Host cell responses to dengue virus Infection
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TOMATIDINE, A NOVEL ANTIVIRAL COMPOUND TOWARDS DENGUE VIRUS

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Abstract

Dengue is the most common arboviral disease worldwide with 96 million symptomatic cases annually. Despite its major impact on global human health and huge economic burden there is no antiviral drug available to treat the disease. The first tetravalent dengue virus vaccine was licensed recently for individuals aged 9 to 45, however, most cases are reported in infants and young children. This, together with the poor efficacy of the vaccine to dengue virus (DENV) serotype 2, stresses the need to continue the search for compounds with antiviral activity to DENV. In this report, we describe tomatidine as a novel compound with potent antiviral properties towards DENV serotype 2. The effective concentration in which a 50 and 90% reduction of infectious virus particle production is observed was 0.82 and 1.61 µM following infection of Huh7 cells at MOI 1. The selectivity index is 97.7. Time-of-drug-addition experiments revealed that tomatidine is still active when added 12 hours post-infection, which suggests that the compound predominantly acts at late stages of viral replication. Furthermore, tomatidine was found to control the expression of activating transcription factor 4 (ATF4) yet this protein is not solely responsible for the observed antiviral effect. In summary, we propose that tomatidine serves as a potential candidate for the treatment of dengue given its potent antiviral activity.
1. Introduction

Annually, an estimated 390 million individuals are infected with dengue virus (DENV), of which 96 million individuals develop clinically apparent disease\(^1\). These staggering numbers make DENV the most common viral infection that is transmitted by arthropods worldwide. Clinical disease usually manifests as an acute self-limited illness with symptoms as high fever, severe headache, severe eye pain, muscle and/or bone pain and rash. However, approximately 0.5 to 1 million individuals per year develop severe disease\(^1\). Severe dengue is a potential fatal complication due to capillary leakage, ascites, pleural effusion, severe bleeding and organ impairment\(^2\). DENV is endemic in (sub)tropical regions and most cases are reported in infants and young children\(^3\). Severe disease is predominantly seen in individuals experiencing a secondary DENV infection with another serotype or in infants born to dengue immune mothers\(^4,5\).

In total 4 DENV serotypes exist and all of them can cause disease. Not only virus factors but also host factors have been associated with severe disease manifestations. It is generally believed, however, that \textit{original antigenic sin} of T and B cells play a dominant role in the development of severe disease\(^6\). Low affinity T cells and high numbers of cross-reactive antibodies are postulated to limit efficient clearance of infection. In fact, these cross-reactive antibodies have been shown to enhance DENV titers \textit{in vitro} and \textit{in vivo} via the phenomenon of antibody-dependent enhancement of infection\(^7\). Epidemiological studies indeed confirmed that high DENV viremia correlates with an increased chance to develop severe disease\(^8-10\).

Researchers have attempted to identify antiviral compounds for the treatment of dengue for decades but unfortunately with limited success. Antiviral treatment is aimed at reducing the viral load thereby decreasing the chance to develop severe disease\(^11\). Both direct-acting antivirals as well as host-directed antivirals have been pursued as potential candidates for dengue treatment\(^12,13\). However, despite the large number of compounds that exert antiviral activity \textit{in vitro}, very few compounds have been further developed and evaluated in clinical trials. Moreover, none of these compounds (chloroquine, lovastin, prednisolone, balapiravir and celgosivir,) showed a clear beneficial effect in humans\(^14-19\). These results emphasize the need to follow-up other and identify new compounds that intervene with DENV infection.

Tomatine is a steroidal alkaloid that can be extracted from the skin and leaves of tomatoes. Unripe green tomatoes contain up to 500 mg tomatine per kg whereas ripe red tomatoes have less than 5 mg/kg\(^20\). In nature, tomatine functions as an important defense mechanism for pathogens\(^21\). Tomatidine is an aglycon metabolite of tomatine and was shown to exert a wide array of beneficial biological activities.
such as anti-cancer, anti-inflammatory and improvement of the muscle healthspan by stimulating muscle hypertrophy\textsuperscript{22-24}. Furthermore, anti-microbial properties have been described. For example, tomatidine was found to potently reduce replication of pathogenic \textit{S. Aureus} variants typically seen in cystic fibrosis\textsuperscript{25}. Antiviral activity has been reported for Sunnhemp Rossette virus and Tobacco mosaic virus whereas for herpex simplex virus, human respiratory syncytial and influenza virus tomatidine had no effect on virus replication\textsuperscript{26-29}.

In this study, we evaluated the antiviral properties of tomatidine towards DENV serotype 2. Potent anti-DENV activity was observed in human hepatocarcinoma Huh7 cells. In Huh7 cells, the EC\textsubscript{50} was 0.82 µM (SI index 97.7) following infection at MOI 1. Importantly, potent antiviral activity was still observed when tomatidine was added 12 h post-infection (hpi). Activating transcription factor 4 (ATF4) may contribute to the observed antiviral effect yet the exact mechanism by which tomatidine exerts its antiviral effect remains unknown.

2. Materials and methods

\textbf{Cell culture.} Baby hamster kidney-21 cells clone 15 (BHK-15) was a kind gift from Richard Kuhn (Purdue University). BHK-15 cells were grown in Dulbecco’s minimal essential medium (DMEM) (Gibco, the Netherlands) supplemented with 10% fetal bovine serum (FBS) (Lonza, Basel, Switzerland), 100U/mL penicillin and 100mg/mL streptomycin (PAA Laboratories, Pasching, Austria), 100 µM of non-essential aminoacids (Gibco) and 10mM of hepes (Gibco). Human hepatocarcinoma Huh7 cells (JCRB0403) were a kind gift from Tonya Colpitts (University of South Carolina) and cultured in DMEM/Glutamax supplemented with 10% FBS, 100U/mL penicillin and 100mg/mL streptomycin. Vero WHO (WHO Reference Cell Bank 10-87) were grown in DMEM supplemented with 10% FBS, 100U/mL penicillin and 100mg/mL streptomycin. \textit{Aedes albopictus} C6/36 cells (ATCC: CRL-1660) were maintained in minimal essential medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% FBS, 25 mM HEPES, 7.5% sodium bicarbonate, 100U/mL penicillin and 100mg/mL streptomycin, 200 mM glutamine, and 100 µM nonessential amino acids. All mammalian cells were cultured at 37°C and 5% CO\textsubscript{2} and C6/36 cells were cultured at 28°C and 5% CO\textsubscript{2}.

\textbf{Virus stocks and titration.} DENV serotype 2 strain 16681 was propagated on C6/36 cells as described before\textsuperscript{30}. The number of infectious particles was determined by plaque assay on BHK-15 cells or by focus immunoassay on Vero WHO cells as described before\textsuperscript{31}. For plaque assays, BHK-15 cells were seeded in 12-well plates at a cell density of 9.0x10\textsuperscript{4} cells per well. At 24 h post-seeding, cells were
infected with 10-fold serial dilutions of the sample. At 2 h post-infection (hpi), an overlay of 1% seaplaque agarose (Lonza, Swiss) prepared in MEM was added and plaques were counted 5 days post-infection. Titers are reported as plaque forming units (PFU) per ml. The number of genome equivalent copies (GEC) in a solution was determined by Q-RT-PCR as described previously. Briefly, viral RNA was extracted using a QIAamp viral RNA mini kit (QIAGEN, Venlo, The Netherlands) following manufacturer’s instructions. cDNA was synthesized from viral RNA using Omniscript (QIAGEN) and the primers 5’-ACAGGCTATGGCACTGTTACGAT-3’ (forward) and 5’-TGCAGCAACACCCTCATTG-3’ (reverse). For real-time PCR, the TaqMan probe (5’-FAM-AGTGCTCTCCAAGAACGGGCCTCG-TAMRA-3’, where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine) (Eurogentec, Maastricht, The Netherlands) was used. The number of GEcs was determined using a StepOne Real-Time PCR instrument (Applied Biosystems, Carlsbad, CA) and a standard curve using a quantified cDNA plasmid encoding the DENV structural genes (pSINDENCrME). Chemicals. Tomatidine hydrochloride was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and dissolved in absolute ethanol (EtOH) to a final concentration of 5mM. Aliquots were stored for no longer than three months at -20°C. The final concentration of EtOH was below 0.01% in all infectivity experiments.

Cytotoxicity assay. Cytotoxicity of tomatidine was assessed in vitro by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Huh7 cells were seeded in 96-well plates at a density of 7.0x10³ cells per well, respectively. At 24 h post-seeding, cells were treated with increasing concentrations of tomatidine ranging from 1 to 200 µM. At 24 h, MTT was added at a final concentration of 0.45mg/ml and incubated for 3 h. Subsequently, media was removed and cells were incubated for 1 h at room temperature (RT) with acidic-2-2-propanol. The absorbance was measured with a microplate reader (Biotek, Sinergy, HT, Vermont, USA) at 570 nm. Cell viability was expressed according to the following formula:

Antiviral assays. Huh7 cells were infected with DENV at a multiplicity of infection (MOI) of 1 or 10. Tomatidine (or the equivalent volume of EtOH) was added at different stages of infection. In most experiments, increasing concentrations of tomatidine was added together with the virus to the cells. At 2 hpi, the virus inoculum was removed, cells were washed three times and tomatidine-containing medium was added for the duration of the experiment. In case of pre-treatment experiments, tomatidine was added 1 or 2 h prior to infection. At the time of infection, cells were washed three times before the virus inoculum was added. The condition “during” relates to the presence tomatidine during the infection for 2 h. Also, tomatidine was added 2, 4, 6, 12, 16, 20 hpi. In all experiments, the virus inoculum was removed at
2 hpi, cells were washed three times and incubation was continued. At 24 hpi, cell supernatants were harvested and the titer was determined by plaque assay, focus immunoassay or Q-RT-PCR.

**Virucidal effect.** DENV (1×10⁵ PFU) was incubated for 2 h at room temperature or 37°C in the absence or presence of 10 µM tomatidine in a final volume of 250 µl. Upon incubation, the infectious titer was determined by plaque assay.

**Flow cytometry.** Huh7 cells were trypsinized using 1X Trypsin/EDTA (Gibco). Cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin. Staining was performed with 4G2 antibody and a rabbit anti-mouse IgG coupled to AF647 (Molecular probes, Eugene, Oregon, USA). Flow cytometry was carried out in a FACSCalibur cytometer (BD Biosciences) and analysis was performed with Kaluza 1.1.

**Transfection of siRNAs.** Huh7 cells were seeded in 24-well plates at a cell density of 7.0×10⁴ cells per well. At 24 h post-seeding, cells were transfected with 1.5µl of lipofectamine RNAi/Max (Invitrogen) and 10nM of siRNAs (Dharmacon, ATF4: L-005125-00-0005 and siRNA negative control (siNC): D-001810-01-05 5). When indicated, cells were infected 48 h post transfection.

**Western blot.** Cells were lysed with RIPA Lysis Buffer System (Santa Cruz Biotechnology) and proteins were extracted following manufacturer’s instructions. Bradford assay (Expedeon, Swavesey, UK) was used to determine protein concentration. Samples (50-90 µg protein) were mixed with 5x Laemmli buffer and heated at 95°C for 5 min for denaturation prior to fractionation by SDS-PAGE. Proteins were transferred to Polyvinylidene difluoride membranes (Immobilon-P, Millipore, Darmstad, Germany) and blocked with 5% bovine serum albumin (GE Healthcare) for 10 min. Primary antibodies were incubated overnight at 4°C. The antibody against ATF4 (Cell Signalling, The Netherlands) was diluted 1:1000 and the antibody for GAPDH (Abcam, UK) was diluted 1:10000. Secondary HRP-conjugated antibodies, anti-mouse or anti-rabbit (Thermo Fisher Scientific) were diluted 1:4000. All dilutions were prepared in TBST (TBS-Tween) at 5% of BSA and 0.1% of sodium azide. Pierce ECL western blotting substrate (Thermo Fisher Scientific) or Super Signal West FEMTO (Thermo Fisher Scientific) was used for detection by means of chemiluminescence using LAS-4000 mini camera system (GE Healthcare, Little Chalfont, UK). Image analysis was performed using the Image QuantTL software (GE Healthcare). The band intensity of each protein was normalized to that of GAPDH and expressed as the fold-change over non-treated/mock-infected cells.

**Statistical analysis.** The tomatidine concentration at which 50 and 90% reduction in virus particle production is seen is referred to as EC50 and EC90, respectively. The concentration of tomatidine that caused 50 and 90% cellular cytotoxicity is referred to as CC50 and CC90, respectively. Dose-response curves were fitted by non-linear
regression analysis employing a sigmoidal model. The selectivity index (SI) was determined by the ratio of CC50 to EC50. All data was analyzed in GraphPad Prism software (La Jolla, CA, USA). Data is presented as mean ± SEM. Student T test was used to evaluate statistical differences and a p value ≤0.05 was considered significant with *p≤0.05, **p≤0.01 and ***p≤0.001.

3. Results

The effect of tomatidine on DENV infectivity was determined in the human hepatocarcinoma cell line Huh7. This cell line was chosen as hepatocytes are important target cells during DENV infection and Huh7 cells are permissive to DENV infection. Indeed, at 24 hpi, on average $8.3 \times 10^4$ progeny infectious particles per ml are produced following infection at MOI 1. Fig. 1A shows that tomatidine has potent antiviral activity towards DENV. At a concentration of 10 µM tomatidine, infectious virus particle production was reduced 2.02 Log (99%) when compared to DENV-infected cells treated with equivalent volumes of EtOH. The final concentration of EtOH was below 0.01% and had no effect on virus particle production when compared to non-treated (NT) cells (Fig. 1A). The EC50 and EC90 values are 0.82 and 1.61 µM for MOI 1, respectively. At MOI 10, the EC50 and EC90 values are 0.97 and 5.72 µM, respectively (Fig. 1B). Viability assays were performed in parallel and revealed a CC50 value of 80.2 µM for tomatidine in Huh7 cells (Fig. 1C). This implies that the SI index is 97.7 at MOI 1 and 82.6 at MOI 10. To analyze whether tomatidine has a direct negative (virucidal) effect on the virion, we next incubated $1 \times 10^5$ PFU of DENV with 10 µM tomatidine for 2 h at room temperature or 37ºC and determined the infectious titer by plaque assay. Figure 1D shows that tomatidine does not influence DENV infectivity, indicating that tomatidine is not virucidal.

To better understand the potent antiviral effect of tomatidine, we investigated whether, next to reducing the production of infectious particles, the compound also reduces the number of infected cells. The percentage of infection was determined by flow cytometry using the 4G2 antibody which recognizes the viral E-protein. At MOI 1, the percentage of infection in EtOH-treated control cells was on average 21.9% whereas at tomatidine concentrations of 1 and 10 µM, the percentage of infected cells were 12.7 and 5.1%, respectively (Fig. 2A and 2B). At MOI 10, the percentage of infection in EtOH-treated cells was on average 74.2% while in cells treated with 1 and 10 µM tomatidine we observed on average 64.2 and 43.2% of infected cells, respectively. At higher MOI values, less robust effect of tomatidine is observed as, compared to EtOH, treatment with 10 µM tomatidine reduced the percentage of infection by 76 and 41% at MOI 1 and 10, respectively. The mean fluorescent intensity (MFI) of infected cells treated with 10 µM tomatidine was also decreased in comparison with the EtOH-
tomatidine is antiviral towards dengue virus (Fig. 2B), indicating that E protein expression is reduced by the treatment with tomatidine. This suggests that tomatidine reduces both, the number of infected cells and the level of E protein expression in infected cells. Less pronounced inhibition of infection was observed when the compound was added at 2 hpi, thus, after virus-cell binding, entry and removal of the virus inoculum (Fig. 2C). At 10 µM tomatidine, however, a significant (Fig. 2C) and comparable (Fig. 2B vs Fig. 2C) decrease in the number of infected cells and MFI was observed, suggesting that tomatidine predominantly acts at a late stage of infection.

**FIGURE 1** | Tomatidine reduces the production of infectious DENV particles. (A) Huh7 cells were infected with DENV at MOI 1 and 10. Simultaneously with the infection, cells were treated with 10 µM of tomatidine, the equivalent volume of EtOH or left un-treated (NT). (B) Dose-response curve showing the inhibition of DENV infection at increasing concentrations of tomatidine in relation to the equivalent EtOH-treated control. EC50 and EC90 values were calculated with GraphPad Prism software. (C) Dose response curve showing the cytotoxicity of tomatidine determined by MTT assay performed in triplicate. The CC50 value was calculated with GraphPad Prism software. (D) 1x10^5 PFU of DENV was incubated for 2 h at room temperature RT or 37 °C with 10 µM of tomatidine. The infectivity was determined by plaque assay on BHK-15 cells. Data is presented as mean ± SEM from three independent experiments.
Tomatidine is antiviral towards dengue virus

**FIGURE 2 | Tomatidine decreases the percentage of DENV-infected Huh7 cells.** (A, B) Huh7 cells were infected with DENV at MOI 1 and 10 in the presence of 1 or 10 µM tomatidine as indicated. At 2 hpi, DENV inoculum was removed and incubation was continued in the presence of the compound until harvesting of cells at 24 hpi. As control, cells were infected with DENV in presence of an equal volume of EtOH. (A) Representative dot plots. (B) Quantification of the percentage of infected cells and MFI. (C, D) Tomatidine was added 2 hpi of Huh7 cells with DENV at MOI 1. (C) Representative dot plot. (D) Quantification of the percentage of infected cells and MFI. (A, C) Red numbers indicate the percentage of infected cells and grey numbers the MFI. Data is presented as mean ± SEM from three independent experiments.

To study whether tomatidine indeed acts a late stages of the DENV replication cycle, we next performed time of addition experiments (Fig. 3A). In pre-treatment experiments, cells were incubated with tomatidine for 2 or 1 h after which the compound was washed out and DENV infection was initiated. Alternatively, the compound was added together with the virus to Huh7 cells and at 2 hpi the medium...
was removed, cells were washed and incubation was continued without tomatidine. For post-treatment experiments, the compound was added at 2, 4, 6, 12, 16 and 20 hpi. In all experiments, cells were infected at MOI 1 and tomatidine was added at a final concentration of 10 µM. At each time point, EtOH-treated cells were included. We found that tomatidine reduces DENV infectivity when added pre, during and up to 12 hpi (Fig. 3B). This suggests that tomatidine may interfere with multiple stages of infection. Nevertheless, the strongest antiviral effect was seen when the compound was added post-infection. Indeed, addition of tomatidine to cells at 2, 4, 6, and 12 hpi reduced infectious virus particle production by on average 98.4, 97, 95.5 and 96.6%, respectively. No antiviral effect was observed when the compound was added at 16 hpi. Detailed growth kinetic analysis of DENV in Huh7 cells revealed that initial virus particle production is seen at 18 hpi (Supplementary Fig. S1). Hence, the above results suggest that tomatidine interferes with a step prior to progeny virus secretion. To study this, we next analyzed the number of genome-equivalent copies (GEC) secreted by DENV-infected Huh7 cells treated with 10 µM tomatidine at 12 hpi. Fig. 4 shows that the number of GEC is reduced by 1 Log (90.2%) when compared to EtOH-treated control cells. Thus, the number of secreted infectious particles (Fig. 3B) and GEC (Fig.4) is correspondingly reduced, thereby confirming that tomatidine acts at a step prior to virion secretion.

**FIGURE 3 | Tomatidine reduces DENV infectivity when added up to 12 hpi.** (A) Outline of the experimental set-up. (B) Infectious virus particle production following the conditions presented in (A). The EtOH control was added to all experimental conditions and the average titer is depicted. For the tomatidine samples, data is presented as mean ± SEM from three independent experiments.
Tomatidine has been reported to interfere with various cellular processes, such as inflammation and angiogenesis\textsuperscript{22,23}. Tomatidine was also described to inhibit the expression of genes induced by activating transcription factor 4 (ATF4). ATF4 is an important regulatory molecule in the restoration of cell homeostasis upon several types of stress\textsuperscript{33}. Interestingly, it was recently reported that ATF4 translocates to the nucleus in DENV-infected lung epithelial A549 cells\textsuperscript{34}. Thus, tomatidine may reduce DENV infection by inhibiting ATF4. To test this, we first determined ATF4 protein levels in Huh7 cells upon DENV infection in the presence and absence of tomatidine. We revealed that DENV increased ATF4 expression by 1.8, 2.2 and 3.6-fold at 18, 24 and 30 hpi when compared to time-matched mock-infected cells, respectively (Fig. 5A). Next, we evaluated whether tomatidine reduces the levels of DENV-induced ATF4. Indeed, tomatidine reduced ATF4 levels up to 60% in DENV-infected cells (Fig. 5B). To investigate whether ATF4 affects DENV replication, we next silenced the expression of ATF4 by means of siRNAs and determined infectious virus particle production. Cells were transfected with a pool of 4 siRNAs targeting ATF4 (siATF4) or a non-targeting siRNA negative control (siNC). At 48 h post-transfection, siATF4 transfection reduced ATF4 levels by 85% when compared to the siNC-transfected cells (Fig. 5C). At this point, cells were infected with DENV at MOI 1. Infectious progeny production was determined at 24 and 30 hpi by plaque assay (Fig. 5D). Virus particle production was reduced by 2-fold in siATF4-transfected cells when compared to non-transfected cells and cells transfected with the siRNA control (Fig. 5D). Thus, despite efficient knockdown of ATF4, we only observed a moderate reduction in infectious virus particle production. Given the robust drop in infectious titer in tomatidine-treated cells, we conclude that although ATF4 might contribute to the action of tomatidine, it is not the sole molecule responsible for the observed antiviral effect.
Tomatidine is antiviral towards dengue virus.

Mock DENV Mock DENV Mock DENV
18h 24h 30h
Mock NTF EtOH NTF Tomatidine NTF siNC 20nM siATF4 5nM siATF4 10nM siATF4 20nM

ATF4
GAPDH

Figure 5: Antiviral effect of tomatidine is independent of ATF4. (A) Huh7 cells were mock-infected or infected with DENV at MOI 10. The upper panel shows representative WB images of ATF4 and GAPDH expression at 18, 24 and 30 hpi. Lower panel shows the quantitation ATF4 levels normalized to the time-matched mock-infected cells. (B) Huh7 cells mock-infected or infected with DENV at MOI 10 and treated with the indicated concentrations of tomatidine, the highest equivalent volume of EtOH or left untreated (NT). Upper panel shows a representative WB image visualizing the expression level of ATF4, NS3 and GAPDH. Lower panel shows the quantitation of the normalized ATF4 expression relative to the non-treated DENV-infected cells. (C) Huh7 cells were mock-infected or infected with DENV at MOI 10. At 2 hpi cells were treated with 10 10 µM tomatidine or the equivalent volume of EtOH. NTF denotes for non-transfected. In addition, cells were transfected with 20 nM negative control siRNA (siNC) and 5, 10 and 20 nM siRNA targeting ATF4 (siATF4). At 48 h post-transfection, cells were infected with DENV at MOI 10. The upper panel shows representative WB images of ATF4 and GAPDH expression at 30 hpi. (D) Huh7 cells were transfected with 10 nM siNC, 10nM siATF4 or left non-transfected (NTF). At 48 h post transfection, cells were infected with DENV at MOI 1. Viral titer is presented at 24 and 30 hpi. Data is presented as mean ± SEM from at least three independent experiments.
4. Discussion

We report here that tomatidine has potent antiviral activity towards DENV serotype 2 in Huh7 cells. The EC50 and EC90 values relate to a concentration of 0.82 and 1.61 µM tomatidine following infection at MOI 1, respectively. Tomatidine was not toxic to Huh7 cells and a selectivity index of 97.7 was found. Even at very high MOI values (MOI 10), the EC50 value remained below 1 µM tomatidine. Time-of-drug-addition experiments showed that the efficacy of the compound is still high when added at late stages of infection. The cellular factor ATF4 may contribute to the observed antiviral effect, yet, it is not fully responsible for it.

The EC50 values of tomatidine are in the sub-µM range suggesting that tomatidine belongs to the more potent anti-dengue compounds identified to date. EC50 values are, however, difficult to compare as these have been shown to be dependent on the cell line, virus strain and MOI used. Actually, many studies use a very low MOI (<1) for infection and consequently low EC50 values are observed. For comparison, it would be best to standardize the infectivity protocols. Furthermore, ideally, infection should be based on multiplicity of GEC (MOG) rather than MOI as the MOI might be biased due to the cell line used for titration. Here, we used an MOI of 1 and 10 which corresponds to 72 and 720 MOG, respectively. Even upon the addition of on average 720 GEC per cell the EC50 value remained below 1 µM, indicating that tomatidine exerts potent antiviral activity towards DENV serotype 2.

Time-of-drug-addition experiments suggested that tomatidine acts at several stages of infection. Significant antiviral activity was observed when the compound was present prior to or during virus infection. This suggests that tomatidine interferes with the early steps of the viral replication cycle. Alternatively, tomatidine is internalized by cells and interferes with DENV replication latter in infection. The latter is strengthened by the observation that only a minor reduction in the percentage of infection (up to 4-fold, Fig. 2) is observed when compared to the overall reduction in infectious virus particle production (up to 100-fold, Fig. 1A). Furthermore, the strongest reduction in infection was seen when tomatidine was added to the cells after the removal of the virus inoculum (Fig. 3). In fact, potent antiviral activity was still observed upon addition of the compound at 12 hpi. Tomatidine does not control the infectious properties of progeny virions as the number of infectious particles was equally reduced to that of the number of GEC. Collectively, this suggests tomatidine predominantly intervenes with steps downstream of protein translation/replication but prior to the secretion of progeny virions. Tomatidine might act directly on the viral proteins or indirectly by controlling the expression of a cellular factor that is important in the late stages of infection.
Tomatidine was found to inhibit ATF4 expression\textsuperscript{24} and a recent study showed that ATF4 is translocated to the nucleus upon DENV infection\textsuperscript{34}. Here, we showed that DENV induces the expression of ATF4. Why ATF4 is upregulated during DENV infection remains to be studied though it is tempting to speculate that DENV induces ATF4 to reduce cellular stress thereby allowing protein translation\textsuperscript{33}. However, silencing of ATF4 reduced the production of infectious virus particles by only 50\% whereas in tomatidine-treated cells more than 99\% reduction in virus progeny was observed. Thus, although tomatidine controls ATF4 expression this does not fully explain the potent antiviral activity observed in this study. Future studies should be conducted to unravel the mode of action of tomatidine and dissect whether it functions as a direct- or a host-directed antiviral compound.

Tomatidine shares many physical and biological properties with steroid glycosides yet it is classified separately given the nitrogen in the ring structure. Recently, a few other compounds with a steroid ring structure have been described as antivirals towards DENV. For example, ecdysones derived from Zoanthus spp. were found to inhibit DENV-2 replication in Huh7 cells and were predicted, by molecular docking studies, to associate with the NS5 polymerase of DENV\textsuperscript{35}. Moreover, carbenoxolone disodium was reported to reduce DENV infectivity due to direct virucidal activity of the compound\textsuperscript{36}. Furthermore, coumarins were shown to be potent inhibitors of both DENV as well as Chikungunya virus (CHIKV)\textsuperscript{37}. Likewise, many other DENV inhibitors targeting viral components or directed to host cellular factors have been discovered or developed in the more than a decade hunt for specific antivirals\textsuperscript{12,13}. However, none of these compounds have reached clinical trials, among other reasons because adverse effects in animals and poor pharmacokinetic properties\textsuperscript{38}. Importantly, the use of tomatidine in several mouse models, including pregnant-mice, suggest a favorable safety profile\textsuperscript{39-41}. Furthermore, tomatidine dietary supplement is commercially available in the United States of America, although a proper study of safety and pharmacokinetics in humans is lacking.

This is the first report that shows that tomatidine has antiviral activity towards DENV. The observed EC\textsubscript{50} and cytotoxicity profiles together with the time-of-addition data are promising and suggest tomatidine as a potential candidate for treatment. We are currently assessing the \textit{in vitro} and \textit{in vivo} potency of the compound to other DENV serotypes and flaviviruses.
Supplementary material

SUPPLEMENTARY FIGURE S1 | DENV growth kinetics in Huh7 cells. Huh7 cells were infected with DENV at MOI 1 and 10. At the indicated time points, viral titers were determined by plaque assay. Values represent mean ± SEM from two independent experiments.
References


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