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 in 2015 for individuals aged 9 to 45, however, most cases are reported in infants and young children. This, together with the limited efficacy of the vaccine to dengue virus (DENV) serotype 2, stresses the need to continue the search for compounds with anti-viral activity to DENV. In this report, we describe tomatidine as a novel compound with potent antiviral properties towards all DENV serotypes and the related Zika virus. The strongest effect was observed for DENV-2 with an EC50 and EC90 value of 0.82 and 1.61 μM, respectively, following infection of Huh7 cells at multiplicity of infection of 1. The selectivity index is 97.7. Time-of-drug-addition experiments revealed that tomatidine inhibits virus particle production when added pre, during and up to 12 h post-infection. Subsequent experiments show that tomatidine predominantly acts at a step after virus-cell binding and membrane fusion but prior to the secretion of progeny virions. Tomatidine was found to control the expression of the cellular protein activating transcription factor 4 (ATF4), yet, this protein is not solely responsible for the observed antiviral effect. Here, we propose tomatidine as a 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 (Bhatt et al., 2013). These staggering numbers make DENV the most common viral infection that is transmitted by ar-thropods worldwide. Clinical disease usually manifests as an acute self-limited illness with symptoms such as high fever, severe headache, severe eye pain, muscle and/or bone pain and rash. However, ap-proximately 0.5–1 million individuals per year develop severe disease (Bhatt et al., 2013). Severe dengue is a potential fatal complication due to capillary leakage, ascites, pleural effusion, severe bleeding and organ impairment (World Health Organization, 2009). DENV is endemic in (sub)tropical regions and most cases are reported in infants and young children (Hammond et al., 2005).

DENV belongs to the family Flaviviridae next to other arthropod-borne viruses of clinical importance such as Zika virus (ZIKV) and West Nile Virus (WNV). In total 4 DENV serotypes exist (DENV-1 – 4) and all of them can cause severe disease. Severe disease is predominantly seen in individuals experiencing a secondary DENV infection with another serotype or in infants with waning maternal antibody titers towards dengue (Halstead, 2003; Guzman et al., 2013). It is generally believed that *original antigenic* sin of T and B cells play an important role in the development of severe disease during heterologous re-infection (Halstead et al., 1983). Original antigenic sin implies that the immune response is skewed towards the primary infecting virus serotype through which low affinity T cells and high numbers of cross-reactive antibodies are produced. As a consequence, the infection is less effi-ciently cleared. In fact, these cross-reactive antibodies have been shown to enhance DENV titers in *vitro* and *in vivo* via the phenomenon of an-tibody-dependent enhancement of infection (Halstead, 1977). Fur-thermore, several epidemiological studies reported that high DENV

viremia correlates with an increased chance to develop severe disease (Libraty et al., 2002; Costa et al., 2013; Rothman, 2011).

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 disease burden (Beesetti et al., 2016). Both direct-acting antivirals as well as host-directed antivirals have been pursued as potential candidates for dengue treatment (Lim et al., 2013; Behnam et al., 2016). However, despite the large number of compounds that exert antiviral activity *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) thus far showed a clear beneficial effect in humans (Tricou et al., 2010; Borges et al., 2013; Whitehorn et al., 2012; Tam et al., 2012; Nguyen et al., 2013; Low et al., 2014). 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 (Blankemeyer et al., 1997). In nature, tomatine functions as an important defense mechanism for pathogens (Friedman, 2002). 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 (Yan et al., 2013; Chiu and Lin, 2008; Ebert et al., 2015). Furthermore, anti-microbial properties have been described. For example, tomatidine was found to potently reduce replication of pathogenic *S. Aureus* variants typically seen in cystic fibrosis (Mitchell et al., 2011). 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 (Jain et al., 1990; Thorne et al., 1985; Bailly et al., 2016; Bier et al., 2013).

In this study, we evaluated the antiviral properties of tomatidine towards DENV, ZIKV, and WNV. Potent anti-DENV activity was observed in human hepatocarcinoma Huh7 cells. The EC50 values of all DENV serotypes were in the (sub-)micromolar range. For DENV-2, the SI index is 97.7 following infection of Huh7 cells at a multiplicity of infection (MOI) of 1. The antiviral potency of tomatidine towards DENV was confirmed in adenocarcinoma alveolar epithelial A549 cells. Less potent antiviral activity was observed for ZIKV and no antiviral effect was seen for WNV under the conditions of the experiment. Importantly, potent anti-DENV activity was still observed when tomatidine was added 12 h post-infection (hpi). Activating transcription factor 4 (ATF4) might contribute to the observed antiviral effect yet the exact mechanism by which tomatidine exerts its antiviral effect remains to be elucidated.

2. Materials and methods

2.1. Cell culture

Baby hamster kidney-21 cells clone 15 (BHK-15) were a kind gift from Richard Kuhn (Purdue University) and are not commercially available. Vero ATCC-CCL-81 cells were obtained from ATCC. Both cell lines 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 100 mg/mL streptomycin (PAA Laboratories, Pasching, Austria). For BHK-15 cells, DMEM was also supplemented with 100 μM of non-essential aminoacids (Gibco) and 10 mM of hepes (Gibco). Vero E6 (ATCC: CRL-1586, a kind gift from Gorben Pijlman (Wageningen University)) and Vero-WHO cells (European Collection of Cell Culture # 88020401) were maintained in DMEM containing 5% FBS and 100U/mL penicillin and 100 mg/mL streptomycin. 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 100 mg/mL streptomycin. Adenocarcinoma alveolar epithelial A549 cells (ATCC: CCL-185) were grown in DMEM supplemented with 10% FBS, 100U/mL penicillin and 100 mg/mL streptomycin. *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 100 mg/mL streptomycin, 200 mM glutamine, and 100 μM nonessential amino acids. All mammalian cells were cultured at 37 °C and 5% CO_2 and C6/36 cells were cultured at 28 °C and 5% CO_2.

2.2. Virus stocks and titration

The DENV WHO reference strains: DENV-2 strain 16681, DENV-1 strain 16007, DENV-3 strain H87 and DENV-4 strain 1036 were used in this study. All DENV serotypes were propagated on C6/36 cells, as described before (Zybert et al., 2008). The number of infectious DENV particles was determined by plaque assay on BHK-15 cells or by focus immunoassay on Vero-WHO cells, as described before (Richter et al., 2014). For plaque assays, BHK-15 cells were seeded in 12-well plates at a cell density of 9.0×10^4 cells per well. At 24 h post-seeding, cells were infected with 10-fold serial dilutions of sample. At 2 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. For the focus immunoassay, Vero WHO cells were seeded in 96-well plates at a cell density of 1.3×10^4 cells per well. At 24 h post-seeding, cells were incubated with 10-fold serial dilutions of the virus solution for 1.5 h at 37 °C. Subsequently, an overlay of 1% carboxymethylcellulose (Sigma-Aldrich) prepared in MEM was added and cells were fixed at 3 (DENV-1 and DENV-4) or 4 days post-infection (DENV-2 and DENV-3). Foci were visualized by use of the 4G2 antibody (Merk Millipore, Billerica, Massachusetts, USA) and the goat anti-mouse HRP-labeled antibody (Southern Biotech). Trueblue peroxidase substrate (VWR International, Radnor, Pennsylvania, USA) was used for detection and titers are reported as foci forming units (FFU) per ml. The number of genome equivalent copies (GEC) in a solution was determined by Q-RT-PCR as described previously (van der Schaar et al., 2007). 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′-TGCAGCAAC ACCATCTCATTG-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 system (Applied Biosystems, Carlsbad, CA) and a standard curve using a quantified cDNA plasmid encoding the DENV structural genes (pSINDENCprME) (van der Schaar et al., 2007). For ZIKV, we used a clinical isolate from Surinam (a kind gift from Martijn van Hemert, Leiden University Medical Center) that was passaged seven times on Vero E6 cells to obtain a stock virus. Supernatants were harvested at 5 days post-infection following infection at MOI 0.01 and the virus titer was determined on Vero CCL-81 cells by plaque assay. WNV strain NY99 (gift from Claire Huang, CDC) was produced on C6/36 cells. The infectious virus titer was determined via plaque assay on BHK-15 cells as described above, but with cells fixed and stained at 2 dpi.

2.3. Chemicals

Tomatidine hydrochloride was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and dissolved in absolute ethanol (EtOH) to a final concentration of 5 mM. Naringenin was purchased from Santa
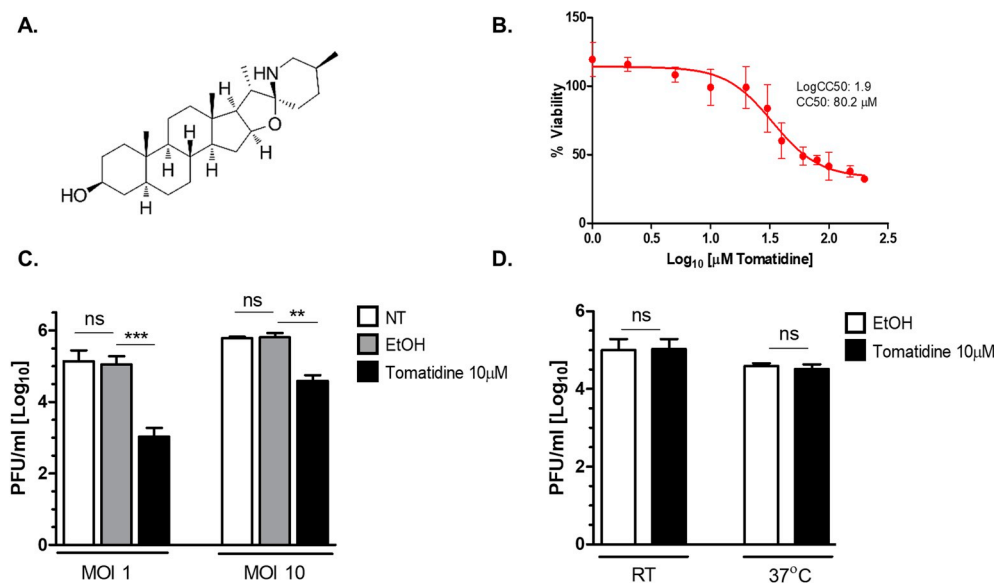
Fig. 1. Tomatidine reduces the production of infectious DENV particles and is not virucidal. (A) Chemical structure of tomatidine. (B) Dose response curve showing the cytotoxicity of tomatidine in Huh7 cells determined by MTT assay performed in triplicate. The CC50 value was calculated with GraphPad Prism software. Data are presented as mean ± SEM from three independent experiments and differences were assessed with Student T test. (C) 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 untreated (NT). (D) DENV (1×10^5 PFU) was incubated for 2 h at room temperature or 37 °C with 10 μM tomatidine or the equivalent volume of EtOH. Upon incubation, the infectious titer was determined by plaque assay in BHK-15 cells. Data are presented as mean ± SEM from three independent experiments and differences were assessed with Student T test.

Cruz Biotechnology (Dallas, Texas, USA) and prepared in absolute EtOH to a final concentration of 50 mM. The structure of tomatidine is shown in Fig. 1A. Aliquots were stored for no longer than three months at −20 °C. The final concentration of EtOH was below 0.2% in all infectivity experiments.

2.4. Cytotoxicity assay

Cytotoxicity of tomatidine was assessed *in vitro* by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Huh7 and A549 cells were seeded in 96-well plates at a density of 7.0×10^3 and 6.0×10^3 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 0.45 mg/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. Cytotoxicity of naringenin on Huh7 cells was determined via the ATPlite Luminescence ATP Detection assay system. Huh7 cells were seeded in white polystyrene 96-well plates and treated with naringenin as described above with concentrations of 10–500 μM. At 24 h post-treatment, 50 μL mammalian cell lysis solution was added per well and incubation was continued for 5 min at room temperature on an orbital shaker. Thereafter, 50 μL substrate solution was added to the wells and following a 5 min incubation as before, the plate was incubated for 10 min in the dark. Luminescence was measured with a microplate reader. In both assays, cytotoxicity was expressed according to the following formula:

$$\% \, Cytotoxicity = \frac{(Abs \, sample) - (Abs \, blank)}{(Abs \, negative \, control) - (Abs \, blank)} * 100$$

2.5. Antiviral assays

Huh7 cells were infected with DENV-1 at MOI 1, DENV-2 at MOI 1, DENV-3 at MOI 0.5 and DENV-4 at MOI 0.1. These MOIs were chosen to ensure an equal number of infected cells under normal infection conditions. For DENV-2, MOI 10 was also used to investigate if tomatidine also affects virus particle production at high MOI values. A549 cells were infected with DENV-2 at MOI 0.1 and 1. Tomatidine (or the equivalent volume of EtOH) was added at different stages of infection.

In most experiments, increasing concentrations of tomatidine were 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 at 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 (DENV-1,2,3) or 30 hpi (DENV-4), cell supernatants were harvested and the PFU, FFU or GEC titers were determined by plaque assay, focus immunoassay or Q-RT-PCR, respectively. Alternatively, Huh7 cells were infected with ZIKV (MOI 5) or WNV (MOI 1). Tomatidine (5 and 10 μM) was added together with the virus to the cells as described above. Supernatants were collected at 24 hpi (ZIKV) or 12 hpi (WNV) and the infectious virus titer was determined via plaque assay.

2.6. Virucidal effect

DENV-2 (1×10^5 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 in BHK-15 cells.

2.7. Flow cytometry

Huh7 or A549 cells were trypsinized using 1X Trypsin/EDTA (Gibco). Cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Tween 20. Staining was performed with the 4G2 antibody (Millipore, Billerica, Massachusetts, USA) diluted 1:400 and a rabbit anti-mouse IgG coupled to AF647 (Molecular probes, Eugene, Oregon, USA) diluted 1:2000. Flow cytometry was carried out in a FACSCalibur cytometer (BD Biosciences) and analysis was performed with Kaluza 1.1.

2.8. Transfection of siRNAs

Huh7 cells were seeded in 24-well plates at a cell density of 7.0×10^4 cells per well. At 24 h post-seeding, cells were transfected with 1.5 μl of lipofectamine RNAi/Max (Invitrogen) and 10 nM of
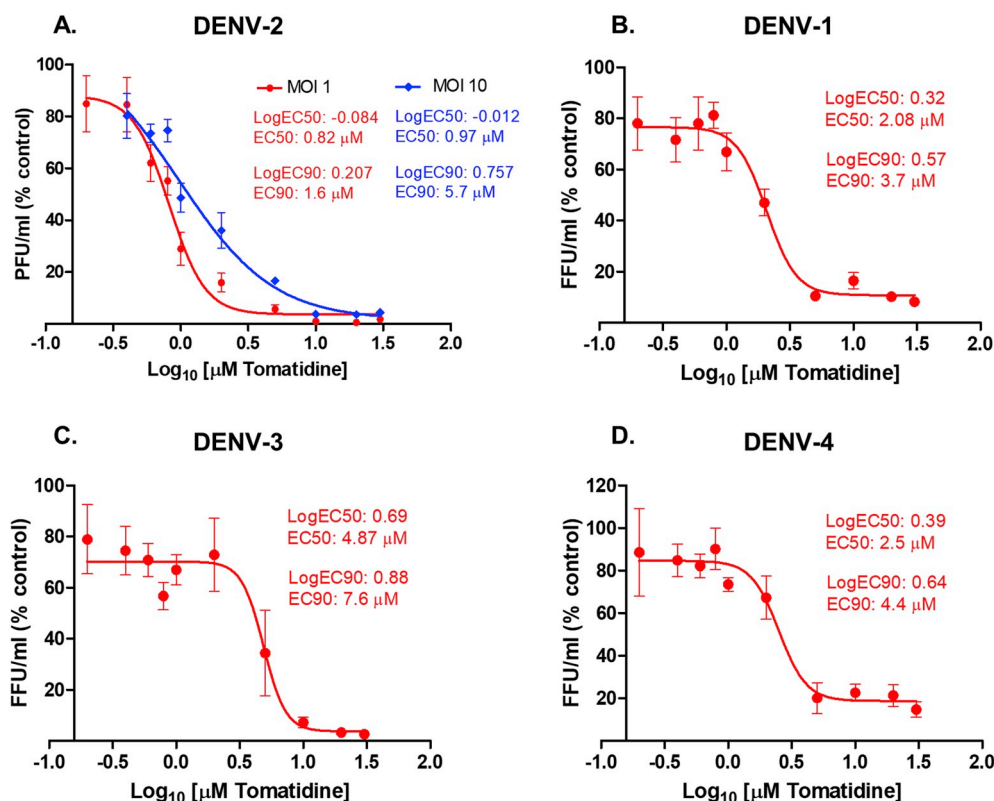
Fig. 2. Dose response curve analysis of tomatine against all four DENV serotypes. Huh7 cells were infected with (A) DENV-2 strain 16681 for 24 h at MOI 1, (B) DENV-1 strain 16007 for 24 h at MOI 1, (C) DENV-3 strain H87 for 24 h at MOI 0.5 and (D) DENV-4 strain 1036 for 30 h at MOI 0.1. In the absence of tomatidine, the infectious titers were 5.04 ± 0.24 Log PFU (DENV-2), 5.51 ± 0.05 Log FFU (DENV-1), 4.97 ± 0.07 Log FFU (DENV-3) and 4.71 ± 0.25 Log FFU (DENV-4). Dose-response curves show 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. Data are presented as mean ± SEM from three independent experiments.

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.

2.9. 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 protein 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. Dilutions were prepared in TBS-Tween at 5% of BSA and 0.1% of sodium azide. Secondary HRP-conjugated antibodies, anti-mouse or anti-rabbit (Thermo Fisher Scientific) were used as recommended by manufacturer. Pierce ECL western blotting substrate (Thermo Fisher Scientific) or Super Signal West FEMTO (Thermo Fisher Scientific) were 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.

2.10. Statistical analysis

The concentration at which tomatidine reduced virus particle production by 50 and 90% 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) reflects the CC50 to EC50 ratio. All data were analyzed in GraphPad Prism software (La Jolla, CA, USA). Data are presented as mean ± SEM. Student T test or one-way ANOVA were 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 all DENV serotypes. First, the cellular cytotoxic effect of tomatidine in Huh7 cells was determined by MTT assay (Fig. 1B). Tomatidine was largely non-toxic in Huh7 cells, the concentration of tomatidine required to reduce cell viability by 50%, i.e. the CC50 value, was 80.2 μM. No cellular cytotoxicity was seen at a concentration of 10 μM tomatidine. Therefore, we next investigated the effect of 10 μM tomatidine on the production of infectious DENV-2 particles in Huh7 cells. Under normal infection conditions, on average 5.14 ± 0.31 Log progeny infectious particles per ml were produced following infection at MOI 1 (Fig. 1C). In the presence of 10 μM tomatidine, infectious virus particle production was reduced by 2.02 Log (99%) when compared to DENV-2 infected control cells that were treated with equivalent volumes of EtOH. Tomatidine was dissolved in absolute ethanol yet the final concentration of EtOH was below 0.2% in all experiments. At these conditions, EtOH did not affect infectious virus particle production (Fig. 1C). Not only following infection at MOI 1 but also at MOI 10, tomatidine was found to cause a significant reduction of 1.44 Log (96%) in infectious virus particle production (Fig. 1C). To rule out that tomatidine has a direct negative (virucidal) effect on the virion, we next incubated 1×10^5 PFU of DENV-2 with 10 μM tomatidine for 2 h at room temperature or 37 °C and determined the infectious titer by plaque assay on BHK-15 cells. Fig. 1D shows that tomatidine does not influence DENV infectivity, indicating that

tomatidine is not virucidal.

To obtain a detailed insight in the efficacy of tomatidine against all four DENV serotypes, we next determined the dose-response curve and calculated the EC50 and EC90 values which reflect the concentration of tomatidine that is required to abolish infectious virus particle production by 50 and 90%, respectively. Fig. 2 shows the EC50 and EC90 values for tomatidine following infection with DENV-2 (Fig. 2A), DENV-1 (Fig. 2B), DENV-3 (Fig. 2C) and DENV-4 (Fig. 2D). For DENV-2, the EC50 and EC90 values were 0.82 and 1.61 μM tomatidine, respectively, following infection at MOI 1. At MOI 10, the EC50 and EC90 values were 0.97 and 5.72 μM, respectively (Fig. 2A). Higher EC50 and EC90 values were observed for DENV-1, 3 and 4. Nevertheless, all values remained in the micromolar range, with EC50 values of 2.08, 4.87 and 2.5 μM and EC90 values of 3.7, 7.6 and 4.4 μM for DENV-1, 3 and 4, respectively (Fig. 2B–D). Of note, plaque assay was used to determine the infectious DENV-2 titers, but in our hands this assay is not suitable to titrate DENV-1, 3 and 4. Therefore, the infectious titers for these viruses were determined by a focus immunoassay and the titer is expressed as focus forming unit (FFU) per mL. Importantly, DENV-2 titers were similar in both assays (Supplementary Fig. S1A) and the correlation between both assays was found statistically significant (Supplementary Fig. S1B).

Based on the above results the SI for DENV-2 is 97.7 at MOI 1 and 82.6 at MOI 10. The SI for DENV-1 is 38.6; for DENV-3 16.7 and for DENV-4 32.1. Due to the pan-dengue antiviral activity, we next evaluated if tomatidine also controls virus particle production of related flaviviruses, including ZIKV and WNV. To this end, Huh7 cells were infected with ZIKV at MOI 5 for 24 h or with WNV at MOI 1 for 12 h in the absence or presence of 5 and 10 μM of tomatidine. For ZIKV, treatment of cells with 10 μM tomatidine moderately (2-fold) but significantly reduced viral titers when compared to the EtOH control (Fig. 3A). No significant antiviral activity was seen at a concentration of 5 μM tomatidine, demonstrating that tomatidine is much less effective in reducing ZIKV titers when compared to DENV. Furthermore, tomatidine did not reduce the production of WNV particles (Fig. 3B). Altogether, these results indicate that tomatidine has potent antiviral activity against all DENV serotypes, moderate antiviral activity for ZIKV and no effect on WNV.

To further illustrate the potent effect of tomatidine on DENV infection, we next performed antiviral assays in parallel with naringenin, a flavonoid recently described by Frabasile and collaborators as a DENV antiviral (Frabasile et al., 2017). In their study, the authors reported an EC50 of 18 μM and CC50 of 311.3 μM for naringenin in Huh7.5 cells. In agreement with their study, we found a CC50 of 326.9 μM in Huh7 cells (Supplementary Fig. S2A). Antiviral activity of naringenin towards DENV-2 was tested using a range of concentrations and we observed ~50% reduction in viral titer at a concentration of 30 μM naringenin (data not shown). Based on these findings we performed additional antiviral assays using 15, 30 and 60 μM naringenin and confirmed that the EC50 is surrounding 30 μM naringenin in Huh7 cells

(Supplementary Fig. S2B). This indicates that at low concentrations, tomatidine is more effective in reducing DENV titers than naringenin.

To better understand the potent antiviral effect of tomatidine, we performed time-of-addition assays (Fig. 4A). In these experiments, tomatidine was added to Huh7 cells pre-, during or post-infection. In pre-treatment experiments, cells were incubated with tomatidine for 2 or 1 h after which the compound was washed out and DENV-2 infection was initiated. In the 'during' condition, 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. In 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 observed that tomatidine reduced DENV-2 infectivity when added pre, during and up to 12 hpi (Fig. 4B). When cells were pre-incubated with tomatidine for 2 and 1 h, infectious virus particle production was reduced by on average 61 and 51.8%, respectively; and when cells were solely treated during infection, a reduction of on average 84.6% was observed. 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 and 20 hpi. To confirm that tomatidine interferes with the production of progeny virus particles we next evaluated the number of GEC secreted by DENV-2-infected Huh7 cells in presence and absence of 10 μM tomatidine added at 12 hpi. Fig. 4C shows that the number of GEC is reduced by on average 1 Log (from 7.86 ± 0.16 to 6.85 ± 0.18 Log GEC/ml) when compared to EtOH-treated control cells. The reduction in infectious titer (96.6%: Fig. 4B, bar number 8) is slightly higher than the reduction in GEC (90.2%: Fig. 4C) suggesting that tomatidine may interfere with the assembly and/or maturation of progeny virions. It is however not very likely that tomatidine directly interferes with secretion of progeny virions as detailed growth kinetic analysis of DENV-2 in Huh7 cells revealed that initial virus particle production is seen at 18 hpi (Supplementary Fig. S3) while tomatidine shows effect till 12 hpi.

The time-of-addition experiments suggest that tomatidine might also act on the early stages of infection as a significant reduction in infectious virus titer was observed in the pre- and during conditions of the experiment. Previous single virus tracking experiments demonstrated that more than 90% of DENV particles induce membrane fusion within 17 min after addition to cells (van der Schaar et al., 2007). To evaluate whether tomatidine interferes with virus cell-binding, entry and membrane fusion, we compared the percentage of DENV-2-infected cells treated with tomatidine during infection with cells treated at 2 hpi (thus after virus-cell binding, entry and removal of the virus inoculum). The number of infected cells was determined at 24 hpi by flow cytometry using the 4G2 antibody which recognizes the viral E-protein. At MOI 1, the percentage of infected cells treated with EtOH was on average 22 ± 5.04 whereas in presence of 10 μM tomatidine, the percentage of infected cells was reduced to on average 5.1 ± 0.52,
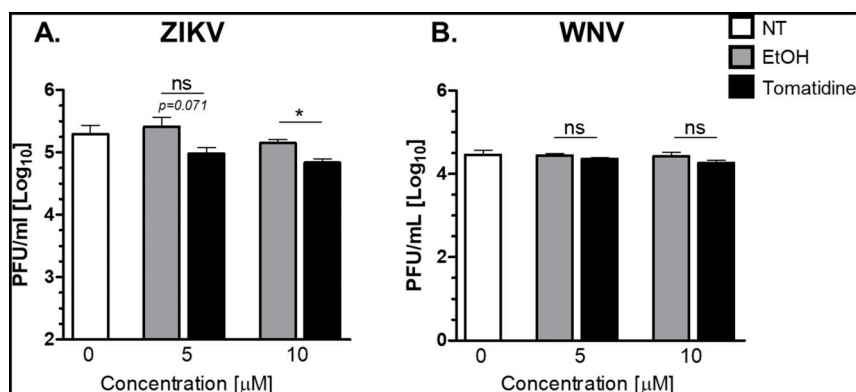
Fig. 3. Effect of tomatidine on ZIKV and WNV infectious particle production. Huh7 cells were infected with (A) ZIKV (clinical isolate/Surinam, 2016) at MOI 1 for 24 h and with (B) WNV (strain NY99) at MOI 1 for 12h. Simultaneously with the infection, cells were treated with 1 and 10 μM of tomatidine, the equivalent volume of EtOH or left un-treated (NT). Treatment was continued for the duration of the experiment and viral titers were determined by plaque assay. Data are presented as mean ± SEM from three independent experiments and differences were assessed with Student T test.
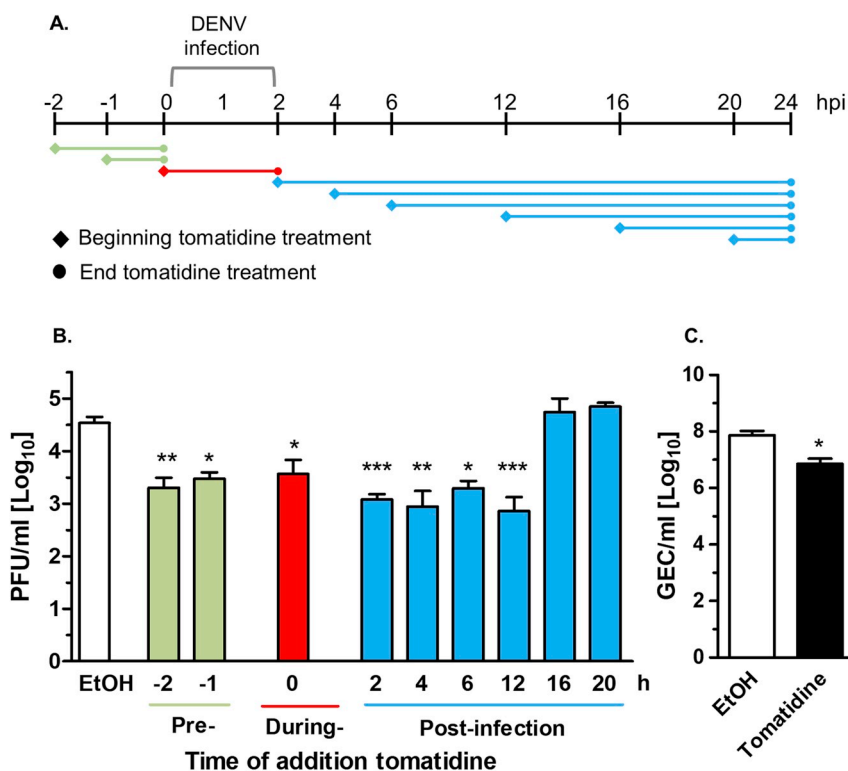
Fig. 4. Tomatidine reduces DENV infectivity when added up to 12 hpi. (A) Outline of the experimental set-up. (B) DENV-2 infectious virus particle production following the conditions presented in (A). The EtOH control was added to all experimental conditions and the average titer is depicted. For the tomatidine-treated samples, data are presented as mean ± SEM from three independent experiments. Differences were assessed by one way ANOVA and Dunnet's post-hoc test. (C) Number of GEC secreted at 24 hpi by Huh7 cells infected with DENV-2 at MOI 1 and treated with 10 μM tomatidine at 12 hpi. Data are presented as mean ± SEM from three independent experiments and differences were assessed with Student T test.

which corresponds to an inhibition of 76.8% (Fig. 5). Interestingly, when EtOH or tomatidine were added to the culture at 2 hpi the percentages of infection were 23.6 ± 2.37 and 8.9 ± 1.81, which translates into an inhibition of 62.2%. Although the percentage of inhibition is slightly lower when tomatidine is added at 2 hpi, this difference is not statistical significant. This suggests that tomatidine predominantly acts at a stage post-cell entry and membrane fusion. Indeed, next to a reduction in the percentage of infected cells, we also observed a significant decrease in the expression level (based on mean fluorescence intensity, MFI) of the E protein in DENV-infected cells treated with tomatidine (Fig. 5B). The reduction in MFI was found independently of the time at which tomatidine was added. Altogether the results suggest that upon addition of tomatidine to cells, the compound is rapidly internalized and likely exerts its antiviral function at a step after virus-cell entry and membrane fusion but prior to progeny virion secretion.
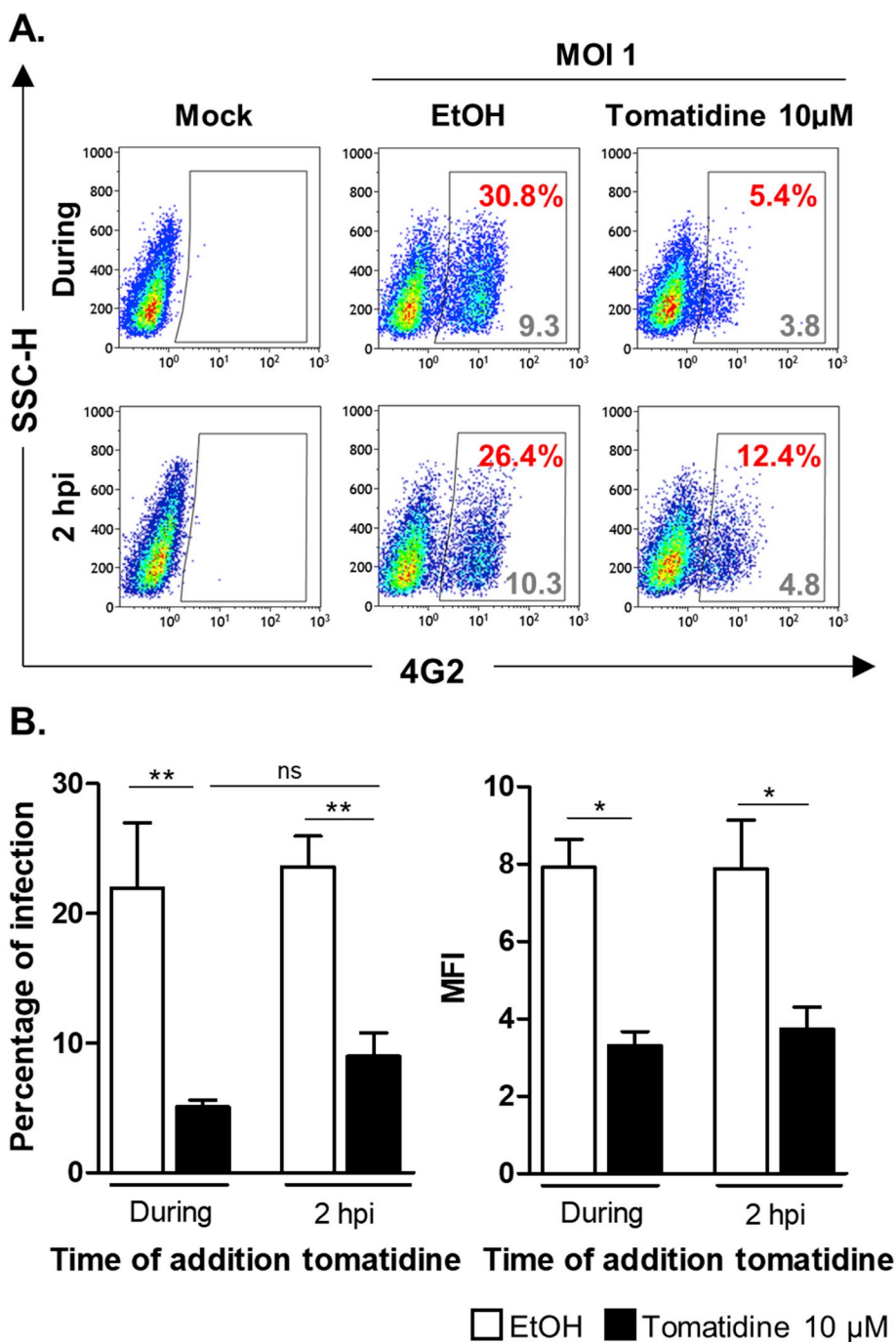
Next, we investigated whether tomatidine also exerts antiviral activity towards DENV in another cell line. Human alveolar epithelial (A549) cells were chosen as these are highly permissive to DENV infection. At MOI 1, 48.9 ± 3.82% of the cells were infected and 5.06 ± 0.23 Log infectious DENV-2 particles per ml were produced at 24 hpi (Fig. 6). Given the high susceptibility of DENV to A549 cells, we decided to determine the antiviral effect of tomatidine following infection at MOI 1 and MOI 0.1. In cells infected at MOI 1 and treated with 10 μM tomatidine, the viral titer was reduced 1.46 Log, which corresponds to a decrease in infectious virus particle production of 96% (Fig. 6A). Infection of A549 cells at MOI 0.1 in presence of tomatidine did not yield viral plaques indicating that the viral titer was reduced at least 3.68 Log. The detection limit of our plaque assay is 40 PFU/ml. Furthermore, and in line with the data obtained in Huh7 cells, tomatidine was found to reduce both the number of infected cells (Fig. 6B) and the level of E protein expression in infected cells (Fig. 6C). The extent of reduction is dependent on the MOI used for infection, the strongest effect being observed at MOI 0.1. In parallel, the CC50 value of tomatidine in A549 cells was determined (Fig. 6D). The CC50 was 180.2 μM, indicating that tomatidine is less toxic to A549 cells in comparison to Huh7 cells (compare Fig. 6D with 2E). Collectively, our data demonstrate that tomatidine exerts potent antiviral activity

towards DENV-2 in at least two distinct human cell lines.

Tomatidine has been reported to interfere with various cellular processes, such as inflammation and angiogenesis (Yan et al., 2013; Chiu and Lin, 2008). 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 (Pakos-Zebrucka et al., 2016). Interestingly, it was recently reported that ATF4 translocates to the nucleus in DENV-infected lung epithelial A549 cells (Fraser et al., 2016). Thus, tomatidine may reduce DENV infection by inhibiting ATF4. To test this, we first determined ATF4 protein levels in Huh7 cells upon DENV-2 infection in the presence and absence of tomatidine. We revealed that DENV-2 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. 7A). Next, we evaluated whether tomatidine reduces the levels of DENV-induced ATF4. Indeed, tomatidine reduced ATF4 levels up to 60% in DENV-2-infected cells (Fig. 7B). 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. 7C). 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. 7D). 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. 7D). Thus, despite efficient knockdown of ATF4, we 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.

4. Discussion

We report here that tomatidine has antiviral activity towards all

A.

DENV serotypes and ZIKV but not for WNV in Huh7 cells. For DENV-2, the EC50 and EC90 values in Huh7 cells 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. The EC50 and EC90 values for the other serotypes were slightly higher but still remained in the micromolar range. 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 (Lim et al., 2013). 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. Here we chose to standardize on the basis of an equal number of infected cells under normal infection conditions. The potency of tomatidine was found the highest for DENV-2, then DENV-1, DENV-4, and DENV-3. Furthermore, the closely related ZIKV, that shares around 43% of amino acid identity across the viral polyprotein with all four DENV serotypes (Lazear and Diamond, 2016), was also inhibited by tomatidine. Ongoing studies regarding the mode of action of tomatidine and the identification of the functional groups of tomatidine might further enhance the potency of the compound. Intriguingly, no antiviral activity was seen for WNV which suggests that the compound acts in a virus or virus group specific manner.

Time-of-drug-addition experiments suggested that tomatidine acts
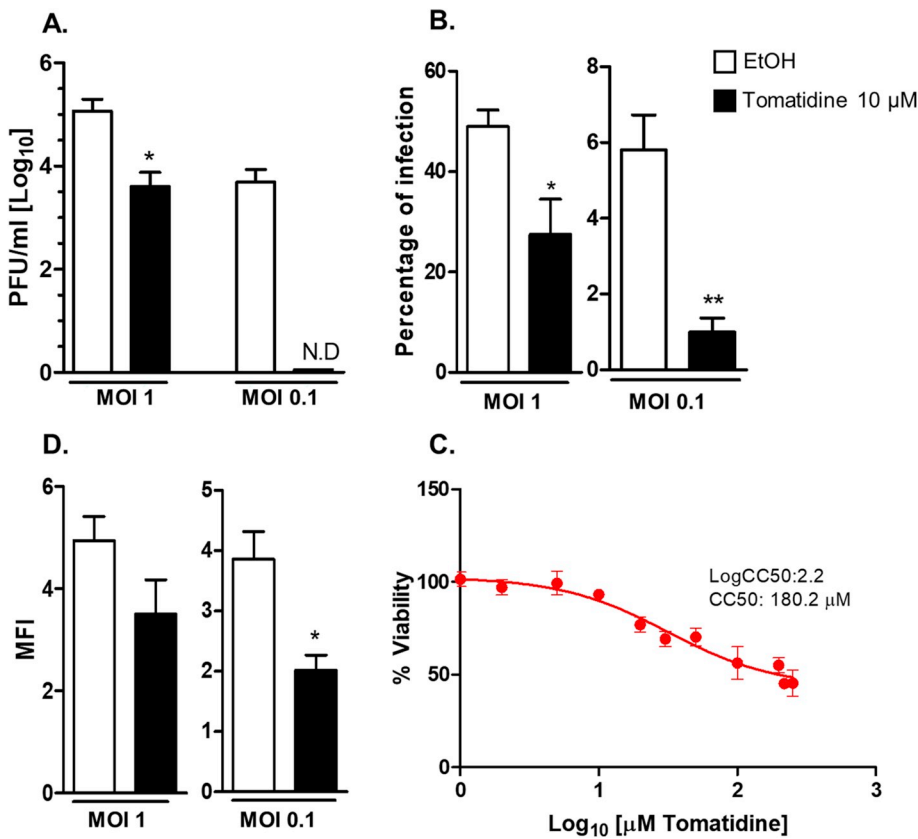
Fig. 6. Tomatidine is antiviral towards DENV in A549 cells. (A–C) A549 cells were infected with DENV-2 at MOI 1 and 0.1. Simultaneously with the infection, cells were treated with 10 μM of tomatidine or the equivalent volume of EtOH. Viral titers (A) were determined by plaque assay in BHK-15 cells, the percentage of infected cells (B) and the MFI (C) were determined by flow cytometry. Data are presented as mean ± SEM from three independent experiments and differences were assessed with Student T test. (D) Dose response curve showing the cytotoxicity of tomatidine in A549 cells determined by MTT assay performed in triplicate. The CC50 value was calculated with GraphPad Prism software.
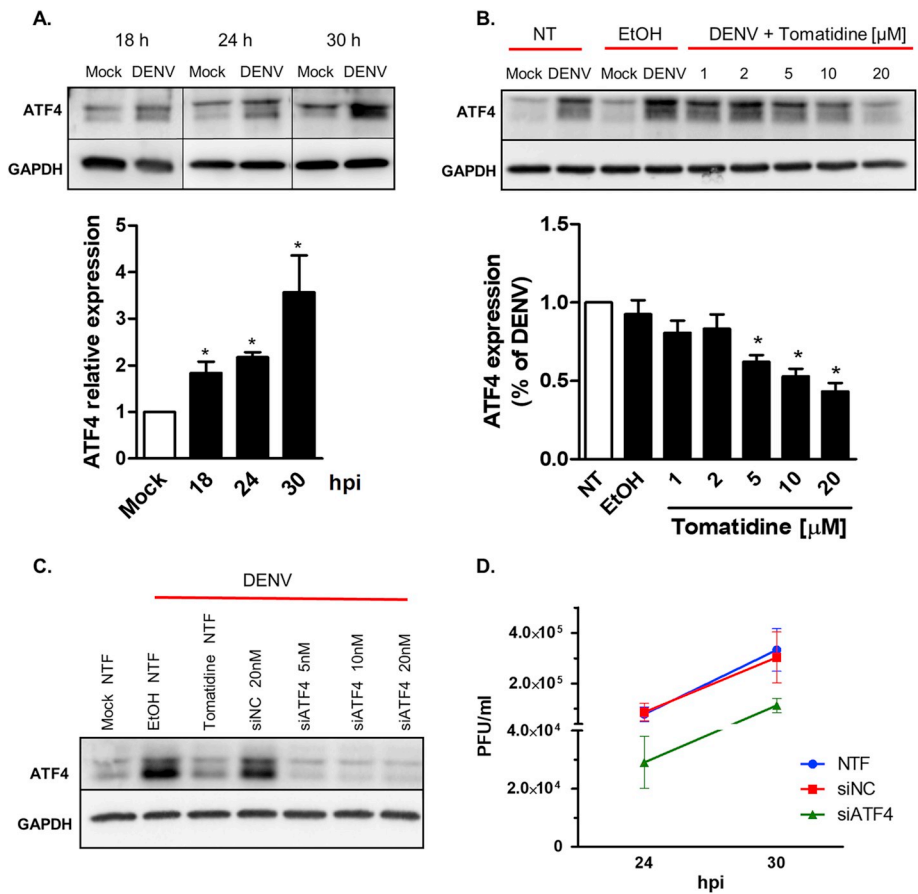
Fig. 7. Antiviral effect of tomatidine is independent of ATF4. (A) Huh7 cells were mock-infected or infected with DENV-2 at MOI 10. The upper panel shows representative WB images of ATF4 and GAPDH expression at 18, 24 and 30 hpi. Lower panel shows the quantitation ATF4 levels normalized to the time-matched mock-infected cells. (B) Huh7 cells mock-infected or infected with DENV-2 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-2 at MOI 10. At 2 hpi cells were treated with 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-2 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, 10 nM siATF4 or left non-transfected (NTF). At 48 h post transfection, cells were infected with DENV-2 at MOI 1. Viral titer is presented at 24 and 30 hpi. Data are presented as mean ± SEM from at least three independent experiments.

at early and late stages of infection. Subsequent experiments, however, revealed that there is only a minor reduction in the percentage of infection (up to 4-fold, Fig. 5) when compared to the overall reduction in infectious virus particle production (up to 100-fold, Fig. 1B). Furthermore, the reduction in the percentage of infected cells was also seen when the compound was added after virus cell entry and removal of virus inoculum. This suggests that tomatidine does not directly interfere with virus-cell binding, internalization and membrane fusion. Rather it suggests that tomatidine is internalized by cells and interferes with DENV replication at latter stages in infection. Indeed, potent antiviral activity was still observed upon addition of the compound at 12 hpi. No antiviral activity was seen upon addition of tomatidine at 16 hpi suggesting that tomatidine cannot directly interfere with virus secretion as initial virus particle production is seen at 18 hpi. Collectively, this suggests that tomatidine predominantly intervenes with steps downstream of virus cell entry and membrane fusion but prior to 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 (Ebert et al., 2015) and a recent study showed that ATF4 is translocated to the nucleus upon DENV infection (Fraser et al., 2016). 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 synthesis (Pakos-Zebrucka et al., 2016). Indeed, silencing of ATF4 reduced the production of infectious virus particles by 50%. Nevertheless, 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 (Cheng et al., 2016). Moreover, carbenoxolone disodium was reported to reduce DENV infectivity due to direct virucidal activity of the compound (Pu et al., 2017). Furthermore, coumarins were shown to be potent inhibitors of both DENV as well as Chikungunya virus (Gómez-Calderón et al., 2017). 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 (Lim et al., 2013; Behnam et al., 2016). However, none of these compounds have reached clinical trials, among other reasons because adverse effects in animals and poor pharmacokinetic properties (Kaptein and Neyts, 2016). A proper study of safety and pharmacokinetics of tomatidine in humans is lacking, even though it is commercially available in the United States of America as a dietary supplement. The high SI observed in our *in vitro* study and the safe use of tomatidine in pregnant mice (Friedman et al., 2003; Fujiwara et al., 2012; Dyle et al., 2014) are promising data with regard to the potential use of tomatidine in humans. Nevertheless, a formal study of absorption, distribution, metabolism, and excretion is still required.

This is the first report that shows that tomatidine has antiviral activity towards all DENV serotypes. The observed EC50 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 *in vivo* potency of the compound against DENV as well as further dissecting the molecular mechanism of its antiviral activity.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.11.011.

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