Antiviral Research 82 (2009) 110–114

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Structure-based discovery of dengue virus protease inhibitors☆

Suzanne M. Tomlinson[a], Robert D. Malmstrom[a], Andrew Russo[a], Niklaus Mueller[b], Yuan-Ping Pang[c], Stanley J. Watowich[a,*]

[a] Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555, United States
[b] Department of Neurology, University of Colorado Health Sciences Center, Aurora, CO 80045, United States
[c] Computer-Aided Molecular Design Laboratory, Mayo Clinic, Rochester, MN 55905, United States

ARTICLE INFO

Article history:
Received 23 October 2008
Received in revised form 5 January 2009
Accepted 8 February 2009

Keywords:
Dengue virus
NS2B-NS3
Protease
Small-molecule inhibitor
Flavivirus

ABSTRACT

Dengue virus belongs to the family *Flaviviridae* and is a major emerging pathogen for which the development of vaccines and antiviral therapy has seen little success. The NS3 viral protease is a potential target for antiviral drugs since it is required for virus replication. The goal of this study was to identify novel dengue virus (type 2; DEN2V) protease inhibitors for eventual development as effective anti-flaviviral drugs. The EUDOC docking program was used to computationally screen a small-molecule library for compounds that dock into the P1 pocket and the catalytic site of the DEN2V NS3 protease domain apo-structure [Murthy, K., Clum, S., Padmanabhan, R., 1999. Crystal structure and insights into interaction of the active site with substrates by molecular modeling and structural analysis of mutational effects. J. Biol. Chem. 274, 5573–5580] and the Bowman–Birk inhibitor-bound structure [Murthy, K., Judge, K., DeLucas, L., Padmanabhan, R., 2000. Crystal structure of dengue virus NS3 protease in complex with a Bowman–Birk inhibitor: implications for flaviviral polyprotein processing and drug design. J. Mol. Biol. 301, 759–767]. The top 20 computer-identified hits that demonstrated the most favorable scoring "energies" were selected for *in vitro* assessment of protease inhibition. Preliminary protease activity assays demonstrated that more than half of the tested compounds were soluble and exhibited *in vitro* inhibition of the DEN2V protease. Two of these compounds also inhibited viral replication in cell culture experiments, and thus are promising compounds for further development.

The mosquito-borne dengue virus (DENV), a member of the family *Flaviviridae* and genus *Flavivirus*, appears to be expanding its range, increasing in disease severity, and has recently been deemed by the US National Institutes of Health as a potential serious public health threat to the United States (Morens and Fauci, 2008). The U.S. Centers for Disease Control and Prevention estimate that ~2.5 billion people worldwide are at risk for dengue infection; annually 50–100 million dengue infections occur with ~500,000 cases of dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) resulting in ~25,000 deaths. Primary dengue fever is caused by any of four distinct serotypes of the virus (dengue virus type 1–4). It is postulated that subsequent infection by a different serotype may promote more serious forms of the disease such as DHF/DSS (Alvarez et al., 2006; Halstead, 2003). There are no approved vaccines or antiviral therapies to combat this disease. The increasing spread and severity of DENV infections emphasize the importance of drug discovery strategies that efficiently and cost-effectively identify antiviral drug leads for development into potent drugs.

The ~11 kB positive-strand RNA genome of DENV is transcribed and translated as a single polyprotein that is co- and post-translationally cleaved by cellular and viral proteases (Gubler et al., 1996). The N-terminal region of the nonstructural 3 protein (NS3) is a serine protease (Bazan and Fletterick, 1989; Chambers et al., 1990) that binds a required NS2B cofactor and cleaves the polyprotein after dibasic residues in the NS2A-NS2B, NS2B-NS3, NS3-NS4A, and NS4B-NS5 cleavage sites (Chambers et al., 1990). The NS2B-NS3 protease is required for viral replication (Falgout et al., 1991) and serves as a promising target for DENV antiviral drug development (Leyssen et al., 2000; Sampath and Padmanabhan, 2009). While other studies have identified protease inhibitors by either high-throughput screening or assaying compounds that mimic the peptide substrate (Chanprapaph et al., 2005; Ganesh et al., 2005; Leung et al., 2001; Yin et al., 2006a,b), this study used a virtual screen to

Abbreviations: DENV, dengue virus; DEN2V, dengue 2 virus; NS, nonstructural; NS2B-NS3pro, NS3 protease with NS2B cofactor; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; GKR, glycine–lysine–arginine; AMC, 7-amino-4-methylcoumarin.

☆ This research was supported in part by a subcontract to SJW from NIH/NIAID NGA 5 U54 AI057156-03, The Welch Foundation H-1642 (SJW), a training fellowship to SMT from the Computational and Structural Biology in Biodefense Training Program of the W. M. Keck Center for Interdisciplinary Bioscience Training of the Gulf Coast Consortia (NIAID Grant No. 1 T32 AI065396-01), and the Defense Advanced Research Projects Agency DAAD19-01-1-0322 (YPP).

* Corresponding author. Tel.: +1 409 747 4749; fax: +1 409 747 4745.
E-mail address: watowich@xray.utmb.edu (S.J. Watowich).

0166-3542/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2009.02.190

predict which chemicals in a commercially available library could inhibit the dengue virus protease. Testing of computer-predicted hits using a rapid *in vitro* DEN2V protease assay confirmed the activity of several compounds that inhibited the NS2B-NS3 protease; two compounds further demonstrated antiviral activity in cell-based replication assays.

The increased availability of three-dimensional protein structures and chemical compound libraries has expanded the role of high-throughput computational screening in discovering new drug leads (e.g., Laird and Blake, 2004). The chemical library chosen for this screen was a subset of an in-house database from Mayo Clinic that contained 2.5 million three-dimensional structures of small molecules that were reportedly available from chemical vendors. Two additional filters were imposed on this dataset. First, due to concerns regarding cellular uptake and cell membrane impermeability to ions (Ghose and Viswanadhan, 2001; Alberts et al., 2002) only neutral non-zwitterionic compounds (at pH 7.4) were passed to the filtered chemical library. Second, only compounds readily purchasable from highly reputable chemical vendors were retained in the filtered chemical library. Using the EUDOC program (Pang et al., 2001, 2008; Wang and Pang, 2007), we computationally screened this filtered chemical library against two previously reported dengue virus type 2 (DEN2V) protease crystal structures, the NS3 protease domain alone (PDB identifier 1BEF; Murthy et al., 1999) and the NS3 protease domain complexed with the Bowman–Birk inhibitor (a soybean protein that has been shown to inhibit the dengue protease) (PDB identifier 1DF9; Murthy et al., 2000). The EUDOC program performed virtual screens by systematically and separately docking each small molecule from our chemical library into the protease active site and the P1 pocket. Docked conformations of each compound were assigned an intermolecular interaction "energy" score that included charge–charge and Van der Waals interaction energy terms (Pang et al., 2001). From the ensemble of docked conformations for each ligand, the conformation with the lowest "energy" score was retained as representative of the bound structure. Twenty potential protease inhibitors that had some of the lowest energy scores were purchased from large chemical suppliers (e.g., Sigma–Aldrich) for further characterization.

Compounds identified by the EUDOC program as potential protease inhibitors were tested for solubility and protease inhibition activity. Of the 20 potential inhibitors that were purchased, 13 were soluble at 100 μM in aqueous cleavage buffer (200 mM Tris [pH 9.0], 20% glycerol) and used in subsequent NS2B-NS3pro inhibition tests. Potential inhibitors were initially dissolved in DMSO and diluted with cleavage buffer to their desired final concentration. In addition to analysis by light scattering to determine compound solubility, protease inhibition assays were performed before and after filtering and high-speed centrifugation to remove precipitated compounds. Poorly soluble compounds generally produced false positive results, in that protease inhibition appeared to occur in pre-centrifuged solutions that contained precipitants (Fig. 1A, inhibitors 13 and 154). Soluble compounds generated similar protease inhibition results both before and after centrifugation (Fig. 1A, inhibitor 6). Compounds with poor solubility were removed from consideration to prevent false positives, and all assays were performed on

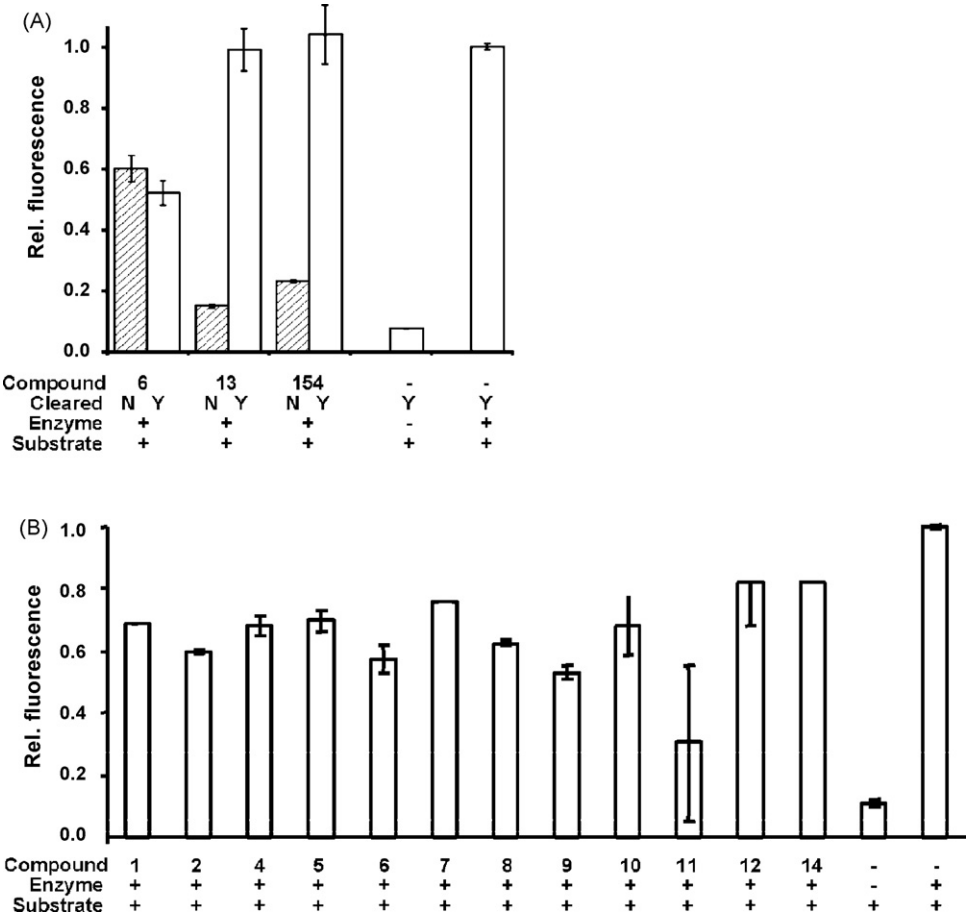


Fig. 1. *In vitro* DEN2V NS2B-NS3 protease inhibition assay. (A) Representative protease inhibition assays performed both before and after centrifugation and filtration to "clear" the solutions of precipitated compounds. (B) Protease inhibition assay of soluble compounds in cleared solutions; compounds were predicted by EUDOC virtual screening as potential DEN2V inhibitors. Compounds were assayed for *in vitro* protease inhibition along with "no inhibitor" (i.e., protease + substrate) and no protease (i.e., substrate alone) controls. Protease activities of each reaction were normalized to the "no inhibitor" control.

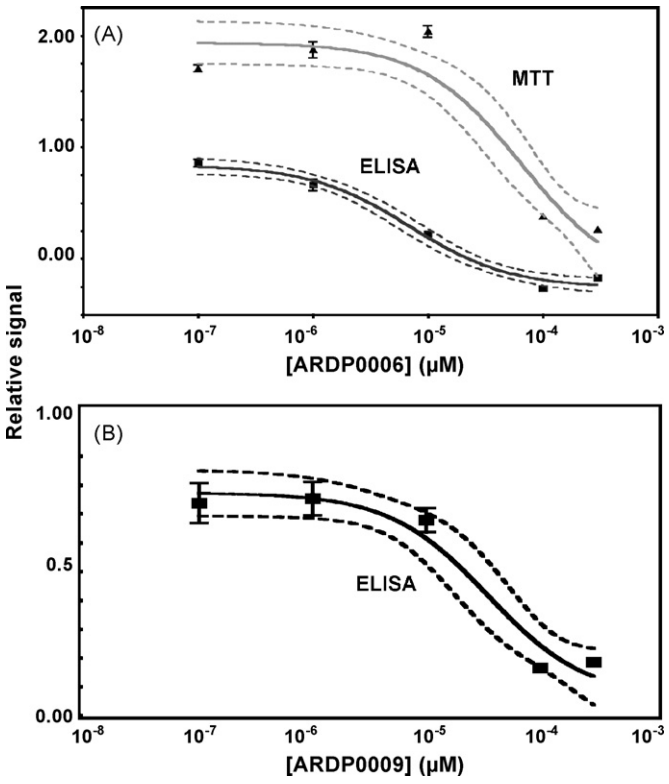


Fig. 2. Dose–response curves for compounds that demonstrated antiviral activity in cell-based ELISA replication assays. Toxicity was measured using a standard MTT assay. Curves were fit using Graphpad Prism 4. (A) Efficacy and toxicity dose–response curves for ARDP0006. (B) Efficacy dose–response curve for ARDP0009. Since this compound had no observed toxicity at the concentrations tested, its toxicity curve was not included.

solutions that were "cleared" by centrifugation to remove precipitants. *In vitro* DEN2V protease activity assays have been previously described (Leung et al., 2001; Yusof et al., 2000). The DEN2V NS2B cofactor linked to the protease domain of NS3 (NS2B-NS3pro; plasmid obtained from Dr. Padmanabhan, Georgetown University) was expressed and purified as previously described (Yusof et al., 2000). Protease activity was confirmed by cleavage of a dibasic fluorophore-linked peptide substrate, Boc-Gly-Lys-Arg-AMC (Fig. 1, substrate + enzyme control relative to substrate control). DEN2V NS2B-NS3pro (100 nM) was incubated with each soluble compound (100 μM, 1% DMSO) and 100 μM Boc-GKR-AMC (Bachem, USA) for 30 min at room temperature. Protease activity was determined by monitoring fluorescence intensity from AMC (excitation 280 nm, emission 465 nm) generated by cleavage from the peptide substrate. Protease reactions performed with 100 μM aprotinin, a known broad-spectrum serine protease inhibitor, showed fluorescence levels that were similar to that of the "substrate alone" background control (data not shown).

In vitro assay results indicated that more than half of the tested compounds showed reduced *in vitro* NS2B-NS3pro activity as compared to the "no inhibitor" control (Fig. 1B). The five compounds with the greatest *in vitro* inhibition of DEN2V protease (Fig. 1B) were further characterized for the ability to reduce DEN2V replication in cell cultures. These virus replication studies used the New Guinea C (Sabin, 1952; ATCC, Manassas, Virginia) and Thailand/16681/1964 (Halstead and Simasthien, 1970) strains of DEN2V. To test small molecules for inhibition of viral replication, we optimized an ELISA-like cell-based replication assay that monitored production of DEN2V viral proteins. For this assay, confluent LLC-MK2 cells in 96-well plates were infected with DEN2V at MOI 0.2. Final concentrations of compounds ranged from 10 nM to 300 μM, and were added to the cell monolayer at the time of infection. All assays were performed in quadruplicate. After 48 h incubation at 37 °C, cells were fixed, treated with polyclonal mouse DEN2V primary antibody (R. Tesh, Arbovirus Reference Collection, UTMB), washed, and incubated with HRP-conjugated anti-mouse secondary antibody. Following secondary antibody incubation, cells were washed and developed with 50 μL per well of the ELISA substrate 3,3′,5,5′-tetramethylenzidine (Sigma). Reactions were incubated at room temperature for 5 min, stopped with 50 μL 1N HCl, and results determined by measuring optical density at 450 nm in a microplate reader (Fig. 2).

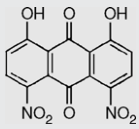
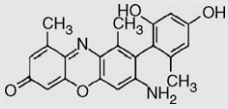
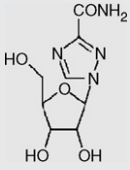
In parallel experiments, MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] toxicity assays were performed to determine possible cellular toxicity of the compounds. In these assays, compound was removed and MTT added to the cells. MTT reduction in the mitochondria of living cells yielded purple crystals which when solubilized can be quantified spectroscopically to determine drug toxicity as a function of living cells (Mosmann, 1983; Gerlier and Thomasset, 1986). Effective concentrations (EC_{50}) and cytotoxic concentrations (CC_{50}) were calculated from nonlinear regression fitting (GraphPad Prism 4, Graphpad, SanDiego, CA) of signal vs. concentration data points to the standard dose–response equation $Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{(\text{Log}(EC50)-X)})$. In this equation, X was the log of compound concentration, Y was the response signal, and bottom and top refer to plateaus of the sigmoid response curve (Fig. 2).

Using the above cell-based virus replication assay, the small molecules ARDP0006 and ARDP0009 were observed to have the best efficacy among the tested compounds at inhibiting DEN2V replication (Table 1). ARDP0009 had no apparent toxicity at the concentrations tested thus a lower limit to its toxicity was based on the maximum tested concentration. Selectivity indices (SIs; selectivity index = CC_{50}/EC_{50}) calculated for ARDP0006 and ARDP0009 were comparable to the SI calculated for ribavirin (Table 1), a nucleoside analog that has demonstrated inhibition of the DENV 2′-O-methyltransferase NS5 (Benarroch et al., 2004) and hepatitis C virus replication when used in combination with interferon.

The EUDOC-predicted bound conformations of ARDP0006 and ARDP0009, the two compounds that were active against DEN2V in cell culture, were analyzed to identify intermolecular contacts that might contribute to the observed inhibition. ARDP0006 was predicted to interact with active site and P1 pocket residues, including Gln 35 (adjacent to the active site), His 51 and Ser 135 of the active site, and Gly 151 and Gly 153 of the P1 pocket (Fig. 3A). Additional contacts (<4 Å) were made between ARDP0006 and protease residues Ser 131, Pro 132, Gly 133, Thr 134, Asn 152, and Val 155. ARDP0009 was also predicted to interact with the protease active site and P1 pocket residues, including Gln 35, His 51, Ser 135, Tyr 150, Gly 151, and Gly 153 (Fig. 3B). Additional contacts (<4 Å) were made between ARDP0009 and protease residues Leu 115, Asn 152, Val 155, and Ala 160. Both small-molecule inhibitors were also docked into the recently solved structure of active DEN2V NS2B-NS3 protease (PDB identifier 2FOM; Erbel et al., 2006). Although the NS2B-NS3 protease complex and the NS3 protease had several significant structural differences, docking predictions identified similar intermolecular contacts between the inhibitors and the DEN2V protease structures. The intermolecular contact analysis supported the inference that protease inhibition observed *in vitro* was responsible for inhibition of virus replication observed in the cell-based assays. However, further investigations will be required to unequivocally delineate the molecular mechanism of virus replication inhibition of our protease inhibitors.

In summary, we report a strategy that correctly predicted the protease inhibitory activity of several small molecules and assays to rapidly validate the computer predictions. This strategy incorporated virtual screening of a chemical library to identify small

Table 1
Inhibition of dengue virus in cell-based replication assays.

Compound	Structure	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
ARDP0006		4.2 ± 1.9	69 ± 4	16.6
ARDP0009		35 ± 8	>300	>8.6
Ribavirin (non-protease Inhibitor)		37 ± 1	>300	>8.1

[a] EC_{50} represents the concentration at which the ELISA signal has been reduced 50% in the cell-based replication assay.
[b] CC_{50} represents the concentration at which 50% of the cells have died in the toxicity (MTT) assay.
[c] SI (selectivity index) represents the CC_{50} divided by the EC_{50}.

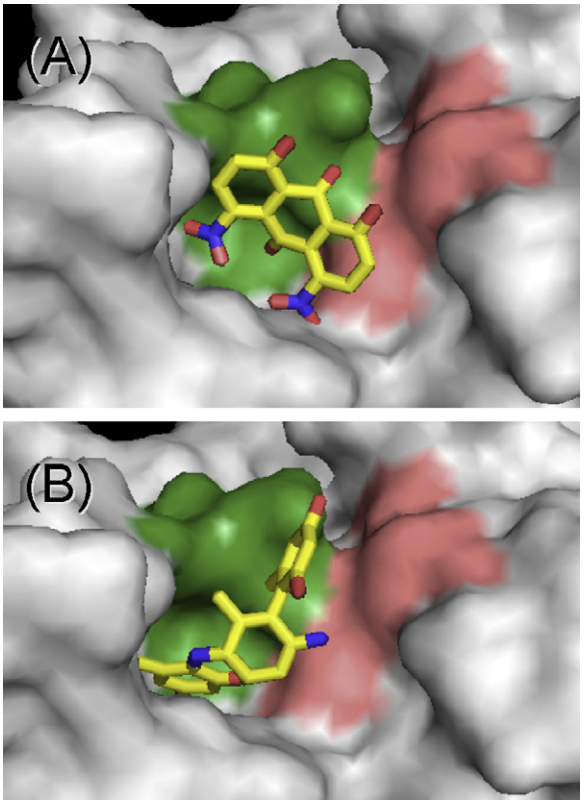


Fig. 3. EUDOC-predicted models of bound conformations of compounds that demonstrated dengue antiviral activity in cell culture. (A) ARDP0006 and (B) ARDP0009 were predicted to interact with active site (pink) and P1 pocket (green) residues of the DEN2V protease. Images were generated with SWISS PDB (Guex and Peitsch, 1997). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

molecules that fit into defined target sites (active site and P1 pocket) on the DEN2V NS3 protease crystal structure. These computer "hits" were tested for solubility and *in vitro* protease inhibition to establish activity and mechanism of inhibition. Several EUDOC "hits" demonstrated *in vitro* inhibition of the DEN2V NS3 protease. Significantly, these compounds were subsequently analyzed for antiviral activity in cell-based replication assays, in which two protease inhibitors showed dengue antiviral activity in cell culture. While others have reported limited *in vitro* studies to identify DEN2V protease inhibitors (Ganesh et al., 2005), we extended these studies to analyze protease inhibitors for cellular toxicity and inhibition of viral replication in cell culture. This strategy reflects a logical progression for early stage drug discovery that can be used to successfully identify candidate antiviral drugs.

Acknowledgements

We thank Drs. A. Barrett, S. Gilbertson, J. Halpert, J. Lee, and J. Stevens for helpful discussions. We thank Drs. S. Weaver and N. Vasilakis for providing dengue 2 strain 16681, Dr. R. Tesh for the dengue 2 antibodies, and Dr. Padmanabhan for the plasmid expressing dengue NS2B-NS3pro.

References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Principles of membrane transport. In: Molecular Biology of the Cell, 4th ed. Garland Science, New York, New York, pp. 616–619.
Alvarez, M., Rodriguez-Roche, R., Bernardo, L., Vasquez, S., Morier, L., Gonzalez, D., Castro, O., Kouri, G., Halstead, S., Guzman, M., 2006. Dengue hemorrhagic fever caused by sequential dengue 1–3 virus infections over a long time interval: Havana epidemic, 2001–2002. Am. J. Trop. Med. Hyg. 75, 113–117.
Bazan, J., Fletterick, R., 1989. Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses. Virology 171, 637–639.
Benarroch, D., Egolff, M., Mulard, L., Guerreiro, C., Romette, J., Canard, B., 2004. A structural basis for the inhibition of the NS5 dengue virus mRNA 2′-O-methyltransferase domain by ribavirin 5′-triphosphate. J. Biol. Chem. 279, 35638–35643.
Chambers, T., Weir, R., Grakoui, A., McCourt, D., Bazan, J., Fletterick, R., Rice, C., 1990. Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. Proc. Natl. Acad. Sci. U.S.A. 87, 8898–8902.
Chanprapaph, S., Saparpakorn, P., Sangma, C., Niyomrattanakit, P., Hannongbua, S., Angsuthanasombat, C., Katzenmeier, G., 2005. Competitive inhibition of the dengue virus NS3 serine protease by synthetic peptides representing polyprotein cleavage sites. Biochem. Biophys. Res. Commun. 330, 1237–1246.
Erbel, P., Schiering, N., D'Arcy, A., Renatus, M., Kroemer, M., Lim, S., Yin, Z., Keller, T., Vasudevan, S., Hommel, U., 2006. Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus. Nat. Struct. Mol. Biol. 13, 372–373.
Falgout, B., Pethel, M., Zhang, Y., Lai, C., 1991. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. J. Virol. 65, 2467–2475.
Gubler, D., Juno, G., Markoff, L., 1996. Flaviviruses. In: Fields, B., Knipe, D., Howley, P., Chanock, R., Melnick, J., Monath, T., Roizman, B., Strauss, S. (Eds.), Field's Virology, vol. 1, 3rd ed. Lippincott William & Wilkins, Philadelphia, PA, pp. 961–1034.
Ganesh, V.K., Muller, N., Judge, K., Luan, C.-H., Padmanabhan, R., Murthy, K.H.M., 2005. Identification and characterization of nonsubstrate based inhibitors of the essential dengue and West Nile virus proteases. Bioorg. Med. Chem. 13, 257–264.

Gerlier, D., Thomasset, N., 1986. Use of MTT colorimetric assay to measure cell activation. J. Immunol. Methods 94, 57–63.
Ghose, A., Viswanadhan, V., 2001. Fast continuum electrostatics methods for structure-based ligand design. In: Combinatorial Library Design and Evaluation: Principles, Software, Tools, and Applications in Drug Discovery. CRC Press, New York, New York, p. 223.
Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714–2723.
Halstead, S., 2003. Neutralization and antibody-dependent enhancement of dengue viruses. Adv. Virus Res. 60, 421–467.
Halstead, S.B., Simasthien, P., 1970. Observations related to the pathogenesis of dengue hemorrhagic fever. II. Antigenic and biological properties of dengue viruses and their association with disease response in the host. Yale J. Biol. Med. 42, 276–292.
Laird, E., Blake, J., 2004. Structure-based generation of viable leads from small combinatorial libraries. Curr. Opin. Drug Discov. Dev. 7, 354–359.
Leung, D., Schroder, K., White, H., Fang, N., Stoermer, M., Abbenante, G., Martin, J., Young, P., Fairlie, D., 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.
Leyssen, P., De Clercq, E., Neyts, J., 2000. Perspectives for the treatment of infections with Flaviviridae. Clin. Microbiol. Rev. 13, 67–82.
Morens, D., Fauci, A., 2008. Dengue and hemorrhagic fever: a potential threat to public health in the United States. JAMA 299, 214–216.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
Murthy, K., Clum, S., Padmanabhan, R., 1999. Crystal structure and insights into interaction of the active site with substrates by molecular modeling and structural analysis of mutational effects. J. Biol. Chem. 274, 5573–5580.
Murthy, K., Judge, K., DeLucas, L., Padmanabhan, R., 2000. Crystal structure of dengue virus NS3 protease in complex with a Bowman–Birk inhibitor: implications for flaviviral polyprotein processing and drug design. J. Mol. Biol. 301, 759–767.
Pang, Y.-P., Perola, E., Xu, K., Prendergast, F., 2001. EUDOC: a computer program for identification of drug interaction sites in macromolecules and drug leads from chemical databases. J. Comput. Chem. 22, 1750–1771.
Pang, Y.-P., et al., 2008. EUDOC on Blue Gene: accelerating the transfer of drug discoveries from laboratory to patient. IBM J. Res. Dev. 52, 69–81.
Sabin, A., 1952. Research on dengue during World War II. Am. J. Trop. Med. Hyg. 1, 30–50.
Sampath, A., Padmanabhan, R., 2009. Molecular targets for flavivirus drug discovery. Antivir. Res. 81, 6–15.
Wang, Q., Pang, Y.-P., 2007. Accurate reproduction of 161 small-molecule complex crystal structures using the EUDOC program: expanding the use of EUDOC to supramolecular chemistry. PLoS ONE 2, e531.
Yin, Z., Patel, S.J., Wang, W.-L., Wang, G., Chan, W.-L., Rao, K.R.R., Alam, J., Jeyaraj, D.A., Ngew, X., Patel, V., Beer, D., Lim, S.P., Vasudevan, S.G., Keller, T.H., 2006a. Peptide inhibitors of dengue virus NS3 protease. Part 1. Warhead. Bioorg. Med. Chem. Lett. 16, 36–39.
Yin, Z., Patel, S.J., Wang, W.-L., Chan, W.-L., Rao, K.R.R., Wang, G., Ngew, X., Patel, V., Beer, D., Knox, J.E., Ma, N.L., Ehrhardt, C., Lim, S.P., Vasudevan, S.G., Keller, T.H., 2006b. Peptide inhibitors of dengue virus NS3 protease. Part 2. SAR study of tetrapeptide aldehyde inhibitors. Bioorg. Med. Chem. Lett. 16, 40–43.
Yusof, R., Clum, S., Wetzel, M., Murthy, K., Padmanabhan, R., 2000. Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids in vitro. J. Biol. Chem. 275, 9963–9969.