SINV E1 gene amplification. The primers used for CHIKV E1 gene amplification were: 5′-AAGTACACT GTGCAGCTGAGT-3′ (forward) and 5′-GCATAGCACCACGATTAGA ATC-3′ (reverse). The GAPDH internal control primers were: 5′-ACCA CAGTCCATGCCATCAC-3′ (forward), 5′-TCCACCACCCTGTTGCTGTA −3′ (reverse).

3. Results

3.1. TCLL purification

TCLL was isolated and purified from seeds of tamarind using affinity and ion-exchange chromatography. Firstly, the crude extract was obtained from the soaked tamarind seeds and centrifuged at 12,000 ×*g* for 20 min at 4 °C. The supernatant was collected. Affi-Gel Blue matrix was used and elution of the protein was done in a stepwise gradient of NaCl. The protein was eluted out in the 0.5 M NaCl fraction and dialyzed by using buffer (50 mM Tris-HCl pH 7.4) to remove the excessive salt. Secondly, the dialyzed sample was subjected to anion-exchange (DEAE) matrix and the eluted protein was obtained in a 0.1 M NaCl fraction. The protein was concentrated and further purified using gel-filtration chromatography. The presence of single band on 15% SDS-PAGE stained using silver stain confirmed the purity of the protein (Fig. 1). The molecular weight of the TCLL protein was ~33 kDa. The protein was concentrated up to 10 mg/ml.

3.2. Cell cytotoxicity testing

The maximal non-toxic dose (MNTD) for both TCLL and NAG was determined using MTT assay. BHK-21 cells were seeded in 96-well plate and different concentrations of the TCLL (5–500 μM) and NAG (1–25 mM) were added, and then MTT assay was performed at 36 h post treatment. TCLL did not show any toxicity up to 250 μM as > 80% cells were viable. In case of NAG, up to 25 mM concentration, no effect on cell viability was seen. The MNTD value of TCLL and NAG was found to be 250 μM and 25 mM, respectively with > 80% cell viability (Fig. 2). All the antiviral assays were performed at a concentration lower than MNTD value i.e. at 100 μM and 1 mM of TCLL and NAG, respectively.

3.3. TCLL and alphavirus interaction using ELISA

TCLL is known to bind to the glycans/sugar molecules, so we hypothesized that it may bind to the N-glycan rich surface of alphaviruses and further block the virus entry into the cells. An indirect ELISA assay was used to explore the binding of TCLL protein with SINV or CHIKV. The binding of TCLL to virus was checked by primary anti-E2 alphavirus glycoprotein monoclonal antibody and secondary horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 antibody. An increase in absorbance with the increasing concentration of TCLL was observed, suggesting the interaction of TCLL protein with the virus glycans in a concentration-dependent manner.

To further confirm the interaction of TCLL with the viral glycans, TCLL was pre-incubated with NAG before its incubation with virus. An insignificant increase in absorbance was seen, confirming the binding of TCLL to virus is due to its binding with glycans. As a negative control, BSA was used instead of TCLL and in another control, only TCLL protein was used without the virus. In both these controls, an insignificant increase in absorbance was seen. Also, when BSA was pre-incubated with TCLL in place of NAG, the rescue of TCLL was not observed and a significant increase in absorbance was seen (Fig. 3). For normalization, all the obtained readings were subtracted from the readings having no TCLL.

3.4. Luciferase based assay for SINV

Luciferase reporter system was used to measure the antiviral activity of TCLL. The amount of luciferase-derived signal correlates with the virus infection. SINV with firefly luciferase reporter gene was pre-incubated with TCLL for 1 h and this mixture was used to infect BHK-21 cells and luminescence reading was taken at different intervals of time at 4, 6, 8 and 12 h. A significant reduction in luciferase-derived signal was observed as compared to the virus control, suggesting that TCLL binds to virus glycans and virus is not able to bind or infect the cells and further not able to replicate. In a separate set, TCLL was pre-incubated with NAG before its incubation with the virus and finally added to the cells and an insignificant decrease in luciferase-derived signal was observed. These results showed that the antiviral activity of TCLL is due to its ability to target the virus glycans as when NAG is provided externally, the antiviral effect of TCLL was reduced to a significant extent (Fig. 4a). To further investigate whether TCLL inhibits virus infection by interacting with the cells, the BHK-21 cells were pre-incubated with TCLL for 1 h prior to the virus infection. An insignificant reduction in luciferase-derived signal was seen on pre-incubation of cells with TCLL indicating that TCLL did not interact with the cell surface receptors required for the virus entry (Fig. 4b).

3.5. TCLL reduces virus infectivity in a dose-dependent manner

The reduction in infectivity of virus in the presence of TCLL was investigated using plaque assay. TCLL-treated SINV and CHIKV lost infectivity to nearly 55% and 64% as compared to untreated virus control, respectively. However, in case of NAG-treated TCLL, nearly 18% and 14% loss in infectivity was observed in case of SINV and CHIKV, respectively (Fig. 5a, b). This study supported our hypothesis that TCLL is interacting with glycosylation sites present on the envelope of alphaviruses and subsequently reducing the virus infectivity as the

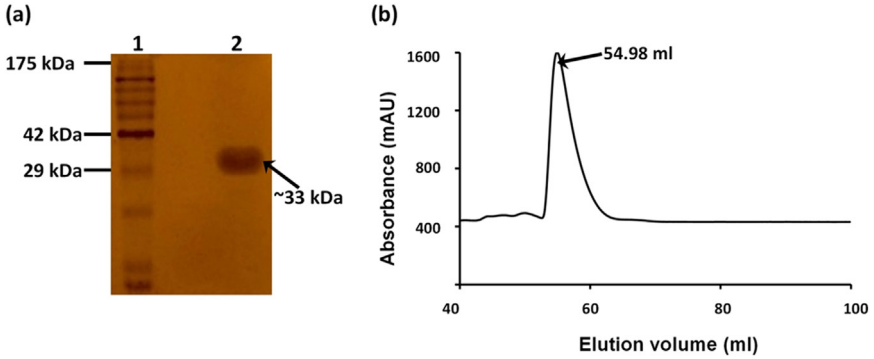

Fig. 1. Purification and gel-filtration profile of Chitinase (chi)-like lectin from *Tamarindus indica* (TCLL). (a) 15% SDS-PAGE gel stained with silver stain where; Lanes: 1, Protein marker; 2, Purified TCLL protein (~33 kDa). (b) The size-exclusion chromatography profile of the TCLL protein eluting out at ~ 54.98 ml.

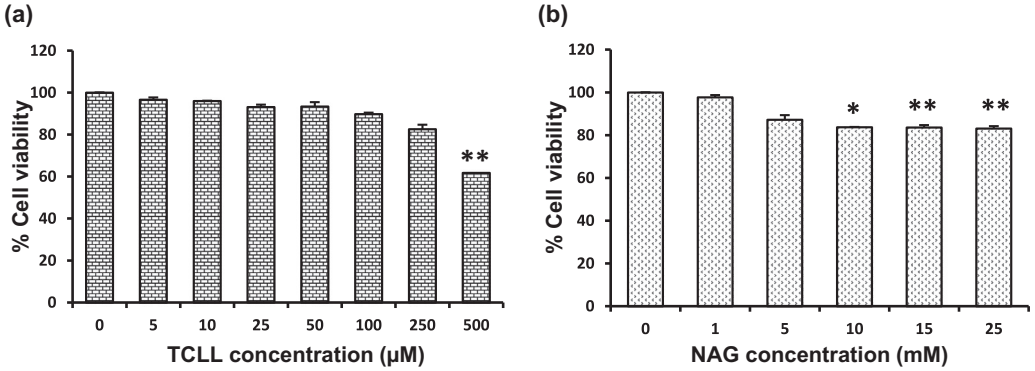

Fig. 2. Cytotoxicity assay. Percentage of BHK-21 cell viability at indicated doses when treated with (a) TCLL and (b) *N*-acetylglucosamine (NAG). Error bars represent the standard deviation from three different experiments. The statistical significance is analyzed by one-way ANOVA test and Dunnett's method (*P < 0.05 and **P < 0.01).

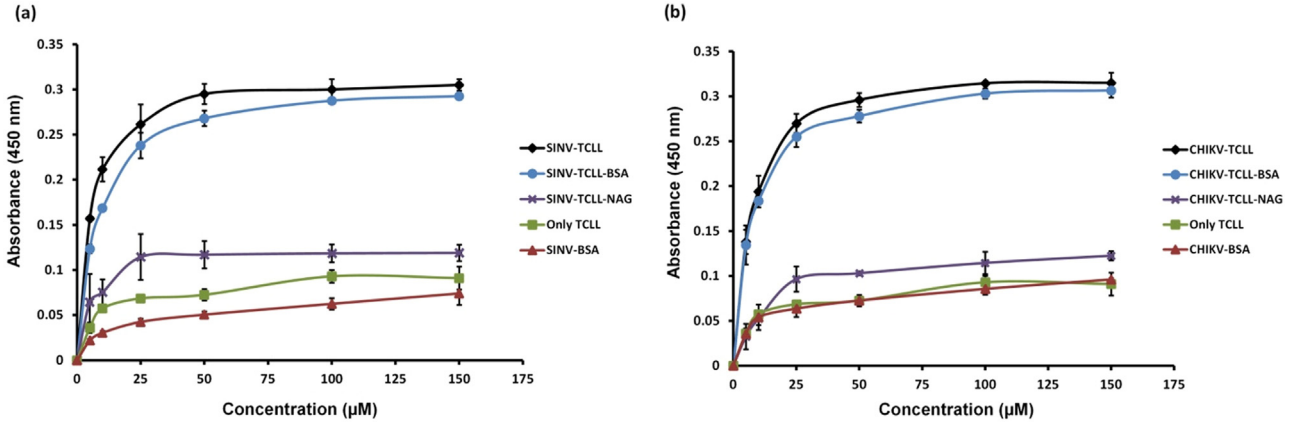

Fig. 3. TCLL interaction with SINV and CHIKV using ELISA assay. Indirect ELISA was performed using different concentrations of TCLL with (a) SINV (b) CHIKV using primary mouse anti-E2 alphavirus glycoprotein antibody and HRP-conjugated anti-mouse IgG1 secondary antibody. The different colored lines represent; where Black (♦) = Virus and TCLL; Blue (•) = BSA-incubated TCLL and virus; Purple (×) = NAG-incubated TCLL and virus; Green (■) = TCLL protein in the absence of virus; Red (▲) = BSA and virus. The absorbance values represent an average of two readings plotted against TCLL concentration. Error bars represent the standard deviation.

virus is not able to bind to the cells.

To explore the dose-dependence, SINV or CHIKV viruses were incubated with increasing dose of TCLL from 1 to 250 μM at room temperature for 1 h. The pre-incubated viruses were incubated with cells and the supernatant was collected at 24 hpi and tittered using conventional plaque reduction assay. A significant dose-dependent reduction in SINV and CHIKV virus titers was observed as compared to the viral control (Fig. 5c, d). Further, SINV and CHIKV viruses were incubated with 100 μM TCLL at different time periods of 0.5, 1, 4 and 8 h to check the time-dependence of the phenotype. The TCLL-treated virus resulted in loss of infectivity with as little as 0.5 h of incubation when compared to untreated control. However, in case of 1, 4 and 8 h samples, the reduction in virus titers is almost the same.

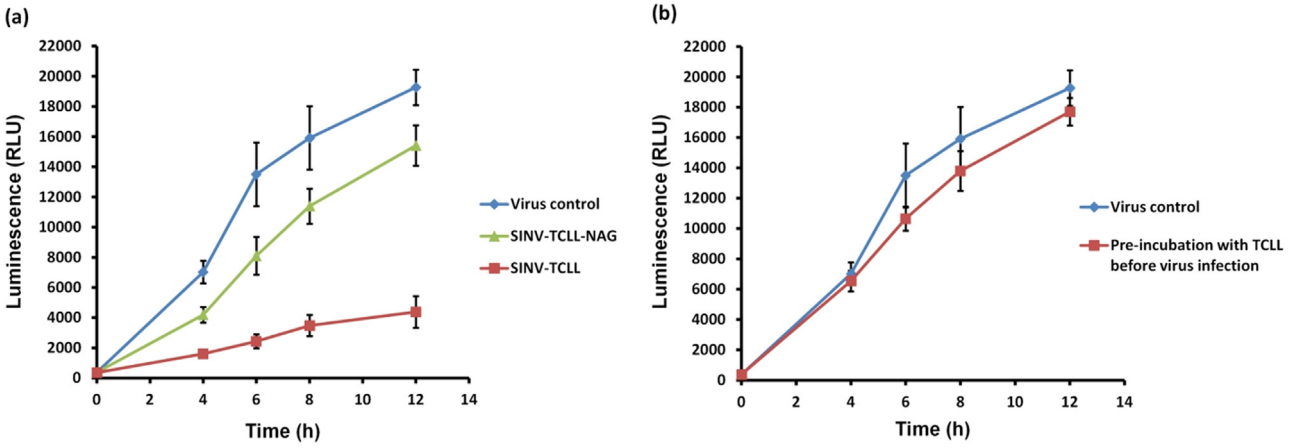

Fig. 4. Luciferase based assay. (a) Firefly luciferase-expressing SINV was incubated with TCLL or NAG-incubated TCLL. The mixture was added to the BHK-21 cells and cell lysates was analyzed for luciferase activity at indicated time points. The different colored lines represent; where Blue (♦) = Untreated virus control; Green (▲) = NAG-incubated TCLL and virus; Red (■) = TCLL and virus mixture. (b) Inhibition of firefly luciferase-expressing SINV virus infection upon pre-treatment of BHK-21 cells with TCLL. The different colored lines represent; where Blue (♦) = Untreated virus control; Red (■) = Cells pre-incubated with TCLL for 1 h prior to virus infection. The error bars represent the standard deviation from two experimental repeats.

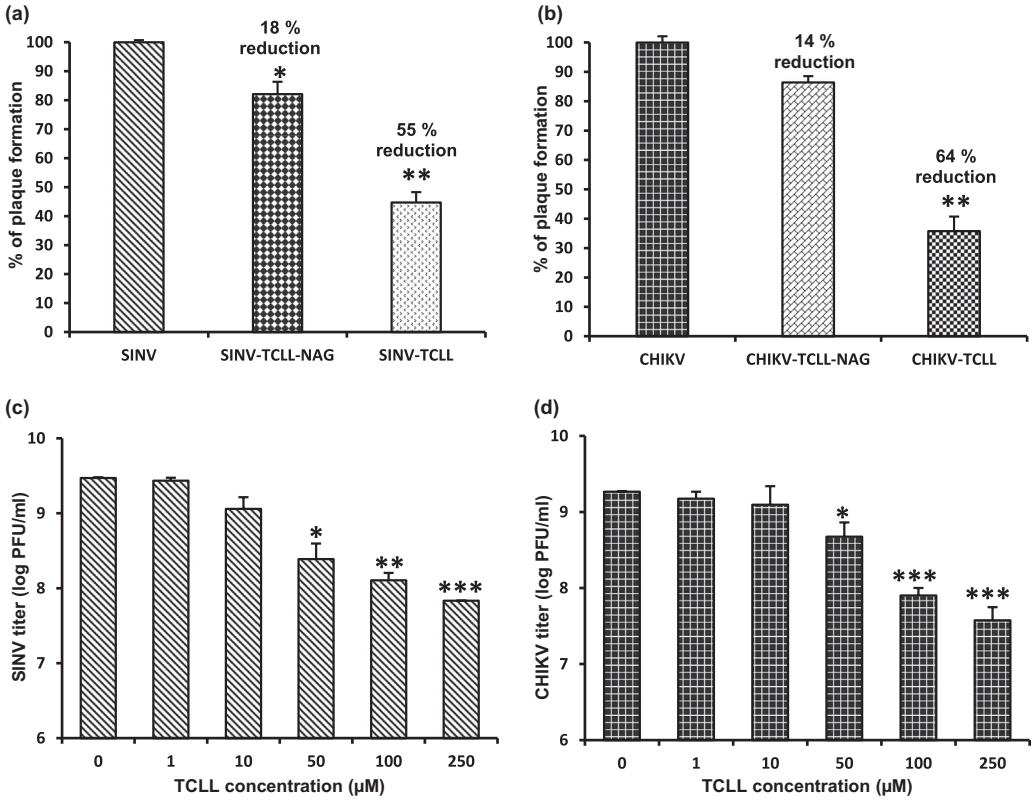

Fig. 5. Inhibition of SINV and CHIKV by TCLL. (a) SINV and (b) CHIKV viruses at an MOI of 0.1 were directly incubated with TCLL or NAG-incubated TCLL for 1 h at room temperature. The viral titers were determined using standard plaque assay. Further, the (c) SINV and (d) CHIKV titers were assessed by standard plaque reduction assay from the supernatant collected at 24 hpi in the presence of increasing concentration of TCLL along with untreated virus controls. Error bars represent the mean from two independent experiments. The statistical significance is analyzed by one-way ANOVA test and Dunnett's method (*P < 0.05, **P < 0.01 and ***P < 0.001).

3.6. Quantitative Real-Time PCR (qPCR)

The antiviral effect of TCLL was confirmed by measuring SINV and CHIKV RNA levels in the infected cells using qPCR. The reduction in viral RNA level 24 hpi was seen when the cells were treated with TCLL. The SINV and CHIKV viral RNA levels upon treatment with 100 μM TCLL were reduced to nearly 37% and 45%, respectively; whereas in case of NAG-treated TCLL, the SINV and CHIKV RNA levels were reduced to nearly 18% and 20%, respectively (Fig. 6).

4. Discussion

Chikungunya virus is a mosquito-borne pathogenic virus that causes mild to severe disease in humans and livestock (Charrel et al., 2014; Chevillon et al., 2008). Since the reemergence of CHIKV in 2005, it has caused severe outbreaks in various part of the world. The development of therapeutics or antiviral molecules against them is in progress (Aggarwal et al., 2017; Kaur et al., 2018; Sharma et al., 2016). However till date, an effective antiviral therapeutics against them is still lacking. Thus, there is a need to discover antiviral compounds for the treatment of alphavirus infections.

The alphavirus propagation and its pathogenesis via virus surface glycoprotein interactions with the host cell receptors is a complex process. Targeting and blocking the interaction of virus with the host cells provides an opportunity to develop an antiviral strategy against viruses (Favacho et al., 2007; Luo et al., 2007; Lusvarghi and Bewley, 2016; Mounce et al., 2017; Swanson et al., 2010; Tsai et al., 2004, 2003; Van der Meer et al., 2007). Enveloped alphaviruses contain the membrane-anchored surface glycoproteins for recognition and interaction with the host's receptors. Thus, viruses including alphaviruses can be inhibited by targeting this virus binding to cell surfaces leading to loss of viral infection. The crystal structures of E2-E1 glycoprotein of SINV and the structures of glycoprotein E3-E2-E1 complex (mature) and the p62-E1 heterodimer (immature) of CHIKV have showed the presence of N-glycan sites for NAG. The residues N196, N318 in SINV and residues N263, N141 in CHIKV, respectively are glycosylated (Li et al., 2010; Voss et al., 2010). The mutagenesis and neutralizing antibody escape mutations in these glycoproteins have shown the role of key residues in virus receptor binding, neurovirulence, mosquito vector adaptation etc. (Agapov et al., 1994; Hammar et al., 2003; Heil et al., 2001; Lee et al.,

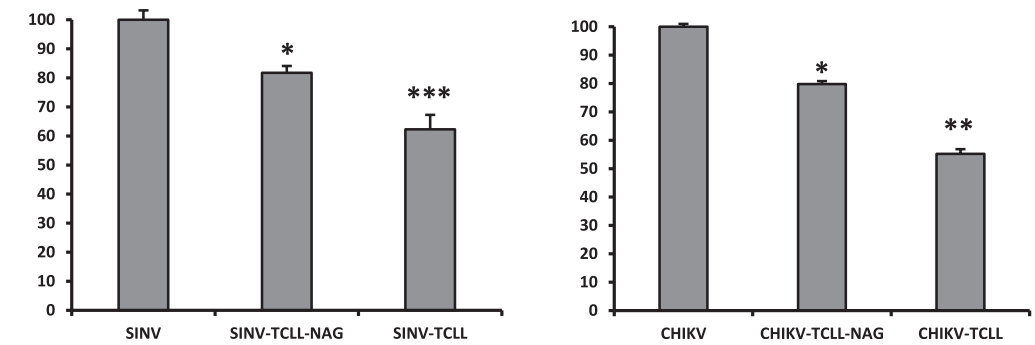

Fig. 6. Reduction in SINV and CHIKV RNA copies in the presence of TCLL were measured by qPCR. The number of copies of (a) SINV and (b) CHIKV viral RNA in the presence of TCLL from infected cells at 24 hpi was quantified and compared with the virus control. Error bars represent the standard deviation from three different experiments. The statistical significance is measured by one-way ANOVA test and Dunnett's method (*P < 0.05, **P < 0.01 and ***P < 0.001).

2002; Meyer and Johnston, 1993; Pierro et al., 2008; Strauss et al., 1991; Tucker and Griffin, 1991; Vrati et al., 1988; Wang et al., 2003).

The mechanism of inhibition by TCLL, a chitinase (chi)-like lectin lies in its ability to bind to N-glycans found on the envelope of alphaviruses and preventing the virus attachment to the cells and thus, virus entry. This conclusion comes from the ELISA assay which confirmed the interaction of TCLL with the glycans present on the alphavirus envelope. These glycans were recognized by the anti-E2 alphavirus glycoprotein monoclonal antibody. The addition of NAG inhibited the interaction of TCLL with glycans which further confirmed that the binding is with the glycans of virus envelope. The ELISA assay confirmed that TCLL binds to the SINV and CHIKV glycosylated envelope in a concentration-dependent manner.

SINV with firefly luciferase reporter system was used to explore the antiviral potential of TCLL and the amount of luciferase-derived signal produced in the cells with time correlates with the virus infection. The luciferase-derived signal was highly reduced in the presence of TCLL indicating that TCLL binds to the virus envelope and reduces virus infection by blocking virus entry to the cells. Additionally, in the presence of NAG, the antiviral effect of TCLL is lost or reduced to a larger extent showing that NAG, a monosaccharide, saturates/blocks the binding sites on the TCLL, rendering TCLL unable to bind to the glycans of the alphavirus envelope and thus, the antiviral effect of TCLL is lost. Further, TCLL did not interact with the cell surface receptors required for the virus entry as no significant reduction in luciferase-derived signal was seen on pre-incubation of cells with TCLL.

The reduction in virus titers upon direct treatment of TCLL with SINV and CHIKV indicated that the inhibitory and/or antiviral effect of TCLL is through its direct interference with virus attachment to the cells which results in a reduction of virus infectivity. Further, it was found that TCLL reduces the virus infectivity in a dose-dependent manner for both SINV and CHIKV viruses. The time-dependence of TCLL phenotype was checked up to 4 h of infection and it was found that TCLL-treated virus resulted in loss of infectivity with as little as 0.5 h of incubation when compared to untreated control. In the presence of TCLL, the SINV and CHIKV RNA levels found to be reduced to nearly 37% and 45%, respectively; whereas, in presence of NAG the inhibitory activity of TCLL is highly reduced.

In conclusion, the present study investigates the inhibitory and/or antiviral activity of TCLL against alphaviruses. The interaction of TCLL with N-glycans of virus envelope was confirmed by ELISA assay. The luciferase based assay was used to check the inhibitory effect of TCLL. The antiviral effect of TCLL was investigated using cytopathogenic effect reduction (CPE) assay and/or plaque reduction assay using various virus-infected cell lines such as BHK-21 or Vero monkey cell lines. TCLL showed a significant reduction in virus titers in a dose-dependent manner which confirmed the antiviral activity of TCLL. The reduction in viral RNA levels in presence of TCLL validated the antiviral activity of TCLL. Several lectins such as Griffithsin (GRFT), cyanovirin-N (CV-N), BanLec are being investigated and developed as antiviral therapeutics against HIV and other viruses. Their use has been mostly suggested as antiviral microbicides. To reduce the toxicity and immunogenicity, various methods such as addition of the polyethylene glycol chains to proteins (PEGylated) or rationally engineering of therapeutic proteins is being probed. Thus, lectins including TCLL have a great potential to be used as virucidal agents. This study along with the availability of the three- dimensional structure of TCLL provides its usefulness for further investigations towards the development of lectin-based antiviral therapeutics.

Acknowledgements

This research was supported by the Department of Biotechnology (DBT), New Delhi, India [grant number: BT/PR21935/NNT/28/1217/2017]. R.K. thanks University Grant Commission (UGC), India; Neetu and R.M. thank Council of Scientific and Industrial Research (CSIR), India for providing the financial support. Authors thank Biotech Consortium India Limited, Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India, New Delhi, India for their assistance in filing the patent for antiviral composition of tamarind chi-like lectin (Patent file no. 201811019298, dated 23rd May 2018).

Conflicts of Interest

The authors declare no conflicts of interest.

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