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In silico screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with antiviral activity against multiple flaviviruses

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ABSTRACT

The flaviviruses comprise a large group of related viruses, many of which pose a significant global human health threat, most notably the dengue viruses (DENV), West Nile virus (WNV) and yellow fever virus (YFV). Flaviviruses enter host cells via fusion of the viral and cellular membranes, a process mediated by the major viral envelope protein E as it undergoes a low pH induced conformational change in the endosomal compartment of the host cell. This essential entry stage in the flavivirus life cycle provides an attractive target for the development of antiviral agents. We performed an *in silico* docking screen of the Maybridge chemical database within a previously described ligand binding pocket in the dengue E protein structure that is thought to play a key role in the conformational transitions that lead to membrane fusion. The biological activity of selected compounds identified from this screen revealed low micromolar antiviral potency against dengue virus for two of the compounds. Our results also provide the first evidence that compounds selected to bind to this ligand binding site on the flavivirus E protein abrogate fusion activity. Interestingly, one of these compounds also has antiviral activity against both WNV (kunjin strain) and YFV.

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1. Introduction

The flavivirus family contains many human pathogens of global importance, most notably the dengue viruses (DENV), West Nile virus (WNV), yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV). DENV and YFV are spread globally and are a major concern for human health (Jacobs and Young, 1998; Monath, 2008), particularly in tropical regions, while WNV has dramatically expanded its global distribution over the last decade following its introduction to the Americas in 1999 (Lanciotti et al., 1999). Despite the fact that together they account for more than one hundred million human infections annually, no effective therapeutic options are currently available. While a safe vaccine is available for YFV and candidate vaccines are now in clinical trials for both DENV and WNV, the development of antiviral agents as therapeutic options, particularly in outbreak control situations, is warranted.

A number of inhibitory agents for the flaviviruses directed against a wide range of both cellular and viral targets have already been reported. These include those that inhibit nucleoside triphosphate synthesis (Morrey et al., 2002), suppression of viral RNA synthesis (Puig-Basagoiti et al., 2006), inhibitors of NS3 protein helicase and protease activities (Knox et al., 2006; Leung et al., 2001; Zhang et al., 2003), suppression of virion secretion and infectivity (Courageot et al., 2000; Whitby et al., 2005; Wu et al., 2002), assembly and maturation of the DENV (Chu and Yang, 2007), monoclonal antibodies (Brandriss et al., 1986; Kimura-Kuroda and Yasui, 1988; Mathews and Roehrig, 1984; Shimoni et al., 2001), RNA silencing (Adelman et al., 2002; Bai et al., 2005; McCown et al., 2003), peptides targeting the E protein (Bai et al., 2007; Hrobowski et al., 2005), host cell receptor binding (Marks et al., 2001), and recently, the rational design of small molecules that interfere with the struc-

Abbreviations: BSA, bovine serum albumin; MTT, methyl thiazole tetrazolium; pfu, plaque-forming units; m.o.i., multiplicity of infection; PBS, phosphate buffered saline; DENV, dengue viruses; YFV, yellow fever virus; WNV, West Nile virus; KUNV, kunjin virus; VS, virtual screening.

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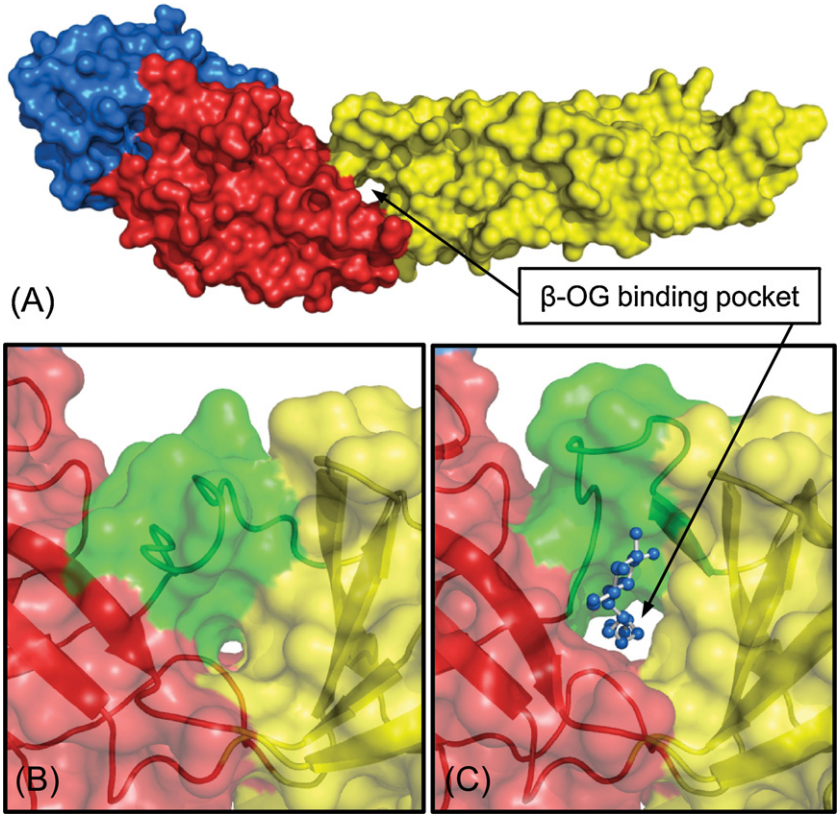

Fig. 1. Structure of the DENV E ectodomain highlighting the β-OG binding site. (A) Surface representation of the E protein ectodomain colored by domain: DI (red), DII (yellow) and DIII (blue). The β-OG molecule in this crystal structure (PDB code, 1OKE) has been removed to reveal the hydrophobic channel into which it is bound. (B) Transparent surface representation of the hinge domain in the structure not bound by β-OG. The kl loop connecting the two domains is colored in green. (C) Transparent surface representation of the hinge domain in the structure bound by β-OG (blue ball-and-stick model). Comparison of these structures reveals that the kl loop is shifted by up to 10 Å by ligand binding. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

tural changes of the envelope protein during viral fusion (Li et al., 2008; Yang et al., 2007; Yennamalli et al., 2009; Zhou et al., 2008). However, none of these small molecule inhibitors have yet entered clinical trials.

Members of the flavivirus family gain entry into host cells via a three-stage process: receptor binding, uptake by receptor-mediated endocytosis and low pH mediated fusion between the viral and host cell endosomal membranes, thereby releasing viral genomic RNA into the cell cytoplasm and initiating the viral replicative cycle. Much of this initial process is mediated by the virion envelope protein E, which is responsible both for host cell receptor engagement as well as viral and host cell membrane fusion (Anderson et al., 1992; Guirakhoo et al., 1991; Heinz and Allison, 2001). The essential nature of this entry step to the viral life cycle makes it an obvious target for antiviral drug design. The E protein consists of three structural domains (I, II and III colored red, yellow and blue, respectively in Fig. 1A) (Rey et al., 1995). Domain II (DII) contains the fusion peptide loop responsible for insertion into the host cell membrane following pH induced conformational changes in the structure of E while domain III (DIII) is involved in receptor binding (Heinz and Allison, 2001).

The fusion of viral envelope and cell membranes is catalyzed by complex structural changes in the dengue virus E protein. At the beginning of this process the low pH environment of the endosome catalyzes the protonation of selected, highly conserved histidine residues in E which triggers a reversible dissociation of the virion surface E protein dimers (Fritz et al., 2008; Kampmann et al., 2006). Exposure of the fusion peptide loop at the tip of DII is then facilitated by the movement of DII around a flexible hinge domain at the DI–II junction, allowing its insertion into the host cell membrane (Bressanelli et al., 2004; Modis et al., 2003; Zhang et al., 2004). In this transitionary stage, the E protein connects the viral envelope with the cellular membrane. Both membranes are ultimately pulled into proximity for fusion while the E protein re-associates into trimers. After merging of the membranes, E protein homotrimers are irreversibly formed (Allison et al., 1995; Modis et al., 2004).

Crystallization of the DENV E ectodomain led to the identification of two distinct crystal structures, one with (Fig. 1C) and one without (Fig. 1B) a molecule of *n*-octyl-β-D-glucoside (β-OG) bound in a hydrophobic pocket in the DI–DII hinge domain (Modis et al., 2003). The binding of β-OG into this pocket dramatically alters the conformation of one of the loops connecting the two domains (highlighted in green in Fig. 1B and C), implying an induced-fit binding that opens up the pocket into a hydrophobic channel that traverses the short axis of the molecule at this position (Fig. 1A). The finding of altered fusion phenotype and virulence attenuation for viruses with mutations to residues in this region further supports the pivotal role played by this hinge domain in viral infectivity (Hurrelbrink and McMinn, 2001; Lee et al., 1997; Monath et al., 2002). The β-OG pocket is therefore a logical target for the development of small molecule inhibitors that have the potential to bind and perturb the normal conformational transitions around this region of the E protein that drives membrane fusion.

Here we report the de novo discovery by virtual screening (using the GOLD docking algorithm; Verdonk et al., 2003), Maybridge databases and the β-OG binding site in the dengue virus E pro-

tein as the target of small molecule inhibitors of dengue virus with low micromolar potency. One of these has generic antiviral activity against DENV, YFV, and WNV_{KUN} (IC_{50} 1.2, 1.6 and 3.8 μM, respectively).

2. Materials and methods

2.1. Virtual screening

To prepare the protein for docking, hydrogens were added to the β-OG bound form of the E protein (Protein Data Bank code: 1OKE) (Modis et al., 2003) and the protonation of acidic and basic residues was adjusted to their pH 7 states using InsightII (Accelrys).

Prior to virtual screening, the vendor-supplied Maybridge chemical database (2002 edition) was filtered to remove salts and counterions and then converted into 3D coordinates using Corina (Version 3, Molecular Networks GmbH Computerchemie) giving a database of approximately 135,000 compounds. These were docked into the crystal structure (PDB: 1OKE) of the dengue virus E protein ectodomain using GOLD (v.2.1) (Verdonk et al., 2003) with the "GOLD-library screening" setting. The top 10% of molecules ranked for best fit were then re-docked using the standard default setting with 10 independent dockings for each molecule performed. The molecules were again ranked and selected according to their affinity based on scoring function in "GOLD" yielding a final set of 359 compounds.

2.2. Rational selection of ligands

To select which of these *in silico* compounds would be purchased and tested, the logarithm of the octanol/water distribution coefficient ($\log D$) of all top scoring compounds was calculated with Pallas (Compudrug Pty Ltd.). Compounds with a $\log D$ between 2 and 4 were visually inspected and then grouped according to their chemical structure, potential synthetic tractability, chemical stability and structural diversity. In this way, compound selection incorporated predicted solubility and drug-like character (Lipinski et al., 1997, 2001). Five compounds were selected and purchased from Maybridge based on these considerations for testing of inhibitory potential in an *in vitro* viral proliferation assay (plaque reduction assay).

2.3. Viruses and cell culture

2.3.1. Virus strains

Viral strains used for the biological studies were DENV2 strain NGC (Gruenberg et al., 1988), WNN_{KUN} (West Nile virus, kunjin strain; Coia et al., 1988; Hall et al., 2001) and the YFV 17D vaccine strain (Hahn et al., 1987). DENV, WNV_{KUN} and YFV were propagated using the Vero cell line (African green monkey kidney epithelial cells) and Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 3% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. Sub-culturing was performed after the cells reached 90% confluence.

2.3.2. MTT cell cytotoxicity assay

The cytotoxicity of the compounds obtained from Maybridge (www.maybridge.com) was assessed using a methylthiazoletetrazolium (MTT) cytotoxicity assay. The test compounds were serially diluted in a 96-well plate with concentrations ranging from 100 to 0.8 μM. After dilution, 50 μl of test compound containing 2% DMSO was added to Vero cell monolayers and incubated for 2 h at 37 °C + 5% CO_2. Then 20 μl of 5 mg/ml MTT solution was added to each well and the plate was incubated for 4 h at 37 °C + 5% CO_2. After incubation, 150 μl of 0.04 M hydrochloric acid in 2-propanol was added to each well and the plates were analyzed in a plate reader (Labsystems MultiskanEX) at 540 nm.

2.3.3. Antiviral inhibition assays

Stock solutions of test compounds were first prepared at 100 mM in 100% DMSO and sterilized by passage through 22 nm syringe filters, then diluted with DMSO to 8 mM. In preparation for the plaque assay, the compounds were serially diluted in serum free tissue culture media to compound concentrations of 200, 100, 50, 25, 12.5, 6, 3, 1.5 and 1 μM in a final DMSO concentration of 2%. In the final dilution step, sufficient virus to produce 20–40 plaques per well in a 96-well plate was added into the compound containing preparation. After 30 min incubation at 4 °C, 50 μl of the compound-virus suspension was used to infect cells which were then incubated for 2 h at 37 °C. Following this incubation period the compound-virus inoculum was removed and the cell monolayers were overlaid with M199 cell culture medium supplemented with 1.5% carboxymethylcellulose (CMC) and 2% FBS. DENV infected plates were incubated for 5 days, West Nile (kunjin) virus infected plates for 3 days and yellow fever virus infected plates for 6 days.

Plaques were visualized either by immunostaining (DENV) or crystal violet (CV) staining (WNV_{KUN} and YFV). For immunostaining, the overlay media was removed after completion of the incubation period, monolayers were washed twice with PBS and cells fixed at 20 °C for 20 min with 200 μl of ice-cold 20% PBS in 80% acetone followed by air drying. Monolayers were washed twice for 5 min with PBS containing 0.1% bovine serum albumin (BSA) and then blocked with 200 μl of the same buffer for 20 min at room temperature. Fifty microlitres of a primary antibody cocktail containing 1:200 dilution in PBS/0.1% BSA of the anti-NS1 monoclonal antibody 1H7 was added per well and the plates incubated at room temperature for 1 h. The antibody solution was removed and the wells were washed three times for 10 min with PBS/0.1% BSA. Goat-antimouse secondary antibody conjugated to peroxidase (Invitrogen, Australia) was diluted 1:200 and 50 μl added to each well. After 1 h incubation at room temperature, the antibody was removed and the wells were washed as before. The plaques were visualized using 200 μl of Sigmafast-DAB dye (Sigma, Australia), prepared as per the manufacturer's instructions. Following development for 20–30 min in the dark, the 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution was removed and the cells were rinsed with tap water and dried. Plates were scanned and plaques counted manually.

For CV staining, the CMC medium was aspirated, the cells were washed twice with PBS and then fixed with 20% formaldehyde in PBS for 20 min. The plates were aspirated, washed and dried at room temperature. Once dry, plates were stained for 30 min with a solution of 0.1% CV, washed and allowed to dry before manually counting the plaques. The compound concentration resulting in 50% plaque reduction was defined as IC_{50}.

2.3.4. Fusion inhibition assay

The fusion assay used in this study is based on a modification of a "fusion from within" (FFWI) assay previously described (Randolph and Stollar, 1990). This assay operates on the principle that fusion or syncytia of infected cells can be induced under low pH conditions. Monolayers of insect (C6/36) cells on coverslips were infected with DENV (at an m.o.i. of 0.5) and incubated for 24 h at 28 °C. Media was then removed and replaced with fusion media at either neutral or acidic pH (serum free media plus 15 mM MES, 20 mM HEPES, 0.1% BSA, pH 7.0 and 5.5, respectively) and containing either the appropriate concentration of inhibitor or left as the mock control. Cultures were incubated for 3 h at 40 °C (determined as optimal for syncytium induction) with the appropriate pH media being replaced every hour (with and without inhibitor as appropriate). At the end of the incubation period the cells were fixed with 4%

paraformaldehyde in PBS and then permeabilized with 0.1% Triton X-100. Syncytia were visualized by immunofluorescence using a cocktail of anti-prM (2H2) and anti-E (3H5) monoclonal antibodies with the cell nuclei counter-stained with DAPI. Coverslips were mounted on slides and examined using a confocal microscope (Zeiss). To calculate the fusion index, syncytia (fluorescently tagged multi-nucleated cells) and total cells (DAPI staining for nuclei) were counted with the fusion index = 1 − # infected cells/# nuclei.

3. Results

3.1. Compound database screening and selection

The unexpected finding of a bound molecule of *n*-octyl-β-D-glucoside (β-OG) in a hydrophobic pocket in the hinge domain of the dengue E protein crystal structure, pointed to this site as a potential target for small molecule inhibitors that may interfere with the conformational changes required in E for viral entry (Modis et al., 2003). We reasoned that this detergent molecule would also serve to provide a preliminary assessment of how effectively the program GOLD would perform in a virtual docking screen of compounds into this binding pocket. The bound β-OG in the crystal structure, (PDB code: 1OKE) was removed and then GOLD used in ten separate analyses to re-dock the detergent molecule back into the pocket using the same parameters to be employed in the virtual screen. All of the docking solutions placed β-OG close to that found in the crystal structure. A GOLD score of 40 was obtained for the best docking solution for β-OG compared with the crystal structure (within 1.0 Å root mean square distance [RMSD]) and this provided our benchmark for the selection of compounds from the database screening.

A two-tiered database docking screen was then performed. In the first screen a filtered selection of the Maybridge database containing about 135,000 compounds was docked into the β-OG binding site using the library screening settings of GOLD. A panel of 359 compounds achieved a GOLD score of more than 40. In the second docking run, 10 independent dockings for each of these 359 molecules were performed with the standard default settings, and 67 molecules reached GOLD scores of 58 or more. Comparing these scores with our β-OG standard suggested that these 67 compounds may have a 30% higher affinity to the binding site than β-OG itself.

Compounds were then selected based on a number of criteria, including commercial availability, synthetic tractability for potential modifications, chemical structural class, predicted solubility (Pallas) and toxicity. All selected compounds have as a common feature a chain of aromatic rings which carry up to three N, O or S substitutions with some of these halogenated by Cl and F. These aromatic rings are linked in a chain-like manner connected by polar and non-polar linkers. The rings and linkers, however, differ in their relative size and charge. Five candidate compounds (referred to herein as A1–A5) that represent the different shapes and charge distributions of the screening hits were chosen for biological testing and further analysis (Fig. 2).

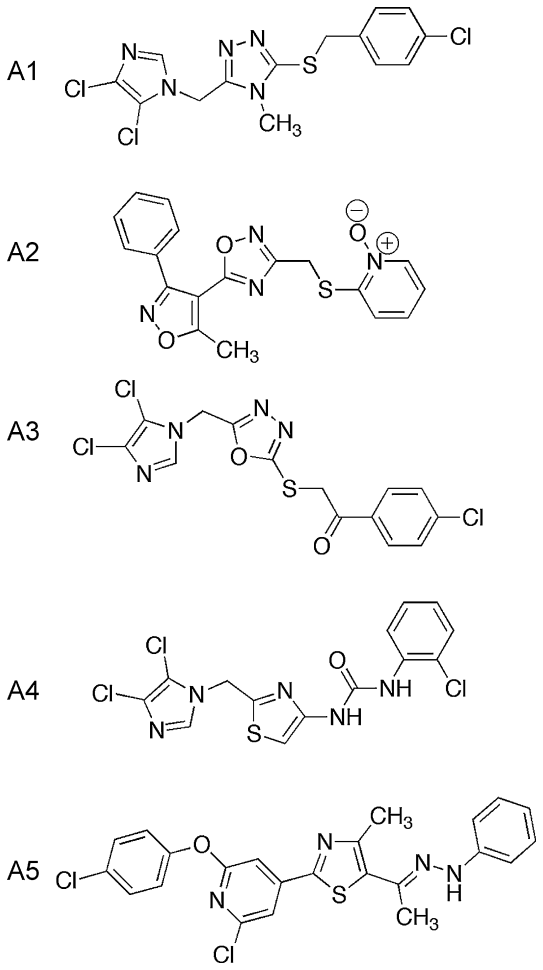

Fig. 2. Chemical structures of the molecules, A1–A5 identified by virtual screening and selected for biological testing.

3.2. Biological testing of compounds A1–A5

3.2.1. Antiviral activity

Compounds were initially screened for cytotoxicity (MTT assay, see Section 2.3) in Vero cells, the cell line chosen for the antiviral assays given the efficiency of plaquing. Compounds were prepared as stock solutions in neat DMSO from which a dilution series was then prepared, also in neat DMSO. A final dilution in tissue culture medium for cell culture testing ensured that the diluted compounds were all added in medium in which the final concentration of DMSO did not exceed 2%. None of the five compounds showed significant cytotoxicity at concentrations up to 100 μM (Table 1). Beyond this concentration all compounds showed varying signs of precipitation, presumably reflecting their hydrophobic character. The antiviral activity of the five compounds was then tested in standard virus plaque reduction assays (see Section 2.3.3). The hydrophobic pocket in the hinge region between domains I and II is thought to be a highly conserved structure amongst the flaviviruses having been observed in the crystal structures of TBEV (Rey et al., 1995), DENV (Modis et al., 2003) and WNV (Kanai et al., 2006; Nybakken et al., 2006) pre-fusion forms of the E protein. Therefore, we examined the generic antiviral potential of the compounds selected in the virtual screen using the DENV E protein structure against WNV_{KUN} and YFV as well as DENV. Compound A5 showed low micromolar inhibitory activity against each of these three flaviviruses, while A4 was found to be active against both DENV and YFV (Fig. 3).

Table 1
Antiviral activity of compounds identified from the virtual screen.

Compound	IC_{50} (μM)				CC_{50} (μM)[a]	SI[b] (DENV)
	DENV	WNV_{KUN}	YFV	RSV		
A1	>100	>100	>100	>100	>100	
A2	>100	>100	>100	>100	>100	
A3	>100	>100	>100	>100	>100	
A4	32 ± 17	>100	16 ± 8	>100	>100	>3
A5	1.2 ± 0.7	3.8 ± 2.9	1.6 ± 1.5	>100	>100	>83

[a] Host cell toxicity.
[b] Selectivity index (SI = CC_{50}/IC_{50}) for DENV.

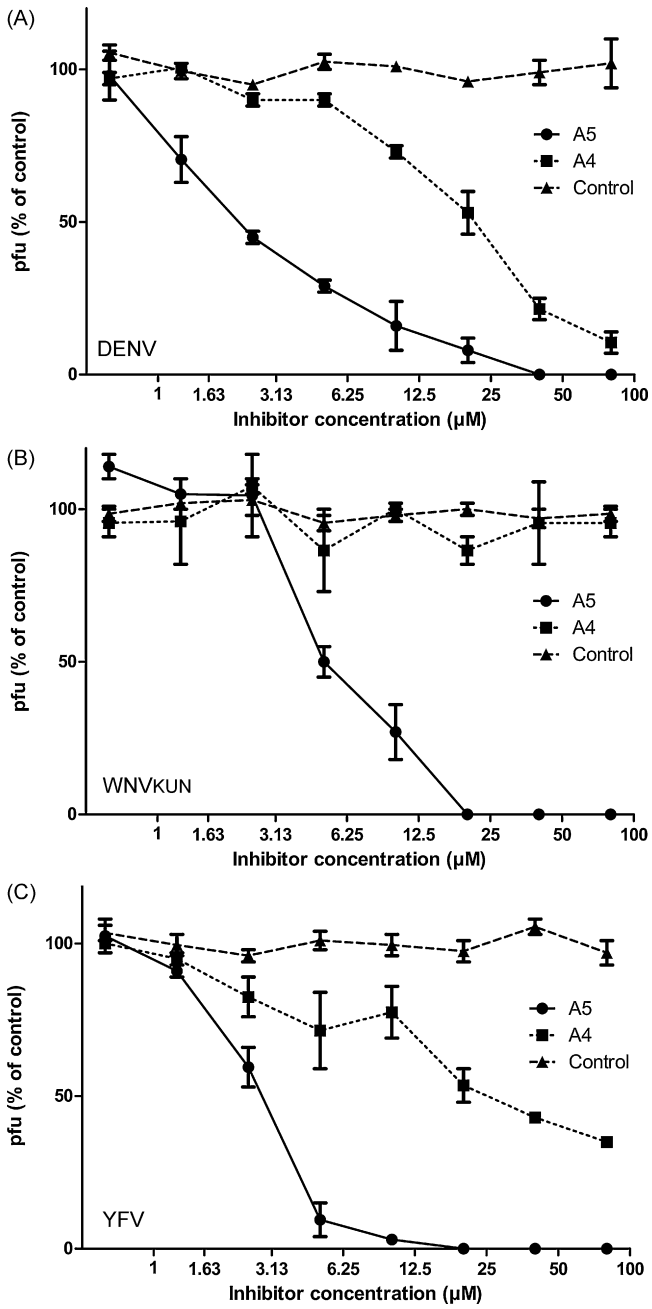

Fig. 3. Antiviral activity of compounds A4 and A5. (A) Dengue 2 virus, (B) West Nile virus (kunjin strain) and (C) yellow fever virus (17D vaccine strain). The graphs show plaque-forming units (pfu) as a percentage of mock-treated controls and as a function of compound concentration. The assays were performed in duplicate with error bars showing standard deviations. Symbols refer to: A5, closed circles (solid line); A4, closed squares (dotted line); mock-treated controls, closed triangles (dashed line).

IC_{50} values for A5 against the three flaviviruses ranged from 1.2 to 3.8 μM, while the IC_{50} for A4 against DENV and YFV was 16 and 32 μM, respectively (Table 1). Pre-incubation of A5 with virus at 37 °C prior to plaquing resulted in essentially a similar inhibitory profile. Compounds A1, A2 and A3 were not active at or below 100 μM in any of these antiviral assays (Table 1). We also examined whether the inhibitory activity of A4 and A5 may simply be the result of a non-specific virucidal effect by assessing their antiviral potential against an unrelated enveloped virus respiratory syncytial virus (RSV). No significant reduction in RSV replication was observed (Table 1).

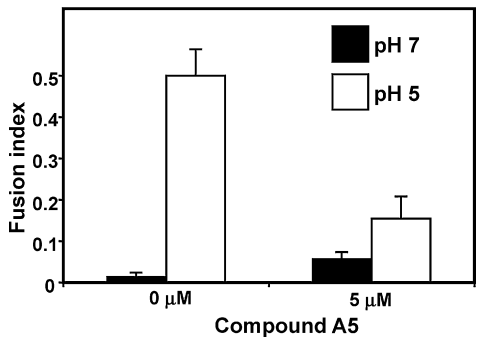

Fig. 4. Fusion inhibition by compound A5. Fusion (quantified by measuring syncytium formation—fusion index = 1 – # infected cells/# nuclei) of DENV infected insect cells (C6/36) was induced following exposure to media adjusted to pH 5.0 (white bars) but not pH 7.0 (black bars). Syncytium formation was inhibited significantly in the presence of 5 μM compound A5. Each bar represents the mean of three replicates with error bars showing standard deviation.

3.2.2. Fusion inhibition

Several reports published since this study was conducted have also used the hydrophobic pocket within the hinge domain of the DENV E protein crystal structure as the target for virtual screening of compound libraries and reported antiviral activity (Li et al., 2008; Yang et al., 2007). Each study has been based on the premise that ligand binding in this key hydrophobic pocket will likely disrupt the low pH triggered conformational changes in E that are necessary to drive viral and cell membrane fusion. However, despite evidence of antiviral activity, none of these studies have directly measured inhibition of virus-mediated fusion. The compound with the most potent antiviral activity identified in the present study (A5) was therefore tested in an infected cell based fusion assay (see Section 2.3.4) in order to examine whether its antiviral activity was the result of inhibition of E protein-mediated fusion. This assay is based on the induction of syncytia (quantified as a fusion index) in infected insect cells (C6/36 cells) following low pH triggering of membrane fusion by DENV E protein expressed on the cell surface.

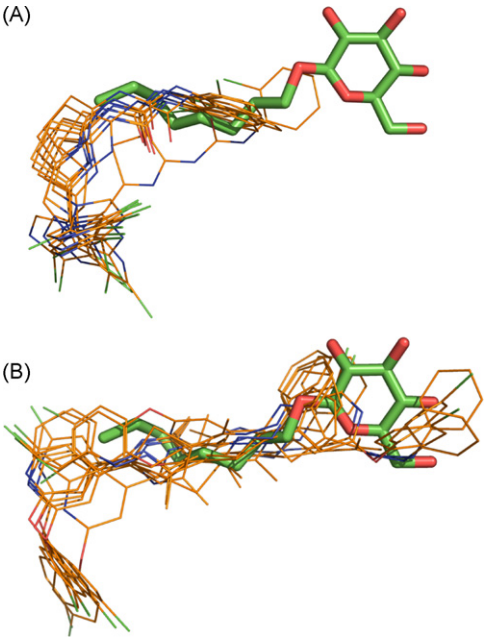

Fig. 5. Docking studies showing the relative placement of (A) A4 and (B) A5 into the β-OG binding pocket. β-OG (green stick) shown in presence of ten structures obtained by docking (gold sticks).

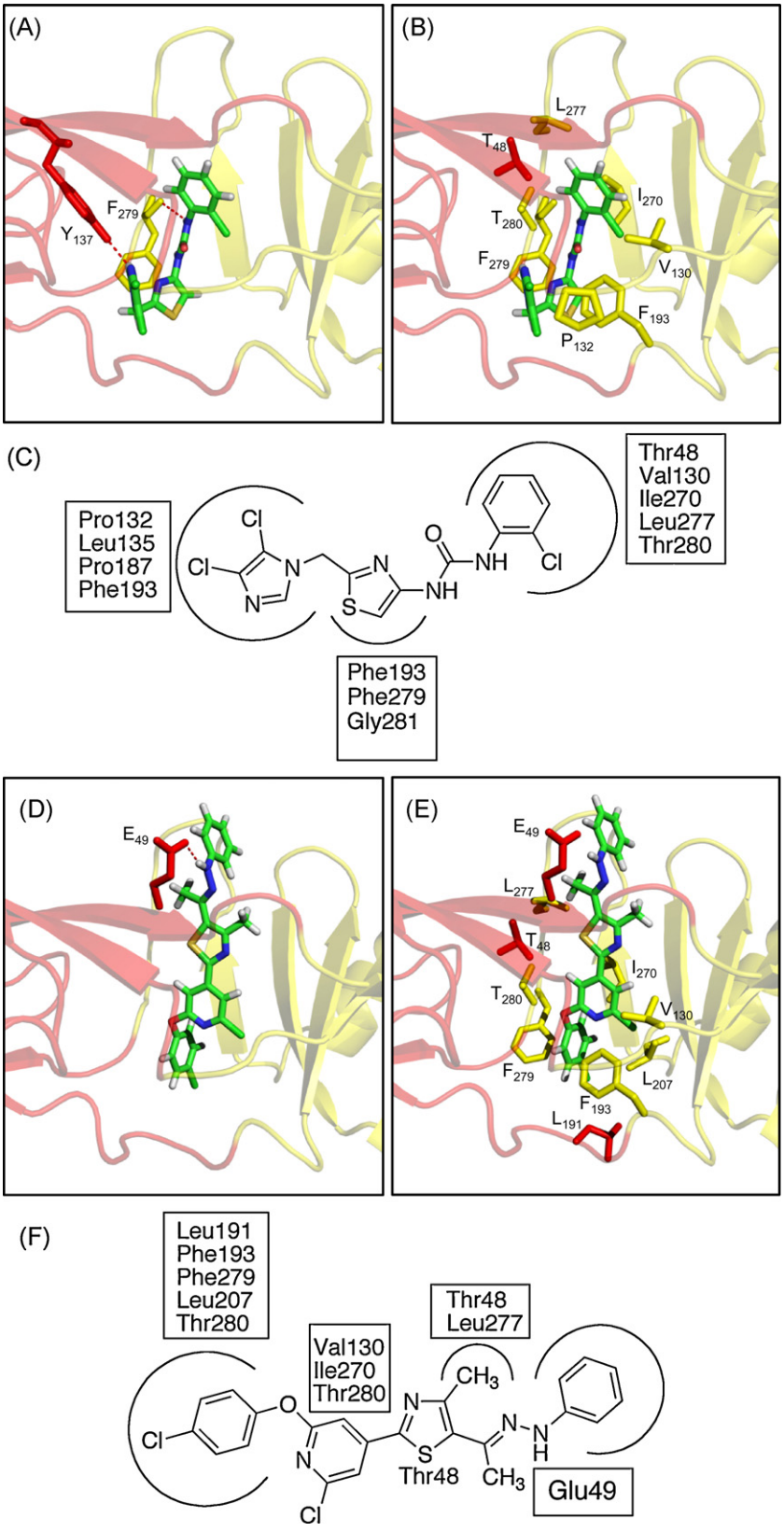

Fig. 6. Proposed binding orientations of compounds A4 and A5 in the β-OG binding pocket of the DENV E protein. (A) Docked conformation of A4 (green sticks) with E protein shown as red (DI) and yellow (DII) ribbons and key hydrogen bonding residues (Tyr137, Phe279) as sticks, (B) 3D representation and (C) 2D schematic, of the main residues (red and yellow sticks in B) of the E protein involved in van der Waals interactions with A4. (D) Docked conformation of A5 (green sticks) with E protein shown as red (DI) and yellow (DII) ribbons and key hydrogen bonding residue (Glu49) as a stick. (E) 3D representation and (F) 2D schematic of the main residues (red and yellow sticks in E) of the E protein involved in van der Waals interactions with A5.

Insect cells are employed in this assay as flaviviruses fail to induce significant syncytium formation in mammalian cells. Infected cells were exposed to low pH media in the absence or presence of compound A5 at 5 μM. An approximately 60% reduction in fusion in the presence of 5 μM A5 supports the premise that this compound does indeed inhibit E protein-mediated fusion (Fig. 4).

3.3. Docking of compounds and binding site interactions

In order to elucidate likely interactions between the compounds and their binding site, independent docking solutions of all five compounds were compared with the binding of β-OG used as reference. The docking outcomes for A1, A2 and A3 were scattered and do not show a common binding mode (data not shown). By contrast, nine out of ten docking outcomes for A4 had a conserved binding mode (Fig. 5A), with a key feature being the prediction of hydrogen bonds between (a) the imidazole nitrogen and the hydroxyl group of Tyr137 and (b) a urea NY and the backbone carbonyl oxygen of Phe279 (Fig. 6A). In addition, several key ligand–protein hydrophobic interactions were observed (Fig. 6B and C). The dichloroimidazole ring occupies a pocket bounded by Pro132, Leu135, Pro187 and Phe193. The central thiazole unit makes hydrophobic contact with Phe193, Phe279 and Gly281, and the terminal chlorobenzene ring occupies a hydrophobic pocket containing the side chains of Thr48, Val130, Ile270, Leu277 and Thr280. The docking outcomes for A5 are slightly more varied than for A4. Six out of ten docking outcomes for A5 predict a similar binding orientation to that of β-OG (Fig. 5B). The phenyl hydrazone group is predicted to bind in a position near to that observed for the solvent-exposed head group of β-OG and thus makes no hydrophobic contacts with the protein. Rather, the NH group of the hydrazone forms a hydrogen bond with the side chain carboxylate group of Glu49 (Fig. 6D). The central thiazole makes hydrophobic contacts with Thr48 and Leu277 and lies in a similar region to the chlorophenyl group of A4. The pyridine ring is predicted to form an extended planar hydrophobic surface with the thiazole and extends towards the region defined by Val130, Ile270, and Thr280. The chlorophenoxy group binds in a separate pocket defined by Leu191, Phe193, Phe279, Leu207 and Thr280 (Fig. 6E and F).

4. Discussion

The DENV E protein plays an essential role in viral replication, through both its binding to cell surface receptors and in mediating viral-cell membrane fusion. With the availability of a number of crystal structures, it has therefore been a suitable target for structure-based drug design. The hydrophobic β-OG binding pocket identified in the hinge region between domains I and II in the first DENV E crystal structure has been a particular focus of attention (Modis et al., 2003).

In this study, we performed a virtual screen of approximately 135,000 compounds selected from the Maybridge database by docking them into the β-OG binding site in the crystal structure of the pre-fusion form of the DENV E protein. Five compounds were selected for further analysis and biological testing and two of these, A4 and A5 were found to be low micromolar inhibitors of dengue virus replication. A5 was also found to inhibit the replication of both WNV_{KUN} and YFV at low micromolar concentrations, suggesting a generic antiviral potential against the flaviviruses for these compounds, and possibly also their derivatives. This is not entirely surprising given the functionally important and highly conserved nature of the domain I/II hinge region hydrophobic pocket targeted (Bressanelli et al., 2004). Furthermore, using an infected cell based fusion assay, we showed that the most potent antiviral compound did indeed inhibit dengue virus-mediated fusion. This is the first time, to our knowledge, that candidate inhibitors targeting the E protein hydrophobic pocket have been shown to directly impact on the fusion phenotype.

The similarities and slight differences between compounds A1–A5 in shape and charge distribution coupled with their differential antiviral potency were used to systematically explore the properties of the ligand binding pocket and draw conclusions regarding their biological activity. A1 and A3 both possess a dichloroimidazole group on their central oxadiazole/thiazole ring, similar to A4 with similar length linker regions before the terminal aromatic moiety. However, the absence of viral inhibition for these compounds suggests that the chloro-substituted imidazole group alone is not responsible for this activity and that most likely the hydrogen bonding capability of the urea component of A4 is necessary for activity. A2 carries on its central oxadiazole group a 5-methyl-3-phenylisoxazole similar in size to the 2-chloro-6-(4-chlorophenoxy)-pyridine group of A5. Both groups are structurally comparable, however only A5 is biologically active, suggesting that the 2-chloro-6-(4-chlorophenoxy)-pyridine or similar groups do not determine compound activity. The large hydrophobic groups at position 2 of the central thiazole ring are shared by A4 and A5; however, activity appears to also be determined by the presence of a polar linker between position 4 or 5 and another hydrophobic group. If the hydrogen bond donating linker in A4 and A5 is replaced by neutral or hydrogen bond acceptors as in A1–A3, the antiviral activity is lost. The overall architecture of A4 and A5 is very similar. The two central rings of C5 (pyridine and thiazole) and the central ring of C4 (thiazole) are aromatic and N-substituted. Hence, these rings have similar chemical properties and are likely to maintain similar interactions. Furthermore, the chlorobenzene-substituted urea linker of A4 is very similar to the phenylhydrazone of A5 with both linkers having NH hydrogen bond donors. However, the docking studies suggest they bind in slightly different locations as the hydrogen bonds are not conserved. The main difference between the compounds is their molecular size; A5 has one aromatic ring more than A4 and extends further out into the solvent-exposed side of the β-OG binding channel. This additional ring seems to cause a different placement of A5 within the target site relative to A4 with the docking results predicting that the dichloroimidazole, thiazole and chlorophenyl rings of A4 bind similarly to the chlorophenyl, chloropyridine and thiazole rings of A5, respectively.

The dominant structural similarity between the active compounds, A4 and A5 is the central thiazole ring with comparisons between the structures suggesting that this thiazole ring linked via urea/hydrazone to a large aromatic group are the key components for antiviral activity. In support of this interpretation, a recent study in which a virtual screen was performed of the NCI compound library using the DENV E protein hinge domain pocket as the target, also concluded that the most active compound hits shared the common feature of a central thiazole ring (Li et al., 2008). These two studies point to structural templates comprising a central thiazole ring as being promising candidates for further inhibitor development.

5. Conclusion

Virtual screening of databases of commercially available compounds against the DENV E protein has identified two lead compounds that have antiviral and fusion-inhibitory activity. They also share a central thiazole ring as a potential template for synthetic elaboration to even more active compounds. Our finding that one of the dengue virus inhibitors also inhibits the replication of other flaviviruses suggests the exciting possibility that antivirals with generic activity against this group of medically important viruses may be possible. We are currently using templates based

on these results for the development of more potent antiviral compounds.

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References

Adelman, Z.N., Sanchez-Vargas, I., Travanty, E.A., Carlson, J.O., Beaty, B.J., Blair, C.D., Olson, K.E., 2002. RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome. J. Virol. 76, 12925–12933.

Allison, S.L., Schalich, J., Stiasny, K., Mandl, C.W., Kunz, C., Heinz, F.X., 1995. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. J. Virol. 69, 695–700.

Anderson, R., King, A.D., Innis, B.L., 1992. Correlation of E protein binding with cell susceptibility to dengue 4 virus infection. J. Gen. Virol. 73, 2155–2159.

Bai, F., Town, T., Pradhan, D., Cox, J., Ashish, Ledizet, M., Anderson, J.F., Flavell, R.A., Krueger, J.K., Koski, R.A., Fikrig, E., 2007. Antiviral peptides targeting the West Nile virus envelope protein. J. Virol. 81, 2047–2055.

Bai, F., Wang, T., Pal, U., Bao, F., Gould, L.H., Fikrig, E., 2005. Use of RNA interference to prevent lethal murine West Nile virus infection. J. Infect. Dis. 191, 1148–1154.

Brandriss, M.W., Schlesinger, J.J., Walsh, E.E., Briselli, M., 1986. Lethal 17D yellow fever encephalitis in mice. I. Passive protection by monoclonal antibodies to the envelope proteins of 17D yellow fever and dengue 2 viruses. J. Gen. Virol. 67, 229–234.

Bressanelli, S., Stiasny, K., Allison, S.L., Stura, E.A., Duquerroy, S., Lescar, J., Heinz, F.X., Rey, F.A., 2004. Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. EMBO J. 23, 728–738.

Chu, J.J., Yang, P.L., 2007. c-Src protein kinase inhibitors block assembly and maturation of dengue virus. Proc. Natl. Acad. Sci. U.S.A. 104, 3520–3525.

Coia, G., Parker, M.D., Speight, G., Byrne, M.E., Westaway, E.G., 1988. Nucleotide and complete amino acid sequences of Kunjin virus: definitive gene order and characteristics of the virus-specified proteins. J. Gen. Virol. 69, 1–21.

Courageot, M.P., Frenkiel, M.P., Dos Santos, C.D., Deubel, V., Desprès, P., 2000. Alpha-glucosidase inhibitors reduce dengue virus production by affecting the initial steps of virion morphogenesis in the endoplasmic reticulum. J. Virol. 74, 564–572.

Fritz, R., Stiasny, K., Heinz, F.X., 2008. Identification of specific histidines as pH sensors in flavivirus membrane fusion. J. Cell. Biol. 183, 353–361.

Gruenberg, A., Woo, W.S., Biedrzycka, A., Wright, P.J., 1988. Partial nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue virus type 2 New Guinea C and PUO-218 strains. J. Gen. Virol. 69, 1391–1398.

Guirakhoo, F., Heinz, F.X., Mandl, C.W., Holzmann, H., Kunz, C., 1991. Fusion activity of flaviviruses: comparison of mature and immature (prM-containing) tick-borne encephalitis virions. J. Gen. Virol. 72, 1323–1329.

Hahn, C.S., Dalrymple, J.M., Strauss, J.H., Rice, C.M., 1987. Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it. Proc. Natl. Acad. Sci. U.S.A. 84, 2019–2023.

Hall, R.A., Scherret, J.H., Mackenzie, J.S., 2001. Kunjin virus: an Australian variant of West Nile? Ann. N.Y. Acad. Sci. 951, 153–160.

Heinz, F.X., Allison, S.L., 2001. The machinery for flavivirus fusion with host cell membranes. Curr. Opin. Microbiol. 4, 450–455.

Hrobowski, Y.M., Garry, R.F., Michael, S.F., 2005. Peptide inhibitors of dengue virus and West Nile virus infectivity. Virol. J. 2, 49.

Hurrelbrink, R.J., McMinn, P.C., 2001. Attenuation of Murray Valley encephalitis virus by site-directed mutagenesis of the hinge and putative receptor-binding regions of the envelope protein. J. Virol. 75, 7692–7702.

Jacobs, M.G., Young, P.R., 1998. Dengue: a continuing challenge for molecular biology. Curr. Opin. Infect. Dis. 11, 319–324.

Kampmann, T., Mueller, D.S., Mark, A.E., Young, P.R., Kobe, B., 2006. The role of histidine residues in low-pH-mediated viral membrane fusion. Structure 14, 1481–1487.

Kanai, R., Kar, K., Anthony, K., Gould, L.H., Ledizet, M., Fikrig, E., Marasco, W.A., Koski, R.A., Modis, Y., 2006. Crystal structure of West Nile virus envelope glycoprotein reveals viral surface epitopes. J. Virol. 80, 11000–11008.

Kimura-Kuroda, J., Yasui, K., 1988. Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. J. Immunol. 141, 3606–3610.

Knox, J.E., Ma, N.L., Yin, Z., Patel, S.J., Wang, W.L., Chan, W.L., Ranga Rao, K.R., Wang, G., Ngew, X., Patel, V., Beer, D., Lim, S.P., Vasudevan, S.G., Keller, T.H., 2006. Peptide inhibitors of West Nile NS3 protease: SAR study of tetrapeptide aldehyde inhibitors. J. Med. Chem. 49, 6585–6590.

Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K.E., Crabtree, M.B., Scherret, J.H., Hall, R.A., MacKenzie, J.S., Cropp, C.B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., Savage, H.M., Stone, W., McNamara, T., Gubler, D.J., 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 286, 2333–2337.

Lee, E., Weir, R.C., Dalgarno, L., 1997. Changes in the dengue virus major envelope protein on passaging and their localization on the three-dimensional structure of the protein. Virology 232, 281–290.

Leung, D., Schroder, K., White, H., Fang, N.X., Stoermer, M.J., Abbenante, G., Martin, J.L., Young, P.R., Fairlie, D.P., 2001. Activity of recombinant dengue 2 virus NS3 protease in the presence of a truncated NS2B co-factor, small peptide substrates, and inhibitors. J. Biol. Chem. 276, 45762–45771.

Li, Z., Khaliq, M., Zhou, Z., Post, C.B., Kuhn, R.J., Cushman, M., 2008. Design, synthesis, and biological evaluation of antiviral agents targeting flavivirus envelope proteins. J. Med. Chem. 51, 4660–4671.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. 23, 3–25.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. 46, 3–26.

Marks, R.M., Lu, H., Sundaresan, R., Toida, T., Suzuki, A., Imanari, T., Hernaiz, M.J., Linhardt, R.J., 2001. Probing the interaction of dengue virus envelope protein with heparin: assessment of glycosaminoglycan-derived inhibitors. J. Med. Chem. 44, 2178–2187.

Mathews, J.H., Roehrig, J.T., 1984. Elucidation of the topography and determination of the protective epitopes on the E glycoprotein of Saint Louis encephalitis virus by passive transfer with monoclonal antibodies. J. Immunol. 132, 1533–1537.

McCown, M., Diamond, M.S., Pekosz, A., 2003. The utility of siRNA transcripts produced by RNA polymerase I in down regulating viral gene expression and replication of negative- and positive-strand RNA viruses. Virology 313, 514–524.

Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc. Natl. Acad. Sci. U.S.A. 100, 6986–6991.

Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2004. Structure of the dengue virus envelope protein after membrane fusion. Nature 427, 313–319.

Monath, T.P., 2008. Treatment of yellow fever. Antiviral Res. 78, 116–124.

Monath, T.P., Arroyo, J., Levenbook, I., Zhang, Z.X., Catalan, J., Draper, K., Guirakhoo, F., 2002. Single mutation in the flavivirus envelope protein hinge region increases neurovirulence for mice and monkeys but decreases viscerotropism for monkeys: relevance to development and safety testing of live, attenuated vaccines. J. Virol. 76, 1932–1943.

Morrey, J.D., Smee, D.F., Sidwell, R.W., Tseng, C., 2002. Identification of active antiviral compounds against a New York isolate of West Nile virus. Antiviral Res. 55, 107–116.

Nybakken, G.E., Nelson, C.A., Chen, B.R., Diamond, M.S., Fremont, D.H., 2006. Crystal structure of the West Nile virus envelope glycoprotein. J. Virol. 80, 11467–11474.

Puig-Basagoiti, F., Tilgner, M., Forshey, B.M., Philpott, S.M., Espina, N.G., Wentworth, D.E., Goebel, S.J., Masters, P.S., Falgout, B., Ren, P., Ferguson, D.M., Shi, P.Y., 2006. Triaryl pyrazoline compound inhibits flavivirus RNA replication. Antimicrob. Agents Chemother. 50, 1320–1329.

Randolph, V.B., Stollar, V., 1990. Low pH-induced cell fusion in flavivirus-infected Aedes albopictus cell cultures. J. Gen. Virol. 71, 1845–1850.

Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., Harrison, S.C., 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2A resolution. Nature 375, 291–298.

Shimoni, Z., Niven, M.J., Pitlick, S., Bulvik, S., 2001. Treatment of West Nile virus encephalitis with intravenous immunoglobulin. Emerg. Infect. Dis. 7, 759.

Verdonk, M.L., Cole, J.C., Hartshorn, M.J., Murray, C.W., Taylor, R.D., 2003. Improved protein–ligand docking using GOLD. Proteins 52, 609–623.

Whitby, K., Pierson, T.C., Geiss, B., Lane, K., Engle, M., Zhou, Y., Doms, R.W., Diamond, M.S., 2005. Castanospermine, a potent inhibitor of dengue virus infection in vitro and in vivo. J. Virol. 79, 8698–8706.

Wu, S.F., Lee, C.J., Liao, C.L., Dwek, R.A., Zitzmann, N., Lin, Y.L., 2002. Antiviral effects of an iminosugar derivative on flavivirus infections. J. Virol. 76, 3596–3604.

Yang, J.M., Chen, Y.F., Tu, Y.Y., Yen, K.R., Yang, Y.L., 2007. Combinatorial computational approaches to identify tetracycline derivatives as flavivirus inhibitors. PLoS ONE 2, e428.

Yennamalli, R., Subbarao, N., Kampmann, T., McGeary, R.P., Young, P.R., Kobe, B., 2009. Identification of novel target sites and an inhibitor of the dengue virus E protein. J. Comput. Aided Mol. Des. 23, 333–341.

Zhang, N., Chen, H.M., Koch, V., Schmitz, H., Minczuk, M., Stepien, P., Fattom, A.I., Naso, R.B., Kalicharran, K., Borowski, P., Hosmane, R.S., 2003. Potent inhibition of NTPase/helicase of the West Nile Virus by ring-expanded ("fat") nucleoside analogues. J. Med. Chem. 46, 4776–4789.

Zhang, Y., Zhang, W., Ogata, S., Clements, D., Strauss, J.H., Baker, T.S., Kuhn, R.J., Rossmann, M.G., 2004. Conformational changes of the flavivirus E glycoprotein. Structure 12, 1607–1618.

Zhou, Z., Khaliq, M., Suk, J.E., Patkar, C., Li, L., Kuhn, R.J., Post, C.B., 2008. Antiviral compounds discovered by virtual screening of small-molecule libraries against dengue virus E protein. ACS Chem. Biol. 3, 765–775.