A fluorescence quenching assay to discriminate between specific and nonspecific inhibitors of dengue virus protease

Bodenreider, C; Beer, D; Keller, T H; Sonntag, S; Wen, D; Yap, L; Yau, Y H; Shochat, S G; Huang, D; Zhou, T; Caflisch, A; Su, X C; Ozawa, K; Otting, G; Vasudevan, S G; Lescar, J; Lim, S P

Bodenreider, C; Beer, D; Keller, T H; Sonntag, S; Wen, D; Yap, L; Yau, Y H; Shochat, S G; Huang, D; Zhou, T; Caflisch, A; Su, X C; Ozawa, K; Otting, G; Vasudevan, S G; Lescar, J; Lim, S P (2009). A fluorescence quenching assay to discriminate between specific and nonspecific inhibitors of dengue virus protease. Analytical Biochemistry, 395(2):195-204.

Postprint available at:
http://www.zora.uzh.ch

Posted at the Zurich Open Repository and Archive, University of Zurich.
http://www.zora.uzh.ch

Originally published at:
A fluorescence quenching assay to discriminate between specific and nonspecific inhibitors of dengue virus protease

Abstract

In drug discovery, the occurrence of false positives is a major hurdle in the search for lead compounds that can be developed into drugs. A small-molecular-weight compound that inhibits dengue virus protease at low micromolar levels was identified in a screening campaign. Binding to the enzyme was confirmed by isothermal titration calorimetry (ITC) and nuclear magnetic resonance (NMR). However, a structure-activity relationship study that ensued did not yield more potent leads. To further characterize the parental compound and its analogues, we developed a high-speed, low-cost, quantitative fluorescence quenching assay. We observed that specific analogues quenched dengue protease fluorescence and showed variation in IC(50) values. In contrast, nonspecifically binding compounds did not quench its fluorescence and showed similar IC(50) values with steep dose-response curves. We validated the assay using single Trp-to-Ala protease mutants and the competitive protease inhibitor aprotinin. Specific compounds detected in the binding assay were further analyzed by competitive ITC, NMR, and surface plasmon resonance, and the assay's utility in comparison with these biophysical methods is discussed. The sensitivity of this assay makes it highly useful for hit finding and validation in drug discovery. Furthermore, the technique can be readily adapted for studying other protein-ligand interactions.
Identification of lead-like and non-lead-like inhibitors for dengue virus NS2B/NS3 protease by tryptophan fluorescence

Christophe Bodenreider¹, David Beer¹, Thomas H. Keller¹, Sebastian Sonntag¹, Daying Wen¹, LiJian Yap², Yin Hoe Yau², Susana Geifman Shochat², Danzhi Huang³, Ting Zhou³, Amedeo Caflisch³, Xun-Cheng Su⁴, Kiyoshi Ozawa⁴, Gottfried Otting⁴, Subhash G. Vasudevan¹,†, Julien Lescar⁴, and Siew Pheng Lim¹,*

¹Novartis Institute for Tropical Diseases, 10 Biopolis Road, Chromos, Singapore 138670.
²School of Biological Sciences, Nanyang Technological University, 60, Nanyang Drive, Singapore 637551.
³University of Zürich, Department of Biochemistry, Winterthurerstrasse 190, CH-8057, Zürich, Switzerland
⁴The Australian National University, Research School of Chemistry, Canberra ACT 0200, Australia

Running title: Fluorescence binding assay to detect promiscuous inhibitors for dengue virus protease
*To whom correspondence should be addressed

Phone: +65 6722 2924. Fax:+65 6722 2916. Email: siew_pheng.lim@novartis.com

†Current address: School Emerging Infectious Diseases Program, Duke-NUS Graduate Medical School, Singapore 169547.

Abbreviations: AMC, 7-amino-4-methyl coumarin; BPTI, Bovine Pancreatic Trypsin Inhibitor; bz, benzoyl; CF40-gly-NS3pro185, NS2B (amino acids 1394-1440) fused to NS3 protease domain (amino acids 1476-1660) via 9 amino acids (Gly_{4}-Ser-Gly_{4}); DENV1-4, dengue virus serotypes 1-4; FRET, Förster resonance energy transfer; HTS, high-throughput screening; IC_{50}, concentration of compound that inhibits 50% of enzyme activity; IPTG, isopropyl β-D-thiogalactoside; IFE, inner filter effect; ITC, isothermal titration calorimetry; n, nle, norleucine; NS2/NS3, non-structural proteins 2/3; PAGE, polyacrylamide gel electrophoresis; RFU, relative fluorescence units; SAR, structure-activity relationship; SDS, sodium dodecyl sulphate; SPR, surface plasmon resonance; WNV, West Nile virus; WT, wild type; YFV, Yellow Fever virus.
ABSTRACT

A small molecular weight compound that inhibits dengue virus (DENV) protease at low micro-molar levels was identified from follow-up studies in a screening campaign. Isothermal titration calorimetry (ITC) and nuclear magnetic resonance confirmed its binding to the enzyme. A structure-activity-relationship study that ensued did not yield more potent leads. For better characterization, the parental compound and its analogues were tested against DENV types 1-4 and West Nile virus proteases. In order to discriminate between specific and non-specific inhibition, we developed a high-speed, low-cost, and quantitative fluorescence quenching assay. We observed that non-specifically binding compounds did not quench DENV protease fluorescence, and showed similar IC$_{50}$ values with steep dose-response curves. In contrast, specific inhibitors quenched DENV protease fluorescence and showed variation in IC$_{50}$ values. Specific compounds detected in the fluorescent binding assay were verified by competitive ITC and further analyzed by surface plasmon resonance. The utility of this fluorescence binding assay in comparison with other biophysical methods tested is discussed. The sensitivity of this binding assay makes it highly useful for hit-finding and hit-to-lead activities in drug discovery.
INTRODUCTION

High throughput screening (HTS) is a major method in drug discovery for new small lead molecules. In high-throughput enzyme inhibition assays, over a million compounds are usually tested for their abilities to inactivate the target enzyme. Often, the process involves two consecutive screens (1, 2). The first screen identifies compounds that inhibit the enzymatic activity and is usually performed at a single compound concentration. The second screen confirms the positive hits from the former by measuring IC\textsubscript{50} values i.e. the concentration of compound needed to obtain 50% inhibition of the enzyme in the in vitro assay. After the second round, one may still be left with hundreds of lead candidates, many of which could be false positives (3).

Often one or more candidate lead compound classes are chosen as starting points for structure-activity relationship (SAR) studies. Compounds are chemically derivatized in order to improve their inhibitory capacity or IC\textsubscript{50}. IC\textsubscript{50} measurements alone, however, contain no information on the inhibitory mechanism. It has been widely documented that an IC\textsubscript{50} value can arise from many mechanisms besides specific inhibition such as target sequestration, compound aggregation or interference with the enzymatic assay (4). IC\textsubscript{50} values may thus serve as a disqualifying parameter (if the compound is only weakly active) but should not be the only qualifying parameter for identification of a lead compound.

It is crucial, therefore, to have additional screening methods in drug discovery to determine the specificity of a compound for the target in order to deliver high quality candidate compounds for lead generation. Since dozens of compounds are usually evaluated during hit confirmation and hit-to-lead chemistry, additional screening methods have to satisfy three requirements: (i) low cost (in terms of amounts of compound and protein needed), (ii) high throughput and (iii) the ability to identify specific binding.

The best method to characterize inhibition mechanism is via enzyme kinetics studies. In general this cannot be done systematically since the throughput is very low and analyses of mechanisms of complex enzymes are challenging. Established biochemical methods to identify non-specific inhibitors include
the variation of enzyme concentrations and the addition of non-ionic detergent (such as triton-X-100) or bovine serum albumin (BSA) in the enzymatic assay (3). In particular, additives such as triton X-100 can discriminate specific from non-specific inhibitors that act via formation of colloidal aggregates (5, 6). Not all enzyme assays, however, tolerate triton-X-100, which is also not necessarily compatible with robotic screening platforms. In addition, as the promiscuous nature of some inhibitors depends on the protein target, this method cannot eliminate all false positives (3).

Other known screening methods employ biophysical techniques to detect binding of compounds to the target enzyme. A compound with a good IC$_{50}$ value often has a good binding affinity (or low dissociation constant $K_D$) (7). Table 1 presents a non-exhaustive list of techniques used to measure binding. Isothermal titration calorimetry (ITC) is one of the best methods for binding studies as it yields binding and thermodynamic parameters that can be highly useful for guiding SAR and compound design (8). Despite the recent development of high-throughput and small-volume calorimeters, ITC remains a costly method in terms of quantity of protein needed.

Nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography can yield very valuable structural information about the binding sites but requires too much protein and compound for acceptable throughput. ITC, NMR and crystallography also share the disadvantage of requiring compounds often at concentrations above 100 µM. As compounds used in drug discovery tend to be highly hydrophobic in nature they are frequently not very soluble at these concentrations in aqueous buffer, thereby limiting the types of compounds that can be analyzed by these methods.

Surface plasmon resonance (SPR) could be a method of choice since good throughput is easily achievable (9). However, immobilized proteins may not retain their native characteristics. Finally, since proteins are stabilized by ligand binding, this effect can be measured by thermal denaturation experiments (10). Although the throughput of this method is good and costs are low, this technique does not assess specificity of binding as proteins may also be stabilized by non-specific ligands.

This article reports the development of a selective and high-throughput binding assay in the context of anti-dengue drug discovery. Dengue virus (DENV) protease is an obvious target for anti-dengue drug
development(11, 12). It plays a key role in the replication of the virus by cleaving the viral polyprotein precursor after translation. The protease domain is contained in non-structural protein 3 (NS3)(13) and its activity is greatly enhanced by interactions with the NS2B protein which acts as its co-factor(14, 15).

Several efforts to find inhibitors for different flavivirus proteases (West Nile virus (WNV), dengue and Yellow Fever) have been reported, with several studies focusing on peptidic substrate based inhibitors(16-21) while others identified non-peptidic inhibitors from in vitro screening (22-24) or in silico high-throughput docking(25). None of these compounds has the appropriate properties for drug development, either because the scaffold is too labile(19-21, 23) or the inhibitors bind too weakly(22, 24).

Our attempts to identify a non-peptidic inhibitor of dengue protease led to establishment of a simple and efficient binding assay based on tryptophan fluorescence as described herein. It provides quantitative compound binding affinities and identifies promiscuous inhibitors in an inexpensive way that is compatible with a high-throughput setup.
MATERIALS AND METHODS

Chemistry. All compounds generated in Figure 1 were determined to have >95% purity by HPLC. See supplementary information.

Materials. All compounds (1-9, Bz-nKRR-H) were chemically synthesized in-house. Fluorogenic peptide substrate Bz-Nle-Lys-Arg-Arg-AMC was purchased from LSU Health Sciences Center (New Orleans, LA) and bovine pancreatic trypsin inhibitor was purchased from Sigma-Aldrich (St Louis, MO). PCR was carried out using Turbo Pfu polymerase from Stratagene (La Jolla, CA). Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Research Biolabs (Singapore).

Cloning of DENV2 CF40-gly-NS3pro185 W mutant constructs. All W mutants were generated using overlapping PCR using the plasmid DENV2 TSV01 pET15b-CF40-gly-NS3pro185 as a template(26). To obtain cNS2B with W mutations, PCR was carried out using the forward primer NS2BcfXhoI-F (26) and reverse primers W5A-REV, W50A-REV, W69A-REV, W83A-REV and W89A-REV respectively (Supporting information Table S1). To obtain NS3pro185 with W mutations, PCR was carried out using the forward primers W5A-FOR, W50A-FOR, W69A-FOR, W83A-FOR, and W89A-FOR, respectively (Supporting information Table S1), and reverse primer NS3pro185BamHI-R(26). The two products were joined in the second round of PCR using the primer-pair NS2BcfXhoI-F and NS3pro185BamHI-R to generate the individual CF40-gly-NS3pro185 W mutants. The overlapped PCR products were digested with XhoI and BamHI, and ligated into the same sites in pET15b.

Protein expression and purification. Expression and purification of DENV2 CF40-gly-NS3pro185 has been described previously(26). Briefly, E. coli BL21-CodonPlus(DE3)-RIL (Stratagene) transformed with these plasmids were grown in Luria-Bertani (LB) broth, supplemented with ampicillin (100 mg/ml) and chloramphenicol (50 mg/ml) at 37 °C until OD₆₀₀ reached approximately 0.5. Protein expression was induced with 0.4 mM IPTG at 16 °C for 20 h. Cells were harvested by centrifugation and
resuspended in 4 ml of phosphate buffered saline, containing 1% Triton X-100 (PBT). Cells were lysed by sonication and debris removed by centrifugation at 35,000 rpm for 30 min. The resulting protein solution was filtered through a 0.22 µm filter and loaded onto a 5 ml HiTrap chelating HP (Amersham Biosciences, Piscataway, NJ) column equilibrated with lysis buffer. The resin was washed with 10 column volumes lysis buffer before bound proteins were eluted from the column with lysis buffer and a linear gradient of imidazole from 20-300 mM in the same buffer. Peak fractions were analyzed by 10% SDS-PAGE. Positive fractions were pooled, desalted and concentrated with spin concentrators (Amicon Ultra-15ml, molecular weight cut-off 10,000 Da; Millipore, Billerica, MA).

Enzymatic Assay. The assay has been described previously (18). Briefly, activities of cNS2B/NS3pro complexes were measured in a Safire\textsuperscript{2} plate reader (Tecan) ($\lambda_{\text{exc}} = 385$ nM; emission: $\lambda_{\text{exc}} = 465$ nM) and performed in a final volume of 50 µl containing 50 mM Tris/HCl, pH 7.5, 1 mM CHAPS, 20% glycerol and 50 µM Bz-Nle-Lys-Arg-Arg-AMC at 37 °C. Control reactions contained 50 nM of CF40-gly-NS3pro185. The proteolytic reaction was monitored by an increase in fluorescence (RFU/min) which was subsequently converted to M·s\textsuperscript{-1} from a standard AMC calibration curve. Progression curves were fitted to Michaelis-Menten kinetics by nonlinear regression using GraphPad Prism. Steady-state kinetic constants of each substrate were determined from triplicate measurements and reported as mean ± Standard Error (SE).

Inhibitors were assayed in a 96-well plate format using 50 mM Tris/HCl, pH 7.5, 1 mM CHAPS in a final volume of 50 µl. Typically, protease (40 nM) was pre-incubated with 0-100 µM concentrations of test compounds at 37 °C for 30 min. The reaction was initiated by the addition of 20 µM substrate Bz-nKRR-AMC. Reaction progress was monitored continuously by following the increase in fluorescence on a Tecan Safire\textsuperscript{2} plate reader. IC\textsubscript{50} values of inhibitors were derived by fitting the calculated initial velocities to a non-linear regression curve using GraphPad Prism software. Each point of the IC\textsubscript{50} curve was measured in duplicate during a single experiment.
Buffer. The buffer used for absorbance spectra, titration, and ITC was 50 mM Tris/HCl, pH 7.5, 50 mM NaCl.

Absorbance Spectra. Absorbance spectra of compounds were measured on a Tecan Safire². All compounds were diluted in 90 µl buffer to a final concentration of 100 µM on a UV-star 96-well microplate (Greiner).

Fluorescence-monitored Titrations. Two protein solutions were prepared (2-5 µM protein with and without 40 µM compound) and were mixed in a microplate to obtain 12 different compound concentrations ranging from 0-40 µM in about 3.5 µM steps. 90 µl of each dilution were thereafter transferred to a UV-star Greiner microplate 96-wells. After 1 h incubation at room temperature, fluorescence was measured at 25 °C on a Tecan Safire² with λ_{exc}=280 nm and λ_{em}=340 nm. The setting for slit widths depended on the protein concentration used. For 3 µM protein the slits were 10 and 20 mm for excitation and emission, respectively. At the end of the measurements, 11 µM of BPTI were added to the wells containing 40 µM compound and the fluorescence was re-measured. Binding curves were analyzed according the following two-state model describing the formation of a 1:1 complex:

\[ P + L \xrightleftharpoons[\text{Kd}]{LD} PL \]

The corresponding binding equation is

\[ \text{Signal} = \text{Offset} + \text{Amp} \cdot \frac{(Kd + Pt + Lt) - \sqrt{(Kd + Pt + Lt)^2 - 4Lt.Pt}}{2.Pt} \]

Equation 2

where \( K_D \) is the equilibrium dissociation constant, Pt and Lt are the total protein and ligand concentrations, respectively, and Amp is the amplitude of the measured response. This equation was used to analyze the binding isotherms. The data were fitted using the non-linear least square option of the GraphPad Prism software.
**Isothermal Titration Calorimetry.** Calorimetry experiments were performed with a Auto-ITC microcalorimeter (Microcal). All titrations were done at 25 °C. For standard experiments, 6 µM of NS2B/NS3 protease was titrated with 10 µl injections of 80 µM BPTI. Each titration comprised an initial 1 µl injection. The stirring speed used was 300 rpm, and the reference power was 10 µcal/sec. The heat of the last injection of each titration series was subtracted from the titration data to account for the heat of dilution. For competition experiments, compounds were added to the protein in the cell. DMSO was added to the BPTI solutions in the syringe to obtain equal DMSO concentrations in the cell and in the syringe. $K_D$ values of the compounds from competition experiments with BPTI were estimated using the relationship:

$$K_{D \text{--apparent}} = \frac{K_{D \text{--BPTI}}}{K_{D \text{--compound}}} \cdot [\text{Compound}] + K_{D \text{--BPTI}} \quad \text{Equation 3}$$

where $K_{D \text{--compound}}$ is the true $K_D$ value for the protease-compound association, $K_{D \text{--BPTI}}$ is the $K_D$ for the protease-BPTI association in the absence of compound and $K_{D \text{--apparent}}$ is the apparent $K_D$ for protease-BPTI association in the presence of compounds.

**SPR biosensor measurements.** Measurements were done on a Biacore 3000 instrument (Biacore, Uppsala, Sweden). DEN2 CF40-gly-NS3pro186 with a C-terminal Cys residue was immobilized via the engineered cysteine to a level of 7000RU on a carboxymethyl-dextran sensor surface (CM5) using ligand-thiol coupling chemistry, according to the following procedure, where each step was performed sequentially at a flow rate of 10 µl/min: (i) 7-min injection of a mixture of 0.1 M NHS and 0.4 M EDC, (ii) 3-min injection of 80 mM PDEA, (iii) 3.5-min injection of 1.0 M ethanolamine-HCl, (iv) 5-min injection of CF40-gly-NS3pro186 in sodium acetate, pH 4.0, (v) 7-min injection of 50 mM cysteine in 1 M NaCl. The control surface was treated in an identical way, omitting the injection of the protein. The inhibitors were diluted with 50 mM phosphate buffer, pH 7.4, and 5% DMSO (final concentration). The same buffer was used as running buffer throughout the experiment. 0.078 to 12.5 µM inhibitors were
injected for 1 min sequentially over the reference and test flow-cells at a flow rate of 30 µl/min. Raw sensorgrams were reduced and solvent-corrected using a DMSO calibration curve(28, 29) using the Scubber software package (BioLogic Software, Campbell, Australia). Binding affinities were evaluated by fitting the data to the 1:1 Langmuir and steady-state models using Biaevaluation 4.1.
RESULTS

HTS/Choice of Lead Compound/Structure of Inhibitor. We previously reported the development of a highly sensitive and robust in vitro assay using the substrate, Bz-nKRR-AMC, for monitoring dengue single-chain NS2B/NS3 protease activity(26). The assay was used to test peptidic inhibitors against DENV and WNV proteases(17, 19, 20). We adapted it into a 1536-well format and used it to screen our in-house library comprising approximately $10^6$ compounds. One class of compound was observed to inhibit DENV2 and WNV proteases with IC$_{50}$ values of 2 and 3 µM respectively. However, this compound class and its synthetic intermediates were highly insoluble and the synthesis and testing of analogues proved to be challenging (data not shown).

A preliminary search for structurally related compounds yielded a second compound, 1, with a more readily amenable scaffold (IC$_{50}$ for DENV and WNV = 6 and 24.5 µM respectively; Figure 1). Binding of 1 to the catalytic pocket was verified by NMR spectroscopy, where the presence of 1 led to a dramatically improved $^{15}$N-HSQC spectrum of the DENV2 protease (Figure S1). Although, in the absence of resonance assignments of the protease, this does not prove binding of 1 to the catalytic pocket, the same observation was made in $^{15}$N-HSQC spectra of the WNV protease. For the WNV protease, NMR resonance assignments are available and intermolecular NOEs showed that 1 binds to the catalytic pocket (Su et al., manuscript submitted).

We subsequently initiated chemistry efforts using compound 1 as lead compound with the goal to generate a more potent DENV protease inhibitor. In addition, in the absence of a crystal structure of DENV protease-inhibitor complex, binding mode of compound 1 was obtained by automatic docking with a suite of programs SEED/FFLD and CHARMM minimization(30-33; Figure 2A) using the structure of WNV protease bound with a tetrapeptide aldehyde inhibitor Bz-nKRR-H(34). The central phthalazine ring is located in a cavity of S1 pocket and forms π-π interaction with the phenyl group of Tyr161. Both charged imidazoline groups are involved in salt bridge or hydrogen bonds with several residues (Asp129, Gly159 and Asn84). Furthermore, one of the NH group linking the phenyl and phthalazine moieties forms a hydrogen bond with Pro131 backbone carbonyl oxygen. These results are
in agreement with intermolecular NOEs reported for the homologous compound 5 (Su et al., manuscript submitted).

The predicted binding mode of compound 1 in the protease catalytic site (Figure 2A), as well as quantum-mechanical calculations to determine the lowest-energy state of compound 1 and derivatives thereof, were used to design more analogues.

Based on these data, about 130 compounds were subsequently synthesized. Several analogues inhibited DENV and WNV proteases at IC\textsubscript{50} values in the low micromolar range (representative compounds are shown in Tables 2 and 3). Yet, despite extensive efforts, none of the analogues synthesized possessed sub-micromolar inhibitory activities and no clear SAR emerged from these studies. It is not unusual that micromolar leads with unfavorable physical properties (e.g. solubility) show flat SAR and turn out to be unsuitable for lead optimization. In this case, however, we had clear evidence that the lead compound was binding to the target protein and therefore the absence of SAR was puzzling.

\textit{IC\textsubscript{50} values for different DENV serotypes.} To expand our understanding of the inhibitory properties of this class of compounds, we tested a subset on proteases from the four different dengue serotypes (DENV1, 2, 3 and 4). NS2B/NS3 proteases from DENV1-4 share between 50-70\% sequence identity\textsuperscript{(35)} and NS3 amino acid residues forming the substrate binding pockets S1, S2 and S3 are mostly conserved\textsuperscript{28}. Differences between the catalytic pockets of DENV1-4 arise from non-conserved NS2B amino acid residues that participate in formation of S2 and S3 pockets\textsuperscript{31}. These variations could conceivably alter the binding affinities of inhibitors, by changing the NS2B structure or its ability to re-organize upon ligand binding\textsuperscript{(34)}. Indeed IC\textsubscript{50} values obtained with the peptidic inhibitor Bz-nKRR-H, varied from 1.4 to 11.8 \textmu M across DENV1-4 (Table 2). This is in line with our previous finding that despite similar preferences for P1-P4 residues in substrate sequences, their overall catalytic efficacies are not the same\textsuperscript{(26)}.
Amongst several structurally similar compounds, we found two different types of behaviors. One group, represented by inhibitors 1, 3, 5 and 8 showed selectivity amongst the different proteases with inhibition curves that exhibited Hill slopes ranging from 1-2. The second group represented by compounds 2 and 7 had similar inhibitory effects on different proteases and consistently displayed Hill slopes much larger than 1 (Table 2), suggesting that they act as non-specific inhibitors. Compounds 4, 6 and 9 were control compounds that were poorly or non-inhibitory (Table 2).

Tryptophan Fluorescence. DENV2 NS2B/NS3pro contains five tryptophans (W5, W50, W69, W83, W89) in the NS3pro moiety and one tryptophan in the NS2B co-factor (W23). Since no 3D structure of DENV2 NS2B/NS3pro in complex with a ligand is available, Figure 2B illustrates the locations of the tryptophan residues in the structure of the homologous WNV NS2B/NS3pro (PDB accession code 2FP7(34)). All tryptophan residues of DENV2 NS2B/NS3pro are also present in WNV NS2B/NS3pro(34). None of the tryptophans is in the protease catalytic pocket and the model of the complex with compound 1 suggests the absence of any direct contacts between 1 and any of the tryptophan residues. Nonetheless, to test whether tryptophan fluorescence can be used to monitor compound binding, we utilized 1 as a reference compound. The absorbance spectrum of 1 has two main absorption bands at 300 and 380 nm and overlaps with tryptophan emission, usually at 305-360 nm (Figure 3). When bound to the protease active site, compound 1 can potentially influence the fluorescence of the nearby tryptophans by a Förster resonance energy transfer (FRET). Since 1 has negligible intrinsic fluorescence (data not shown), its binding can be followed by monitoring the decrease in fluorescence signal intensity of the tryptophan residues.

Binding of compound 1. We titrated 4 μM DENV2 NS2B/NS3pro with 1 (from 0 to 40 μM) and monitored fluorescence emission at 340 nm (excitation at 280 nm). Addition of 1 led to fluorescence quenching of the tryptophans in the protease (Figure 4A). The effect was not caused by a change in the environment of the tryptophan side chains, as their emission wavelength maximum (λ<sub>max</sub>) remained
unchanged. A plot of fluorescence intensity versus inhibitor concentration showed a classical binding isotherm (Figure 4B). Nevertheless, the observed shallow shape of the binding isotherm indicated that the protein concentration used was comparable to or below the $K_D$ value of the inhibitor, thus preventing a good estimate of binding stoichiometry. Therefore, 1:1 binding stoichiometry was assumed for all fits to allow comparison between different compounds. The $K_D$ value obtained for 1 was $6 \pm 2.6 \mu M$ and is comparable to its IC$_{50}$ value obtained in the enzyme assay (Table 2).

Binding of 1 to NS2B/NS3pro catalytic pocket was further proven by using bovine pancreatic trypsin inhibitor (BPTI or aprotinin)\(^{(36)}\), which is a known competitive ligand of the protease. BPTI did not induce any fluorescence change upon binding to the protease (data not shown). When BPTI was added to wells containing DENV2 NS2B/NS3pro and compound 1, we observed an increase of fluorescence (Figure 4B). This indicates that BPTI can displace the binding of 1, and confirms that the latter binds in the same pocket as BPTI. Competitive binding was also observed with the peptidic inhibitor Bz-nKRR-H \(^{(34)}\) (data not shown).

Which Tryptophan is targeted in the quenching experiments? In order to decipher which tryptophan residue was targeted by the quenching experiments, we systematically mutated the five tryptophan residues of DENV2 NS3pro (W5, W50, W69, W83, W89) to alanine and titrated the mutant proteins with compound 1. Normalized titration curves revealed that the mutants and WT showed similar $K_D$ values for 1 ranging from 5-8 $\mu M$ (Figure 4C), with the exception of the W83A mutant (not shown). The crystal structures of DENV and WNV proteases show that W83 is buried, suggesting that W83A mutation destabilizes the protein structure. W83A was also the least active mutant, with only 1% of the WT activity (data not shown). Thus the role of W83 in the fluorescence studies could not be accurately assessed.

In contrast, the other mutants allowed us to pinpoint tryptophan residues affected by inhibitor binding. Most of the fluorescence change from binding of compound 1 could be attributed to W50 as mutating this residue to alanine resulted in the greatest loss in fluorescence intensity (Figure 4C). This is not
surprising since W50 is in direct proximity of the binding site(34) (residue H51 is part of the catalytic triad). Thus, the titration experiment with W50A further ascertained that 1 was binding to the catalytic pocket. W69 and W89 are also involved in compound induced FRET as mutating these to alanine likewise resulted in significant loss in fluorescence signal. In contrast, fluorescence was unchanged in W5A, implying that this residue plays a negligible role in the signal change.

Compounds binding to DENV2 protease. For SAR analysis, fluorescence titrations were performed with 63 compounds. Some representative absorbance spectra of the compounds are shown in Figure 3. Since the parent compound 1 had the optical properties required to quench tryptophan fluorescence, most of the structurally related analogues (all bearing the central phthalazine rings) from the SAR study had the same property. Six of these titrations are represented in Figure 5A and the \( K_D \) values obtained are listed in Table 3. These compounds spanned a wide range of IC\(_{50}\) values (from 1 to 55 \( \mu \)M; Table 3). To our surprise, only one out of four compounds with good IC\(_{50}\) values showed good binding (3, IC\(_{50}\) = 1.1 \( \mu \)M, \( K_D \) = 7.7 \( \mu \)M). The binding affinity of 3 was similar to 1, despite 5-fold difference in their IC\(_{50}\) values. Of the remaining three compounds, one showed intermediate binding (5, IC\(_{50}\) = 5.8 \( \mu \)M, \( K_D \) = 18.6 \( \mu \)M) whilst two were poor binders (2, IC\(_{50}\) = 2.3 \( \mu \)M, \( K_D \) = 100 \( \mu \)M and and 7, IC\(_{50}\) = 1.6 \( \mu \)M, \( K_D \) = 100 \( \mu \)M). Titration of these compounds with BPTI resulted in displacement of 3 and 5 only, indicating that only these compounds are competitive binders and not compounds 2 and 7. These results are in agreement with the steep Hill slopes observed in the inhibition curves of DENV 1-4 proteases for the latter two compounds. On the other hand, \( K_D \) values for two compounds that showed intermediate binding (4, and 8, \( K_D \) = 22.9 and 16.3 \( \mu \)M respectively) correlated well with their IC\(_{50}\) values and likewise, two poorly inhibitory compounds (6 and 9; IC\(_{50}\) = 55 and >100 \( \mu \)M) were also found to be weak or non-binders. When BPTI was added to the wells after each titration, competition was observed for compounds 4, 6, and 8, but not 9, demonstrating their different binding specificities.
Compounds binding to WNV protease. WNV and DENV proteases are homologous proteins but show different sensitivity towards different compounds. For example, compound 6 displayed a relatively low IC$_{50}$ value for WNV protease (6.2 µM) but not for DENV2 (55 µM; Table 3). This raised the question whether the tryptophan fluorescence assay would detect these differences. The same fluorescence quenching experiments can be applied for WNV and DENV proteases since tryptophans are present in both proteins at the same positions. We titrated WNV with the compounds as before and obtained a $K_D$ value for compound 1 that was similar to its IC$_{50}$ value (Figure 5B; Table 3). Interestingly, two compounds which were weak inhibitors and binders to DENV2 protease displayed good IC$_{50}$ values and good binding affinities to WNV protease (compounds 4 and 6; $K_D$ = 4.6 and 7 µM, respectively). Conversely, some of the compounds which were more potent against DENV than WNV, bound only weakly to the WNV protease and showed poor $K_D$ values for the latter (compounds 3 and 5, $K_D$= 90 and 100 µM respectively). Finally, compounds 2 and 7 which showed comparable IC$_{50}$ values in DENV2 and WNV, also bound poorly to WNV ($K_D$ = 100 µM).

Isothermal Titration Calorimetry. ITC was used to corroborate the binding affinities obtained by tryptophan fluorescence. Due to low compound binding affinities and low solubilities, no satisfactory data were obtained in ITC experiments where the compounds were directly titrated to the DENV2 protease. To circumvent this experimental barrier, we performed competition ITC using BPTI. Titration of 6 µM protease with 80 µM BPTI resulted in exothermic binding and gave $K_D$ = 19 nM, n = 1.19 and $\Delta H = -5.5$ kcal/mol (Figure 6). Competition experiments were performed by titrating the protein-BPTI complex with different compounds. Figure 6 shows representative examples of titrations performed with compounds 3, 8, and 9. Compound 9 was used as a control since it did not inhibit the protease activity and did not show any binding to the protease by fluorescence (Table 3). Addition of 100 µM of compound 9 did not induce any significant change on the apparent $K_D$ or the binding enthalpy of BPTI (30 nM and -5.4 kcal/mol). In contrast, 100 µM compound 3, and 150 µM compound 8 resulted in the respective changes in BPTI binding: $K_D$ = 125 nM and $\Delta H = -3$ kcal/mol, and $K_D$= 77 nM and $\Delta H = -2.6$
kcal/mol. Other compounds were also tested for competition by ITC (Table 3). In summary, based on changes observed in the apparent $K_D$ and/or on the binding enthalpy for BPTI, compounds that clearly competed with BPTI binding were: 1, 3, 5, and 8 whilst non-competing compounds were 2 and 9. Results from ITC experiments concurred with the previous fluorescence quenching data as compounds 1, 3, 5 and 8 were identified as specific protease binders. Conversely, compounds which showed no binding in fluorescence are also negative in ITC (2, 7).

Surface plasmon resonance. Binding affinities between compounds 1, 2, 3, 5, 8, 9 and DENV2 protease were evaluated using an SPR biosensor. DENV2 protease was immobilized on the matrix via an engineered C-terminal cysteine and increasing concentrations of compounds were passed across (from 78 nM to 12.5 µM). The results are summarized in Table 3. Generally, the compounds showed fast on- and off-rates in this concentration range. Compounds 1 and 8 gave satisfactory sensorgrams allowing a straightforward analysis. Binding affinity for compound 1 was evaluated by fitting the data to the 1:1 Langmuir (Figure S2) and steady-state models (data not shown), which returned similar $K_D$ values of 3.75 µM and 2 µM, respectively. However, at concentrations above 12.5 µM, we observed deviations from the 1:1 fit presumably due to non-specific binding to the immobilized protein or carboxy methyl matrix. A competition assay showed that BPTI completely displaced 1 from its binding site on the protease (Figure S3). This corroborated with results from other biophysical methods tested that compound 1 shares part of the binding site of BPTI.

Following steady-state model, compound 8 yielded a $K_D$ value of 4.3 µM. Compounds 2, 3, 5 and 9 displayed evidence of non-specific interactions. Following addition of 150mM NaCl to the phosphate buffer, specific binding was observed for 3 ($K_D = 0.3$ µM), but not for compound 5 ($K_D = >100$ µM). Non-specific binding persisted for compounds 2 and 9.

DISCUSSION
This study highlights the importance in drug discovery to confirm IC_{50} measurements with binding assays. The tryptophan fluorescence quenching assay presented here provided information on the binding mode of the compounds, including their binding site and the tightness of association. Using this assay, we systematically measured the binding affinities of 63 compounds generated from the SAR study and compared the \( K_D \) values obtained with their IC_{50} values (data not shown). We observed that in a number of instances, small modifications of compound structures led to dramatic changes in specificity of inhibition. These changes were not always detectable by IC_{50} measurements. Nine key compounds were further characterised and are presented in this report.

**Tryptophan fluorescence quenching.** Our experiments revealed that the change in tryptophan fluorescence upon protease-compound binding was caused by a FRET mechanism where the compounds acted as tryptophan fluorescence acceptors. Binding of compounds did not induce any apparent conformational changes in the immediate vicinity of tryptophan side chains as no change in emission maximum was observed (Figure 4A). Our studies with Trp-to-Ala mutants showed that fluorescence change induced upon compound binding was mostly due to W50 in the NS3 moiety. W50 is the tryptophan nearest to the active site, and in the co-complexed WNV protease-Bz-nKRR-H crystal structure (34), the C-\( \alpha \) distance between W50 and the center of the peptide is about 10-15 Å. Is this distance sufficiently short for a FRET? Förster distances \( R_F \) (where \( R_F \) corresponds to the distance where 50% FRET efficacy is observed) of 6-12 and 21 Å have been reported for Trp-Trp homo-transfer and Trp-Dansyl pairs(37), respectively. As the distance between W50 and the compound falls within this range, the significant FRET observed is fully expected.

A potential pitfall associated with fluorescence spectroscopy is the inner filter effect (IFE) which arises when the absorbance of the sample becomes too high at the excitation and emission wavelengths. IFE causes a non-linearity of the fluorescence signal which can be estimated by

\[
F_{obs} = F \cdot 10^{-\frac{A_{obs} + A_{exc}}{2}} \quad \text{Equation 1}
\]
where $F_{obs}$ is the measured fluorescence, $F$ is the fluorescence after IFE correction, and $Abs_{exc}$ and $Abs_{em}$ are the absorbance at the excitation and emission wavelength, respectively(38). The compounds tested in this study absorbed both at 280 and 340 nm so that, in principle, IFE could have contributed to the signal change observed. A control experiment where 15 µM solutions of N-acetyl-tryptophanamide were titrated with four of the compounds showed, however, that IFE is only minimally responsible for the fluorescence decrease (Figure S4). Therefore the hyperbolic shapes observed in the titration experiments of the protease were indeed due to specific binding.

**Correlation between IC$_{50}$ values and specific binding.** Our goal in developing the TRP-fluorescence binding assay was to screen out non-specific inhibitors and to ascertain if compounds were binding competitively to the enzyme. A total of 63 compounds were tested in our study for their inhibitory capabilities (IC$_{50}$) and their binding affinities ($K_D$). This approach identified promiscuous inhibitors and, using BPTI, we demonstrated that only a subset of compounds with low $K_D$ were truly competitive binders (data not shown). On the other hand, attempts to eliminate non-specific binders by addition of Triton-X-100 in the enzyme assay was not very successful, as even the reference inhibitor, Bz-nKRR-H, showed at least five-fold change in IC$_{50}$ values in the presence of the detergent (data not shown).

Compound 1, which was the parental compound from which SAR was developed, showed comparable IC$_{50}$ (6±2.6 µM) and $K_D$ values from Trp fluorescence (7.5±3.2 µM) and ITC (7.3 µM) as well as SPR (3.75 µM) for DENV2 protease. We further observed competition of 1 with BPTI by ITC, SPR and fluorescence quenching. NMR experiments confirmed that it interacted with the DENV2 protease active site (Figure S1). Compound 1 showed some differences in affinities for different dengue serotypes (IC$_{50}$ value varied between 6 µM for DENV2 and 33 µM for DENV4) and the Hill slopes obtained were close to 1, indicating a specific mode of inhibition(7, 39). The same is true for compounds 3 and 8.

On the other hand, compound 2 displayed one of the best IC$_{50}$ values in our SAR analysis (2.64±0.44 µM for DENV2 protease). Despite its potency, no binding was detected by Trp fluorescence and addition of BPTI after the titration did not induce any decrease in fluorescence intensity. This was
confirmed by ITC where no competition with BPTI was observed and again by the absence of binding in SPR. Hence it is likely that compound 2 is a non-specific inhibitor. This reasoning also applies to compound 7. Careful inspection of the enzyme inhibition data for WNV and DENV1-4 proteases revealed two variables as particularly useful criteria of specific binding. Firstly, the inhibition curves obtained with 2 and 7 displayed steep transitions. This translated into steep Hill slopes which strongly suggested that they act by a non-specific mechanism(7, 39). Relying solely on Hill slopes, however, may be misleading. For example, compounds 3, 5 and 8 gave Hill slopes of about 2 (Table 2), yet data from fluorescence quenching experiments and ITC clearly showed that they were specific inhibitors. These examples reinforce our conclusion that it is important to use a repertoire of biochemical and biophysical techniques to accurately determine compound binding specificity during drug screening.

Secondly, IC\textsubscript{50} values obtained with compounds 2 and 7 were very similar for WNV and DENV1-4, indicating that they inhibited all proteases equally well. This is unexpected considering that the S1 site of WNV protease catalytic pocket differs from those of DENV1-4 proteases and NS2B residues forming S2 and S3 sites are also not well conserved between WNV and the different DENV serotypes. Moreover, the transition state analogue Bz-nKRR-H showed different inhibition properties with proteases from the different dengue serotypes (Table 2) and WNV(17). We conclude that in the present SAR, similar IC\textsubscript{50} values observed for the different serotypes, coupled with high Hill slopes, indicated non-specific inhibition.

\textit{Use of tryptophan fluorescence for drug discovery.} The tryptophan fluorescence assay presented here proved to be a very useful tool for our drug discovery effort as it discriminated between specific and non-specific compounds from our SAR study. The method has many advantages. Firstly, small amounts of purified recombinant proteins can be readily used without need for further labeling or tagging. Secondly, fluorescence spectroscopy tolerates a wide range of buffer conditions, salts, detergents, or reducing agents. Thirdly, the binding assay can be done in 96- or 384-well formats. Automation would further increase throughput. Fourthly, the sensitivity of this method allows measurements of $K_D$ values
in the range from 0.1 to 20 µM. Below 0.1 µM, the shape of the binding curve would not permit a precise determination of the $K_D$ value whereas above 20 µM, inner filter effect would obscure fluorescence decrease induced by ligand-protein binding. Assays sensitive to this range of $K_D$ values are of great value in the critical drug discovery phases of hit-to-lead conversion and early lead optimization.

Comparison with other biophysical methods. As described in this manuscript, we also explored other biophysical techniques besides tryptophan fluorescence to evaluate the binding characteristics of compounds from our SAR study. The major issue we encountered was low compound solubility which prevented meaningful analysis by NMR and ITC. Consequently these methods offered a mostly qualitative observation (binding or no binding), whilst quantitative binding parameters (rather than binary answers) are paramount for understanding SAR. Another drawback of these techniques is that compounds that are poorly soluble at high concentrations will be eliminated in the studies even if they are specific inhibitors. With SPR, we observed a correlation with $K_D$ measured by fluorescence for most of the compounds, except for compound 5. We don’t know the reason for this discrepancy. The compound could have aggregated from non-specific interactions with the immobilized protease or chip matrix.

In contrast, we rarely faced solubility or aggregation problems in the fluorescence quenching assay with the concentrations of compounds tested (up to 40 µM) and were able to quantify the binding affinities of most specific compounds.

CONCLUSION

As tryptophans are present in most proteins, the Trp fluorescence quenching assay presented here is applicable to other studies of protein-compound interaction. If Trp residues are absent or far from the binding site, they may be introduced by site-directed mutagenesis. The simplest manner to detect compound binding is via direct compound quenching of tryptophan fluorescence as shown in this report or by observing changes in tryptophan emission maximal. Most compounds in drug discovery follow the
Lipinski rules\(^{(40)}\) and are largely hydrophobic, often aromatic, molecules. Absorbance spectra of such compounds often span the spectral range from 270 to 400 nm, making them suitable quenchers of tryptophan fluorescence. In the case where a class of compounds lack these spectroscopic properties, a known inhibitor that induces a fluorescence change upon binding could be used as a competitor. The ease of implementing this technique makes it well suited for drug discovery needs.
ACKNOWLEDGMENTS

We thank Lim Siew Choo for assistance with the cloning of mutant trp constructs and Dariusk Ekonomiuk and Shahul Nilar for performing some of the docking runs. NMR work carried out in the laboratory of Gottfried Otting was funded by the Australian Research Council.
REFERENCES


### Table 1. Examples of techniques used to measure binding.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Cost</th>
<th>Throughput</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITC</td>
<td>High</td>
<td>Low</td>
<td>Yes</td>
</tr>
<tr>
<td>X-Ray</td>
<td>Very High</td>
<td>Very Low</td>
<td>Yes</td>
</tr>
<tr>
<td>Nuclear Magnetic Resonance</td>
<td>Very High</td>
<td>Low</td>
<td>Yes</td>
</tr>
<tr>
<td>Thermal shift</td>
<td>Low</td>
<td>High</td>
<td>No</td>
</tr>
<tr>
<td>Biacore</td>
<td>Medium</td>
<td>High</td>
<td>Yes</td>
</tr>
</tbody>
</table>

### Table 2. IC$_{50}$ values and Hill slopes obtained with different compounds and proteases (DENV1-4).

<table>
<thead>
<tr>
<th>Compound</th>
<th>DENV1</th>
<th>DENV2</th>
<th>DENV3</th>
<th>DENV4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (µM)</td>
<td>Hill Slope</td>
<td>IC$_{50}$ (µM)</td>
<td>Hill Slope</td>
</tr>
<tr>
<td>1</td>
<td>36.4</td>
<td>1.0</td>
<td>6.06</td>
<td>0.85</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>2.4</td>
<td>2.3±0.6</td>
<td>5.1</td>
</tr>
<tr>
<td>3</td>
<td>10.7</td>
<td>1.8</td>
<td>1.1±0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>233</td>
<td>1.4</td>
<td>27.4</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>12.3</td>
<td>2.4</td>
<td>5.8±2.3</td>
<td>1.6</td>
</tr>
<tr>
<td>6</td>
<td>170</td>
<td>0.9</td>
<td>55</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>2.72</td>
<td>2.64</td>
<td>1.6±0.8</td>
<td>3.2</td>
</tr>
<tr>
<td>8</td>
<td>21.4</td>
<td>1.8</td>
<td>10.2±6</td>
<td>0.9</td>
</tr>
<tr>
<td>9</td>
<td>&gt;100</td>
<td>NA</td>
<td>&gt;100</td>
<td>NA</td>
</tr>
<tr>
<td>Bz-nKRR-H</td>
<td>11.8±0.9</td>
<td>1.05</td>
<td>8.9±0.5</td>
<td>1.06</td>
</tr>
</tbody>
</table>
Table 3. IC$_{50}$ and $K_D$ values of different compounds for DENV2 protease and WNV protease.

<table>
<thead>
<tr>
<th>Compound</th>
<th>DENV2 IC$_{50}$ (µM)</th>
<th>DENV2 $K_D$, µM$^a$</th>
<th>DENV2 Competition ITC$^b$</th>
<th>DENV2 $K_D$ (µM) by ITC$^c$</th>
<th>DENV2 $K_D$ (µM) by SPR</th>
<th>WNV IC$_{50}$ (µM)</th>
<th>Hill Slope</th>
<th>WNV $K_D$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6±2.6</td>
<td>7.2±3.2 (Y)</td>
<td>Y</td>
<td>7.3</td>
<td>3.7</td>
<td>24.5</td>
<td>3.5</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>2.3±0.6</td>
<td>100 (N)</td>
<td>N</td>
<td>N</td>
<td>NB</td>
<td>3.6±1.8</td>
<td>2.6</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>1.1±0.2</td>
<td>7.7±2 (Y)</td>
<td>Y</td>
<td>13</td>
<td>0.3</td>
<td>22</td>
<td>1.4</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>27.4</td>
<td>22.9 (Y)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>5.2</td>
<td>1.0</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>5.8±2.3</td>
<td>18.6 (Y)</td>
<td>Y</td>
<td>17</td>
<td>&gt;100</td>
<td>10.6</td>
<td>1.9</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>54 (Y)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>6.2</td>
<td>1.1</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>1.6±0.8</td>
<td>100 (N)</td>
<td>N</td>
<td>N</td>
<td>nd</td>
<td>2.2</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>10.2±6</td>
<td>16.3±0.3 (Y)</td>
<td>Y</td>
<td>40</td>
<td>4.3</td>
<td>16</td>
<td>2.0</td>
<td>nd</td>
</tr>
<tr>
<td>9</td>
<td>&gt;100</td>
<td>100 (N)</td>
<td>N</td>
<td>N</td>
<td>NB</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

$^a$Y(yes) and N(no) indicates whether competition was observed or not between compound and BPTI.

$^b$“Competition ITC” reflects a qualitative judgment of the competition data based on both change in $K_D$ and change in binding enthalpy. Y and N indicates whether competition was observed or not between compound and BPTI.

$^c$For compound 1, the $K_D$ value was calculated based on a single concentration of compound 1 while the $K_D$ values for compounds 3 and 8 were determined from several compound concentrations using equation 1.
**FIGURE LEGENDS**

**Figure 1.** Structures of phthalazine-based compounds. The central phthalazine ring is shown on the left. Compounds are numbered from 1-9 where 1 represents the parental compound and 2-9 are representative modified derivatives described in the report.

**Figure 2.** Binding of compound 1 in WNV NS3/NS2B protease obtained by automatic docking with FFLD and CHARMM minimization. (A) Close up view of WNV NS3/NS2B in complex with compound 1. The protein surface (PDB code 2FP7) is colored according to atomic partial charges. Compound 1 is colored by atom type and the nonpolar hydrogens is not shown for clarity. The central phthalazine ring is located in a cavity of S1 pocket and forms $\pi - \pi$ interaction with the phenyl group of Tyr161. Both charged imidazoline groups are involved in salt bridge or hydrogen bonds with several residues (Asp129, Gly159 and Asn84). One of the NH group linking the phenyl and phthalazine moieties forms a hydrogen bond with Pro131 backbone carbonyl oxygen. (B) Location of tryptophan residues in WNV NS2B/3 protease. Binding pose of compound 1 is shown by green sticks. NS2B is depicted as blue ribbons and NS3 as red ribbons. Tryptophan residues are represented as yellow sticks and are labeled. The catalytic triad and the conserved residues of NS3 at the S1, S2, and S3 sites are colored in magenta. The figures were made using PyMOL (http://pymol.sourceforge.net/).

**Figure 3.** Absorbance spectra of 100 µM compounds measured on a 96-wells plate. Compound numbers are indicated in the figure.

**Figure 4.** Titration of DENV2 protease with compound 1. (A) Fluorescence spectra of DENV2 protease at varying protein concentrations. The kinks in the spectra at 400 nm are an artifact of the microplate reader. (B) Titration of WT DENV2 protease with compound 1 monitored by fluorescence at 340 nm. The solid line is the fit of the data to equation 3. Symbol (○) shows the level of fluorescence reached after addition of 11 µM BPTI to the well containing WT protein and 40 µM compound 1. (C) Titration of WT and W mutants with compound 1. Normalized fluorescence at 340 nm (measured fluorescence (F) divided by the fluorescence measured in the absence of compound (F₀)). The solid lines are the fit of the
data to equation 3. WT (●), W5A (○), W50A (◼), W69A (△), W89A (◇). Following the same order, the resulting $K_D$ were: 4.7, 5.15, 6.2, 6.6, and 7.8 µM.

**Figure 5.** Fluorescence titrations performed with several compounds. Titration data were normalized by dividing the measured fluorescence ($F$) by the fluorescence measured in the absence of compound ($F_0$). (A) Titration of DENV2 protease; (B) Titration of WNV protease. Solid lines show the fit of the data to equation 3. Titrations with different compounds are represented as follows: 1 (△), 2 (●), 3 (◇), 4 (◼), 5 (◆), 6 (□), 7 (○).

**Figure 6.** ITC Competition Titration. Titrations of 6 µM DENV2 protease with 28 consecutive 10 µl injections of 80 µM BPTI at 25 °C were performed in the presence of varying concentrations of compounds. Typical titrations are shown in the absence of compound (○) and in the presence of 150 µM compound 8 (◼), 100 µM compound 3 (△), and 100 µM compound 9 (▼).
<table>
<thead>
<tr>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>(5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>(6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>(8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>(9)</td>
</tr>
</tbody>
</table>
Figure 3
Figure 4

A

Increasing [compound 1]

B

C

[compound 1] μM

Fluorescence Intensity, AU

Fluorescence, AU
Figure 5

A

B
Figure 6
SUPPORTING INFORMATION AVAILABLE

Oligonucleotide sequences of mutagenesis primers, $^{15}$N-HSQC spectra of DENV2 protease with and without compound 1, SPR studies of the binding of compound 1 and titration of N-acetyl-tryptophanamide with compounds, chemistry and compound syntheses. This material is available free of charge via the internet at http://pubs.acs.org.