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A small molecule fusion inhibitor of dengue virus

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ABSTRACT

The dengue virus envelope protein plays an essential role in viral entry by mediating fusion between the viral and host membranes. The crystal structure of the envelope protein shows a pocket (located at a "hinge" between Domains I and II) that can be occupied by ligand *n*-octyl-β-D-glucoside (βOG). Compounds blocking the βOG pocket are thought to interfere with conformational changes in the envelope protein that are essential for fusion. Two fusion assays were developed to examine the anti-fusion activities of compounds. The first assay measures the cellular internalization of propidium iodide upon membrane fusion. The second assay measures the protease activity of trypsin upon fusion between dengue virions and trypsin-containing liposomes. We performed an in silico virtual screening for small molecules that can potentially bind to the βOG pocket and tested these candidate molecules in the two fusion assays. We identified one compound that inhibits dengue fusion in both assays with an IC_{50} of 6.8 μM and reduces viral titers with an EC_{50} of 9.8 μM. Time-of-addition experiments showed that the compound was only active when present during viral infection but not when added 1 h later, in agreement with a mechanism of action through fusion inhibition.

1. Introduction

Dengue virus (DENV) is an enveloped virus belonging to *Flaviviridae* family which includes members such as yellow fever virus, West Nile virus, and tick-borne encephalitis virus (Chambers et al., 1990). It is transmitted to humans via the bite of an infected mosquito (Vasilakis and Weaver, 2008; Wang et al., 2000). There is an increasing number of reported dengue cases and an emerging trend of dengue fever affecting the affluent as much as the poor in tropical countries (Mathers et al., 2007; Normile, 2007). The four serotypes of DENV (serotypes 1–4) can cause a range of symptoms from a mild fever to severe hemorrhagic manifestations (Chambers and Monath, 2003; Kuno et al., 1998). There has been an on-going effort to develop a vaccine for dengue fever since the 1940s (Hatch et al., 2008; Westaway and Blok, 1997). Vaccine development for DENV is challenging due to the antibody-dependent enhancement concern (Halstead, 1970) and the need to create a tetra-valent vaccine for protection against the four serotypes (Halstead, 1988; Hatch et al., 2008; Whitehead et al., 2007). An alternative approach to vaccine development is to develop antiviral compounds to treat dengue infection. The multiple steps in DENV infection cycle such as viral entry, viral membrane fusion, replication, virus particle assembly and maturation are all potential antiviral targets.

DENV enters the cell by receptor-mediated endocytosis (Acosta et al., 2008; Hernandez et al., 1996) followed by viral envelope protein (E-protein)-mediated membrane fusion. The low pH-dependent fusion event between the viral membrane and the host endosome membrane releases the viral genetic material into the cytoplasm (Mukhopadhyay et al., 2005). The DENV E-protein is divided into three main domains, with the central domain (Domain I) flanked by the immunoglobulin-like C-terminal domain (Domain III) and the dimerization N-terminal domain (Domain II) which carries the fusion peptide (Modis et al., 2004). The DENV E-protein belongs to the class II category of viral fusion proteins which consist mainly of β-sheets, compared to class I fusion proteins which contain a more α-helical content (Kielian, 2006). Other distinct characteristics of class II fusion proteins are an internal fusion peptide and the requirement for low pH initiation of viral fusion (Kielian and Rey, 2006).

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Upon lowering of the pH, the E-protein undergoes major conformational changes, with the particular region between Domains II and I, known as the "hinge" springing upwards to bring the fusion peptide closer to the host membrane for fusion to occur (Bressanelli et al., 2004). This hinge region is believed to be important during the early event of fusion. In one crystallization study, a small detergent molecule, *n*-octyl-β-D-glucoside (βOG), was reported to occupy a hydrophobic pocket near the hinge region; mutations within this binding pocket resulted in alteration of pH threshold for fusion (Modis et al., 2003). This has been interpreted as an avenue to look for occupants in this potentially druggable pocket to inhibit dengue viral fusion.

In this report, we describe a screening program aimed at finding small molecules that can inhibit DENV E-protein-mediated membrane fusion. By combining in silico virtual screening with a medium-throughput 96-well based functional assay and a low-throughput secondary fusion assay, we have identified a novel compound that can inhibit DENV membrane fusion and has antiviral activity.

2. Materials and methods

2.1. Virus and cells

Two types of dengue serotype 2 virus strains, TSV01 and New Guinea C (NGC), were used in this project. These viruses were cultured and harvested from *Aedes albopictus* mosquito C6/36 cells grown in RPMI-1640 media (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and antibiotics, at 28 °C. Infection assays were performed using BHK21 cells grown in RPMI with 10% FBS and antibiotics, at 37 °C in 5% CO_2.

2.2. Antibodies and reagents

Mouse anti-dengue capsid antibody was produced from hybridomas that were a gift from Philippe Gallay, the Scripps Research Institute, and mouse anti-dengue Envelope antibody 4G2 was produced from hybridomas obtained from the American Type Culture Collection (ATCC). Secondary anti-mouse antibody conjugated to horse radish peroxidase was purchased from Sigma (Sigma–Aldrich). All chemicals and reagents were purchased from Sigma unless otherwise stated.

2.3. Virtual screening approach to construct focused library

The βOG ligand was used to define the ligand-binding site, and potential interaction grids around this site were generated. A docking set of the Novartis in-house compound archive was generated. From over 1 million compounds, 85,000 were removed as structural duplicates, possessing molecular weight >660, >2 undefined chiral centers or >12 freely rotatable bonds. The remaining compounds were expanded for unknown chirality and alternative charge states using Pipeline Pilot (Accelrys Software Inc., San Diego, CA), generating 2.4 million structures to dock. These were docked using Glide SP (standard precision, Schrödinger LLC) on a Linux cluster. No constraints, e.g. a compulsory specific hydrogen-bond formation, were applied. Pipeline Pilot was used to sort the compounds by Glide_gscore and the top 3000 scoring compounds were kept. These were re-scored using consensus scoring (CScore) as implemented in Sybyl (Tripos, Inc., St. Louis, MI). The scoring results were normalized and combined. Low-scoring isomers (e.g. same molecules, different charge state) were removed and the remainder clustered with Pipeline Pilot using Functional Class FingerPrint, 6-bond radius fingerprints and a similarity distance threshold of 0.4 to remove some of the very similar compounds. The remaining 1367 top-scoring unique poses were then visually inspected until acceptable compounds were selected. The same procedure was carried out for docking the Novartis in-house natural product collection except that the chemical property limits for acceptable compounds was relaxed considerably, e.g. up to 15 rotatable bonds were accepted. In total, a library of 365 high-scoring, chemically reasonable small molecules was generated for the primary fusion assay.

2.4. Indirect immunofluorescence labeling

C6/36 cells were infected with TSV01 DENV with a multiplicity of infection (MOI) of 1 for 2 days at 28 °C. The infected cells were fixed with 2% paraformaldehyde in PBS for 15 min, washed with PBS, incubated with 100 mM glycine in PBS for 10 min, incubated with 1% FBS in PBS for 1 h, and labeled with monoclonal antibody 4G2 against the E-protein overnight. This was followed by PBS washings before labeling with the secondary antibody, FITC-labeled goat anti-mouse IgG. The cells were counterstained with DAPI to visualize the nuclei. The labeled cells were observed through a laser-scanning microscope (Leica FW 4000) with a 63× oil-immersion objective.

2.5. Primary screening: cell-based fusion assay in a 96-well format

The primary assay was adapted from a method previously described by Randolph and Stollar (1990). C6/36 cells with a cell density of 1.5×10^5 cells/well were seeded together with TSV01 DENV at an MOI of 0.1 in 96-well plates (Nunc, Denmark). Three days post-infection, the individual compounds, obtained from the Novartis compound collection, were added to the infected cells and incubated for 1 h at 28 °C. The medium was acidified to induce fusion by adding 5 μL of 0.5 M 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.0), and incubated for 1 h at 37 °C. The fused cells were then stained with propidium iodide (0.025 mg/mL) for 30 min at 28 °C before proceeding to fluorescence reading with a Tecan Safire II plate reader using an excitation wavelength of 537 nm and an emission wavelength of 617 nm, with a bottom fluorescence measurement, and three readings per well. The quality of the assay can be determined by the dimensionless Z' value, based on positive and negative control samples. The calculation, $Z' = 1 - (3 \times SD_{blank} + 3 \times SD_{positive})/(|Mean_{blank} - Mean_{positive}|)$, takes into account the signal dynamic range and the data variation associated with the signal measurement (Zhang et al., 1999). Only assays with a Z' value above 0.5, indicating a good dynamic range and a low variation, were accepted.

2.6. Purification of dengue viruses

The experimental procedures were performed as previously described (Kuhn et al., 2002). Briefly, the virus was centrifuged overnight (15 h) using a Beckman type 19 rotor at 30,000 × *g*. The virus pellets were then allowed to soak in HNE buffer (5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4) for 4 h on ice before thorough re-suspension by pipetting. Purification was carried out using a discontinuous 15–55% density gradient set up with OPTIPREP™ medium in a Beckman SW41 rotor at 4 °C for 2 h at 125,000 × *g*. The 20–25% section was harvested, aliquoted, and stored at −80 °C. The fractions harvested were subjected to plaque assay to assess for the amount of infectious particles present.

2.7. Virus-liposome fusion assay

The assay was modified from previously described experiment (White and Helenius, 1980). Liposome was prepared from a phospholipid (Avanti Polar Lipids, Alabaster, AL) mixture with a

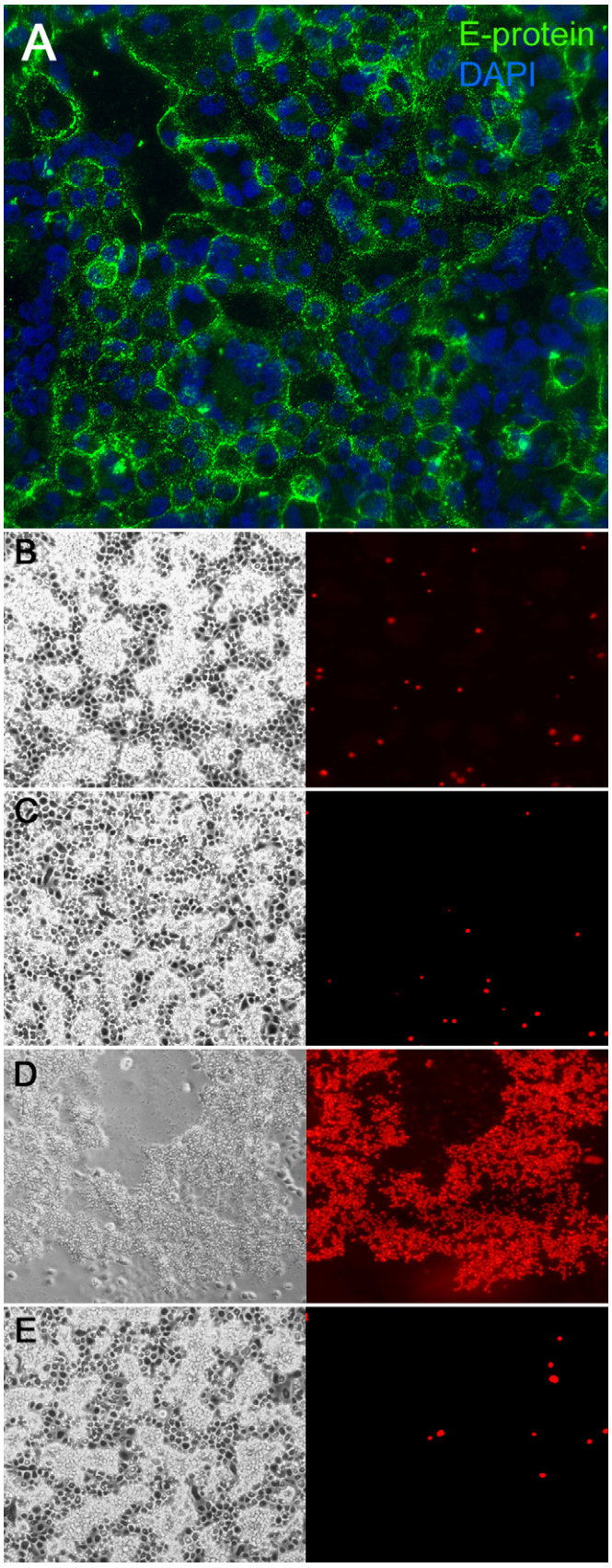

Fig. 1. Low pH induced fusion of dengue infected C6/36 cells mediated by viral E-protein on the cell surface. (A) C6/36 cells infected with dengue virus display viral E-protein on the cell surface shown by immunofluorescent labeling of fixed non-permeabilized cells (green). Nuclei were counterstained with DAPI (blue). (B) Uninfected C6/36 cells did not fuse under acidic conditions, as shown in phase contrast (left panels), and did not exhibit appreciable propidium iodide (PI) staining (right panels). (C) Infected cells 3 days post-infection kept at neutral pH also did not fuse and did not show PI staining. (D) However, infected cells showed widespread fusion under acidic conditions resulting in PI staining of the syncytiated cell mass. (E) Infected cells 3 days post-infection that were pre-incubated for 1 h with antibody 4G2 against the fusion loop of the E-protein, did not fuse and did not show either syncytium formation or PI staining after acidification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

1:1:1:1.5 molar ratio of phosphatidylcholine (PC; from egg yolk, Avanti), phosphatidylethanolamine (PE; prepared by transphosphatidylation from egg PC, Avanti), sphingomyelin (Sph; from bovine brain) and cholesterol (amounts used: 1 μmol PC, 1 μmol PE, 1 μmol Sph, 1.5 μmol Chol). The lipid mixture was then dried under a stream of nitrogen gas and reconstituted in HNE buffer containing 10 mg/mL of trypsin. The lipid mixture was extruded through two pieces of polycarbonate membrane (0.2 μM hole size, Whatman, Clifton, NJ) with a Mini-Extruder (Avanti, Polar Lipids, Inc., Alabaster, AL). Liposomes were stored at 4 °C and used within 1 week. Fusion reactions were performed in 0.1 mL final volume containing 100,000 particles of purified TSV01 virus and 1 mM of trypsin-containing liposome. The samples were adjusted to the indicated pH values by adding a small pre-titrated volume of 0.1 M MES and 0.2 M acetic acid, and incubated for 2 min at 37 °C for fusion to occur. The reaction was then neutralized to pH 8.0 using a pre-titrated volume of 0.1 M NaOH, and incubated at 37 °C for an hour to allow trypsin digestion. The integrity of viral capsid was determined by SDS-PAGE of the reaction mixture followed by immuno-blotting using an anti-capsid antibody.

2.8. Plaque assay

BHK21 cells (ATCC) 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 dilutions of viral supernatant were prepared in RPMI-1640 medium containing 2% FBS and antibiotics, and were loaded (0.2 mL) onto each well of BHK21 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) 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.

2.9. Antiviral activity assay

BHK21 cells were seeded with a cell density of 2×10^4 cells/well into a 96-well plate 1 day before infection. The compound was added at different timepoints and incubated with the cells for 1 h before, during or after infection with DENV-2 strain NGC at an MOI of 1. Virus was harvested from the cell supernatant 3 days post-infection and analyzed by quantitative real-time RT-PCR.

2.10. Quantitative real-time RT-PCR

Total RNA was extracted from viral supernatant using the RNeasy kit from Qiagen. A reverse transcription was performed using the SuperScript™ III Reverse Transcriptase kit (Invitrogen) with random hexamer primers (1.5 μg) (Roche, Switzerland). The reaction was carried out at 25 °C for 10 min, 50 °C for 60 min and 75 °C for 15 min. For real-time PCR, a 20 μL reaction mix was set up using 4 μL of cDNA, 10 μL of iQ™ SYBR® Green Supermix (BioRad) and 10 nM of primers. The sequences of the primers used were: Forward 5′-ACA AGT CGA ACA ACC TGG TCC AT-3′ and Reverse 5′-GCC GCA CCA TTG GTC TTC TC-3′ (Laue et al., 1999). PCR reactions were

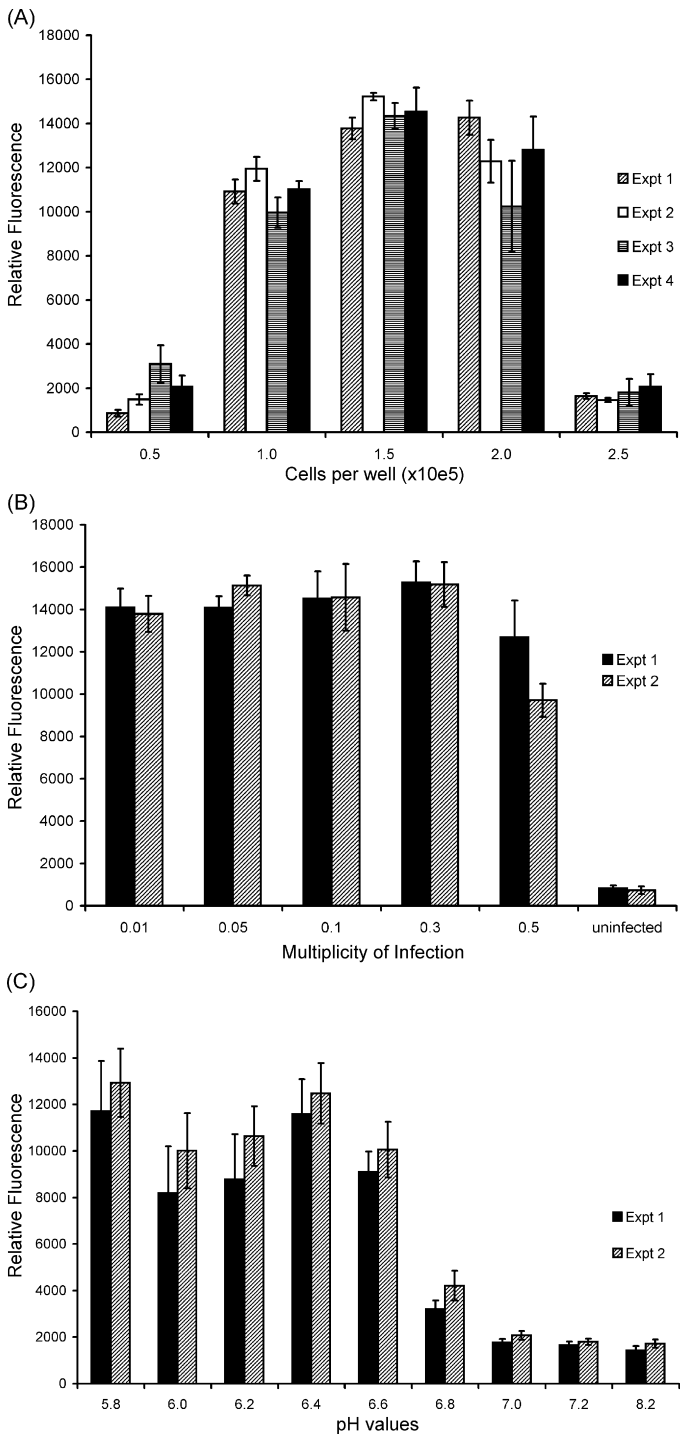

Fig. 2. Characterization and optimization of the primary cell–cell fusion assay. (A) The wells of a 96-well plate were seeded with different cell densities and subsequently infected with dengue virus using an MOI of 0.1, followed by the acidification, staining and fluorescent readout on day 3 post-infection. Results shown are from four independent experiments, using two batches of cell stocks (P10 and P17). (B) Wells were seeded with a density of 1.5×10^5 cells/well and subsequently infected with different MOI, followed by the acidification, staining and readout on day 3 post-infection. Results are from two independent experiments. (C) Wells were seeded with a density of 1.5×10^5 cells/well and infected with dengue virus at an MOI of 0.1. On day 3 post-infection cells were exposed to media of different pH before staining and readout. Results are from two independent experiments. Error bars represent standard deviations.

then cycled at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s in the iCycler Thermal Cycler from the iQ5 Real-Time PCR Detection System (BioRad). A standard curve was established using a dilution series of NGC viral RNA extracted from a known titer as determined by plaque assay.

2.11. Cytotoxicity determination

BHK21 cells were seeded with a density of 2×10^4 cells/well into a 96-well plate 1 day before incubating them with increasing concentration of compound for 3 days at 37 °C. The CellTiter® Glo assay kit (Promega Corporation, Madison, WI) was then used to determine the viability of the cells by quantifying the cellular ATP level via a luciferase-based reporter system.

2.12. Data analysis

All dose–response curves, IC_{50} and CC_{50} values were determined using Prism software (GraphPadPrism4, San Diego, CA).

3. Results

3.1. In silico virtual screening to build a focused library of potential dengue E-protein binding compounds

The DENV E-protein structure published by Modis et al. (2003) was selected for docking because of a βOG molecule found inside the ligand-binding pocket. Two molecules of E-protein form a head-to-tail dimer. Each E-protein molecule forms its own βOG-binding pocket without direct contributions from the neighboring molecule to the βOG pocket formation. Although both chains are fully defined, the B chain was selected as the docking model as it possesses overall lower temperature factors, implying that its coordinates were more accurately determined. All solvent molecules were removed. There are two glycosylation sites on each chain. Since the glycosylation sites are far away (>25 Å) from the βOG-binding site, they were removed for virtual screening. The Protein Preparation module of Maestro (Schrödinger LLC, Portland, OR) was used to prepare the protein for docking. The protein structure was energy minimized and hydrogen atoms added, and their positions were determined to maximize internal hydrogen-bond formation. A total of 2.4 million small molecules were analyzed using in silico-screening, as described in Section 2. The screening resulted in a focused compound library of 365 small molecules that could potentially bind into the βOG-binding pocket.

3.2. A medium-throughput cell-based fusion assay for primary screening a focused compound library

Randolph and Stollar (1990) reported that dengue virus-infected C6/36 cells undergo cell-cell fusion upon low pH exposure. Immunofluorescent labeling of non-permeabilized dengue virus-infected cells allows visualization E-protein on the cell surface while avoiding labeling of E-protein present in the cell interior. As presented in Fig. 1A, we can show E-protein abundantly expressed on the cell surface of infected C6/36 cells, including the interface between adjacent cellular membranes. Based on this system we developed a fluorescent cell-based fusion system, first mentioned in Rajamanonmani et al. (2009). Here we describe how we developed this system into a quantitative assay suitable for screening compound libraries in a 96-well plate format. Incubating at low pH of infected cells resulted in widespread syncytiated cell fusion (Fig. 1D). To quantify the formation of syncytiated cells we developed a propidium iodide (PI) staining method. This method is based on the observations that viral protein-induced membrane fusion can damage membrane integrity (Bonnafous and Stegmann, 2000;

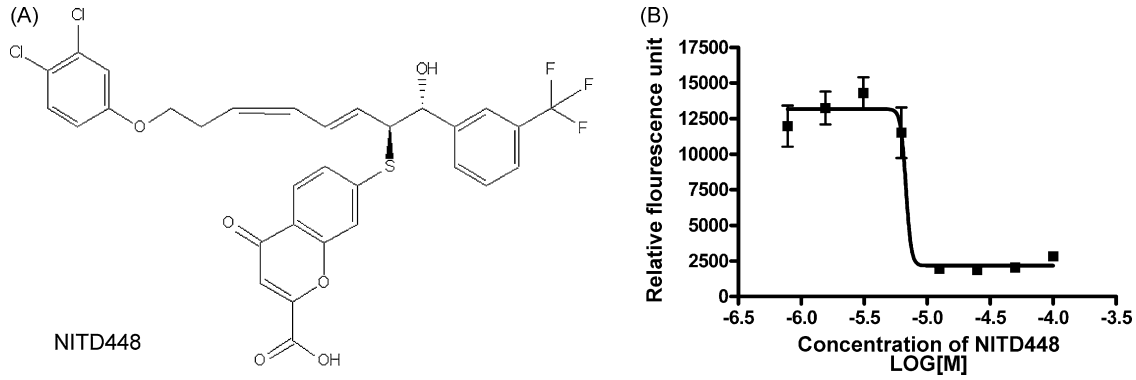

Fig. 3. Compound structure and inhibition of fusion in primary assay. (A) The molecular structure of fusion inhibitor NITD448. (B) Compound NITD448 inhibited fusion in the cell–cell fusion assay with an IC_{50} of 6.8 μM. Error bars represent standard deviations.

Frolov et al., 2003). PI dye cannot penetrate intact cellular membranes, resulting in very little staining in uninfected cells (Fig. 1B) or in infected cells maintained at neutral pH (Fig. 1C). However, infected cells exposed to low pH showed bright PI labeling of the syncytiated cell mass (Fig. 1D), allowing a fluorescent readout of cell–cell fusion. As a control, treatment with 4G2, a monoclonal antibody known to bind the fusion loop of the E-protein (Crill and Chang, 2004; Henchal et al., 1982) before exposure to low pH, inhibited cell fusion and PI staining (Fig. 1E). In contrast, treatment of the infected cells with 9F12, a monoclonal antibody against Domain III of the E-protein which is away from the fusion domain, did not interfere with fusion at low pH (data now shown).

Optimization experiments were performed to develop this cell-based fusion assay into a robust 96-well format. Cell seeding density was found to be an important factor in obtaining a reliable cell fusion signal, with an optimum between 1×10^4 and 2×10^4 cells/well (Fig. 2A). The assay signal was relatively insensitive to the MOI from 0.01 to 0.3, whereas a higher MOI of 0.5 reduced the assay signal (Fig. 2B). As expected, cell fusion was found to be pH-dependent, starting at an acidic pH value lower than 6.6 (Fig. 2C).

Using the above cell-based fusion assay, we tested the 365 small molecules identified from the in silico docking in triplicate at 25 μM. The assay showed a good Z' factor of 0.7. It was found that compound NITD448 (Fig. 3A) reproducibly inhibited the PI-mediated fluorescent staining in a dose–responsive manner, with a 50% inhibitory effect (IC_{50}) of 6.8 μM (Fig. 3B). These results suggested that NITD448 may be an inhibitor of dengue E-protein-mediated membrane fusion.

3.3. Compound NITD448 inhibits E-protein-mediated membrane fusion in a low-throughput liposome-based fusion assay

Based on liposome fusion assays using isotope-labeled virus (White and Helenius, 1980), we developed a non-radioactive content-mixing fusion assay using immunodetection of nucleocapsid protein. Purified dengue virus was mixed with liposomes containing the protease trypsin. Membrane fusion between these two particles allowed the trypsin to access the interior of the virions resulting in digestion of the capsid (C) protein. Western blot analysis showed intact C-protein when the mixture of virus and liposomes was kept at neutral pH; in contrast, degradation of C-protein was observed when the mixture was exposed to low pH, but only if trypsin was present in the liposomes (Fig. 4A). Addition of the 4G2 antibody against the E-protein was able to prevent degradation of C-protein at low pH in a dose-dependent way. The results indicate that the assay reliably monitors the pH-dependent E-protein membrane fusion.

Compound NITD448 was tested in this liposome-virus fusion assay and showed a clear dose-dependent inhibition of membrane fusion (Fig. 4B). Taken together, the results strongly indicate that compound NITD448 can inhibit E-protein-mediated membrane fusion.

3.4. Antiviral activity of compound NITD448

To determine the antiviral activity of compound NITD448, we added the compound at various concentrations to cells being infected with DENV-2 NGC strain, incubated for 3 days, and determined the concentration of virus produced in the supernatant. Compound NITD448 was found to inhibit dengue infection with an EC_{50} value of 9.8 μM and complete suppression of virus production at 25 μM (Fig. 5A). The cytotoxicity of compound CGP was determined at a CC_{50} of 49 μM, resulting in a selectivity index of 5 (Fig. 5B).

Next, we performed time-of-addition experiments to investigate the mode of viral inhibition. Compound NITD448 was added to cells 1 h before, during, or 1 h post-virus infection. As shown in Fig. 5C, the compound did not reduce virus production when present just before or after infection. Only when NITD448 was present during the 1 h virus infection of the cells was the viral production strongly decreased. The results again indicate that the compound inhibits membrane fusion during virus entry into the cells.

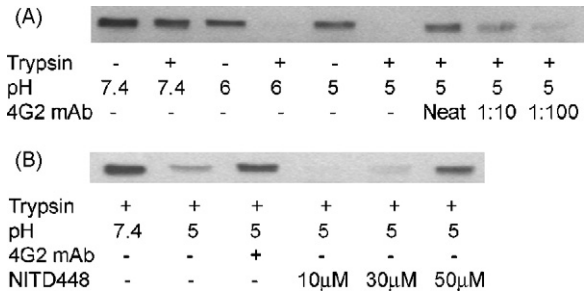

Fig. 4. Inhibition of fusion in secondary assay. (A and B) Mixtures of purified dengue virus and liposomes were exposed to various conditions and analyzed for the presence of intact viral capsid protein. (A) Capsid protein was not degraded if liposomes did not contain trypsin regardless of pH (lanes 1, 3 and 5), but if liposomes contained trypsin the viral capsid protein was degraded when the mixture was exposed to low pH causing fusion between liposomes and dengue virions (lanes 2, 4 and 6). This degradation could be prevented in a dose-dependent manner if the mixture was pre-incubated with antibody 4G2 against the fusion loop of the Envelope protein (lanes 7–9). (The label 'Neat' indicates undiluted antibody.) (B) Similarly, compound NITD448 could inhibit fusion and trypsin degradation of capsid in a dose-dependent manner.

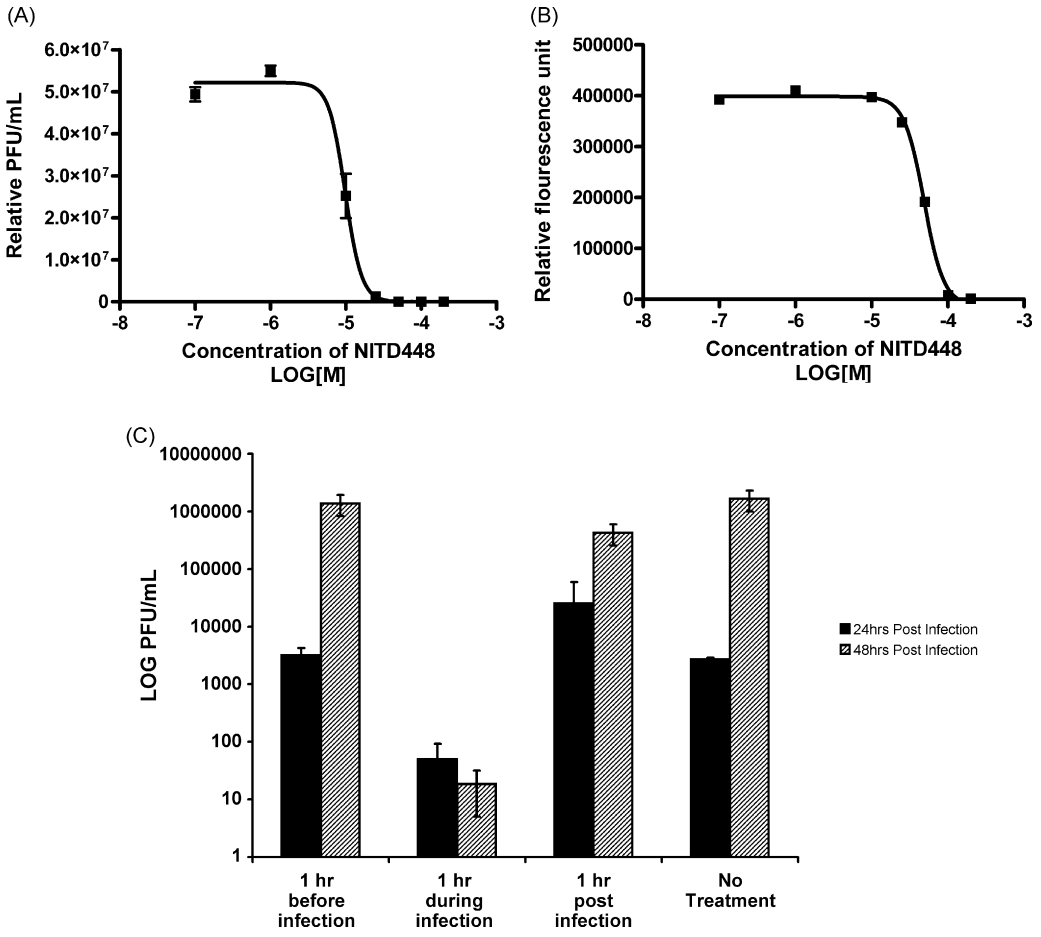

Fig. 5. Antiviral activity of compound NITD448. (A) Cells were treated with a concentration range of compound NITD448 during infection with dengue virus for 3 days. Supernatant was subsequently harvested and analyzed by quantitative RT-PCR expressed in equivalents of plaque forming units (PFU) per mL. The compound showed a dose-dependent inhibition of viral yield with an EC_{50} of 9.8 μM. (B) Cells were treated at a concentration range of compound NITD448 during infection with dengue virus for 3 days. Infected cells were then lysed and quantified using CellTiter® Glo assay kit for cell cytotoxicity. The compound showed a CC_{50} of 48.7 μM. (C) Reduction of virus yield could only be observed when NITD448 was present during the 1 h of infection, i.e. when virus and cells were incubated together for 1 h, but not when cells were incubated 1 h before or 1 h after infection with compound. Error bars represent standard deviations.

4. Discussion

In our efforts towards developing an antiviral treatment for dengue we established a screening program aimed at identifying dengue E-protein-mediated membrane fusion inhibitors. Previous studies only looked at antiviral activity of molecules that have the potential to bind to the E-protein as predicted from in silico docking (Li et al., 2008; Wang et al., 2009; Yennamalli et al., 2009). Our approach combines in silico studies with functional assays able to detect inhibition of membrane fusion. A two step screening approach was developed employing a medium-throughput primary assay and a subsequent low-throughput secondary assay. The primary assay was based on previously published (Randolph and Stollar, 1990) cell–cell membrane fusion of dengue virus-infected cells, leading to syncytium formation, brought about by cell-surface-expressed E-protein. We introduced a convenient detection method for quantification of syncytium formation using a fluorescent dye,

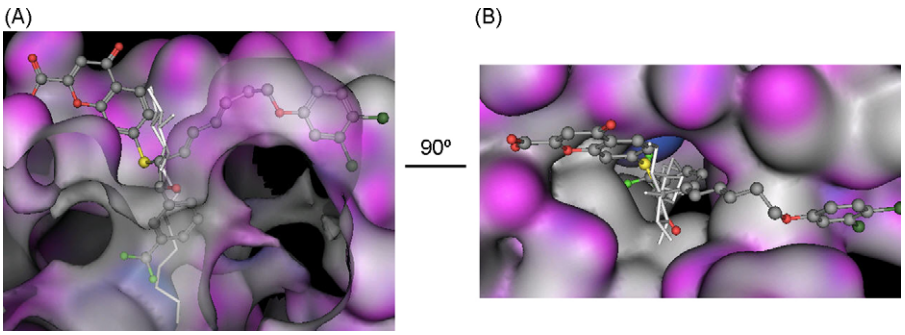

Fig. 6. Putative binding mode of NITD448. The proposed binding modes by GLIDE for NITD448 (ball-and-stick) in DEN2 E-protein (PDB# 1OKE): (A) side view; (B) top view. The protein surface is coded grey and red for hydrophobic and hydrogen bonding, respectively. The βOG molecule in 1OKE is depicted as white sticks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and optimized and characterized this assay for 96-well screening.

A secondary assay was established based on a cell-free system containing purified dengue virus and liposomes, allowing detection of content mixing of these two membrane enveloped particles as a readout of the fusion event. By using antibody detection of capsid protein in Western blot we avoided the need for producing and purifying radioactively labeled dengue virus, significantly simplifying the assay.

Since our primary assay capacity did not allow high throughput screening, we composed a focused library based on the potential of compounds to bind inside a putative binding pocket identified in the E-protein (Modis et al., 2003). Screening this focused library resulted in three primary hits of which one could be confirmed in the secondary assay, showing dose-dependent inhibition of fusion.

The confirmed fusion inhibitor NITD448 proved to have antiviral properties at an EC_{50} of 9.8 μM, comparable to the IC_{50} of 6.8 μM found when titrated in the cell-based fusion assay.

This antiviral effect was only observed if the compound was present during the viral entry phase of infection, not if the compound was present before or after viral entry, consistent with the mechanism of action of a fusion inhibitor.

NITD448 is a novel structure, different from dengue entry inhibitors published before (Li et al., 2008; Wang et al., 2009; Yennamalli et al., 2009). GLIDE docking suggests that the carboxylate on the chromenone ring of NITD448 interacts with Lys128 and Gln52 of the E-protein, with the trifluoro-phenyl motif of the molecule well-buried into the hydrophobic βOG pocket (Fig. 6), similar to the chloro-phenyl-thiophene tail of the previously reported dengue entry inhibitors (Wang et al., 2009). Compound NITD448 is, to our knowledge, the first reported small molecule with confirmed fusion inhibition activity against dengue. It may be the starting point for further development of more potent compounds with higher selectivity index, and can be used as a tool in future screening campaigns for fusion inhibitors. In addition, it may be of value in studying the multi-step class II membrane fusion process.

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