Inhibition of dengue virus by curcuminoids

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A B S T R A C T

The dengue virus is considered to be a globally important human pathogen prevalent in tropical and subtropical regions of the world. According to a recent estimate, the disease burden due to DENV infections is ~390 million infections per year globally in ~100 countries including the southern US, Puerto Rico and Hawaii, resulting in nearly ~25,000 deaths mostly among children. Despite the significant morbidity and mortality that results from DENV infections, there is currently no effective chemotherapeutic treatment for DENV infections. We identified curcumin as an inhibitor of DENV2 NS2B/NS3protease in a previous high-throughput screening (HTS) campaign. We synthesized four analogues of curcumin (curcuminoids) and tested the in vitro protease inhibition activity and inhibition of replication by cell-based assays. The results revealed that curcumin is a weak inhibitor of the viral protease. However, the analogues exhibited more potent inhibition of DENV infectivity in plaque assays suggesting that the cellular pathway(s) required for viral replication and/or assembly are targeted by these compounds. Further analysis shows that inhibition of genes involved in lipid biosynthesis, and of actin polymerization by curcuminoids, are likely to be involved as their mode of action in DENV2-infected cells. Three of the curcumin derivatives possess good selectivity indices (SI) (> 10) when compared to the parent curcumin.

1. Introduction

Dengue virus (DENV) is a member of mosquito-borne flavivirus genus of *Flaviviridae* family which consists of > 70 human pathogens causing considerable morbidity and mortality in tropical and subtropical regions of the world (for reviews, see (Beasley, 2005; Gould and Solomon, 2008; Lindenbach et al., 2007)). There are four serotypes of dengue virus (DENV1-4) that together cause 390 million infections annually (Bhatt et al., 2013). DENV is primarily transmitted by the *Aedes aegypti* and *Aedes albopictus* mosquitos. Currently, there is no FDA-approved vaccine or antiviral drug available for control or treatment of dengue infections. Primary DENV infection by a single serotype causes self-limiting disease known as dengue fever with symptoms including fever, headache, joint pain and a skin rash, whereas a secondary infection by another serotype results in severe dengue disease through antibody dependent enhancement with symptoms including thrombocytopenia, hemoconcentration due to capillary leak leading to hemorrhage in internal organs and shock syndrome (Garcia et al., 2010; Halstead et al., 2005).

Flaviviruses have a single positive strand RNA genome flanked by highly structured 5′ and 3′ untranslated regions (UTRs) which play important roles in translation and replication of viral RNA (Lindenbach et al., 2007). RNA encodes a single polyprotein that is cleaved by viral and host proteases to produce three structural proteins (C, prM and E) and eight nonstructural proteins including the cleavage product of NS4A (NS1, NS2A, NS2B, NS3, NS4A, NS4A-2K, NS4B and NS5) (for a review (Padmanabhan and Strongin, 2010),). Previous HTS campaign using purified DENV2 NS2B-NS3pro as a target and subsequent cherry picks for validation by secondary assays identified curcumin as a potential hit that inhibited the purified viral protease NS2B-NS3pro (unpublished results; see also (Balasubramanian et al., 2016)). Subsequently, four curcuminoids were synthesized and characterized for their antiviral activity.

Here we report the antiviral activities of curcumin and the four analogues against purified DENV2 NS2B-NS3pro as well as in stable DENV2 BHK-21/Renilla luciferase reporter replicon expressing cells and DENV2-infected BHK-21 cells. Our results indicate that curcumin and the four analogues were more potent in inhibition of viral replication and infectivity (plaque) assays than their inhibition of protease activity in vitro. The cyclopentanone analogue of curcumin (CC4) was the most active and less toxic compared to curcumin. These results suggested that curcumin and analogues might target cellular pathways required for viral replication. A previous study reported that cellular pathways, actin polymerization and fatty acid biosynthesis, are involved in endoplasmic membrane expansion and formation of viral replication factories in the infected cells (Heaton and Randall, 2011b)

Table 1
Chemical structures and molecular weights of compounds used in this study.

Label Name	Chemical Structure	Molecular Weight
Curcumin (CC1)		$C_{21}H_{20}O_6$ Mol. Wt. = 368.3
Bis-demethoxy curcumin (CC2)		$C_{19}H_{16}O_4$ Mol. Wt. = 308.3
Acyclic analogue (CC3)		$C_{19}H_{18}O_5$ Mol. Wt. = 326.35
Cyclopentanone analogue (CC4)		$C_{21}H_{20}O_5$ Mol. Wt. = 352.39
Cyclohexanone analogue (CC5)		$C_{22}H_{22}O_5$ Mol. Wt. = 366.41

(Padilla et al., 2014). Curcumin inhibits hepatitis C virus replication via suppression of the transcription factor, AKt- Sterol regulatory element binding protein −1 (SREBP-1) (Kim et al., 2010). Since SREBP-1 is required for lipid biosynthesis and its downregulation affects downstream genes such as fatty acid synthase (FASN) and acetyl CoA carboxylase (ACC), in this study, we examined the role of curcuminoids on FASN and ACC gene expression by qRT-PCR in DENV2-infected cells. Our results indicate that the curcuminoids inhibit lipogenesis and thereby inhibiting DENV replication. The curcuminoids tested in this study also lead to actin depolymerization similar to curcumin (Dhar et al., 2015) in DENV2-infected cells. Actin plays important roles in entry, viral replication and assembly.

2. Materials and methods

2.1. Curcuminoids

Curcumin was purchased from *Alfa Aesar (part of Thermo Fisher, Inc)* which contained 95% total curcuminoid from turmeric, (botanical name, Rhizome) (CC1; Table 1; CAS number 458-37-7; Stock No. B21573-09). Bisdemethoxycurcumin (CC2; Table 1) was purchased from TCI America (Cat No B3347). Derivatives CC3, CC4 and CC5 (Table 1) were synthesized using the protocol described in Section 2.2.

2.2. General procedure for the chemical synthesis of curcuminoids CC3, CC4, and CC5

The chemical synthesis of curcuminoids was accomplished by a slight modification of the previously reported procedure (Du et al., 2006). Glacial acetic acid (50 mL) was saturated with hydrogen chloride gas for 1 h at 0 °C. A mixture of vanillin (3.04 g, 20 mmol, 2.0 equivalent) and the appropriate ketone acetone, 580 mg, for CC3, cyclopentanone, 841 mg, for CC4, and cyclohexanone, 981 mg, for CC5, each containing 10 mmol (1.0 equivalent) was slowly added. The reaction mixture was stirred at 0 °C for 15 min, it was slowly brought to ambient temperature and stirred at ambient temperature for 48 h. The reaction mixture was poured into ice-cold water (200 mL). The

precipitate was filtered using vacuum filtration, washed with ice-cold water (200 mL) and dried. The crude product was purified using silica gel flash column chromatography. Gradient elution (from 30% to 60% ethyl acetate in hexanes) resulted in the isolation of the desired curcuminoids as bright yellow solids in yields of 1.59 g (59%), 3.02 g (79%), 3.24 g (82%) for CC3, CC4, and CC5 respectively (for more details on synthesis refer Supporting information). ^{1}H, ^{13}C-NMR and IR spectra of CC3, CC4, and CC5 (Figs. S1–S6) were consistent with the proposed structure and previously reported literature. The spectroscopic as well as chromatographic analysis revealed that the synthetic curcuminoids were > 95% pure.

2.3. In vitro protease assay

The DENV2 NS2B-NS3pro expression plasmid encoding the protease precursor used in this study contains the hydrophilic domain of NS2B cofactor (48 amino acids) and the NS3pro domain (185 residues) (Yon et al., 2005). The cloning, expression and purification of the viral NS2B-NS3pro were reported previously (Lai et al., 2014; Mueller et al., 2007; Yusof et al., 2000). In this study, we evaluated the efficacy of curcumin and four curcuminoids. Standard reaction mixture (100 μl) containing 200 mM Tris·HCl (pH 9.5), 6 mM NaCl, 30% glycerol, 25 nM DENV2 protease, 10 μM or 25 μM inhibitors in DMSO (final DMSO concentration 1%) were incubated 15 min at 25 °C followed by addition of 25 μM protease substrate (Bz)-Nle-Lys-Arg-Arg-AMC. The amount of AMC (7-Amino-4-methylcoumarin) released from the peptide substrate was quantified by measuring the fluorescence ($\lambda_{ex.}$ = 380 nm, $\lambda_{em.}$ = 460 nm) in a monochromater based spectrofluorometer (SpectroMax Gemini EM Microplate reader from Molecular Devices). A potent serine protease inhibitor, aprotinin, was used as a positive control and 1% DMSO (no inhibitor; 100% activity) served as a negative control. For determination of IC50 values, standard protease assays were performed in the presence of different inhibitor concentrations (1.56, 3.12, 6.25, 12.5, 25, 50, 75, 100, 200 μM). The percent inhibition activities after 30 min in the presence of various inhibitor concentrations were plotted using the GraphPad Prism v5.04 using the four-parameter nonlinear regression analysis (Hill slope method).

2.4. BHK/DENV2 replicon assay

The replicon-based assay covers the stages of viral life cycle including viral translation, viral replication consisting of both plus- and minus-sense RNA synthesis. The assay does not include stages such as viral entry, assembly, and maturation due to the absence of genes encoding the structural proteins, C, prM, and E, which are replaced by *Renilla* luciferase (*Rluc*) reporter gene (Alvarez et al., 2005; Boonyasuppayakorn et al., 2014; Ng et al., 2007). This assay uses a Baby Hamster Kidney (BHK) cell line that stably expresses a DENV2 replicon with *Rluc* reporter gene (Boonyasuppayakorn et al., 2014). The replicating RNA can be quantified by measuring the luciferase activity using a kit (Promega) and a luminometer (Berthold Technologies., LB-960) (Boonyasuppayakorn et al., 2014) and the viral RNA can be quantified by quantitative reverse transcriptase-PCR (qRT-PCR).

BHK-21/DENV2 replicon-expressing cells were maintained in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 I.U./ml penicillin, 100 µg/ml streptomycin (Penicillin + Streptomycin), and 300 µg/ml G418 (Fisher Scientific, Pittsburgh, PA). Briefly, BHK-21/DENV2 replicon expressing cells ($\sim 5 \times 10^3$ in 100 µl) were seeded onto 96-well plates and incubated at 37 °C for 24 h in a humidified incubator with 5% CO_2. Curcuminoids (CC1–CC5) were added at 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM in 1% DMSO in triplicate wells. Cells were incubated at 37 °C for 24 h, washed, lysed, and Rluc activities were measured as described (Boonyasuppayakorn et al., 2014; Manzano et al., 2011). The results were confirmed by two independent experiments. The *Rluc* activities were reported as percent inhibition relative to 1% DMSO (0% inhibition) and mycophenolic acid (100% inhibition) as controls (Diamond et al., 2002; Takhampunya et al., 2006).

2.5. Cell-based cytotoxicity assay

The viability of cells in the presence of varying concentration of curcuminoids was measured using the Cell Counting Kit-8 (Dojindo, Rockville, MD) and the concentration at which the cell viability was reduced by 50% (CC_{50}) was determined. The assay is based on a highly soluble, non-toxic tetrazolium salt (WST-8), which is reduced by dehydrogenase activities in cells to give a yellow-colored formazan dye. Briefly, BHK-21 (5×10^3) cells (100 µl) in wells of a 96-well plates were treated with varying concentrations (200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 µM) of curcuminoids (CC1–CC5). After incubation for ~ 24 h at 37 °C under 5% CO_2, 10 µL of 1X CCK-8 solution was directly added to all the wells. The plates were incubated further at 37 °C under 5% CO_2 for 4 h and the absorbance at 450 nm was read using a spectrophotometer (Molecular Devices). The results were confirmed by two independent experiments. CC_{50} values were calculated using the GraphPad Prism v5.04 software. Selectivity index (SI) values were calculated as the ratio of CC_{50}/EC_{50}.

2.6. Plaque assays

Briefly, BHK-21 cells (1×10^5) in MEM-α medium with 10% FBS were seeded onto each well of 24-well cell culture plates. After the cells were about 80% confluent, they were infected with DENV2 in MEM-α medium supplemented with 2% FBS (multiplicity of infection, MOI of 1 for 1 h). After infection, OPTI-MEM with various drug concentrations (0.07–10 µM) was added to the wells. BHK-21 cells treated with only 1% DMSO served as negative control (100% infectivity) and cells treated with 1 µM mycophenolic acid (0% infectivity) served as the positive control. After 24 h the supernatants were collected and analyzed by plaque assays as described below.

LLC-MK2 cells, plated (1×10^5 cells/ml/well) in 24-well plates, were incubated at 37 °C until reaching 90% confluence. Supernatants collected as described above were serially diluted in OPTI-MEM and used for infecting duplicate wells for 2 h. Cells were overlaid with l X
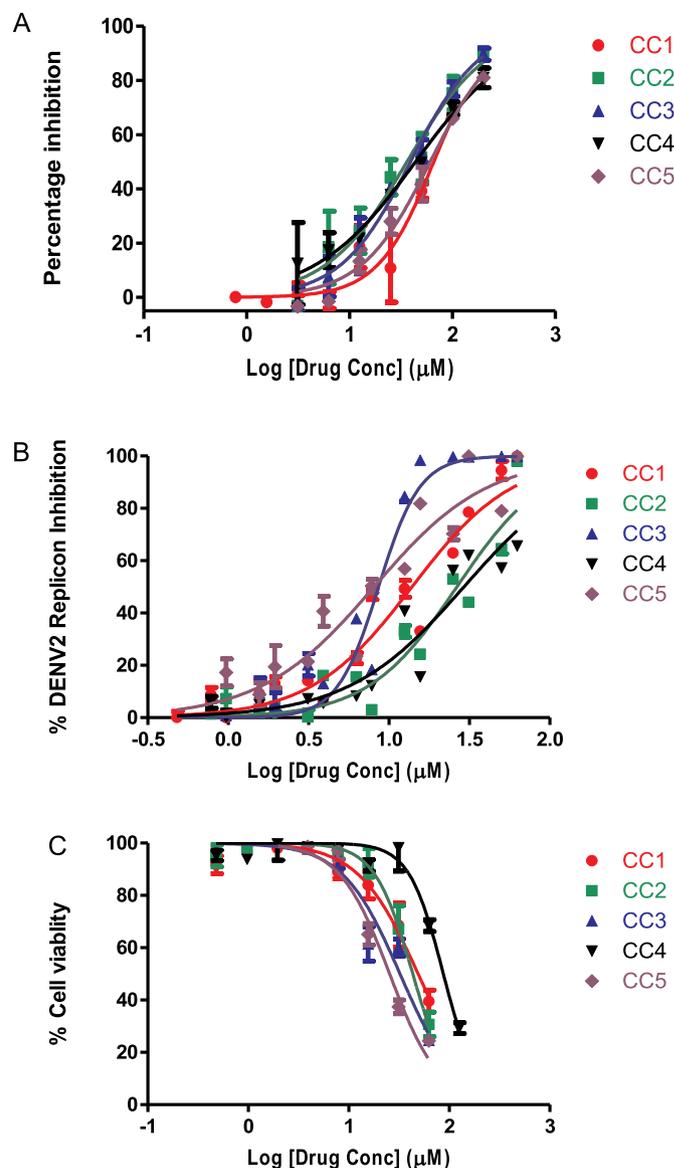

Fig. 1. Inhibition of DENV2 replicon replication by curcuminoids (A) BHK-21/DENV2 replicon expressing cells ($\sim 5 \times 10^3$ in 100 µl) were seeded onto 96-well plates and incubated at 37 °C for 24 h in a humidified incubator with 5% CO_2. Compounds were added at 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM in 1% DMSO in triplicate wells. Cells were incubated at 37 °C for 24 h, washed, lysed, and Rluc activities were measured. The results were confirmed by two independent experiments. (B) Cytotoxicity was measured in the same replicon expressing cells treated with the various concentrations of compounds at 24 h using the Cell Counting Kit −8 following the manufacturer's instructions.

DMEM supplemented with 10% FBS containing 1.5% carboxymethyl cellulose (CMC) and were incubated for 7 days at 37 °C under 5% CO_2. Plaques were visually examined after fixing the cells with 4% paraformaldehyde and staining with 1% crystal violet for 30 min. The number of plaque forming units (PFU) per ml was determined (Balasubramanian et al., 2016; Boonyasuppayakorn et al., 2014). The EC50(PFU) was calculated from two independent experiments.

2.7. qRT-PCR

Human hepatocarcinoma (Huh 7.5) cells were infected with DENV2 (MOI of 1) and treated with 5 µM of a curcuminoid (CC1–CC5) for 24 h at 37 °C under 5% CO_2. Uninfected Huh 7.5 cells were also treated
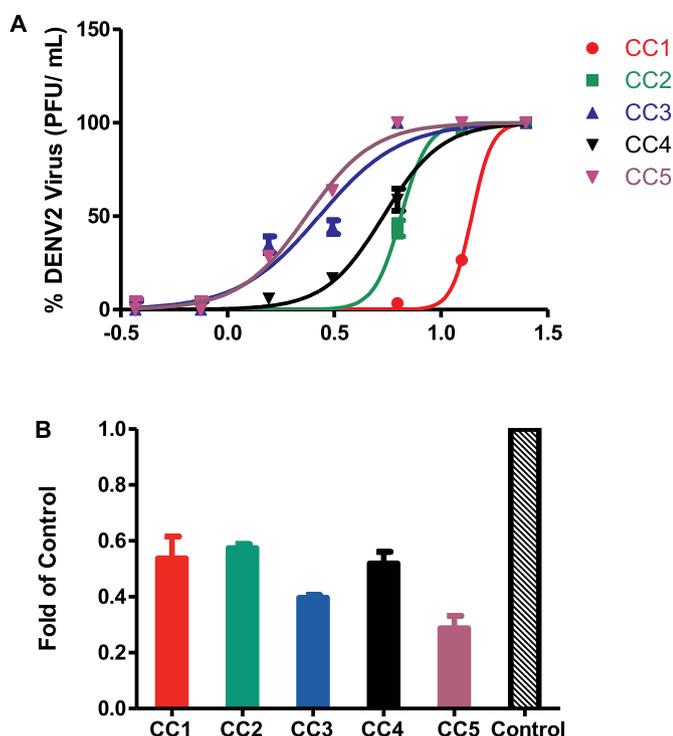
Fig. 2. Inhibition of DENV2 replication analyzed by plaque assays and qRT-PCR. (A) $EC_{50(PFU)}$ values for inhibition of DENV2 infectivity by curcuminoids. BHK-21 cells were infected with DENV2 (MOI, of 1) and treated with varying concentrations of compounds. Incubation was done at 37 °C and the supernatants were collected at 48 h. The virus titers were determined by plaque assays using LLC- MK2 cells. EC_{50} values were calculated using GraphPad Prism v5.04 software. (B) qRT-PCR was performed as described in the Materials and Methods. The fold reduction of viral RNA copy number in DENV2-infected and drug-treated cells as compared to control DENV2-infected cells was determined.

similarly with 5 μM curcuminoids. The total RNAs were extracted using TRIzol reagent (Life technologies) according to the manufacturer's protocol. DMSO (1%)- treated cells served as negative control. RNA concentrations were determined using BioSpec-nano (Shimadzu Corporation, Japan). Reverse transcription reaction was carried out in 20 μL containing 1 μg RNA. Each qRT-PCR carried out in duplicate contained 12.5 μL of the SYBR Green Master Mix reagent (iQ supermix-Biorad), 0.5 μL of primers (20 nM) specifically designed to anneal to the in vitro transcripts (2 μL) from DENV2 NS5 (Manzano et al., 2011), human fatty acid synthase (FASN) and human acetyl-CoA carboxylase (ACC) cDNAs in 9.5 μL of diethylpyrocarbonate-treated water to a final volume of 25 μL. The PCR primers used for human ACC were: forward, CTGTTGGCTCAGATACACTC, and reverse, GCCACAGTGAAATCTCGTT. The PCR primers for human FASN were: forward, GTGGGAAGGTGTA CCAGTG, and reverse, GGATGCCCTGGAAATGAG. The amplification protocol consisted of the following steps: 95 °C for 2 min, followed by 50 cycles at 95 °C for 10 s, 50 °C for 30 s and 72 °C for 30 s and the melt curve analysis was carried out as described (Balasubramanian et al., 2016; Boonyasuppayakorn et al., 2014). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as a reference control. The primer sequences for human GAPDH were: forward, AGGGCTGC TTTTAACTCTGGT and reverse, CCCCACTTGATTTTGGAGGGA.

2.8. Oil Red O staining of lipid droplets

BHK-21 cells were maintained in MEM containing 10% FBS. The cells were infected with DENV2 at a MOI of 1 for an hour followed by incubation with MEM containing 0.1 mM oleic acid-BSA (03008, Millipore Sigma) and various concentrations of curcumin for 24 h. Cells treated with 1% DMSO were used as negative controls. The cultures
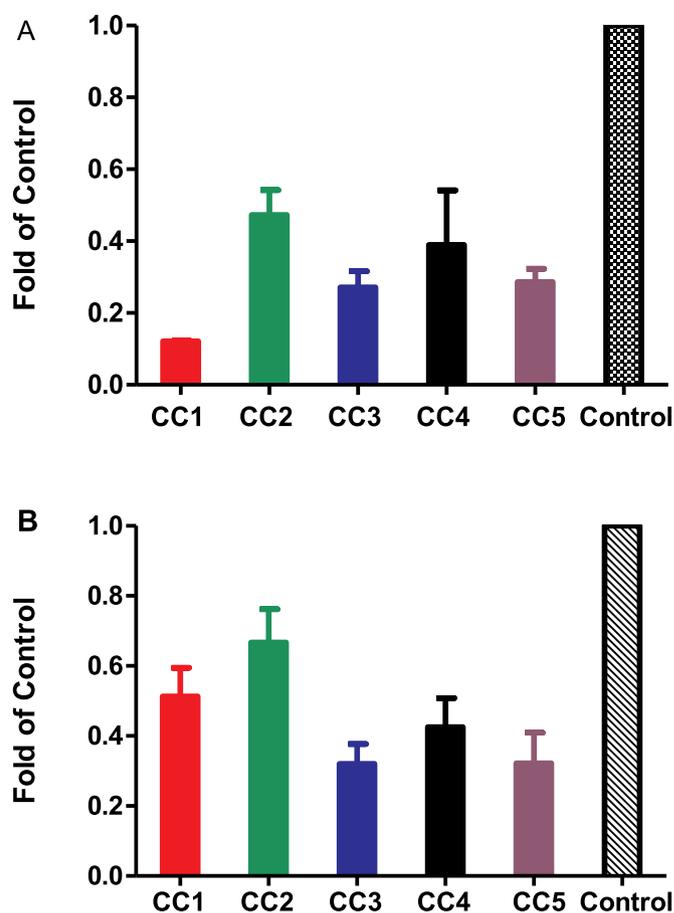

Fig. 3. qRT-PCR to evaluate the effect of curcuminoids on lipogenesis: (A) qRT-PCR was performed as described in the Materials and Methods. The fold reduction of acetyl-CoA carboxylase (ACC) mRNA copy number in DENV2-infected and drug-treated cells versus the control DENV2-infected cells was determined. (B). The fold reduction of fatty acid synthase (FASN) mRNA copy number in DENV2-infected and drug -treated cells versus the control DENV2 infected cells was determined.
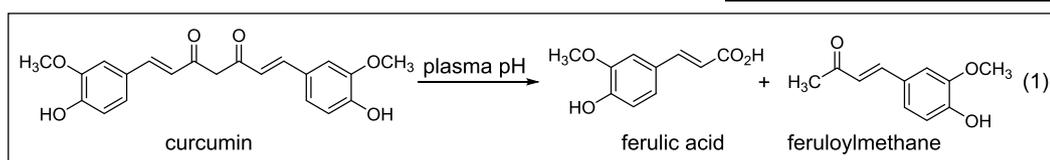
were washed 3 times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min. Oil Red O (ORO) working solution was made by mixing 2.4 ml of ORO (0.3% in isopropanol) with 1.6 ml of distilled water and it was filtered (Cui et al., 2010). Cells were incubated with 0.5 mL of the ORO for 15 min, the solution was removed and rinsed three times with PBS. The images were photographed at 40 × magnification. The ORO was extracted from the stained cells using 500 μl of 100% isopropanol from each well, and two aliquots of 200 μl were transferred to black-walled 96-well microtiter plates. The absorbance of the dye was measured at 405 nm using a plate reader. All tests were performed in duplicate and the means were calculated (Cui et al., 2010).

2.9. Actin staining with phalloidin

BHK-21 cells were grown in MEM containing 10% FBS. Cells were seeded in 24-well plates (5 × 10^4/well) and incubated in a humidified CO_2 incubator for 24 h. Cells were infected with DENV2 at a MOI of 1 and then treated with 5 μM of curcuminoids dissolved in DMSO (1%) for 24 h. DMSO (1%) alone treated cells were used as controls. The cultures were washed 3 times with PBS and were fixed with 4% formaldehyde for 10 min. Alexa Fluor 488 Phalloidin (Thermo Fischer Scientific) was used to stain the F-actin and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The stained cells were visualized under a fluorescent microscope and images were photographed.

3. Results and discussion

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is derived from the spice turmeric, *Curcuma longa*, which is a member of the ginger family and it is a yellow-colored compound primarily found in the roots of turmeric plant (Goel et al., 2008; Jacob et al., 2007; Moghadamtousi et al., 2014). Curcumin is reported to contain various medicinal properties some of which are against cancer, diabetes, microbial infections including those caused by viruses. Curcumin has already been shown to be effective against several enveloped viruses (such as HIV, influenza A virus, herpes simplex virus, hepatitis B virus, Japanese encephalitis virus and DENV) (Chen et al., 2013) and on non-enveloped viruses, coxsackievirus B3 and enterovirus 71 (Chen et al., 2013; Qin et al., 2014). Recently curcumin has also been shown to inhibit Zika and Chikungunya viruses by inhibiting cell binding (Mounce et al., 2017). Curcumin is known to be an inhibitor of DENV2 with pre-treatment or co-treatment with the virus but failed to inhibit when added post entry (Chen et al., 2013). However, it exhibits poor stability at the plasma pH and undergoes a facile retro-aldol decomposition into ferulic acid (Equation (1)) (Anand et al., 2007; Wang et al., 1997). Conversion of the β-diketone moiety in curcumin into monoketone analogues is known to minimize retro-aldol decomposition. We designed curcuminoids CC3, CC4, and CC5 in order to improve their stability at plasma pH.

infectivity in the formation of infectious particles measured by plaque assays as the concentrations causing 50% reduction in PFU ($EC_{50(PFU)}$). The $EC_{50(PFU)}$ of curcuminoids were: 13.95 ± 2.57 (CC1), 6.49 ± 0.33 (CC2), 2.68 ± 0.51 (CC3), 5.37 ± 0.49 (CC4) and 2.34 ± 0.22 (CC5). All analogues showed greater inhibition in formation of infectious particles as determined by plaque assays when compared to native curcumin. But only one analogue, CC4, was less toxic than native curcumin. The selectivity indices (SI) were greater than 10 for three analogues (Fig. 2A, Table 2). Next, we analyzed the RNA copy number by *qRT-PCR* using RNAs isolated from control DENV2 infected cells in the presence of 1% DMSO alone, and DENV2-infected and curcuminoid (5 μM)-treated cells. The results indicated that in the drug-treated and DENV2-infected cells, the viral RNA copy numbers were reduced when compared to DENV2-infected control in the presence of 1% DMSO alone (Fig. 2B).

These results, taken together, suggested that curcuminoids inhibit DENV by targeting a host pathway essential for viral life cycle. Previous studies indicated that curcumin has broad spectrum antiviral activities against both RNA and DNA viruses targeting diverse pathways. For example, in one study, curcumin was shown to inhibit the hepatitis C virus entry of all genotypes (Barbosa et al., 2015) by affecting the membrane fluidity, virus binding and fusion. However, it had no effect on RNA replication. In this study, we sought to examine whether curcumin and its analogues exerted their antiviral activity through key step

curcumin → plasma pH → ferulic acid + feruloylmethane (1)

(1)

Using an in vitro protease assay, we confirmed that curcumin and its analogues show modest inhibition of protease activity. The IC_{50} values for the curcuminoids mean $\pm$ SD of two replicates were as follows: 66.01 ± 13.12 (CC1), 36.23 ± 9.67 (CC2), 39.17 ± 6.69 (CC3), 43.88 ± 10.14 (CC4) and 60.98 ± 8.7 (CC5) (Fig. 1A), consistent with the finding of the parent compound as an HTS hit. The results show that the inhibitory activity of the parent compound, CC1, is less compared to CC2, CC3, CC4 and CC5 analogues. We analyzed the potency of the curcuminoids using the DENV2 *Rluc* reporter replicon-based assay as described under Materials and Methods. The EC_{50} values for the curcuminoids mean $\pm$ SD of three replicates each from two independent experiments were as follows: 13.91 ± 2.08 (CC1), 26.45 ± 3.67 (CC2), 8.61 ± 0.79 (CC3), 29.25 ± 4.46 (CC4) and 8.07 ± 1.52 (CC5) (Fig. 1B). The CC_{50} values of the curcuminoids mean $\pm$ SD of three replicates each from two independent experiments were: 49.01 ± 8.24 (CC1), 43.37 ± 5.19 (CC2), 32.34 ± 4.72 (CC3), 87.40 ± 9.03 (CC4) and 25.50 ± 2.64 (CC5) (Fig. 1C). Thus, in replicon assays, curcuminoids show only modest inhibition of viral replication.

Next, we analyzed the ability of the curcuminoids to inhibit DENV2

(s) in lipid biosynthesis and metabolism. Several studies previously indicated that HCV and DENV replication and assembly are intimately linked to lipid metabolism (Heaton and Randall, 2010, 2011b). We analyzed the gene expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) in DENV2-infected and curcuminoids-treated or untreated BHK-21 cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a reference gene (Fig. 3). The inhibition of gene expression of ACC and FASN was shown as fold reduction of the control in the absence of curcuminoid treatment. The reduction of DENV2 titers corresponded to the reduction in FASN and ACC. Uninfected control cells showed similar reduction in ACC and FASN (Figs. S7 and S8).

Flavivirus replication and assembly into immature virus particles occur in the membrane compartments of ER which undergo proliferation in infected cells to meet the demand for viral replication and assembly. Lipid droplets (LD), ER-derived lipid-rich cellular organelle, play an important role in storage of excess fatty acids and hydrolysis of neutral lipids for membrane formation and maintenance.

LD's have been best described in adipocytes, they are present in all cell types and their formation can be induced by oleic acid treatment (Pol et al., 2004). Oleic acid has been shown to enhance the effects of
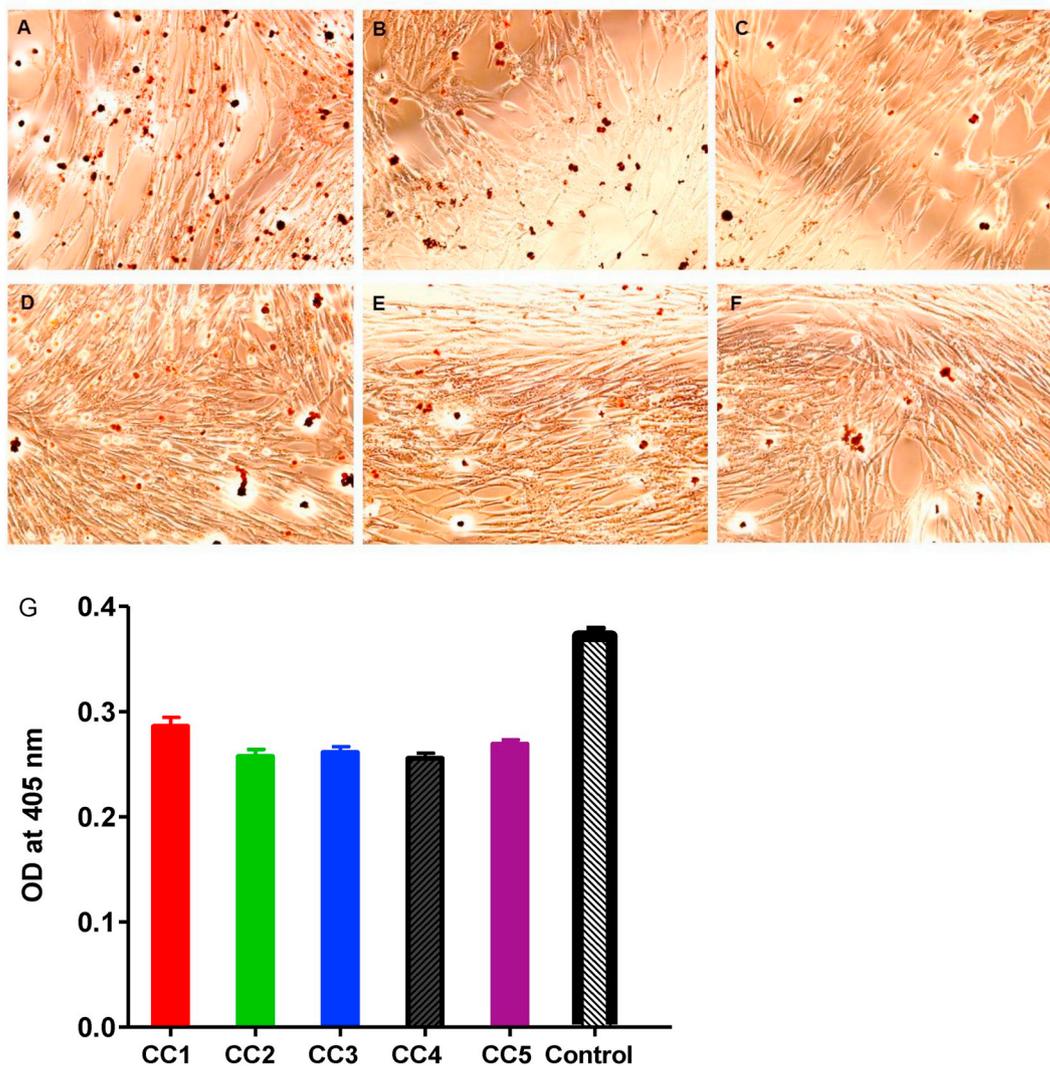
Table 2
Inhibition of DENV2 infectivity and DENV2 subgenomic replicon by curcuminoids.

Compound	$EC_{50\ (PFU)}$	CC_{50}	$SI\ _{(PFU)}$	$EC_{50\ (replicon)}$	$SI\ _{(replicon)}$
CC1	13.95 ± 4.62	49.01 ± 8.24	3.51	13.91 ± 2.08	3.52
CC2	6.49 ± 0.22	43.37 ± 5.19	6.68	26.45 ± 3.67	1.63
CC3	2.68 ± 0.64	32.34 ± 4.72	12.06	8.61 ± 0.79	3.75
CC4	5.37 ± 0.62	87.40 ± 9.03	16.27	29.25 ± 4.46	2.98
CC5	2.34 ± 0.21	25.50 ± 2.64	10.89	8.07 ± 1.52	2.34

The $EC_{50\ (PFU)}$, CC_{50} and $EC_{50\ (replicon)}$ values were determined as described under Materials and Methods.
The $SI\ _{(PFU)}$ values represent the ratios of $CC_{50}/EC_{50\ (PFU)}$ and the $SI\ _{(replicon)}$ values represent the ratios of $CC_{50}/EC_{50\ (replicon)}$.

Fig. 4. LD staining using Oil Red O (ORO). ORO staining protocol described under Materials and Methods was followed. The paraformaldehyde (4%)-fixed cells were stained with ORO, followed by DAPI, and were visualized by fluorescent microscopy. (A) Control oleic acid-treated cells; (B–F): 5 μM curcumioids (CC1- CC5)-treated cells; (G) Quantification of LD after extraction by absorbance at 405 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

DENV2 virus infection (Ramphan et al., 2017). Here we performed LD staining of DENV2 infected cells treated with oleic acid and with curcuminoids and showed that the curcuminoids were able to suppress the oleic acid-induced LD (Fig. 4), the results from two independent experiments were plotted and were found to be statistically significant (pValue < 0.05, Turkey's test). The LD staining was quantified as described under Materials and Methods and results indicated that curcumin affected LD levels in DENV- infected cells (Fig. 4). Curcumin has also been previously shown to suppress FASN expression in adipocytes (Zhao et al., 2011) and reduce the oleic acid-induced lipid accumulation in hepatocytes (Kang et al., 2013).

Flavivirus infection influences the host cell lipids profoundly. Results from lipidomic analysis of membrane fractions associated with DENV replication revealed that 85% of lipids were significantly modified as compared to uninfected control (Perera et al., 2012). DENV infection is highly sensitive to block in the fatty acid synthesis through inhibition of FASN (Heaton et al., 2010). The inhibition of key enzymes in cholesterol synthesis pathway mevalonate diphospho decarboxylase, HMG-CoA synthase and squalene synthetase interfered with DENV replication (Rothwell et al., 2009) indicating the importance of cholesterol in DENV infection. DENV capsid has been previously shown to interact with lipid droplets (Carvalho et al., 2012). DENV virus

infection triggers autophagy which release free fatty acids required to maintain high intracellular ATP favoring viral replication (Heaton and Randall, 2010). Host cell lipids have been found to regulate viral entry into cell and replication (Heaton and Randall, 2011a, b; Mazzon and Mercer, 2014). Curcumin has been demonstrated to reduce lipogenesis in high-fat diet mouse model through regulating SREBP pathway (Ding et al., 2016). Here we show that curcuminoids similarly downregulate the lipogenesis which could affect DENV infection.

Previous studies reported that curcumin alters the actin cytoskeleton and causes disorganization of actin microfilaments concomitant with apoptosis of treated cells (Padilla et al., 2014). DENV have been shown to have close interaction with cytoskeleton elements in the replication cycle (Foo and Chee, 2015; Jitoboam et al., 2016; Kanlaya et al., 2009). Actin filament has been shown to contribute in the DENV2 entry via interaction with DENV E protein (Kanlaya et al., 2009; Yang et al., 2013). A reduction in viral replication of DENV and WNV was also observed by blocking the actin function (Ang et al., 2010; Colpitts et al., 2011). Native curcumin has been shown to inhibit actin polymerization with an IC50 of 16 μM (Dhar et al., 2015) in DENV2-infected cells. To test if the curcuminoids caused actin disorganization, phalloidin staining method was used to image F-actin (Fig. 5) and the results indicate that the curcuminoids caused actin disorganization similar to
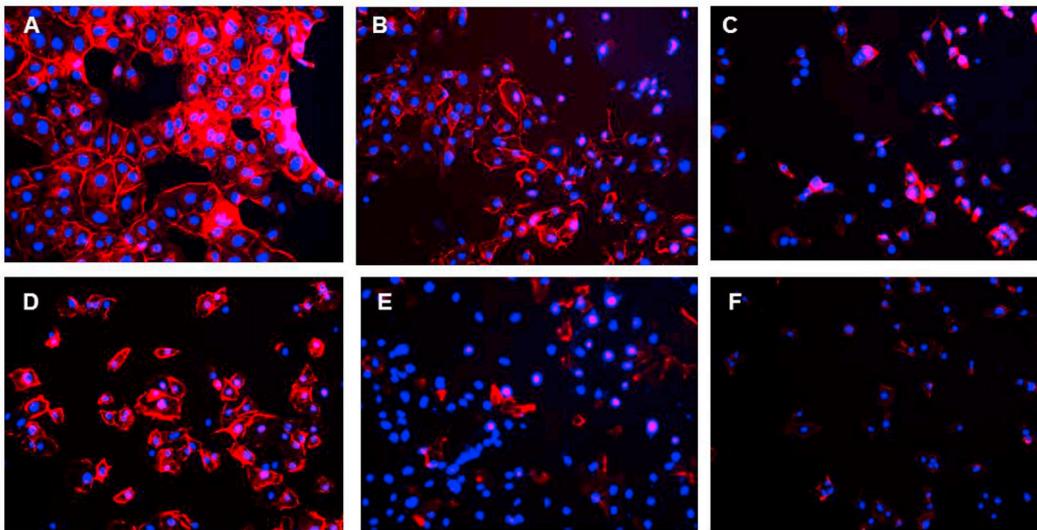

Fig. 5. Actin staining with Alexa Fluor 488 Phalloidin. Actin staining using Alexa Fluor 488 Phalloidin and DAPI staining were performed as described under Materials and Methods and visualized under fluorescent microscope. (A): Control DMSO treated cells; (B–F): 5 μM Curcuminoid (CC1- CC5)-treated cells.

the parent curcumin in DENV2 infected cells. No significant actin disorganization was observed in mock infected and curcuminoid treated cells (Fig. S9).

Thus, we conclude that curcuminoids exhibit their anti-viral activities through a variety of ways on the host cells, but with modest effect in DENV protease. Modulation of host actin and lipids may be involved in regulating the viral binding and entry. Recent studies on DENV mouse models indicated that curcumin treatment reduced the DENV viremia effectively (Ichsyani et al., 2017) indicating that these analogues might be promising in antiviral therapy against DENV.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.12.002.

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