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A Small-Molecule Dengue Virus Entry Inhibitor▽†

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The incidence of dengue fever epidemics has increased dramatically over the last few decades. However, no vaccine or antiviral therapies are available. Therefore, the need for safe and effective antiviral drugs has become imperative. The entry of dengue virus into a host cell is mediated by its major envelope (E) protein. The crystal structure of the E protein reveals a hydrophobic pocket that is presumably important for low-pH-mediated membrane fusion. High-throughput docking with this hydrophobic pocket was performed, and hits were evaluated in cell-based assays. Compound 6 was identified as one of the inhibitors and had an average 50% effective concentration of 119 nM against dengue virus serotype 2 in a human cell line. Mechanism-of-action studies demonstrated that compound 6 acts at an early stage during dengue virus infection. It arrests dengue virus in vesicles that colocalize with endocytosed dextran and inhibits NS3 expression. The inhibitors described in this report can serve as molecular probes for the study of the entry of flavivirus into host cells.

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 and 500,000 cases of dengue hemorrhagic fever occur, particularly in tropical Asia, Latin America, and the Caribbean (5, 22). At present, dengue is endemic in 112 countries around the world (23). However, there is no vaccine or treatment other than vector control and supportive medical care. The development of safe and effective therapeutics is therefore urgently needed.

The etiological agents involved are four serotypes of dengue virus (dengue virus serotype 1 [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 virus (WNV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus. The dengue viral genome is a single-stranded, positive-sense RNA of about 11 kb in length and contains both 5′ and 3′ untranslated regions. The genomic RNA encodes a single polyprotein that is co- and posttranslationally processed by both viral and cellular proteases into three structural proteins, the capsid (C), premembrane (prM), and envelope (E) proteins, and seven nonstructural proteins, the NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 proteins (21).

Infection by dengue virus is initiated by fusion between the viral membrane and the host membrane. The fusion process is mediated by the dengue virus E protein in a pH-dependent manner (36). The dengue virus E protein consists of three domains: central domain I, extended fingerlike domain II, and immunoglobulin-like domain III (24–26, 41). A similar three-domain organization is found in the E proteins of tick-borne encephalitis virus (31) and WNV (15, 28) and the E1 protein of Semliki Forest virus (19). All these E proteins represent a distinct class (class II) of viral fusion proteins that is different from the class I fusion proteins represented by gp120/gp41 of human immunodeficiency virus (HIV) and the hemagglutinin of influenza virus (7, 10, 34). The recently determined crystal structure of the dengue virus E protein reveals a hydrophobic pocket occupied by a detergent molecule (β-*N*-octylglucoside [β-OG]) lying at a hinge region between domains I and II of the E protein, which is important for the low-pH-triggered conformational rearrangement required for fusion (25). The available structural data open up a new avenue for identifying antiviral agents active against early steps of dengue virus infection (30).

Inhibition of enveloped viruses at the stage of viral entry provides a route for therapeutic intervention, as evidenced by the peptidic HIV entry inhibitor enfuviritide (T-20) (16, 35). Other peptides have demonstrated activity against retroviruses in vitro (6, 32) and paramyxoviruses (17, 39, 40). Hrobowski and coworkers have identified peptide inhibitors of dengue virus and WNV infectivity using a physiochemical algorithm (12). Peptidic antivirals, however, suffer from poor absorption from the gastrointestinal tract, necessitating intravenous delivery and high manufacturing costs, making it impractical to treat most vulnerable patients with dengue. We therefore explored the development of nonpeptidic small molecules to inhibit dengue virus entry. We hypothesized that the in silico docking of small-molecule libraries active against the dengue E protein's hydrophobic pocket could be successfully applied to

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the identification of inhibitors of dengue virus entry. This high-throughput docking (HTD) effort led to the 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 (catalog no. CCL-185; ATCC) were maintained in Ham's F-12 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO_2. BHK21 cells were maintained in RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2. C6/36, an *Aedes albopictus* cell line, was maintained in RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin at 28°C in the absence of CO_2. The dengue viruses used in this study were prepared by inoculating monolayers of C6/36 cells grown in RPMI 1640 medium with 5% FBS and 1% penicillin-streptomycin. After incubation at 28°C for 4 to 5 days, the cell culture supernatant was collected after clarification of cell debris and was stored at −80°C.

Docking method. A subset of the Novartis corporate archive was initially selected for use in this virtual screening experiment. The three-dimensional structures were generated by using the CORINA software (Molecular Networks GmbH, Erlangen, Germany). Protonation expansion for compounds containing atoms with pK_a values within the pH range of 5 to 9 was performed with an Ionizer apparatus (Schrödinger, LLC, Portland, OR). The energies of all structures were finally minimized with the Macromodel software (Schrödinger, LLC), according to the Merck molecular force field (9). A library of 586,829 structures was thus obtained.

The dengue virus E glycoprotein structure reported by Modis et al. (25) was retrieved from the Protein Data Bank (PDB entry 1OKE) and was prepared according to the protein preparation module available in the Maestro Modeling package (Schrödinger, LLC). Water molecules were deleted, and the protein structure together with its β-OG ligand were energy minimized within a root mean square deviation limit of 0.30 Å.

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

On the basis of the Glide docking score, the top 10,000 docking hits were initially selected for further analysis. A three-dimensional pharmacophore was applied to filter out docking poses by using the Unity software (Tripos, Inc., St. Louis, MO). This pharmacophore contains two donor spheres (located in front of the backbone carbonyl of residues 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 was conducted in such a way that only docking poses matching at least one donor or acceptor positional constraint (of a possible total of three), in addition to the hydrophobic positional constraint, were kept. A consensus score was then calculated by using a normalized contribution of scoring functions provided by the Cscore software (Tripos, Inc.). Duplicate compounds (multiple protonation states, duplicates docking results) were removed, and the compounds with the highest consensus score were retained. The top 671 compounds were finally retained for visual inspection.

Compound synthesis. Lead compounds 1 to 4 were synthesized by procedures described previously (3). Details regarding the synthesis of compounds 5 to 7 are provided in the supplemental material.

CFI assay. For the cell-based flavivirus immunodetection (CFI) assay, A549 or BHK21 cells were trypsinized and diluted to a concentration of 2×10^5 cells/ml in culture medium containing 2% FBS. A total of 100 μl of the cell suspension (2×10^4 cells) was dispensed into each well of a 96-well tissue culture plate. The cells were grown overnight in culture medium at 37°C with 5% CO_2 and were then infected with dengue virus at a multiplicity of infection (MOI) of 0.3 in the presence of different concentrations of the test compounds for 1 h at 37°C with 5% CO_2. The virus inoculum was removed and replaced with fresh medium containing the test compounds, and the cells were incubated at 37°C with 5% CO_2 for 48 h. The cells were washed once with phosphate-buffered saline (PBS) and fixed with cold methanol for 10 min. After the fixed cells were washed twice with PBS, they were blocked with PBS containing 1% FBS and 0.05% Tween 20 for 1 h at room temperature. A primary antibody (antibody 4G2) solution was then added, and the mixture was incubated for 3 h. The cells were washed three times with PBS, followed by incubation for 1 h with horseradish peroxidase-conjugated anti-mouse immunoglobulin G. After the cells were washed three times with PBS, 3,3′,5,5′-tetramethylbenzidine substrate solution was added to each well, and the reaction was stopped by adding 0.5 M 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 the test compound. The 50% effective concentration (EC_{50}), that is, the concentration of the test compound that decreased the level of viral E protein production by 50%, was calculated by nonlinear regression analysis.

Cell viability assay. The cytotoxicities of the test compounds were measured by a Celltiter-Glo Luminescent cell viability assay, according to the manufacturer's protocol (catalog no. G7570; Promega). The A549 cell preparation and compound addition were performed as described above under "CFI assay." After 48 h of incubation, the luminescent signals for cells treated with the test compounds were compared to those for cells treated with dimethyl sulfoxide (DMSO) to determine the 50% cytotoxic concentration (CC_{50}).

PRA. For the plaque reduction assay (PRA), BHK21 cells were seeded into 24-well plates at a density of 2×10^5 cells/well. Cells were grown overnight in culture medium at 37°C with 5% CO_2 and were then infected with dengue virus at an MOI of 0.0001 in the presence of different concentrations of the test compounds for 1 h at 37°C with 5% CO_2. The virus inoculum was removed and replaced with overlay medium (RPMI 1640, 2% FBS, 1% penicillin-streptomycin, 0.8% methylcellulose) containing the test compounds, and the mixture was incubated at 37°C with 5% CO_2. After 5 days of incubation, the cells were fixed with 10% formaldehyde for 20 min at room temperature, rinsed with tap water, and stained with 1% crystal violet for 5 min. 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 the test compounds. The EC_{50} was calculated by nonlinear regression analysis.

Time-of-addition studies. Two kinds of time-of-addition studies were performed. A549 cell preparation, infection, and viral antigen quantification were performed as described above under "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 2 μM (compound 6) or 50 μM (control compound) simultaneously with virus addition or 20, 40, 60, 75, 90, 105, or 120 min after virus addition.

Immunofluorescence study. A549 cells were seeded onto coverslips at a density of 1×10^5 cells/well. Following incubation overnight at 37°C with 5% CO_2, the cells were infected at an MOI of 25 in the presence or the absence of compound 6 for 7 h. For dextran endocytosis, 20 mg/ml of rhodamine dextran (catalog no. D1817; Invitrogen) was added to the cells at 7 h postinfection and was incubated with the cells for 20 min at 37°C with 5% CO_2. The cells were then fixed with ice-cold methanol for 5 min and blocked with PBS containing 1% FBS and 0.05% Tween 20 overnight at 4°C. The dengue virus E protein was detected by using monoclonal antibody 4G2, and the dengue virus NS3 protein was detected with anti-NS3 antibody. The secondary antibodies used were anti-mouse fluorescein isothiocyanate (FITC) antibody and anti-human tetramethyl rhodamine isocyanate antibody. The coverslips were mounted in Vectashield (hard set with 4′,6-diamidino-2-phenylindole [DAPI]) on glass slides. Labeling was observed on a Leica DM4000B immunofluorescence microscope or a Zeiss LMS 510 Meta confocal microscope.

Evaluation of pH in endosomal compartments. Coverslips and A549 cells were prepared as described above under "Immunofluorescence study." The cells were incubated with compound 6 or bafilomycin A1 in Ham's F-12 medium containing 2% FBS for 4 h at 37°C. Control cells were incubated with 0.9% DMSO. The cells were then incubated for 2 min in Dulbecco's phosphate-buffered saline containing 0.5 μM Lysosensor green DND-189 (catalog no. L-7535; Molecular Probe) at room temperature and washed two times with Dulbecco's phosphate-buffered saline, and pictures of live cells were taken with a Leica DMIRB immunofluorescence microscope.

Compound-virus binding assay. A Micro BioSpin 6 column (Bio-Rad) was used to study the binding of compounds 6 and 7 to dengue virus particles. Dengue virus (~6×10^7 PFU) was incubated with 5 μM compound 6 or 7 at room temperature for 30 or 5 min, respectively, and 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 liquid chromatography-tandem mass spectrometry system consisting of a model 4000 QTrap triple quadrupole/linear ion trap mass spectrometer equipped with a TurboIon-

Spray interface and an Agilent 1100 high-pressure liquid chromatography system with a UV detector. The identification of compound 6 was performed by tandem mass spectrometry in multiple-reaction-monitoring mode by alternating two transition reactions. The compound 7-virus eluent was used to infect A549 cells grown on coverslips. After 2 h of infection, the cells were washed and fixed in formaldehyde for 15 min at room temperature. The fixed cells were blocked with PBS containing 1% FBS and 0.05% Tween 20 overnight. The dengue virus E protein was detected by using monoclonal antibody 4G2, and compound 7 was detected by using streptavidin-FITC.

RESULTS

Molecular docking of E protein. On the basis of visual inspection, the binding site occupied by the β-OG ligand in the X-ray structure reported by Modis et al. (25) appears to be well suited for analysis by an 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 the 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, the backbone polar atoms of residues Thr48 and Ala50 appear to be good candidates, even if none of them makes an H-bond interaction with the β-OG ligand in the reported X-ray structure.

By 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 Modis et al. (25), one can suspect that this binding site should be relatively flexible and adapt its shape to that of the docked ligand. Since one main drawback of current HTD methods is that the protein receptor is considered to be rigid during the docking process, we have used soft Van der Waals contact parameters to artificially take into account the binding site flexibility. While the HTD virtual screening experiment has been carried out without any docking constraint, the 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 to be necessary to filter out docking poses that lacked critical interactions with the β-OG binding site, like hydrophobic contacts in the hydrophobic pocket. A visual inspection of the docking poses with the top scores was 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.

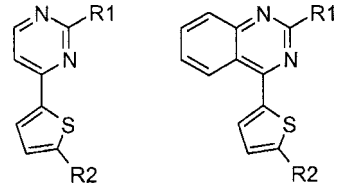
Antiviral activities of docking hits and identification of compound 6. The 111 compounds were next assessed by the CFI assay to examine their activities against dengue virus replication. The assay is based on quantitative immunodetection of dengue virus E protein production in target cells (see Materials and Methods for details). A similar in situ cellular enzyme-linked immunosorbent assay has been reported for the testing of the activities of antiviral agents in several virus systems, including herpes simplex virus, human cytomegalovirus, varicella-zoster virus, Rauscher murine leukemia virus, and influenza A virus (2, 11, 18, 27, 37). Compounds 1 and 2 (Table 1) were identified from the testing. The EC_{50}s of compounds 1 and 2 were calculated to be 1.69 μM and 0.90 μM, respectively. No cytotoxicity was detected at 20 μM.

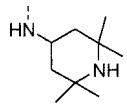
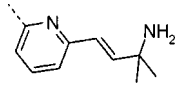
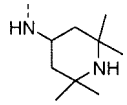
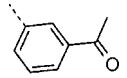
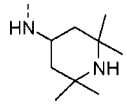
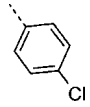
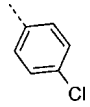
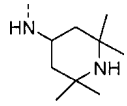
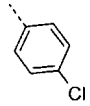
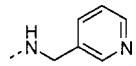
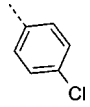
By using compounds 1 and 2 as a starting point, detailed analysis of the structure-activity relationship was undertaken in an attempt to increase the potency of this class of compounds against dengue virus. We found that both amine R1 and aryl group R2 were necessary for antiviral activity. Several substitutions were tolerated on aryl ring R2, with a 4-halo substituent being the optimum (Table 1, compound 3). Both primary and secondary amines as well as both aryl and alkyl amines showed potency, but piperazine was one of the most potent amines (Table 1, compound 4). The change of the pyrimidine ring to a quinazoline ring was very fruitful and further improved the potency (Table 1, compounds 5 and 6). Treatment of A549 cells with compound 6 reduced virus growth and antigen production in a dose-dependent manner (Fig. 1A and B). Virus production was completely inhibited at a concentration of 2.5 μM or above (Fig. 1A). The spectrum of activity of compound 6 against the four serotypes of dengue virus was examined by using the CFI assay. Compound 6 was efficacious against all serotypes of both laboratory strains and clinical isolates, with the EC_{50}s ranging from 0.068 to 0.496 μM (Table 2). The average EC_{50} for the four 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 an early stage during dengue virus infection. To determine the antiviral target of compound 6, time-of-addition experiments were performed by the CFI assay. Serial dilutions of compound 6 or the control compound (7-deaza-2′-*C*-methyladenosine) (29) were added during and/or after 1 h of infection. As shown in Fig. 2A, compound 6 had similar EC_{50}s whether it was added only during the virus infection period or throughout the entire assay. However, it was about 100-fold less active when it was added only after virus infection. Conversely, the control compound, which inhibits the dengue virus NS5 polymerase at a later stage of the virus life cycle, had a different profile. It was inactive when it was 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 during which compound 6 was effective was up to 75 min (Fig. 2C), while the control compound retained activity even after addition at 2 h postinfection. These results suggest that compound 6 acts at an early stage during the 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 studies were performed. As shown in Fig. 3A, at 7 h postinfection, control cells treated with DMSO showed a typical diffused perinuclear staining for both the E protein and the NS3 protein, indicating the initiation of viral protein translation. However, when the cells were treated with compound 6, the E protein showed a punctate distribution throughout the 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 postinfection, excluding the possibility that the inhibition of virus attachment is a possible

TABLE 1. Structures of several dengue virus entry inhibitors



Compound	Core	R1	R2	EC_{50} ± SD (μM)[a]	EC_{50} (μM)[b]	CC_{50} (μM)[c]
1	A			1.69 ± 0.94	0.31	>20
2	A			0.90 ± 0.20	0.30	>20
3	A			0.27 ± 0.11	0.32	>20
4	A			0.09 ± 0.01	0.041	>20
5	B			0.04 ± 0.01	0.084	18.64
6	B			0.07 ± 0.01	0.198	>20

[a] EC_{50}s were determined by the CFI assay. Standard deviations were calculated from at least three independent experiments.
[b] EC_{50}s were determined by the plaque reduction assay.
[c] CC_{50}s were determined by the cell viability assay.

mode of action for compound 6 (data not shown). Furthermore, on the basis of the findings made by confocal microscopy, the E protein appeared to be colocalized with endocytosed dextran, indicating that dengue virus was arrested in endosomes in the presence of compound 6 after receptor-mediated endocytosis (Fig. 3B).

As the fusion of the endosomal and the 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, in this experiment we could use a concentration of only 0.2 μM, at which >50% of viral antigen expression and infectious virus production were blocked (Fig. 1). Bafilomycin A1, a highly specific inhibitor of the vacuolar-type proton pump (4), was used as a control. As shown in Fig. 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 levels of endosomal accumulation of Lysosensor green DND-189, suggesting that compound 6 does not drastically change the endosomal pH at 0.2 μM and that 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 modifications (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, as analyzed by liquid chromatography-mass spectrometry (Fig. 4A). Additionally, when an inactive compound (EC_{50}, >20 μM) with a lipophilicity similar to that of compound 6 was incubated with dengue virus, the background level of the compound was eluted. This finding 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, compound 7 (EC_{50}, 0.234 μM) (Fig. 4B), was synthesized to analyze its colocalization with dengue virus in living cells. Compound 7 was incubated with dengue virus, unincorporated compound was removed with a Micro

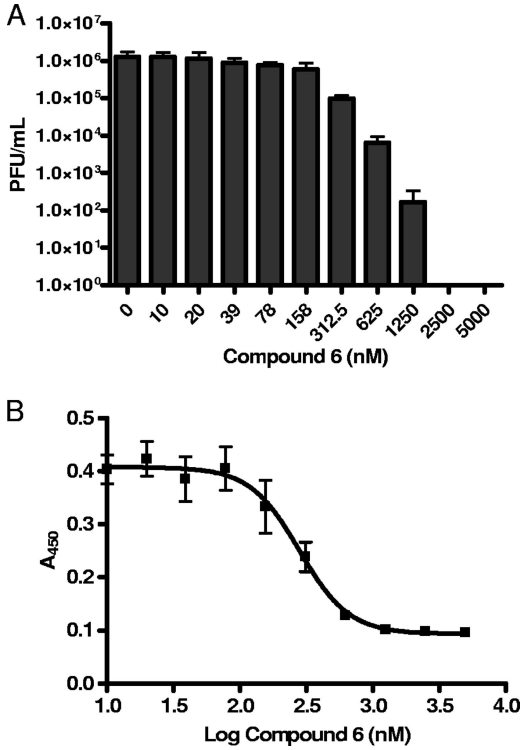


FIG. 1. (A) Effect of compound 6 on the growth of dengue virus in A549 cells. A549 cells were infected with dengue virus (strain NGC) at an MOI of 0.3 in the absence or presence of serial dilutions of compound 6. At 48 h after infection, supernatants were harvested for use in a plaque assay with BHK21 cells. The data are the averages of three independent experiments, 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 averages for duplicate wells for two independent experiments, and the error bars represent the standard errors of the mean.

BioSpin 6 column, and the eluent was then used to infect A549 cells. As shown in Fig. 4C, at 2 h postinfection, most of the E-protein signal colocalized with compound 7 in the cytoplasm in punctated structures.

Docking pose of compound 6 with dengue virus E protein. In the absence of a cocrystal containing compound 6 and dengue virus E protein, docking was carried out by use of the Gold software (14) to understand how the inhibitor may interact with the enzyme. The detailed binding of compound 6 in the

TABLE 2. Spectrum of activity and selectivity of compound 6

Virus	Cell line	Strain	$EC_{50} \pm SD$ (μM)[a]
DENV-1	BHK21	My97-10245[b]	0.108 ± 0.08
DENV-2	A549	NGC	0.119 ± 0.03
DENV-2	BHK21	NGC	0.068 ± 0.01
DENV-3	BHK21	My99-21531[b]	0.496 ± 0.09
DENV-4	BHK21	My01-22713[b]	0.334 ± 0.12
YFV	A549	17D	0.470 ± 0.12
JEV	BHK21	Nakayama	1.42 ± 0.39
WNV	BHK21	B956	0.564 ± 0.17

[a] EC_{50}s were determined by the CFI assay. Standard deviations were calculated from at least three independent experiments.
[b] Clinical isolates.

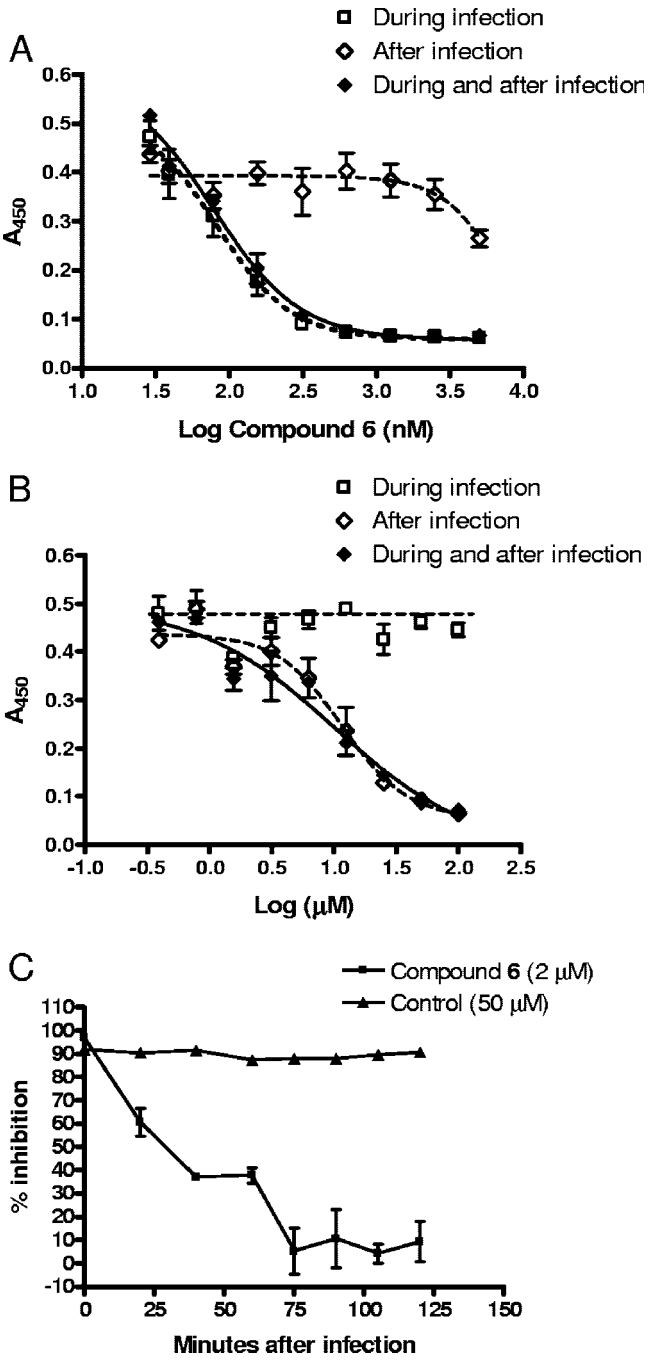


FIG. 2. Time-of-addition studies with compound 6. A549 cells were infected with dengue virus at 37°C for 1 h in the presence (During infection) of serial dilutions of either compound 6 (A) or a control compound (B) and washed to remove unbound virus and compounds, and then fresh medium was added. The amount of E-protein production was determined by the CFI assay after 48 h of incubation. For comparison, a set of samples was treated with compounds only after infection (After infection), and another set of samples was treated with compounds throughout the entire assay (During and after infection). The data are the averages for duplicate wells for two independent experiments, and the error bars represent the standard errors of the means. (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. The data are the averages of three independent experiments, and the error bars represent the standard deviations.

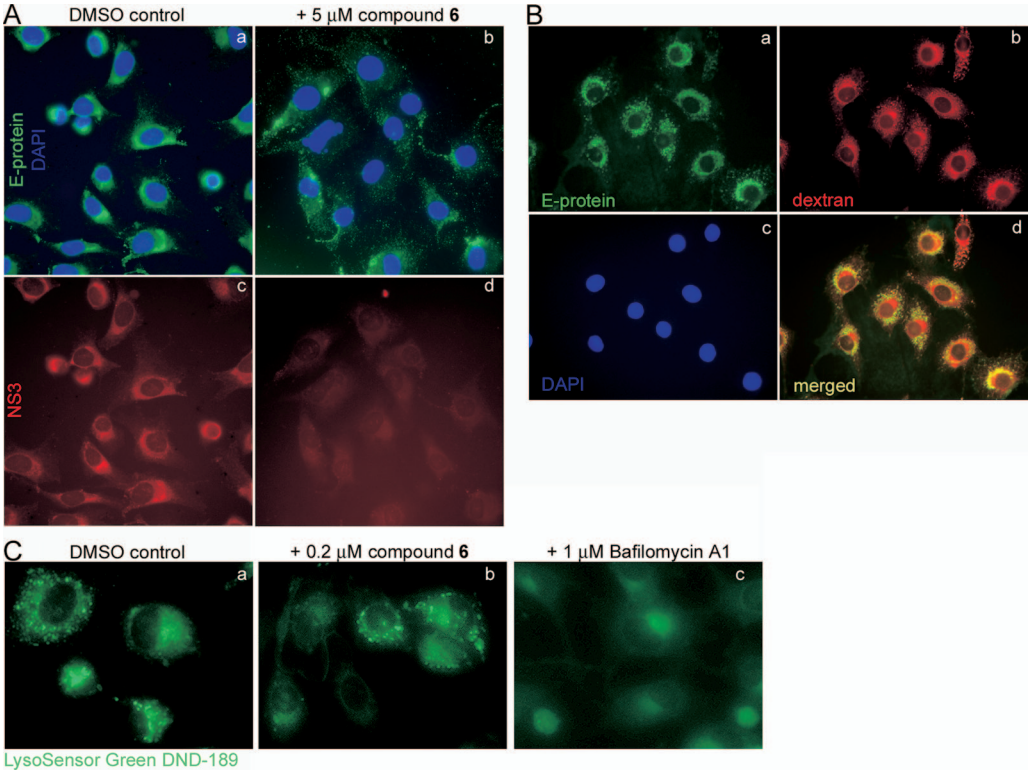


FIG. 3. (A) Compound 6 inhibits the expression of the NS3 protein. A549 cells were infected with dengue virus (strain NGC) at an MOI of 25 in the presence (b and d) or absence (a and c) of compound 6. Infection was monitored by immunofluorescence microscopy with a monoclonal antibody against the E protein and a polyclonal antibody against the NS3 protein. (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 of incubation, the cells were incubated with 20 mg/ml of rhodamine-dextran for 20 min at 37°C with 5% CO_2, followed by immunofluorescence staining with a monoclonal antibody against E protein. (C) Compound 6 treatment does not affect endosomal acidification. A549 cells were treated with DMSO (a), compound 6 (b), or bafilomycin A1 (c). After 4 h of incubation at 37°C with 5% CO_2, the cells were stained with Lysosensor green DND-189.

β-OG pocket was further analyzed by computational chemistry. Docking of compound 6 was performed by use of the Gold software. Compared to the apo structure (PDB entry 1OAN), the presence of the detergent molecule β-OG in the DENV-2 E protein (PDB entry 1OKE) resulted in a remarkable conformation change in the *kl* β-hairpin loop (residues 268 to 280) in domain II. This, in turn, led to the creation of a hydrophobic channel between domains I and II. The entrance of the channel is lined with residues with hydrogen bond donating and accepting capabilities consisting of Lys47, Thr48, Glu49, Lys128, Gln200, Gln271, and Ser274 (Fig. 5). With such an arrangement of protein residues, the octyl chain of β-OG was found to be buried in the hydrophobic pocket, while the glucosyl head group lay at the entrance of this channel.

For compound 6, the binding modes of the best 10 Gold software poses can be categorized into two clusters. As the fitness scores of these poses span a small range (2 units of the Gold software score), it suggests that both binding modes are plausible. In both binding modes, the chloro-phenyl-thiophene tail of compound 6 was well buried in the hydrophobic pocket between domains I and II, occupying the same space where the β-OG octyl tail in PDB entry 1OKE was. The rest of compound 6 (pyridinylmethyl-quinazolinyl-amine) was relatively well exposed to the solvent (Fig. 5). In one mode of binding, the phenyl group of the quinazoline ring had a hydrophobic interaction with Leu198 and Pro53, with the amine hydrogen interacting with the side chain of Glu49 and the pyridine nitrogen hydrogen bonded to the side chain of Gln271. In the other mode of binding, the phenyl group of the quinazoline ring had a hydrophobic interaction with Leu198 and Ala205 and the amine hydrogen interacted with the backbone of Thr48.

Recently, inhibitors of YFV at submicromolar concentrations were identified in cell-based assays. The most potent compound, compound 36, contains a chloro-phenyl-thiazole tail (20), which resembles our lead compound (with thiophene replacing thiazole). The mode of binding for the tails of both compounds is therefore expected to be very similar. Indeed, the docked model of compound 36 suggested that the chloro-phenyl-thiazole tail is buried deep in 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, interference with the virus entry step has become an attractive therapeutic strategy in recent years (1). The proof of concept for entry targets has mostly been obtained from the safe and efficacious HIV fusion inhibitor enfuviritide

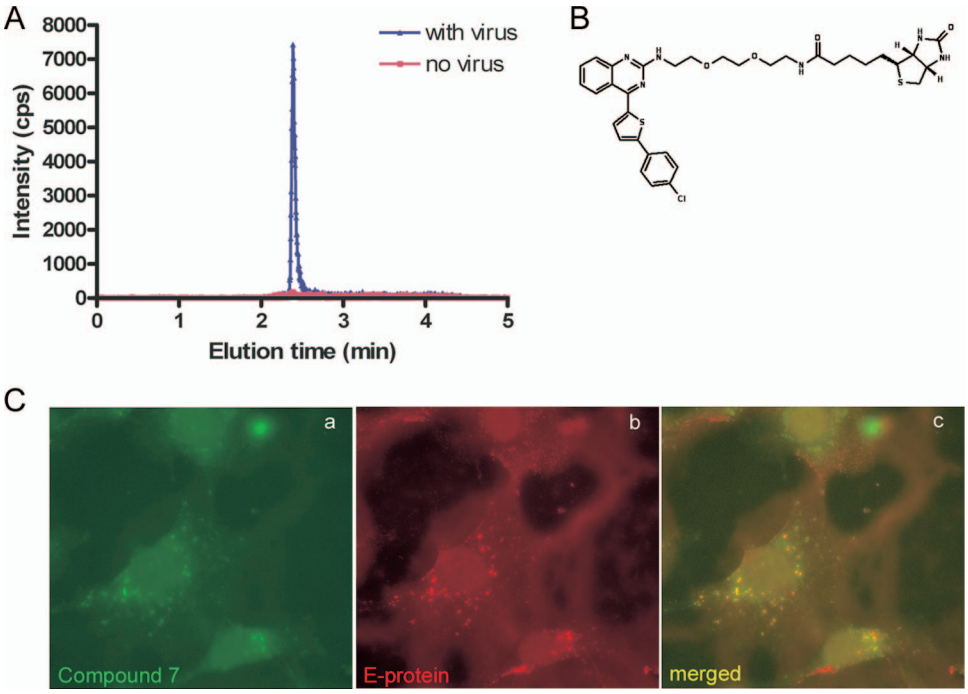


FIG. 4. (A) Representative chromatogram of compound 6 obtained in the multiple-reaction-monitoring mode without or with preincubation with dengue virus. (B) Structure of compound 7. (C) Dengue virus colocalizes with a biotinylated entry inhibitor. A BioSpin column-purified dengue virus-compound 7 complex was used to infect A549 cells. After 2 h of incubation at 37°C, the cells were fixed, stained, and monitored by confocal microscopy. Compound 7 was detected by streptavidin-FITC, and dengue virus was detected by the use of a monoclonal antibody against the E protein.

(T-20) (16, 35). As the dengue virus E protein plays a crucial role in the fusion of the viral membrane with the target cell membrane, inhibition of this early event may lead to the 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 is not available. On the other hand, the discovery of novel lead compounds by virtual screening is a well-established process (13, 38) and is particularly well suited for the identification of compounds that bind to the E protein. Here we report on the

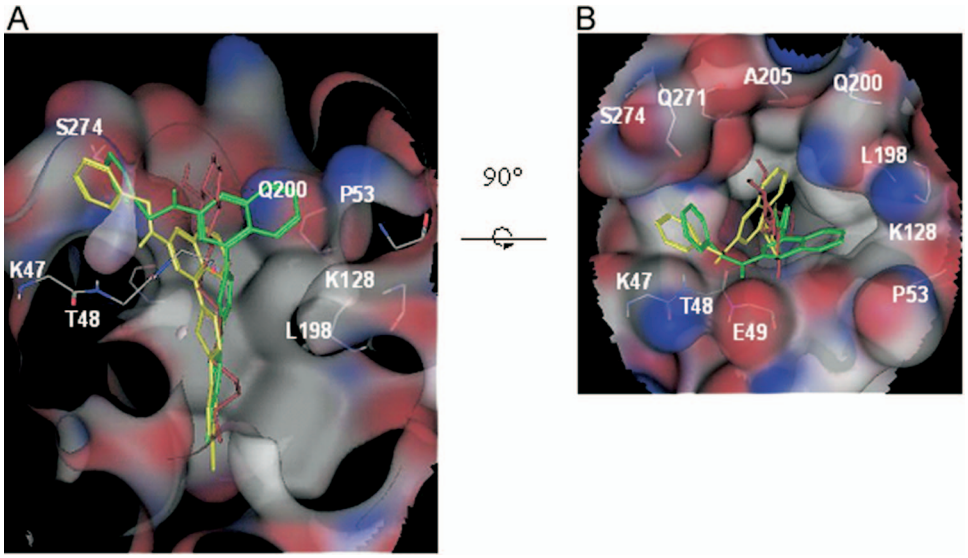


FIG. 5. The proposed binding modes for compound 6 in the open form of the DENV-2 E protein (PDB entry 1OKE). (A) Side view; (B) top view. The protein surface is coded gray, blue, and red for hydrophobic, hydrogen bond donor, and hydrogen bond acceptor, respectively. The two plausible binding modes obtained by use of the Gold program for compound 6 is represented by green and yellow sticks, respectively, with the β-OG in PDB entry 1OKE depicted as a brown stick.

search for dengue virus entry inhibitors by HTD of the E protein. HTD led to the identification of a thiophene-pyrimidine with antiviral activity in the range of submicromolar to low micromolar concentrations. A synthetic chemistry effort yielded compound 6, which had potent activity against a broad range of laboratory and clinical isolates of the four serotypes of dengue virus.

Time-of-addition experiments clearly showed 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 multistep process, the data from immunofluorescence studies further pinpoint the fact that compound 6 blocks dengue virus entry after the virus internalized into endosomes. We speculate that the compound interacts with the virus-endosomal membrane fusion step. Since the fusion step is mainly driven by the E protein in response to low pH and compound 6 had no observed adverse effect on the acidification of the endosomes, it was reasoned that the E protein is the target.

This hypothesis is further supported by a direct virus-compound binding assay, in which compound 6 coeluted with virus particles from a gel filtration spin column. The results of the colocalization of a biotinylated compound, 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 the Gold program in an orientation in which the chloro-phenyl-thiophene tail is well buried and the rest of the molecule is solvent exposed. Apo crystal structures of the DENV-3 and WNV E proteins in the closed form have recently been published (15, 26, 28). As the hydrophobic channel is present only 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 degree of sequence identity between DENV-2 and DENV-3 (68%), it is reasonable to expect that compound 6 might also inhibit DENV-3, which is in line with our experimental results (Table 2). On the other hand, the sequence identity between DENV-2/DENV-3 and WNV is somewhat lower (~40%). Unlike DENV-2 and DENV-3 which crystallize as dimers, the WNV E protein crystallizes as a monomer. Interestingly, it has also been reported that the WNV E protein fails to crystallize in the presence of β-OG, despite repeated attempts (28). All of these findings suggest that compound 6 may behave differently against WNV, which explains why the compound is relatively less active against that virus (Table 2).

Taken together, our data strongly suggest that compound 6 interacts with the E protein and therefore blocks the virus entry process. The precise mode of action remains to be characterized. The 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 molecules that interfere with dengue virus entry in vitro. However, when it was tested in a mouse model of dengue virus viremia (33), precipitation of the compound in the gastrointestinal tract was observed. The compound precipitation thus prohibited the in vivo validation of the E protein interaction and entry blockage as a therapeutic target for combating dengue virus infection. On the other hand, compound 6 constitutes a valuable compound that can be used a tool to dissect the virus entry process mediated by class II fusion proteins. It is hoped that such information will eventually be translated into new approaches to blocking dengue virus infection.

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