A Small Molecule Dengue Virus Entry Inhibitor

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Running Title: Dengue entry inhibitor
Abstract

The incidence of dengue fever epidemics has increased dramatically over the last few decades. However, no vaccine or antiviral therapies is available. Therefore, a need for the search of safe and effective antiviral drugs becomes imperative. Entry of dengue virus into a host cell is mediated by its major envelope protein, E. Crystal structure of E reveals a hydrophobic pocket that is presumably important for low pH-mediated membrane fusion. High throughput docking (HTD) was performed on this hydrophobic pocket and hits were evaluated in cell-based assays. Compound 6 was identified as one of the inhibitors with an average EC$_{50}$ of 119 nanomolar against dengue virus serotype 2 in a human cell line. Mechanism of action studies demonstrate that compound 6 acts at early stage during dengue virus infection. It arrests dengue virus in vesicles co-localizing with endocytosed dextran, and inhibits NS3 expression. Inhibitors disclosed in this report can serve as molecular probes for studying flavivirus entry.
Introduction

Dengue is a mosquito-borne viral disease that has become a major public health concern worldwide in recent years. Annually, 100 million cases of dengue fever (DF) and 500,000 cases of dengue hemorrhagic fever (DHF) occur, particularly in tropical Asia, Latin America, and the Caribbean (5, 21). At present, dengue is endemic in 112 countries around the world (22). However, there is no vaccine or treatment other than vector control and supportive medical care. The development for safe and effective therapeutics is therefore urgently needed.

The etiological agents involved are four serotypes of dengue virus (DENV-1, DENV-2, DENV-3, and DENV-4), which belong to the genus Flavivirus in the family Flaviviridae. Besides dengue virus, many flaviviruses are important human pathogens, including West Nile (WN), yellow fever (YF), Japanese encephalitis (JE), and tick-borne encephalitis (TBE) viruses. The dengue viral genome is a single-stranded, positive-sense RNA of about 11 kb in length, and contains both a 5'- and 3'-untranslated regions. The genomic RNA encodes one single polyprotein that is co- and post-translationally processed by both viral and cellular proteases into three structural proteins, the capsid (C), premembrane (prM), and envelope (E), and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (20).

Infection by dengue virus is initiated by fusion between the viral membrane and the host membrane. The fusion process is mediated by dengue E protein in a pH-dependant manner (35). The dengue E protein consists of three domains, the central domain I, the extended fingerlike domain II, and the
immunoglobin-like domain III (23-25, 41). Similar three-domain organization is found in the E proteins of tick-borne encephalitis virus (30), West Nile virus (14, 27), and E1 protein of Semliki Forest virus (18). All these envelope proteins represent a distinct class (class II) of viral fusion proteins, that is different from class I fusion proteins represented by gp120/gp41 of HIV and the hemagglutinin of influenza virus (7, 9, 33). Recent crystal structure of dengue E protein reveals a hydrophobic pocket occupied by a detergent molecule (β-N-octylglucoside, β-OG) lying at a “hinge” region between domain I and II of E protein, which is important for the low-pH-triggered conformational rearrangement required for fusion (24). The available structural data opens up a new avenue for identifying antiviral agents against early steps of dengue virus infection (29).

Inhibition of enveloped viruses at the stage of viral entry provides a route for therapeutic intervention, as evidenced by the peptidic HIV entry inhibitor T-20 (15, 34). Other peptides have demonstrated activity against retroviruses in vitro (6, 31) and paramyxoviruses (16, 39, 40). Hrobowski and co-workers have identified peptide inhibitors of dengue virus and West Nile virus infectivity using a physio-chemical algorithm (11). Peptidic antivirals, however suffer from poor absorption from the gastrointestinal tract, necessitating intravenous delivery, and high manufacturing cost, making it impractical to treat most vulnerable dengue patients. We therefore explored the development of non-peptidic small molecules to inhibit dengue virus entry. We hypothesized that in silico docking of small molecule libraries against the dengue E protein’s hydrophobic pocket could be successfully applied to identify inhibitors of dengue virus entry. This high
throughput docking (HTD) effort led to identification of small molecules that were able to inhibit the replication of dengue virus in a cellular assay. The initial hits were further evaluated and optimized to identify compound 6 as a lead. Mechanism of action studies indicate that compound 6 blocks dengue virus replication at an early stage of the viral life cycle.
Materials and Methods

Cells and viruses

A549 cells (ATCC, Cat. No. CCL-185) were maintained in Ham's F-12 medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in 5% CO₂. BHK21 cells were maintained in RPMI1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in 5% CO₂. C6/36, an *Aedes albopictus* cell line, was maintained in RPMI1640 medium with 10% FBS and 1% penicillin/streptomycin at 28°C in the absence of CO₂. Dengue viruses used in this study were prepared by inoculating monolayers of C6/36 cells grown in RPMI1640 medium with 5% FBS and 1% penicillin/streptomycin. After incubating at 28°C for 4-5 days, cell culture supernatant was collected after clarification of cell debris, and stored at -80°C.

Docking method

A subset of the Novartis corporate archive was initially selected for this virtual screening experiment. The 3D structures were generated using CORINA (Molecular Networks GmbH, Erlangen, Germany). Protonation expansion for compounds containing atoms with pKa values within the pH range 5-9 was performed with Ionizer (Schrödinger, LLC, Portland, OR, USA). Energy of all structures were finally minimized with Macromodel (Schrödinger, LLC, Portland, OR, USA) according the MMFFs force field (37). A library of 586,829 structures was thus obtained.
Dengue virus envelope glycoprotein structure reported by Modis et al (24) was retrieved from Protein Data Bank (PDB entry 1OKE) and prepared according to the protein preparation module available in the Maestro Modeling package (Schrödinger, LLC, Portland, OR, USA). Water molecules were deleted and the protein structure together with its β-OG ligand were energy minimized within an RMSD limit of 0.30 Å.

The Glide docking suite 2.7 (Schrödinger, LLC, Portland, OR, USA) was used to conduct this HTD screening experiment. Centered on the β-OG ligand location, docking grids were generated according to default parameters. Van der Waals radii scaling factor of non-polar atoms of the protein was set to 0.8. Such value decreases penalties for close contacts and is often used to artificially simulate the flexibility of a binding site. Default docking parameters were applied and all docking calculations were distributed over a linux cluster.

Based on Glide docking score, top 10,000 docking hits were initially selected for further analysis. A 3D pharmacophore was applied to filter out docking poses using Unity program (Tripos, Inc., St. Louis, MO, USA). This pharmacophore contains two donor spheres (located in front of the backbone carbonyl of residue Ala50 and Thr48), one acceptor sphere (located in front of the backbone NH of residue Ala50) and one hydrophobic sphere (located at the center of the N-octyl chain of the β-OG ligand. The pharmacophore query has been conducted in such a way that only docking poses matching at least one donor or acceptor positional constraint (out of a total of three possible) in addition to the hydrophobic positional constraint were kept. A consensus score was then
calculated using a normalized contribution of scoring functions provided by 
Cscore (Tripos, Inc., St. Louis, MO, USA). Duplicate compounds (multiple 
protonation states, duplicates docking results) were removed keeping highest 
consensus score. Top 671 compounds were finally retained for visual inspection.

**Compound synthesis**

Lead compounds 1-4 were synthesized using procedures previously described (3). Details regarding the synthesis of compounds 5-7 are provided in the Supplemental Material.

**Cell-Base Flavivirus Immunodetection (CFI) Assay**

A549 or BHK21 cells were trypsinized and diluted to a concentration of 2x10⁵ 
cells/ml in culture media containing 2% FBS. A 100µl of cell suspension 
(2x10⁴cells) was dispensed per well into one 96-well tissue culture plate (Nunc, 
96-well clear flat bottom, sterile, Nunclone ∆ surface). Cells were grown 
overnight in culture medium at 37°C, 5% CO₂, and then infected with dengue 
virus at an MOI (multiplicity of infection) = 0.3 in the presence of different 
concentrations of test compounds for 1h at 37°C, 5% CO₂. The virus inoculum 
was removed, replaced with fresh medium containing test compounds, and 
incubated at 37°C, 5% CO₂ for 48h. The cells were washed once with PBS, and 
fixed with cold methanol for 10min. After washing twice with PBS, the fixed cells 
were blocked with PBS containing 1% FBS and 0.05% Tween-20 for 1h at room 
temperature. Then primary antibody (4G2) solution was added, and incubated 
for 3h. The cells were washed three times with PBS followed by 1h incubation
with horseradish peroxidase (HRP)-conjugated anti-mouse IgG. After washing three times with PBS, 3,3’,5,5’-tetramethylbenzidine (TMB) substrate solution was added to each well, and the reaction was stopped by adding 0.5M sulfuric acid. The plate was read in Tecan Safire II plate reader at 450 nM for viral antigen quantification. Dose response curves were plotted from the mean absorbance versus the log of the concentration of test compounds. 50% effective concentration (EC$_{50}$), the concentration of the test compound that decreases the viral E protein production by 50%, was calculated by nonlinear regression analysis.

**Cell Viability Assay**

Cytotoxicity of test compounds were measured by Celltiter-Glo Luminescent cell viability assay following manufacturer’s protocol (Promega Cat. No. G7570). A549 cells preparation and compounds addition were performed as described in “CFI assay”. After 48 h incubation the luminescent signals for cells treated with test compounds were compared to those obtained for cells treated with DMSO to determine the 50% cytotoxic concentration (CC$_{50}$).

**Plaque Reduction Assay (PRA)**

BHK21 cells were seeded into 24-well plates at a density of 2x10$^5$ cells/well. Cells were grown overnight in culture medium at 37°C, 5% CO$_2$, and then infected with dengue virus at an MOI=0.0001 in the presence of different concentrations of test compounds for 1h at 37°C,5% CO$_2$. The virus inoculum was removed, replaced with overlay medium (RPMI1640 +2% FBS +1% penicillin/streptomycin +0.8% methyl cellulose) containing test compounds, and
incubated at 37°C, 5% CO₂. After 5 days incubation the cells were fixed by 10% formaldehyde for 20min at room temperature, rinsed with tap water and stained with 1% crystal violet for 5min. The stain was removed by rinsing the cells with tap water, and the viral plaques were counted visually. Dose response curves were plotted from the plaque number versus the log of the concentration of test compounds. EC₅₀, was calculated by nonlinear regression analysis.

**Time-of-addition study**

Two kinds of time-of-addition studies were performed. A549 cells preparation, infection, and viral antigen quantification were performed as described in “CFI assay”. In one experiment, serial dilutions of either compound 6 or a control compound were added during and/or after infection. In the other experiment, test compounds were added at a final concentration of 2µM (compound 6) or 50 µM (the control compound) simultaneously with virus, or 20, 40, 60, 75, 90, 105, and 120 min afterwards.

**Immunofluorescence study**

A549 cells were seeded onto the coverslips at a density of 1x10⁵ cells/well. Following incubation overnight at 37ºC, 5% CO₂, cells were infected at an MOI=25 in the presence or absence of compound 6 for 7 h. For dextran endocytosis, 20mg/mL of rhodamine dextran (Invitrogen Cat. No. D1817) was incubated with cells for 20 min at 37ºC, 5% CO₂ at 7 h post infection. Cells were then fixed with ice-cold methanol for 5 min, and blocked with PBS containing 1% FBS and 0.05% Tween20 (PBS-FT) overnight at 4ºC. Dengue virus E protein was detected using the monoclonal antibody 4G2, and NS3 protein was detected
with anti-NS3 antibody. Secondary antibodies used were anti-mouse-FITC antibody and anti-human-TRITC antibody. Coverslips were mounted in vectashield (Hard-Set with DAPI) on glass slides. Labeling was observed on the Leica DM4000B immunofluorescence microscope or the Zeiss LMS 510 META confocal microscopy.

**Evaluation of endosomal compartments pH**

Coverslips and A549 cells preparation were preformed as described in “Immunofluorescence study”. Cells were incubated with compound 6 or Bafilomycin A1 in F12 media containing 2% FBS for 4 h at 37°C. Control cells were incubated with 0.9% DMSO. Cells were then incubated for 2 min in DPBS containing 0.5 µM Lysosensor Green DND-189 (Molecular Probe cat # L-7535) at room temperature, washed 2 times with DPBS and live cell pictures were taken with the Leica DMIRB immunofluorescence microscope.

**Compound virus binding assay**

Micro Biospin 6 column (Bio-Rad) was used to study the binding of compound 6 and 7 to dengue virus particles. Dengue virus (~6x10^7 PFU) was incubated with 5 µM compound 6 or 7 at room temperature for 30 or 5 min, respectively, the virus/compound mixture was then loaded onto the column. After centrifugation, the eluent was used for further analysis. The compound 6/virus eluent was analyzed on a LC/MS/MS system consisting of a 4000 QTrap® triple quadrupole/linear ion trap (QqQLIT) mass spectrometer equipped with a TurbolonSprayTM interface and Agilent 1100 HPLC system with a UV detector. Compound 6 identification was performed using MS/MS in multiple reaction
monitoring (MRM) mode alternating two transition reactions. The compound 7/virus eluent was used for infecting A549 cells grown on cover slips. After 2h infection, cells were washed and fixed in formaldehyde for 15 min at room temperature. Fixed cells were blocked with PBS-FT overnight. Dengue virus E protein was detected using the monoclonal antibody 4G2, and compound 7 was detected using streptavidin-FITC.
Results

Molecular docking of the E protein

Based on visual inspection, the binding site occupied by the β-OG ligand in the X-ray structure reported by Modis et. al. (24) appears to be well suited for a HTD virtual screening experiment. A clearly defined hydrophobic pocket which is filled by the N-octyl chain of the β-OG ligand is large enough to accommodate usual hydrophobic groups often found in small organic molecules. At the entrance of the pocket, several polar atoms are suitable partners for energetically favorable H-bond interactions. In particular, backbone polar atoms of residues Thr48 and Ala50 appear to be good candidates even if none of them makes H-bond interaction with the β-OG ligand in the reported X-ray structure.

Considering the tertiary structure of the protein in the area of the β-OG binding site and conformational differences with the X-ray structure of the apo form also reported by the same research group, one can suspect that this binding site should be relatively flexible and adapt its shape to the docked ligand. Since one main drawback of current HTD methods is that protein receptor is considered rigid during the docking process, we have used soft Van der Waals contacts parameters to artificially take into account the binding site flexibility. While HTD virtual screening experiment has been carried out without any docking constraint, docking results were filtered according to a set of pharmacophoric features. Due to the limited testing capacity and a preliminary inspection of the docking results, this filtering step was found necessary to filter out docking poses lacking of critical interactions with the β-OG binding site like hydrophobic contacts in the hydrophobic pocket. A visual inspection of the top
scored docking poses were finally carried out to discard unreasonable docking conformations which are often not correctly penalized enough by current scoring functions. As a result, 111 compounds (from a library of 586,829 compounds) were selected for biological testing in a cell-based assay.

**Antiviral activities of docking hits and the identification of compound 6**

The 111 compounds were next assessed in the cell-based flavivirus immunodetection (CFI) assay to examine their activities against dengue virus replication. The assay is based on quantitative immunodetection of dengue E-protein production in target cells (see Materials and Methods for details). Similar in situ cellular ELISA assay has been reported for testing antiviral agents in several virus systems including herpes simplex virus, human cytomegalovirus, varicella-zoster virus, Rauscher murine leukemia virus and influenza A virus (2, 10, 17, 26, 36). Compound 1 and 2 (Table 1) were identified from the testing. The effective concentrations of compound reducing 50% virus antigen production, EC\(_{50}\), were calculated to be 1.69 \(\mu\)M and 0.90 \(\mu\)M, respectively. And no cytotoxicity was detected at 20 \(\mu\)M.

By using Compound 1 and 2 as a starting point, detail analysis of structure activity relationship (SAR) was undertaken in an attempt to increase the potency of this class of compound against dengue virus. We identified that both amine R1 as well as the aryl group R2 were necessary for the antiviral activity. Several substitutions were tolerated on aryl ring R2, with 4-halo substituent being the optimum (3, Table 1). Both primary and secondary, as well as both aryl and alkyl amines showed potency, but piperazine was one of the most potent amines (4,
Table 1). Changing the pyrimidine ring to quinazoline ring was very fruitful further improving the potency (5 and 6, Table 1). Treatment of A549 cells with compound 6 reduced virus growth and antigen production in a dose dependent manner (Figure 1 A and B). Virus production was completely inhibited at a concentration of 2.5 µM or above (Figure 1A). The spectrum of activity of compound 6 against four serotypes of dengue virus was examined by using the CFI assay. Compound 6 was efficacious against all serotypes, both laboratory strains and clinical isolates, with EC₅₀s ranging from 0.068 to 0.496 µM (Table 2). The average EC₅₀ for the 4 viral strains was 0.252 µM. In addition, compound 6 was efficacious against other flaviviruses, including YFV, WNV, and JEV (Table 2).

**Compound 6 acts at early stage during dengue virus infection**

To determine the antiviral target of compound 6, time-of-addition experiments using the CFI assay were performed. Serial dilutions of compound 6 or the control compound (7-deaza-2’-C-methyl-adenosine) (28) were added during and/or after 1 h infection. As shown in Figure 2A, compound 6 had similar EC₅₀s whether added only during the virus infection period or throughout the entire assay. But it was about 100-fold less active when added only after virus infection. Conversely, the control compound, which inhibits dengue NS5 polymerase at a later stage of virus life cycle, had a different profile. It was inactive when added only during the virus infection period. To characterize further the effect of compound 6, its inhibition was measured as a function of time. To have an antiviral effect, compound 6 needed to be present soon after virus addition to the
cells. The period where compound 6 was effective was up to 75 min (Figure 2C). While the control compound, retained activity even after addition at 2h post infection. These results suggest that compound 6 acts at an early stage during dengue virus life cycle, most likely, the entry step.

Dengue virus entry is a series of dynamic events, including virus attachment, receptor-mediated endocytosis, pH-dependent membrane fusion, and virus uncoating. To further define the stage that is blocked by compound 6, immunofluorescence (IF) studies were performed. As shown in Figure 3A, at 7h post infection, control cells treated with DMSO showed a typical diffused perinuclei staining for both the E protein and NS3 protein, indicating initiation of viral protein translation. However, when cells were treated with compound 6, the E protein showed a punctate distribution through cytoplasm, and there was no detectable NS3 labeling. A similar punctate E protein staining was observed for both compound-treated and control cells at 2 h post infection, excluding inhibition of virus attachment as a possible mode of action for compound 6 (data not shown). Furthermore, based on confocal microscopy, E protein appeared to be co-localized with endocytosed dextran indicating that dengue virus was arrested in endosomes in the presence of compound 6 after receptor-mediated endocytosis (Figure 3B).

As fusion of endosomal and dengue viral membrane is a pH-dependent event, we next checked the effect of compound 6 on acidification of the endosomal compartment by staining with lysosensor Green DND-189, an acidotropic probe that is trapped and fluorescent in acidic endosomes. Because
of the intrinsic fluorescent property of compound 6, we could only use a concentration of 0.2 µm in this experiment, at which >50% of viral antigen expression and infectious virus production were blocked (Figure 1). Bafilomycin A1, a highly specific inhibitor for vacuolar-type proton pump (4), was used as a control. As shown in Figure 3C, cells treated with bafilomycin A1 showed almost no labeling of the endosomal compartment by Lysosensor Green DND-189. Whereas cells treated with either DMSO or compound 6 showed similar endosomal accumulation of Lysosensor Green DND-189, suggesting that compound 6 does not drastically change the endosomal pH at 0.2 µM, and its antiviral action presumably occurs by means other than affecting endosome acidification.

**Compound 6 binds with dengue virus particles**

We next wished to address the question of whether the target of compound 6 is the virus, a Micro BioSpin 6 column gel filtration method was used with modification (8). When compound 6 was incubated with dengue virus particles and loaded onto a spin column, it eluted from the column upon centrifugation. In contrast, in the absence of virus, compound 6 was not detected in the elution fraction analyzed by LC-MS (Figure 4A). Additionally, when an inactive compound (EC$_{50}$ >20 µM) with similar lipophilicity was incubated with virus, there was background level of the compound eluted. It suggests that the binding of compound 6 with dengue virus is most likely specific.

To confirm the binding of compound 6 with virus particles, a biotinylated compound 7 (EC$_{50}$ =0.234 µM, Figure 4B), was synthesized to analyze its co-
localization with dengue virus in living cells. Compound 7 was incubated with 
dengue virus, unincorporated compound was removed by Micro BioSpin 6 
column, the eluent was then used for infecting A549 cells. As shown in Figure 
4C, at 2h post infection, most of the E protein signal co-localized with compound 
7 in the cytoplasm in punctuated structures.

**Docking pose of compound 6 with dengue E protein**

In the absence of a co-crystal containing compound 6 and dengue E protein, 
GOLD docking (13) was carried out to understand how the inhibitor may interact 
with the enzyme. The detailed binding of compound 6 in the β-OG pocket was 

further analyzed by computational chemistry. Docking of compound 6 was 

performed with GOLD. Compared to the apo structure (1OAN), the presence of 
the detergent molecule β-OG in the DENV-2 E protein (1OKE) results in a
remarkable conformation change in the κ/β-hairpin loop (residue 268-280) in 
domain II. This in turn leads to the creation of a hydrophobic channel between 
domain I and II. The entrance of the channel is lined with residues of hydrogen 

bond donating/accepting capability consisting of Lys47, Thr48, Glu49, Lys128, 
Gln200, Gln271 and Ser274 (Figure 5). With such arrangement of the protein 
residues, the octyl chain of β-OG is found buried into the hydrophobic pocket, 

while the glucosyl head group lies at the entrance of this channel.

For compound 6, the binding modes of the best 10 GOLD poses can be 
categorized into two clusters. As the fitness scores of these poses span a small 
range (two units of GOLDSCORE), it suggests that both binding modes are
plausible. In both binding modes, the chloro-phenyl-thiophene tail of compound 6 were well-buried into the hydrophobic pocket between domains I and II, occupying the same space where the β-OG octyl tail in 1OKE was. The rest of compound 6 (pyridinylmethyl-quinazolinyl-amine) is relatively well-exposed to the solvent (Figure 5). In one mode of binding, the phenyl group of the quinazoline ring makes hydrophobic interaction with Leu198 and Pro53, with the amine hydrogen interacting with the sidechain of Glu49, and the pyridine nitrogen hydrogen bonded to the sidechain of Gln271. In the other mode of binding, the phenyl group of the quinazoline ring makes hydrophobic interaction with Leu198 and Ala205, and the amine hydrogen interacting with backbone of Thr48.

Recently, sub-micromolar inhibitors against yellow fever virus were identified in cell-based assays. The most potent compound 36 contains a chloro-phenyl-thiazole tail (19), which resembles our lead compound (thiophene replacing thiazole). The mode of binding for the tail for both compounds is therefore expected to be very similar. Indeed, their docked model suggested that the chloro-phenyl-thiazole tail is buried deeply into the hydrophobic channel, which is in agreement with our proposed mode of binding for the chloro-phenyl-thiophene tail of compound 6.
Discussion

In addition to targeting viral enzymes that are indispensable for replication, interfering with virus entry step has become an attractive therapeutic strategy in recent years (1). Proof-of-concept for entry targets has mostly been obtained from the safe and efficacious HIV fusion inhibitor enfuvirtide (T-20) (15, 34). As dengue E protein plays a crucial role in fusion of the viral membrane with the target cell membrane, inhibition of this early event may lead to inhibition of infection. However, an assay that could accurately reflect the complexity of the E protein driven membrane fusion event and be amenable for high-throughput screening (HTS) is not available. On the other hand, the discovery of novel lead compounds via virtual screening is a well established process (12, 38), which is particularly well suited for the identification of compounds binding to the E protein. Herein we report the search for dengue virus entry inhibitors by high throughput docking (HTD) of E protein. HTD lead to the identification of thiophene-pyrimidine with antiviral activity in the range of sub- to low micromolar concentrations. A synthetic chemistry effort yielded compound 6 with potent activity against a broad range of laboratory and clinical isolates of four serotypes of dengue virus.

In the time-of-addition experiments, it clearly shows that compound 6 interacts with an early event in the dengue virus life cycle, which was defined as the entry step. As virus entry is a multi-step process, the data from immunofluorescence studies further pinpoint that compound 6 blocks dengue virus entry after the virus internalized into endosomes. We speculate that the
compound interacts with the viral-endosomal membrane fusion step. Since the fusion step is mainly driven by E protein in response to low pH, and there is no observed adverse effect of compound 6 on the acidification of endosomes, it is reasoned that E protein is the presumable target.

This hypothesis is further supported by a direct virus-compound binding assay, where compound 6 co-eluted with virus particles from a gel filtration spin column. The results of co-localization of a biotinylated compound 7 with virus during infection are also consistent with an interaction between the compound and the E protein.

Compound 6 can be readily modeled into the hydrophobic pocket with GOLD in an orientation where the chloro-phenyl-thiophene tail is well-buried, and the rest of molecule being solvent-exposed. Apo crystal structures of the DENV-3 and WNV E protein, in the “closed” form, have been published recently (14, 25, 27). As the hydrophobic channel is only present in the “open” form, it is impossible to obtain a direct comparison of the β-OG binding site between DENV-2, DENV-3 and WNV. However, given the very high sequence identity between DENV-2 and DENV-3 (68%), it is reasonable to expect that compound 6 might also inhibit DENV-3, which is inline with our experimental result (Table 2). On the other hand, the sequence identity between DENV-2/DENV-3 and WNV is somewhat lower (~40%). Unlike DENV-2/DENV-3 which crystallize as dimers, the WNV E protein crystallizes as a monomer. Interestingly, it has also been reported that WNV E protein fails to crystallize in the presence of β-OG despite repeated attempts (27). All these suggest that compound 6 may behave
differently in WNV, which explains why the compound is relatively less active against this virus (Table 2).

Taken together, our data strongly suggest that compound 6 interacts with the E protein therefore blocking the virus entry process. The precise mode of action remains to be characterized. Generation of compound 6-resistant dengue virus variants will be one of the ways to identify the target. Compound 6 represents a novel class of small molecule to interfere with dengue virus entry \textit{in vitro}. However, when it was tested in a dengue viremia mouse model (32), precipitation of the compound in the gastrointestinal tract was observed, which prohibited the \textit{in vivo} validation of the E protein/entry as a therapeutic target for combating dengue infection. On the other hand, compound 6 constitutes a valuable tool compound for dissecting virus entry process mediated by class II fusion proteins. It is hoped that such information will eventually be translated into new approaches to block dengue virus infection.
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Figure Legends

Figure 1. (A) Effect of compound 6 on the growth of dengue virus in A549 cells. A549 cells were infected with dengue virus (NGC) at an MOI=0.3 in the absence or presence of serial dilutions of compound 6. 48 h after infection supernatants were harvested for plaque assay in BHK21 cells. The data are the average of three independent experiments (n=3) and the error bars represent the standard deviations. (B) Compound 6 dose response curve. A549 cells were infected as described above. The amount of E protein production was determined by the CFI assay. The data are the average of duplicate wells for two independent experiments and the error bars represent the standard error of the mean.

Figure 2. Time-of-addition of compound 6. A549 cells were infected with dengue virus at 37°C for 1 h in the presence (during infection) of serial dilutions of either compound 6 (A) or a control compound (B), washed to remove unbound virus and compounds and then replaced with fresh medium. The amount of E protein production was determined by the CFI assay after 48 h incubation. For comparison, a set of samples were treated with compounds only after infection (after infection), and another set of samples were treated with compounds throughout the entire assay (during and after infection). The data are the average of duplicate wells for two independent experiments and the error bars represent the standard error of the mean. (C) Time course of the effect of compound 6 on dengue virus infection in A549 cells. A549 cells were infected as described above, and the test compounds were added at different time points.
after infection. Inhibition of virus infection was determined by the CFI assay.

- Compound 6 (2 µM); ▲, control compound (50 µM). The data are the average of three independent experiments (n=3) and the error bars represent the standard deviations.

Figure 3. (A) Compound 6 inhibits the expression of NS3. A549 cells were infected with dengue virus (NGC) at an MOI=25 in the presence (b and d) or absence (a and c) of compound 6. Infection was monitored by immunofluorescence microscopy using the monoclonal antibody against E, and the polyclonal antibody against NS3. (B) Dengue virus colocalizes with dextran by confocal microscopy. A549 cells were infected as described above in the presence of 5 µM compound 6. After 7 h incubation, cells were incubated with 20mg/mL of rhodamine-dextran for 20 min at 37°C, 5% CO₂ followed by immunofluorescence staining with the monoclonal antibody against E. (C) Compound 6 treatment does not affect endosomal acidification. A549 cells were treated with DMSO (a), compound 6 (b), or bafilomycin (c). After 4 h incubation at 37°C, 5% CO₂, cells were stained with LysoSensor Green DND-189.

Figure 4. (A) MRM representative chromatogram of compound 6 without (pink line) or with (blue line) preincubation with dengue virus. (B) Structure of compound 7. (C) Dengue virus co-localizes with a biotinylated entry inhibitor. Biospin column purified dengue virus/compound 7 complex was used to infect A549 cells. After 2 h incubation at 37°C, cells were fixed, stained and monitored.
by confocal microscopy. Compound 7 was detected by streptavidin-FITC, and
dengue virus was detected by the monoclonal antibody against E.

Figure 5. The proposed binding modes for compound 6 in the “open” form of the
DENV2 E protein (1OKE). (A) side view (B) top view. The protein surface is
coded grey, blue and red for hydrophobic, hydrogen bond donor and acceptor,
respectively. The two plausible binding modes obtained by GOLD for compound
6 is represented by green and yellow sticks respectively, with the β–OG in 1OKE
depicted as brown stick.
Table 1. Structures of several dengue virus entry inhibitors

![Structures A and B](image)

<table>
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<tr>
<th>Compound</th>
<th>Core</th>
<th>R1</th>
<th>R2</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; ± S.D. (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µM)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td><img src="image" alt="R1" /></td>
<td><img src="image" alt="R2" /></td>
<td>1.69 ± 0.94</td>
<td>0.31</td>
<td>&gt;20</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td><img src="image" alt="R1" /></td>
<td><img src="image" alt="R2" /></td>
<td>0.90 ± 0.20</td>
<td>0.30</td>
<td>&gt;20</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td><img src="image" alt="R1" /></td>
<td><img src="image" alt="R2" /></td>
<td>0.27 ± 0.11</td>
<td>0.32</td>
<td>&gt;20</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td><img src="image" alt="R1" /></td>
<td><img src="image" alt="R2" /></td>
<td>0.09 ± 0.01</td>
<td>0.041</td>
<td>&gt;20</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td><img src="image" alt="R1" /></td>
<td><img src="image" alt="R2" /></td>
<td>0.04 ± 0.01</td>
<td>0.084</td>
<td>18.64</td>
</tr>
<tr>
<td>6</td>
<td>B</td>
<td><img src="image" alt="R1" /></td>
<td><img src="image" alt="R2" /></td>
<td>0.07 ± 0.01</td>
<td>0.198</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

<sup>a</sup> EC<sub>50</sub>s were determined with the CFI assay. Standard deviations were calculated from at least 3 independent experiments.

<sup>b</sup> EC<sub>50</sub>s were determined with the plaque reduction assay.

<sup>c</sup> CC<sub>50</sub>s were determined with the cell viability assay.
Table 2. Spectrum of activity and selectivity of compound 6

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell line</th>
<th>Strain</th>
<th>EC_{50} ± S.D.(µM)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV-1</td>
<td>BHK21</td>
<td>My97-10245^b</td>
<td>0.108 ± 0.08</td>
</tr>
<tr>
<td>DENV-2</td>
<td>A549</td>
<td>NGC</td>
<td>0.119 ± 0.03</td>
</tr>
<tr>
<td>DENV-2</td>
<td>BHK21</td>
<td>NGC</td>
<td>0.068 ± 0.01</td>
</tr>
<tr>
<td>DENV-3</td>
<td>BHK21</td>
<td>My99-21531^b</td>
<td>0.496 ± 0.09</td>
</tr>
<tr>
<td>DENV-4</td>
<td>BHK21</td>
<td>My01-22713^b</td>
<td>0.334 ± 0.12</td>
</tr>
<tr>
<td>YFV</td>
<td>A549</td>
<td>17D</td>
<td>0.470 ± 0.12</td>
</tr>
<tr>
<td>JEV</td>
<td>BHK21</td>
<td>Nakayama</td>
<td>1.42 ± 0.39</td>
</tr>
<tr>
<td>WNV</td>
<td>BHK21</td>
<td>B956</td>
<td>0.564 ± 0.17</td>
</tr>
</tbody>
</table>

^a EC_{50}s were determined with the CFI assay. Standard deviations were calculated from at least 3 independent experiments.

^b Clinical isolates.
Figure 1.

(A) 

(B)
Figure 2.

(A) 

(B) 

(C)
Figure 3.

(A) LysoSensor Green DND-189 + 1 µM Bafilomycin A1 + 0.2 µM compound 6

(B) DMSO control + 5 µM compound 6

(C) DMSO control + 0.2 µM compound 6 + 1 µM Bafilomycin A1

LysoSensor Green DND-189
Figure 4.

(A) 

(B) Compound 7

(C)