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U18666A, an intra-cellular cholesterol transport inhibitor, inhibits dengue virus entry and replication

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ABSTRACT

The level of cholesterol in host cells has been shown to affect viral infection. However, it is still not understood why this level of regulation is important for successful infection. We have shown in this study that dengue virus infection was affected when the cholesterol intake in infected cells was disrupted using a cholesterol transport inhibitor, U18666A. The antiviral effect was found to result from two events: retarded viral trafficking in the cholesterol-loaded late endosomes/lysosomes and suppressed *de novo* sterol biosynthesis in treated infected cells. We also observed an additive antiviral effect of U18666A with C75, a fatty acid synthase inhibitor, suggesting dengue virus relies on both the host cholesterol and fatty acid biosynthesis for successful replication.

1. Introduction

Dengue virus (DENV) belongs to a class of plus strand RNA viruses classified under the family of *Flaviviridae* (Chambers and Monath, 2003). Dengue fever is classified as an emerging disease, with initially less than 10 countries reporting to have DHF (prior to 1970), to more than hundred countries displaying cases of DHF (Gubler, 2002; Gibbons and Vaughn, 2002). Due to the potentially fatal form of this mosquito-borne disease known as Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), there is a need to design an anti-dengue compound that acts across all four serotypes of the virus (Halstead, 1979; Goncalvez et al., 2007).

Compounds have been designed to target viral proteins such as the viral envelope (Costin et al., 2010; Schmidt et al., 2010; Poh et al., 2009; Wang et al., 2009) and viral polymerase (during the viral replication) (Latour et al., 2010; Niyomrattanakit et al., 2010; Yin et al., 2009). Alternative targets being investigated were host targets which have an advantage of having broad serotype reactivity against DENV. Compounds targeting host also have less chance to induce viral resistance. Those targets include glucosidase (Chang et al., 2009; Liang et al., 2006), furin and signal peptidase and host factors leading to the pathogenesis of DHF and DSS (Pastorino et al., 2010; Subramanya et al., 2010; Chen et al., 2008).

Cholesterol is essential to various cellular structures and processes (Cannon et al., 2006). In cells, the level of cholesterol is constantly regulated at three levels: intake of extracellular cholesterol in the form of low-density lipoprotein (LDL) via endocytosis, *de novo* biosynthesis of cholesterol (Goldstein and Brown, 1984) and efflux of cholesterol by ABCA1, a transporter found associated with the cell periphery (Schmitt and Tampé, 2002). Viruses have been shown to affect cholesterol levels during infection (Syed et al., 2010) and when cholesterol is reduced in host cells, viral production is affected (Desplanques et al., 2010; Medigeshi et al., 2008). Enzymes involved in the intermediate steps of biosynthesis such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and mevalonate diphospho decarboxylase (MVD) were shown to be important in viral replication (Mackenzie et al., 2007; Sidorkiewicz et al., 2009; Rothwell et al., 2009). Statins, a widely known class of inhibitors that have been used to lower cholesterol levels in the treatment of cardiovascular diseases by inhibiting HMG-CoA reductase, was also found be to effective against Human Immunodeficiency Virus (HIV), influenza virus and Hepatitis C Virus (HCV) (Oh and Hegele, 2007; Kruger et al., 2006; Bader et al., 2008). In addition, the cholesterol pathway was also recently shown to be important for West Nile virus and DENV infection (Mackenzie et al., 2007; Lee et al., 2008; Rothwell et al., 2009).

U18666A is an amphipathic steroid 3-β-[2-(diethylamine)ethoxy]androst-5-en-17-one. It is a widely used chemical to block the intracellular trafficking of cholesterol and mimic Niemann-Pick type C disease, a hereditary lysosomal storage disease. U18666A

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blocks the exit of free cholesterol from the late endosomal compartment. The amphipathic property of the compound is believed to be the mechanism of action for causing accumulation of cholesterol in late endosomes and lysosomes. U18666A also inhibits cholesterol biosynthesis by inhibiting oxidosqualene cyclase and desmosterol reductase (see review by Cenedella (2009)).

In this study, we used U18666A (Liscum and Faust, 1989) as a tool to study the role of cholesterol during DENV infection. We found that viral particles were trapped in the Lamp-1 positive late endosome/lysosome compartment when cells were treated with U18666A. In addition, viral replication was also affected by U18666A treatment. Further more, we also found an additive anti-viral effect when C75, a fatty acid synthase inhibitor, was used in combination with U81666A, suggesting the role of cholesterol and fatty acid in DENV.

2. Materials and methods

2.1. Virus and cells

Dengue serotype 2 virus strain, New Guinea C (GeneBank Accession No. M29095) was used in this project. This virus were cultured and harvested from *Aedes albopictus* mosquito cell line C6/36. All cell lines were cultured at 37 °C in the presence of 5% CO_2, except for C6/36, which was cultured at 28 °C with 5% CO_2. The A549, BHK21 and C6/36 cell lines were maintained in complete F12 medium or RPMI-1640 (Gibco, Invitrogen, USA) supplemented with 10% Fetal Bovine Serum (FBS, HyClone Laboratories, Logna, Utah, USA) and 100 U/ml Penicillin and Streptomycin (Gibco, Invitrogen, USA). A549 and Huh7 Dengue replicon cell lines were developed in house (Ng et al., 2007) and are maintained in Hams' F12 or DMEM (Gibco, Invitrogen, USA) media respectively supplemented with 10% FBS, 100 U/ml antibiotics and 5 μg/ml puromycin.

2.2. Antibodies and reagents

Mouse anti-dengue envelope antibody 4G2 was produced from hybridomas obtained from the American Type Culture Collection (ATCC HB-112™, USA). Rabbit anti-dengue NS3 antibody was produced in house. Human anti-dengue NS1 was a kind gift from Dennis R. Burton, Scripps Research Institute. Rabbit anti-dengue NS4B antibody was purchased from GeneTex, Inc. (USA). Rabbit anti-EEA1 antibody was from Cell Signaling technology™ (USA). Rabbit anti-Lamp1 antibody was from Abcam (USA). Mouse caveolin antibody was purchased from Invitrogen (USA). Secondary anti-mouse antibody conjugated to Alexa Fluor dyes, were from Molecular Probes (Invitrogen, USA). All chemicals and reagents were from Sigma unless otherwise stated.

2.3. Plaque assay

BHK21 cells were seeded with a cell density of 2×10^5 per well in a 24-well plate (Nunc, Denmark) 1 day prior to infection. Undiluted and 10-fold serial dilutions of viral supernatant were prepared in RPMI-1640 medium containing 2% FBS and antibiotics, and were loaded (0.2 ml) on the cells. After incubating the cells for 1 h at 37 °C (with 5% CO_2), the virus inoculums were replaced with 0.5 ml of 0.8% methylcellulose Aquacide (Calbiochem, USA) containing 2% FBS in RPMI-1640. After 4 days of incubation at 37 °C with 5% CO_2, the cells were fixed with 3.7% formalin, and stained with 1% of crystal violet, followed by visual counting of plaques.

2.4. Dengue infection assay and time of addition experiment of U18666A

BHK21 cells were seeded with a cell density of 2×10^4 cells/well into a 96 well plate 1 day before infection. NGC virus was added at a multiplicity of infection (MOI) of 1 in the presence of 2% FBS and was harvested from the cell supernatant 2 days post infection. Viral titer was quantified by plaque assay. For time of addition experiment of U18666A, cells were pretreated for 16 h with the compound, before virus was added to the cells (16 h pretreatment) or cells were pretreated for 16 h and the compound remained throughout the 48 h of infection (throughout). Alternatively, compound was added only during the 1st hour of infection where most of the entry and fusion occurred (1 h entry), or after the 1st hour of infection (after entry) for the remaining of the infection.

2.5. Indirect immuno-fluorescence labeling

BHK21 cells were infected with NGC DENV with a multiplicity of infection (MOI) of 50 for various time points at 37 °C. The infected cells were fixed with 4% paraformaldehyde in PBS for 20 min, washed with PBS, permeabilized with 0.05% saponin, for 30 min, and labeled with 4G2 against the E-protein together with either anti-EEA1 or anti-Lamp1 antibodies overnight for co-labeling. This was followed by PBS washes before labeling with the secondary antibodies, conjugated with Alexa Fluor 488 and 594 dyes. For cholesterol staining, the cells were fixed with 4% PFA and then stained for 1 h in the dark with 70 μg/ml Filipin III. The labeled cells were observed through a laser-scanning microscope (Leica FW 4000). For detailed colocalization between 4G2 and EEA1 or Lamp1, confocal imagine system (Zeiss LSM510 META) was used.

2.6. Isolation of lipid rafts

Isolation of lipid rafts were performed following a published protocol with modifications (Lee et al., 2008). BHK21 cells were washed two times with ice cold PBS and scraped into Falcon tubes. The pelleted cells were homogenized using a Dounce homogenizer in low salt buffer (LSB) containing 10 mM Tris–HCl, pH 7.5, 25 mM KCl and 5 mM $MgCl_2$ with 1% Triton on ice. Nuclei and unbroken cells were removed by centrifugation at 1000*g* for 8 min. The post nuclei supernatant was then overlaid on a sucrose gradient composed of 10–55–75% and centrifuged for 16 h at 38,000 rpm using a SW41 rotor (Beckman) at 4 °C. The fractions were collected in 1 ml aliquots from the top to the bottom into a total of 10 fractions. Each fraction was cleaned of sucrose and concentrated using a 10-kDa filters (Millipore). The cleaned fractions were loaded on an SDS–PAGE and immuno-blotting with NS3, NS4B and caveolin antibodies.

2.7. Lipid analysis

Control and treated cells were washed with PBS and harvested by scraping. Lipid extracted from cells in chloroform:methanol (1:2) and the organic fraction was dried to lipid film. The dried lipid fraction was re-constituted in chloroform:methanol (1:1) just before mass spectrometry analysis. Sterols were analyzed using an Agilent HPLC 1100 system (Agilent) coupled with an Applied Biosystems 3200 QTrap mass spectrometer (Applied Biosystems, Foster City, CA). In brief, separation of sterols was carried out using an Agilent Zorbax Eclipse XDB-C18 column (i.d. 4.6 × 150 mm) (HPLC conditions were as follows: (1) chloroform:methanol 1:1 (v/v) as the mobile phase at a flow rate of 0.5 ml/min; (2) column temperature: 30 °C; (3) injection volume: 10 ml. The LC-MS instrument was operated in the positive atmospheric pressure chemical ionization (APCI) mode with a vaporizer temperature of 500 °C and corona current of 3 μA. Multiple reaction monitoring (MRM) transitions for endogenous sterols as well as cholesterol-26,26,26,27,27,27-d6 (CDN Isotopes Inc., Quebec, Canada) and zymosterol-2,2,3,4,4-d5 (Avanti Polar Lipids, AL, USA) were used for sterol

quantification (Huang et. al., 2006) . Two independent experiments were carried out and the results were analyzed by student *t*-test (unpaired unequal variance).

2.8. Drug testing in DENV replicon cell lines

For A549 and Huh7 dengue replicon assay, cells were seeded with a density of 20,000 cells/well in a 96 well plate format and cultured with medium containing 2% FBS. To obtain a dose–response curve, a 10 points 2-fold dilution was performed starting with 100 μM for each of the inhibitors. The inhibitors were added to the cells for 48 h before detection of *Renila* luciferase using EnduRen™ (Promega). The Enduren substrate was added at 1:1000 dilution and incubated for 1 h before the luminescence was read using Clarity™ (Bio-Tek, USA) luminometer. Data analysis was performed using Prism v.4 (Graphpad software, San Diego, CA) software. The relative fluorescent unit (RFU) or luminescence reading was plotted against the log transformation of the concentration of the compound and a sigmoidal curve with variable slope was fitted to obtain the 50% effective concentration (EC_{50}) value. Cell viability for all replicons were measured by CellTiter® Glo assay kit (Promega Corporation, Madison, WI, USA) by quantifying the cellular ATP level. All dose–response curves, EC_{50} and CC_{50} values were determined using Prism software (GraphPadPrism4, San Diego, CA, USA) by nonlinear regression analysis.

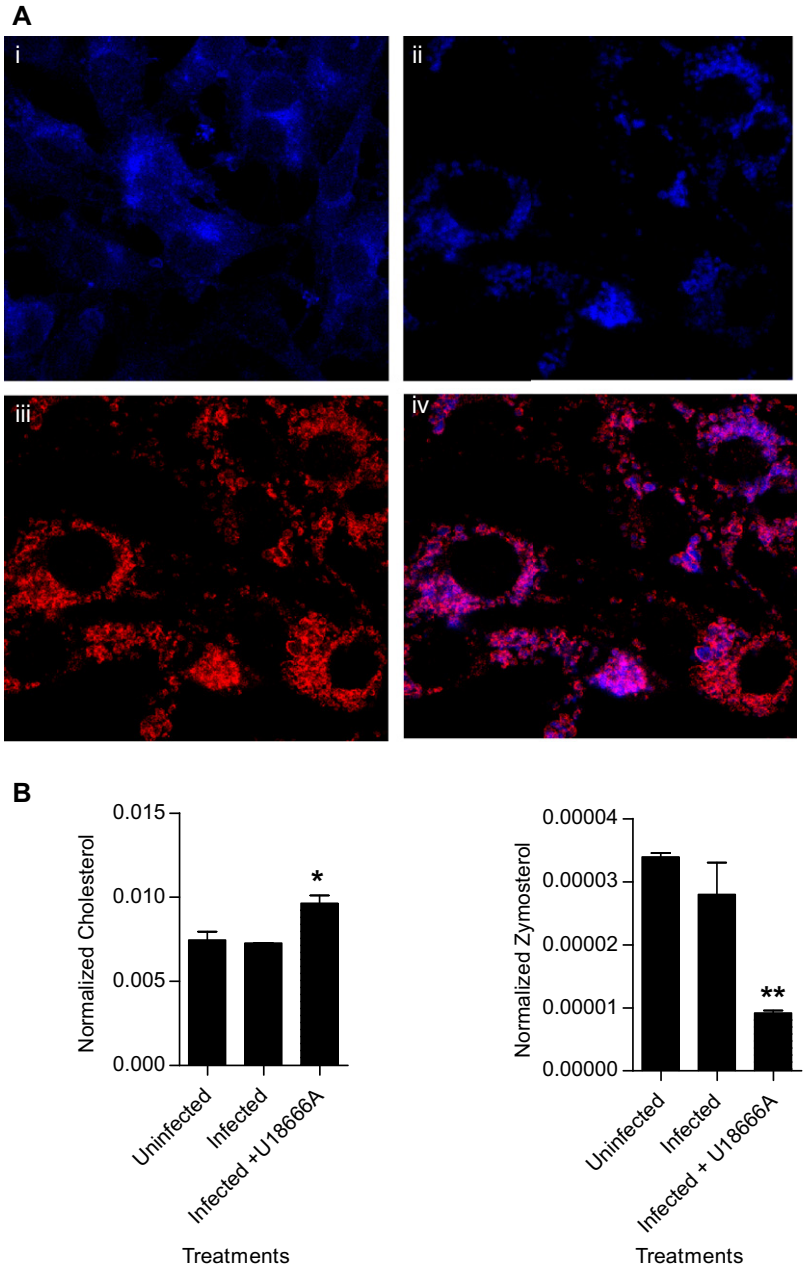

Fig. 1. U18666A arrest cholesterol transport and suppress sterol biosynthesis in host cells. (A) BHK21 cells were treated either with (i) 0.9% DMSO (control) or (ii) 6.15 μM of U18666A for 16 h before fixation and labeling by Filipin III. (iii) Lamp-1 labeling in U18666A treated cells. (iv) Overlay of Filipin and Lamp-1 in U18666A treated cells. (B) BHK21 cells were treated with 0.9% DMSO (control) or 6.15 μM of U18666A and infected with dengue viruses (MOI of 1) for 48 h before lipids were extracted and quantified using GC–MS. Both cholesterol (left panel) and zymosterol (right panel) levels were normalized with total cell protein and expressed as a ratio of cholesterol/protein. The bars present the average of biologically independent duplicate results and standard deviation was shown by error bar. In left panel, $*$ represents a p value of >0.05, and in left panel; $**$ represents a p value of <0.05 by student t-test with unpaired unequal variance.

3. Results

3.1. U18666A showed expected effect on cholesterol accumulation in late endosome/lysosome and inhibition of cholesterol biosynthesis

In order to examine whether dengue virus infection was affected by U18666A compound, we first checked the effect of the compound on the cholesterol distribution in cells. Filipin III, a fluorescent high affinity cholesterol binding chemical was used to stain intracellular cholesterol. Cholesterol is diffusely distributed in the uninfected BHK21 cells (Fig. 1A_i). When cells were treated with U18666A at 6.15 μM for 16 h, a condition well described to block the cholesterol trafficking, we observed an accumulation of cholesterol in punctated intracellular organelles by Filipin staining (Fig. 1A_ii), Filipin labeling in treated cells was co-localizing with the late endosome/lysosome marker Lamp-1 as expected, showing the accumulation of cholesterol in the late endosome and lysosome compartments (Fig. 1A_iii). We then checked the effect of U18666A on cholesterol biosynthesis in cells infected with DENV. By gas phase chromatography–mass spectrometry (GC–MS), we first checked the level of total cholesterol in uninfected versus DENV infected cells (MOI of 1 for 48 h) and found that there was no difference (Fig. 1B, left panel). We then measured the level of total cholesterol in U18666A treated DENV infected cells and found a slight but statistically not significant increase ($p > 0.05$) which is likely due to the accumulation of cholesterol in late endosomes/lysosomes (Fig. 1B, left panel). Since total cholesterol level in cells is constantly balanced by uptake, we further measured zymosterol level, an intermediate product in the sterol biosynthesis pathway, which cannot be compensated by uptake from external media. Zymosterol level was not greatly affected by infection. However, U18666A treatment resulted in a 67% reduction of zymosterol in infected cells (Fig. 1B, right panel $p < 0.05$). These data confirmed the two expected effects of U18666A on cholesterol accumulation in late endosome/lysosome and cholesterol biosynthesis.

3.2. The effect of U18666A on dengue virus infection

U18666A (6.15 μM) was then added to BHK21 cells before and during infection to investigate its effect. When cells were only pretreated for 16 h to allow accumulation of cholesterol in late endosome/lysosome, we observed more than one log of reduction of virus infection, without observing an effect on cell viability (Fig. 2, 16 h pretreatment). We did not see dramatic reduction of virus production when U18666A was given to cells for only 1 h during the virus entry (Fig. 2, 1 h entry), likely because the treatment is not long enough to allow the accumulation of cholesterol in endocytic pathway (data not shown). We also observed more than one log of reduction in viral titer when U18666A was added 1 h after virus infection and stays present for the rest of infection period of 48 h. This suggests that U18666A could also impact on later stage of virus infection, possibly the replication or assembly steps. If we combine both the pretreatment and leave the U18666A compound throughout the infection period, although we observed a 50% reduction of cell number due to the cytotoxicity, a far greater magnitude of viral reduction of more than three logs was observed.

3.3. The effect of U18666A on virus entry and trafficking in cells

We went on to further investigate the inhibitory effect of U18666A compound in the early events of virus entry and trafficking. We used fluorescence microscopy to examine the virus binding on host cell. Virus was added to and incubated with BHK21 cells on ice for 1 h before cells were fixed for immuno-staining. In cold conditions, viruses are able to bind to cell surface but are unable to undergo endocytosis (Helenius, 1980). Using the anti Envelop antibody to detect viral particles, we observed that viruses were still able to bind onto host cells efficiently even if host cells were pretreated with U18666A for 16 h (Fig. 3A).

We then checked where virus particles were localized in the cells after U18666A compound treatment. As shown in Fig. 3B in nontreated cells, 1 h after infection, virus particles were observed in the punctated structures in the cytoplasm (Fig. 3B, NT 1 h). In nontreated cells, Envelop antibody labeling could no longer be detected at 4 and 8 h post infection, presumably because of the fusion of the viral particles and the release of the capsid into the cytoplasm (Fig. 3B, NT 4 h, NT 8 h). Newly synthesized viral envelop proteins re-appeared at the time-point of 12 h post infection, localizing in the peri-nuclear regions of the host cells (Fig. 3B, NT 12 h). However, in cells where the cholesterol transport was arrested by U18666A, we could observe many viral particles at 4 and 8 h post infection (Fig. 3B, U18666A 4 h, U18666A 8 h) and much less staining of the newly synthesized envelop protein at 12 and 24 h post infection (Fig. 3B, U18666A 12 h, U18666A 24 h). Virus labeling at 4 and 8 h post infection in U18666A treated cells were double

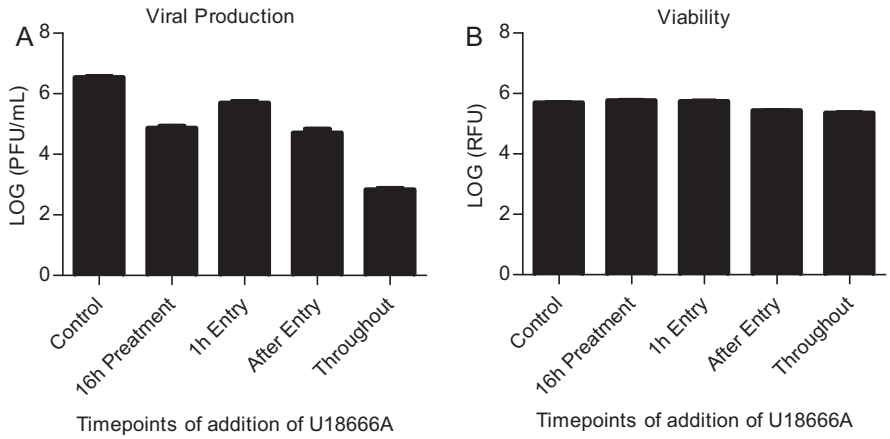

Fig. 2. Time point of addition of U18666A study in dengue infection. (A) BHK21 were treated with U18666A at 6.15 μM at various time-points during the course of infection by dengue viruses at MOI of 1. Viral supernatants were harvested 48 h post infection and viral titer was determined by plaque assay. Independent triplicate results and standard deviation was shown by error bar. Each compound treated group is compared to control group and significant difference was observed for each group based on $p < 0.05$ using student t-test with unpaired unequal variance. (B) The effect of these treatments on the cell viability was quantified using CellTiter® Glo assay kit for cell cytotoxicity. Duplicate experimental data were expressed as average with standard deviation expressed as error bar.

labeled for early endosome or late endosome/lysosome markers, EEA1 or Lamp-1, respectively. As shown in Fig. 4, the envelop labeling of the virus predominantly co-localized with Lamp-1 (Fig. 4ii), but not EEA1 (Fig. 4i). These results showed that viral trafficking was hampered in these cholesterol-loaded endosomes and accumulation of cholesterol in the late endosome/lysosome is inhibitory for dengue virus trafficking. Under the condition where endo-lysosomal compartment is loaded with extra amount of cholesterol, we speculate that it is possible that dengue virus could no longer carry out proper fusion or subsequent uncoating. As a consequence, a reduced level viral genome released into the cytoplasm caused much less virus production at 12 and 24 h post infection.

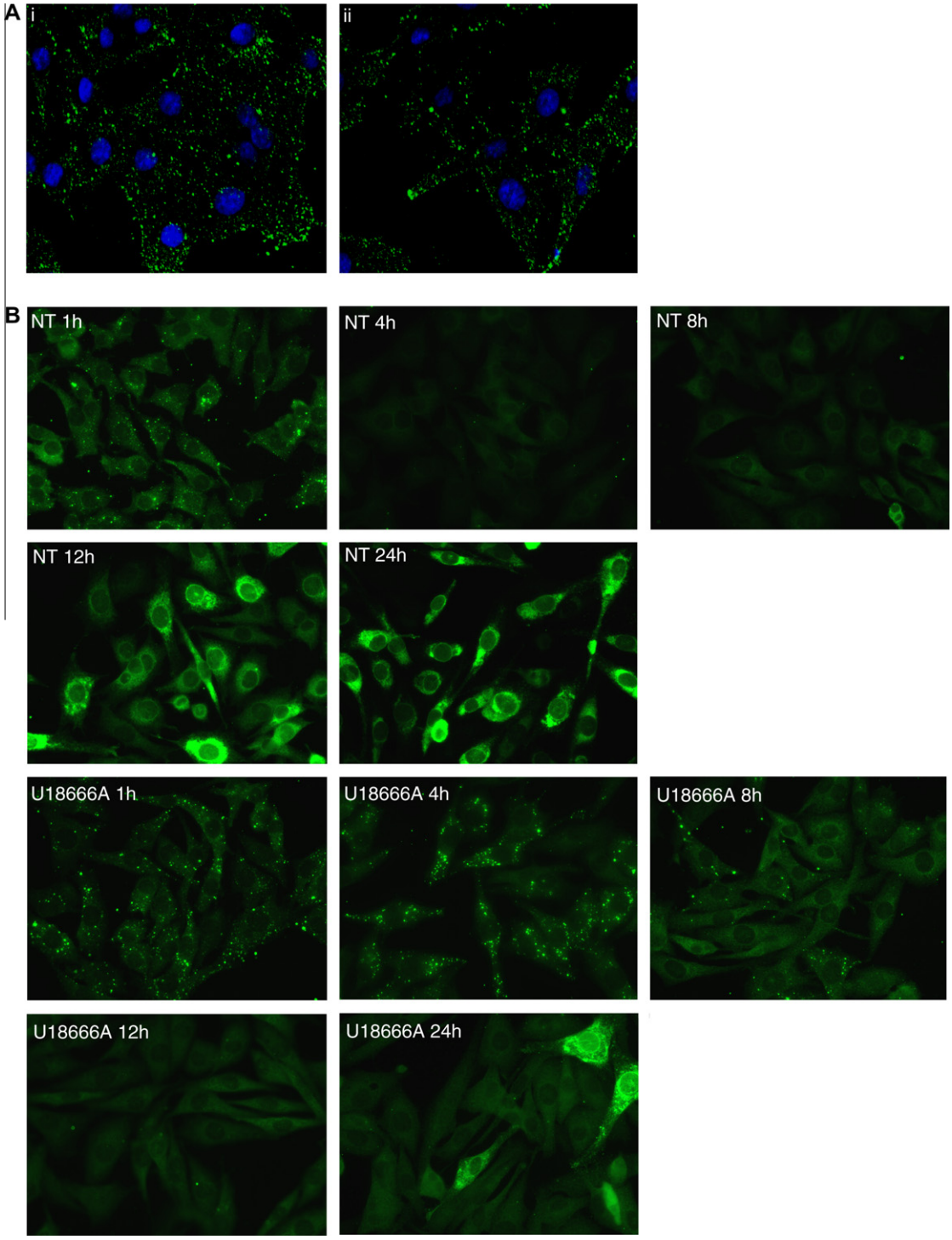

Fig. 3. Characterization of the effect of U18666A on the viral binding and trafficking. (A) For cell binding study: 50 MOI of dengue viruses were added to BHK21 treated either with (i) 0.9% DMSO (NT: nontreated) or (ii) 16 h pre-treatment with U18666A (6.15 μM). This was incubated on ice for 1 h to allow the viruses to bind onto the cell surface. The cells were then washed and fixed, followed by immuno-staining with anti-Env antibody (4G2) (green) to visualize the bound viruses. DAPI (blue) was used as a counter stain for cell nuclei. (B) For viral trafficking study: the control and U18666A-treated cells were infected with dengue viruses and at various time-points of infections (1, 4, 8, 12 and 24 h), cells were fixed and stained with anti-Env antibody (4G2) (green). Immunofluorescence photos were taken with a Leica FW4000 laser-scanning fluorescent microscope.

3.4. The effect of U18666A in replication stage of dengue virus

Since U18666A also inhibits post entry and trafficking steps of the viral cycle, we used DENV replicon to determine whether replication step of the viral cycle was inhibited. Two stable dengue replicon cell lines were used to avoid cell line specific effect. A range of concentrations of U18666A (0–100 μM) were added to the replicon cell lines for 48 h. *Renila* luciferase reporter activity derived from DENV replicon as well as the cytotoxicity (indicated by intracellular level of ATP) were measured. U18666A had an inhibitory effect on both dengue A549 replicon (Fig. 5A) and dengue Huh7 replicon (Fig. 5B), with an effective concentration of 50% reduction (EC_{50}) of 6.2 and 2.9 μM, respectively. The 50% reductions of cell viability (CC_{50}) were 67.6 and 34.4 μM, respectively.

DENV's non-structural proteins were shown by Lee et al. (2008) to be associated with lipid rafts isolated from infected cells. We postulated that U18666A might affect the lipid raft integrity and thereby reduce the association of nonstructural proteins with the lipid rafts, causing inhibition of replication. We set up a lipid raft isolation experiment using a gradient flotation method. In order to see an effect early in the replication, we treated infected cells for 24 h and carried out cold Triton X-100 extraction and raft isolation. We found that in both treated and nontreated cells, NS3 partially localized in fractions 3 and 4, in the same fractions as the lipid raft marker caveolin-1 (Fig. 6). NS4B also partially localized in fraction 3 and 4 but predominantly in fractions 5, 6 and 7. We concluded that U18666A treatment did not show any influence on the association of NS3 and NS4B with lipid raft at 24 h time point, suggesting that the replication complex formation is not affected by U18666A. We have also carried out an ultra-structural study using electron microscopy of the U18666A treated infected cells and could still find the presence of "vesicular packets" induced by replication complexes at 24 h post infection (data not shown).

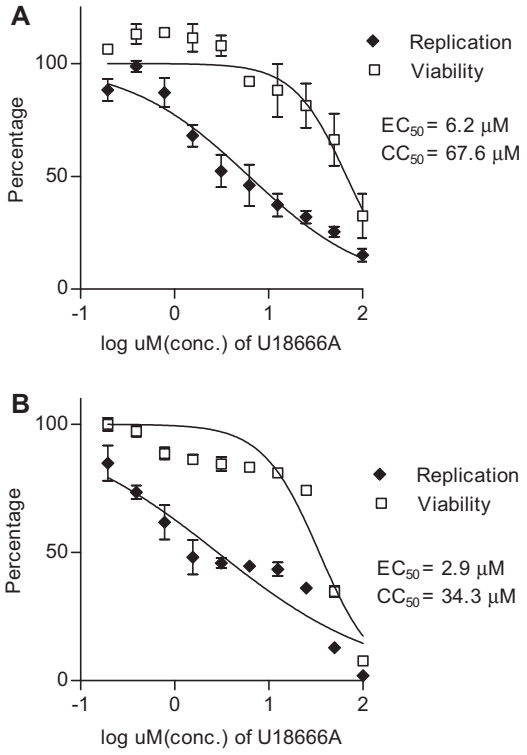

Fig. 5. Inhibition of viral replication. Stably transfected DENV replicons in (A) A549 and (B) Huh7 cell lines were used. Cells were treated with a concentration range of U18666A for 48 h. The replication and viability were measured and expressed as an average percentage of duplicate experimental data with standard deviation expressed as error bar. EC_{50} and CC_{50} are calculated.

3.5. Additive antiviral effect of C75, a fatty acid synthase inhibitor, with U18666A

Recent publications reported the involvement of fatty acid in dengue virus particle formation and replication (Samsa et al., 2009; Heaton et al., 2010). Both studies used C75, a fatty acid synthase inhibitor to reduce DENV replication and viral particle formation. We wanted to see whether fatty acid and cholesterol pathways are both involved in the same step of replication by doing drug combination studies. We first confirmed the inhibition of C75 in

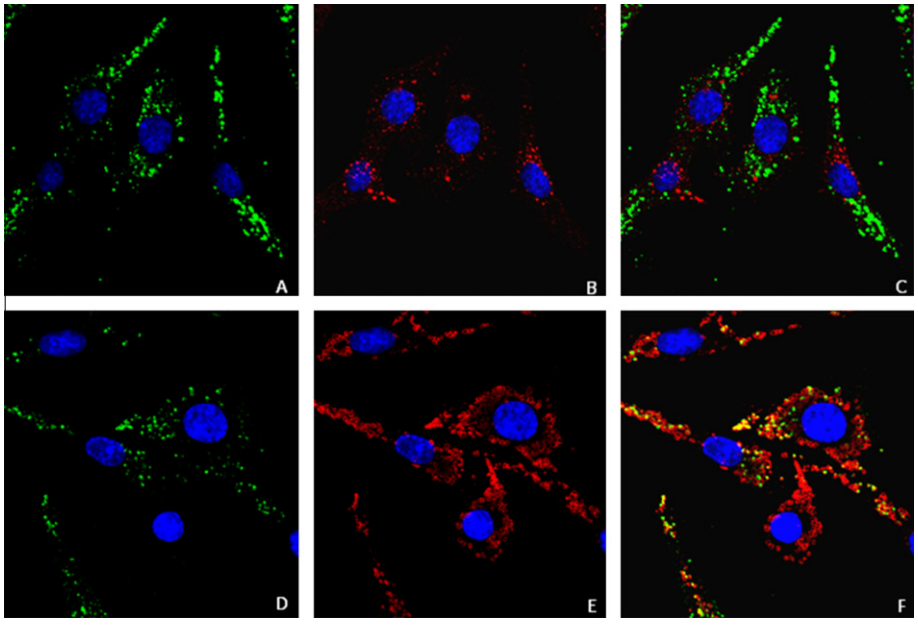

Fig. 4. Association of trapped viruses in Lamp-1 labeled compartments. BHK21 cells were infected and treated with U18666A (6.15 μM) for 4 h and double immuno-stained with (A) anti-Env antibody (4G2) and (B) EEA1 antibody or with (D) anti-Env antibody (4G2) and (E) anti-Lamp-1 antibody. (C) and (F) are overlay images and DAPI (blue) was used as a counter stain for cell nuclei. Immunofluorescence photos were taken by confocal imaging system Zeiss LSM 510 META.

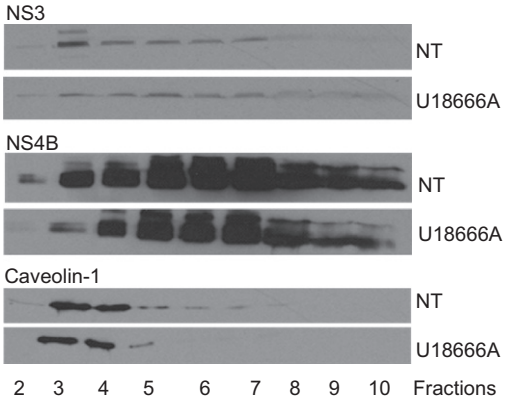

Fig. 6. Association of lipid rafts. BHK21 cells were treated simultaneously with either with 0.9% DMSO (control) or 6.15 μM U18666A and infected with DENV (MOI of 10) for 24 h before harvested for lipid raft extraction in 1% Triton on ice. The detergent resistant fractions were separated from soluble fractions by sucrose density gradient. Nonstructural proteins, NS3 and NS4B, were immuno-blotted for and caveolin-1 was used as a lipid raft maker as shown to be enriched in fractions 3 and 4.

Huh7 DENV replicon (Fig. 7A). It showed an EC_{50} of 5.7 μM and CC_{50} of more than 100 μM. We then chose the concentrations of U18666A and C75 similar to their EC_{50}s and treated the replicon either independently or together. We observed a 46.4% reduction with 1.23 μM of U18666A and 59.7% reduction with 8.3 μM of C75. The overall effect of the combined treatment gave 80.4% reduction. This result showed an overall additive inhibition effect. On top of the single dose combination experiment, we carried out a wider range of different dose combination using MacSynergy II matrix analysis and further confirmed that the two drugs showed an additive effect and not a synergistic effect (data not shown).

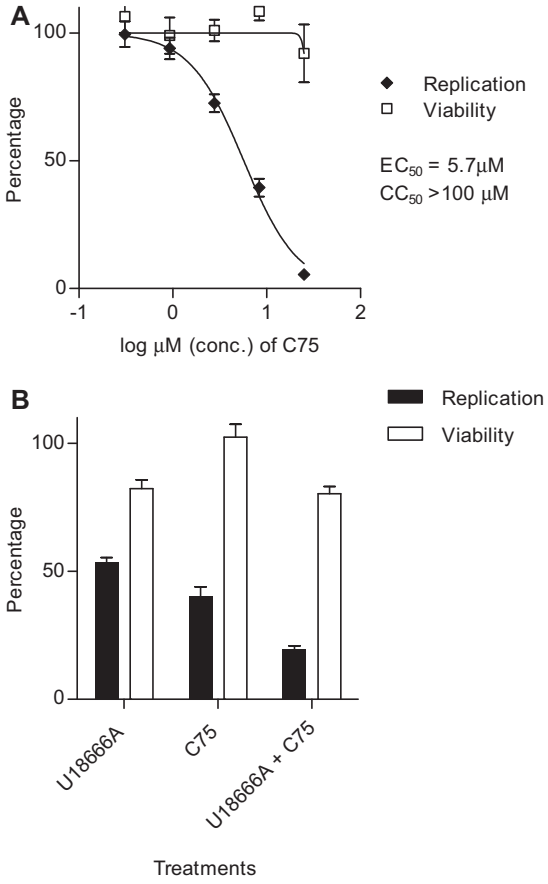

Fig. 7. Additive anti-viral action of C75, a fatty acid synthase inhibitor, with U18666A. (A) Stably transfected DENV replicons in Huh7 cell lines were used. These cells were treated with a concentration range of C75 for 48 h. The replication and viability were measured and expressed as average percentage with standard deviation expressed as error bar. EC_{50} and CC_{50} were also calculated. (B) The stably transfected dengue replicon Huh7 cell line was treated independently or in combination with U18666A (1.2 μM) and C75 (8.3 μM) and determined for inhibition on replicon activity and viability. The replication and viability were measured and expressed as average percentage of eight experimental data with standard deviation expressed as error bar.

4. Discussion

Previous studies on the role of cholesterol in flaviviruses predominantly used statins to inhibit sterol synthesis in host cells and beta methyl cyclodextrin (MBCD) to extract cholesterol from cell plasma membrane. We utilized U18666A in this study because it has been shown to act on host cell cholesterol balance via two mechanisms: (i) by blocking the transport of endocytosed cholesterol in the form of LDL (low-density lipoprotein), resulting in an accumulation of cholesterol in the late endosome/lysosome and (ii) by suppressing the intermediate steps of sterol *de novo* synthesis via oxidosqualene cyclase (Sexton et al., 1983). We asked in this study whether disrupting these two processes in cholesterol homeostasis would have an impact on DENV infection. Plaque assay data indicated that the early event of infection was severely affected by U18666A. The virus binding experiment indicated that perturbation in cholesterol trafficking did not affect the binding onto host cell, which is consistent with earlier studies by others (Reyes-Del et al., 2005). They showed that virus was still able to bind to cells from which plasma membrane cholesterol had been extracted by MBCD. However, we observed the importance of cholesterol in the post-attachment event of infection, which was evidenced in the viral trafficking data. In U18666A treated cells, endocytosed viruses were trapped in the late endosome/lysosome (4 and 8 h post infection) resulted in severe reduction of newly synthesized viral proteins at the later time-points of 12 and 24 h post infection. This could possibly be due to the high level of accumulated cholesterol in the late endosomes/lysosomes caused by U186666A, preventing efficient viral fusion or the subsequent uncoating. However, we cannot exclude the possibility that cholesterol accumulation in endocytic pathway caused a more general effect on late endosome/lysosome function which indirectly affected virus trafficking. But our observation that most of the viral particles were trapped in the late endosome/lysosome compartment also led us to conclude that late stage compartment of the endocytic pathway is more likely to be the organelles for dengue virus to go through and the likely compartment for fusion and the release of the capsid into the cytoplasm. This observation showed that dengue virus does not simply undergo fusion as soon as they enter the cell in the early endosome. They traffic to the late endosome or lysosome where more acidic pH and different lipid composition are required for an efficient fusion to occur.

Furthermore, in this study, we observed that U18666A suppressed level of zymosterol, an intermediate product of cholesterol biosynthesis. Earlier work done on the characterization of cholesterol levels in U18666A treated cells showed a 40% reduction in cholesterol in the ER (endoplasmic reticulum) compartment (Lange et al., 1999). We went on to investigate how U18666A could affect the replication. We postulated that this reduced cholesterol level in ER would affect the replication complex formation or association with the lipid raft. However, by both lipid raft isolation or by ultrastructural study by EM, we found that NS3 and NS4B were still associated with lipid rafts isolated from infected cells treated with U18666A at 24 h post infection.

This prompted us to ask whether other lipids played a role in the replication event. Fatty acids have been recently shown to be important to dengue replication (Heaton et al., 2010). We

confirmed that a fatty acid synthase inhibitor, C75, inhibited dengue replicon. We also found that C75 also gave an additive suppression on dengue replication when used in combination with U18666A. This suggests that both cholesterol and fatty acid affects the replication and that both lipids are likely required for the generation of membrane structure required for replication complex and "vesicular packet" integrity.

In summary, we observed that host cholesterol homeostasis was affected by U18666A and this resulted in two mechanisms of anti-viral action on dengue infection. The role of cholesterol in viral trafficking was evidenced in our observation that viruses were delayed to exit the lamp-1 labeled late endosomes/lysosomes, resulting in impaired release of viral genome into the cytoplasm. Furthermore, inhibition of the host cholesterol biosynthesis as well as fatty acid synthesis resulted in reduced viral replication.

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