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Characterization of 8-hydroxyquinoline derivatives containing aminobenzothiazole as inhibitors of dengue virus type 2 protease *in vitro*

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ABSTRACT

Four serotypes of dengue virus (DENV**1**–**4**), mosquito-borne members of *Flaviviridae* family cause frequent epidemics causing considerable morbidity and mortality in humans throughout tropical regions of the world. There is no vaccine or antiviral therapeutics available for human use. In a previous study, we reported that compounds containing the 8-hydroxyquinoline (8-HQ) scaffold as inhibitors of West Nile virus serine protease. In this study, we analyzed potencies of some compounds with (8-HQ)-aminobenzothiazole derivatives for inhibition of DENV2 protease *in vitro*. We identified analogs **1**–**4** with 2-aminothiazole or 2-aminobenzothiazole scaffold with sub-micromolar potencies (IC_{50}) in the *in vitro* protease assays. The kinetic constant (K_i) for the most potent 8-HQ-aminobenzothiazole inhibitor (compound **1**) with an IC_{50} value of 0.91 ± 0.05 μM was determined to be 2.36 ± 0.13 μM. This compound inhibits the DENV2 NS2B/NS3pro by a competitive mode of inhibition.

1. Introduction

DENV, a member of mosquito-borne *Flaviviridae* family (Flavivirus genus), causes significant morbidity and mortality (for reviews, see Gould and Solomon, 2008; Weaver and Barrett, 2004). DENV virus is the causative agent of dengue fever, dengue hemorrhagic fever, and toxic shock syndrome (Gould and Solomon, 2008). These diseases are prevalent in tropical regions around the world, where the mosquito species, *Aedes aegypti* and *Aedes albopictus* vectors, thrive and infect humans. A total of 50–100 million DENV-related infections occur annually worldwide (Gould and Solomon, 2008). Despite the large burden to human health, basic research into the development of DENV antiviral therapy has been limited. There are currently no vaccines or antiviral therapeutics available for treatment of DENV-infected patients.

DENV encodes a positive-strand RNA of about 11 kb in length with a type 1 cap, m7GpppA (2′-Om) at the 5′-end but lacking a poly(A) tail at the 3′-end. RNA genome is translated to a single polyprotein precursor which is processed to produce 10 mature proteins by co- and post-translational processing (Lindenbach and Rice, 2003). The three proteins, the capsid (C), precursor/mature membrane protein (prM/M), and the envelope (E) form the virion; the seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are expressed in the infected cells and are required for viral replication (for reviews, see (Beasley, 2005; Lindenbach and Rice, 2003).

The two component viral serine protease, NS2B-NS3, plays a crucial role in viral replication as it is required for processing of the polyprotein precursor prior to the assembly of the viral replicase complex (for reviews, see (Padmanabhan and Strongin, 2010; Sampath and Padmanabhan, 2009)). This requirement makes the viral protease as an excellent target for development of antiviral therapeutics. The viral protease cleavage sites have in common a pair of basic amino acids, R and K, followed by G, S, or A at the P1′ position (Lindenbach and Rice, 2003). The serine protease catalytic triad is located within the *N*-terminal 185 amino acids of NS3 protein (Bazan and Fletterick, 1989; Chambers et al., 1990; Preugschat et al., 1990). NS2B is the required cofactor for NS3 protease activity (Chambers et al., 1991; Falgout et al., 1991; Wengler et al., 1991; Zhang et al., 1992). The NS2B is an integral membrane protein in the endoplasmic reticulum (Clum et al., 1997) containing hydrophobic regions flanking a hydrophilic region of ~44 amino acid residues which forms a complex with the NS3 protease domain (Arias et al., 1993; Chambers et al., 1993; Clum et al., 1997; Falgout et al., 1993). Using an *Escherichia coli*-expressed and purified DENV2 NS2B/NS3pro, an *in vitro* protease assay using fluorogenic peptide substrates was established (Yusof et al., 2000) which was further optimized (Li et al., 2005). The crystal structures of the DENV3 NS2B-NS3pro (Noble et al., 2012) or of

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WNV NS2B-NS3pro in a covalent complex with a tetra-peptide substrate-based inhibitor (Erbel et al., 2006) or in a non-covalent complex with the trypsin inhibitor, aprotinin (Aleshin et al., 2007; Noble et al., 2012) have been solved. Similarly, the crystal structures of DENV2 (Erbel et al., 2006) and DENV1 (Chandramouli et al., 2010) without a substrate-based or aprotinin inhibitor have been solved (Aleshin et al., 2007). These studies provide some information regarding the role of cofactor NS2B peptide in activation of the NS3pro domain.

In a previous study, we reported that compounds containing an 8-HQ scaffold with two aryl substitutions at 7 position of the 8-HQ ring inhibited WNV protease (Mueller et al., 2008). In a subsequent study, a number of 8-HQ derivatives were analyzed against WNV protease (Ezgimen et al., 2012). A structure activity relationship (SAR) was revealed in that study regarding the nature of the aryl substitutions at the 7-position and the 8-HQ ring substitutions. The aim of the present study was to select a set of derivatives of 8-HQ scaffold, which exhibited good inhibition of WNV protease, and assay them for their inhibition against the DENV2 NS2B-NS3pro. The 8-HQs with aminothiazole or aminobenzothiazole moiety are shown to inhibit the DENV2 protease and the results are presented. Kinetic analysis was also carried out to determine the mode of inhibition of these compounds which is supported by molecular modeling.

2. Materials and methods

2.1. Materials

The DENV2 NS2B-NS3pro expression plasmid encoding the protease precursor used in this study contains the hydrophilic domain of NS2B cofactor (48 amino acids) and the NS3pro domain (185 residues) (Yon et al., 2005). The expression and purification of the protease were as described previously (Mueller et al., 2007; Yusof et al., 2000). The fluorogenic peptide substrate, Benzoyl (Bz)-norleucine (Nle)-Lys-Arg-Arg-AMC was purchased from Bachem (Torrance, CA). For later experiments, Bz-Nle-Lys-Arg-Arg-AMC was custom-synthesized by NeoBioScience (Cambridge, MA). AMC was purchased from Anaspec, Inc (Fremont, CA). The sources of compounds 13, 20 and 22 (Fig. 1B) are described in (Ezgimen et al., 2012). Compound **1** (M.W. 489.59) is a new compound synthesized in the laboratory using the previously reported method (Phillips et al., 1953) and the structure was confirmed by NMR. The sources of the compounds **2** (M.W. 439.53; II-11-1), **3** (M.W. 438.54; II-11-6), **4** (M.W. 489.59; II-11-5) were the same as compounds **14**, **18**, and **17** previously described (Ezgimen et al., 2012).

2.2. In vitro DENV2 protease assays

Compounds were analyzed by *in vitro* protease assays performed in black 96-well plates. Standard reaction mixture (100 μl) containing 200 mM Tris HCl (pH 9.5), 6 mM NaCl, 30% glycerol, 25 nM DENV2 protease, 10 or 25 μM inhibitors (dissolved in DMSO) were incubated 15 min at 25 °C. Reactions were started by the addition of 5.0 μM *tetra*-peptide substrate, (Bz)-Nle-Gly-Arg-Arg-AMC.

Release of free AMC was measured using a spectrofluorometer (Molecular Devices) at excitation and emission wavelengths of 380 and 460 nm, respectively. Fluorescence values obtained with the no-inhibitor control were taken as 100%, and those in the presence of inhibitors were calculated as the percentage of inhibition of the control using Microsoft Excel and plotted using SigmaPlot 2001 v7.0 software. The background of AMC in the absence of protease was subtracted before the data analysis. All assays were performed in triplicate and repeated twice.

2.3. Determination of IC_{50}

To determine the 50% inhibitory concentration (IC_{50}) of a compound, protease assays were performed as described above except in the presence of various concentrations of an inhibitor (0.005–50 μM), which is dissolved in 100% DMSO and the stock concentration is 5.0 mM. All the final DMSO concentration was less than 2% in each well. The IC_{50} values against DENV2 protease were determined in a buffer containing 200 mM Tris HCl (pH 9.5), 6 mM NaCl, 30% glycerol and 0.1% CHAPS. In our assay, CHAPS detergent was included in the assay buffer to eliminate aggregation-based inhibition by a compound (Feng et al., 2007). Fourteen data points were obtained for inhibitor concentrations in the range of 5, 10, 50 nM, 0.1, 0.5, 1, 2, 4, 6, 8, 10, 20, 25, and 50 μM of selected compounds. IC_{50} values were calculated using the SigmaPlot 2001 v7.0 software.

2.4. Steady-state kinetic analysis

To determine the K_m and V_{max} values of compound **1**, four different concentrations of the inhibitor (0–5 μM) were assayed at twelve *tetra*-peptide substrate concentrations ranging from 0–50 μM. K_i value was calculated from the secondary plot of obtained $K_{m(app)}$ against the concentration of inhibitors.

2.5. Molecular docking of compounds 1, 2, 3, and 4 into DENV3 protease

For molecular docking of the compounds 1–4 into the NS2B-NS3pro, we used the crystal structure of DENV3 pro in complex with a peptide based inhibitor (benzoyl-norleucine-Lys-Arg-Arg-H) that was solved to 2.3 Å resolution (PDB ID: 3U1I) (Noble et al., 2012). In the presence of the peptide substrate-based inhibitor, the DENV3 protease adopts a catalytically active closed conformation in which the hydrophilic beta-hairpin region wraps around the NS3 protease domain (Noble et al., 2012). For molecular docking we used Molegro virtual docker, which has been developed recently and gained significant attention among scientists in the field of structure based drug discovery (Thomsen and Christensen, 2006). The small molecule inhibitors were prepared and optimized using ACD software, Chemsketch. In addition, for consistency, the ligand parameters were also energy minimized using the ligand descriptor option in Molegro. All the water and ligand molecules were deleted from the DENV3 NS2B-NS3pro structure. Hydrogen atoms were added and atomic charges assigned using the protein molecular preparation option in Molegro. Prior to calculating the binding cavities, the side chain conformation of the amino acid residues were minimized globally. We used a grid resolution of 0.5 Å to calculate the cavities in the protein. For docking, a grid resolution of 0.20 Å and a radius of 15 Å around the binding site were used. We used the MolDock optimizer as a search algorithm, and the number of runs was set to 20. A population size of 50, maximum iteration of 1500, scaling factor of 0.50, crossover rate of 0.90 and a variation-based termination scheme for parameter settings were used. The maximum number of poses to generate was set to default value of 5. The solution corresponding to top ranked score from each run was used to create the resulting docked protein:ligand complex structure.

3. Results and discussion

3.1. Inhibition of DENV2 NS2B/NS3pro by 8-HQ-aminobenzothiazole derivatives

In our previous study, we reported identification of small molecule inhibitors of WNV NS2B-NS3pro using a fluorogenic tripep-

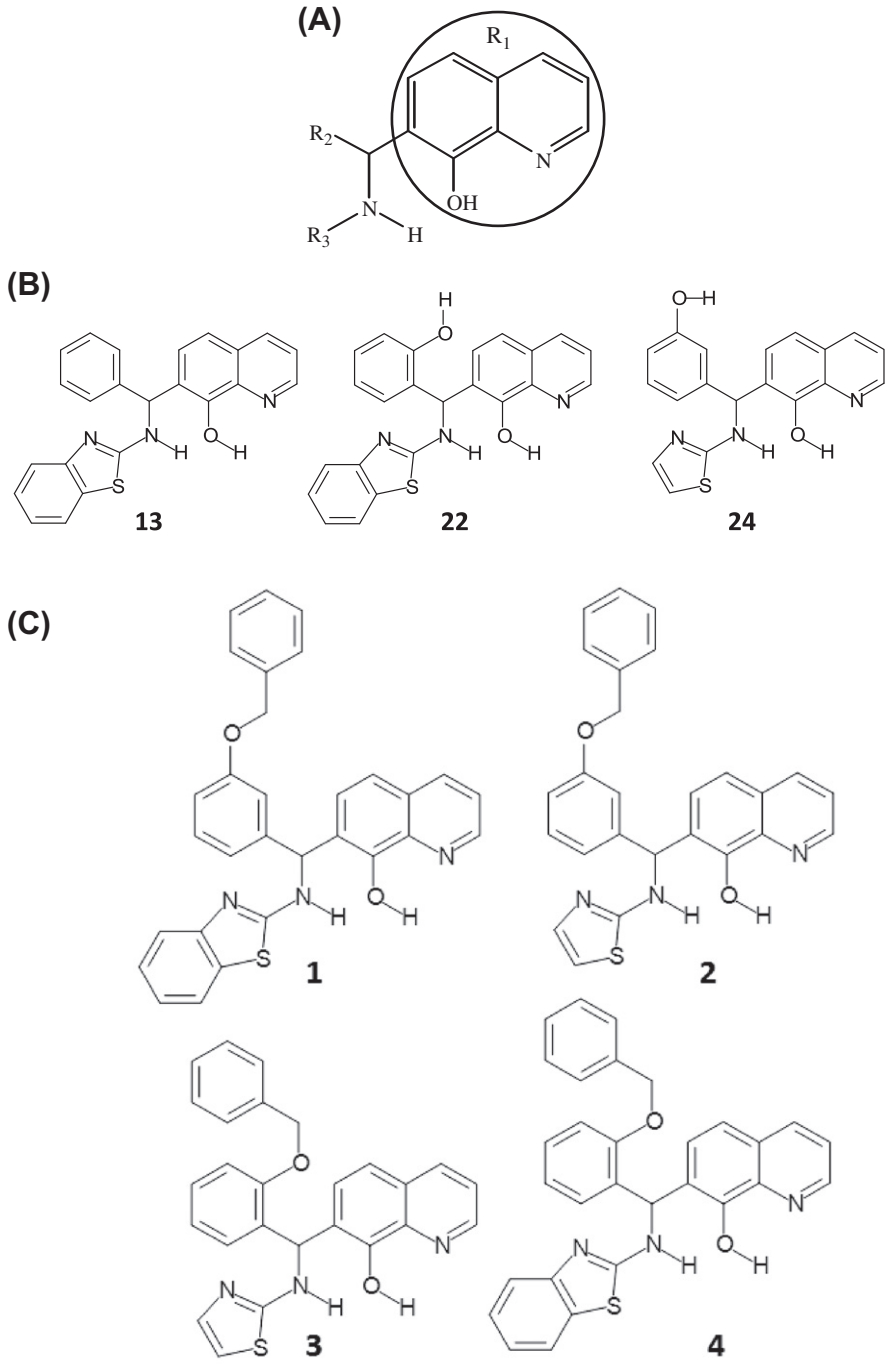

Fig. 1. Structures of 8-HQ derivatives. (A) The general structures of 8-HQ scaffold. (B) The structures of 8-HQ scaffold derivatives previously studied against West Nile virus NS2BH-NS3 protease. (C) The structures of four compounds that were analyzed in this study are shown.

tide substrate, Boc-G-K-R-AMC, in a HTS campaign. The 98 compounds selected as primary "hits" with drug-like physicochemical and pharmacological properties were divided into five groups based on structural similarity. Two groups of compounds are 8-HQ derivatives with substitutions at 7-position that are linked to either an –NH–C═O or an –NH– group (Mueller et al., 2008). The lead compound (Compound **B**) is an 8-HQ derivative belonging to the –NH– group (structure similar to compound **24** in Fig. 1B except with a –OH group in the *ortho* position), was reported to have a K_i value of 3.4 ± 0.6 μM (Mueller et al., 2008). In a recent study, sixteen 8-HQ derivatives (including the lead compound **B** used as a control) with a general structure shown in Fig. 1A with different **R2** and **R3** aryl substitutions were analyzed against West Nile virus NS2B-NS3pro (Ezgimen et al., 2012) using a tripeptide substrate, Boc-G-K-R-AMC. It is noteworthy that the K_m of the WNV protease for the tripeptide (Boc-G-K-R-AMC) substrate was reported as 737 ± 150 μM (Mueller et al., 2007) whereas with the tetra-peptide substrate, Bz-Nle-Lys-Arg-Arg-AMC, the K_m was 24.56 ± 1.43 μM. Therefore, in this study, the *in vitro* assays for WNV and DENV2 proteases were performed using the tetra-peptide substrate which was found to be the optimum substrate for the DENV2 protease (Li et al., 2005).

Of the 16 derivatives of 8-HQ scaffold tested, 9 compounds showed inhibitory activity of >95% whereas among the other seven, the% inhibition were in the range of 20–80%. Fourteen out of 16 compounds had either aminothiazole or benzaminothiazole

Table 1
Percent inhibition of DENV2 NS2B-NS3 protease by compounds containing the 8-HQ scaffold (Fig. 1B) in the presence of 0.1% CHAPS.

Inhibitors	% Inhibition at 10 μM	% Inhibition at 25 μM
13	61.45 ± 1.28	76.69 ± 1.74
22	69.02 ± 1.03	84.52 ± 1.46
24	48.36 ± 1.69	59.74 ± 1.13

Table 2
Percent inhibition and IC_{50} values of compounds **1**, **2**, **3** and **4** against DENV2 NS2B-NS3 protease in the absence (−) and presence (+) of 0.1% CHAPS.

Inhibitors	CHAPS	% Inhibition at 10 μM	% Inhibition at 25 μM	IC_{50} (μM)	Hill slope
1	−	84.64 ± 1.33	90.62 ± 1.03	1.29 ± 0.03	1.14 ± 0.07
	+	87.32 ± 1.25	96.39 ± 1.32	0.91 ± 0.02	1.05 ± 0.05
2	−	78.46 ± 1.06	83.43 ± 1.29	2.99 ± 0.06	1.21 ± 0.06
	+	83.57 ± 1.21	92.92 ± 1.26	2.93 ± 0.07	1.27 ± 0.06
3	−	66.78 ± 1.24	83.12 ± 1.54	4.88 ± 0.15	1.21 ± 0.11
	+	76.92 ± 1.52	92.62 ± 1.47	3.67 ± 0.08	1.18 ± 0.05
4	−	72.57 ± 1.49	85.46 ± 1.09	3.25 ± 0.12	1.24 ± 0.08
	+	82.78 ± 1.06	91.79 ± 1.18	2.34 ± 0.14	1.02 ± 0.05

Table 3
Percent inhibition and IC_{50} values of compounds **1**, **2**, **3** and **4** against WNV NS2B-NS3 protease in the presence of 0.1% CHAPS.

Inhibitors	% Inhibition at 10 μM	% Inhibition at 25 μM	IC_{50} (μM)	Hill slope
1	81.85 ± 1.12	96.79 ± 0.35	2.39 ± 0.04	1.19 ± 0.07
2	72.05 ± 1.38	87.25 ± 0.89	3.46 ± 0.06	1.13 ± 0.06
3	65.77 ± 1.49	83.11 ± 1.08	6.95 ± 0.12	1.19 ± 0.14
4	70.25 ± 1.06	83.65 ± 1.31	5.15 ± 0.09	1.17 ± 0.08

ring as **R3** substitution and phenylmethyl, phenolic hydroxyl, or benzyloxy phenyl as **R2** substitution (Fig. 1A). A rational structure activity relationship (SAR) was revealed. Importantly, it was revealed that the N at 1- position of the 8-HQ ring was shown to be essential for inhibition of the WNV protease because substitution of 8-hydroxyquinoline ring with a naphthalen-1-ol ring (N replaced with –CH–), reduced the inhibitory activity by ~75% (dropped from ~99% to 26%). In this study, we analyzed seven compounds including one novel compound (compound **1**) synthesized for this study (Fig. 1B and C) for their inhibition of DENV2 NS2B/NS3pro. All have aryl substitutions at 7-position with a thiazole or a benzothiazole moiety as an **R3** aryl substitution in the general structure shown in Fig. 1A. The **R2** substitution varied as phenyl, phenylhydroxyl, 2-(benzyloxy)phenyl or 3-(benzyloxy)phenyl moiety (Fig. 1B and C).

The *in vitro* protease assays were performed using the DENV2 protease NS2B/NS3-pro in the absence or the presence of an inhibitor compound at a fixed concentration of 10 and 25 μM and the Bz-Nle-Lys-Arg-Arg-AMC substrate. The inhibitory activities of three compounds (Fig. 1B), **13**, **22**, and **24**, which had ⩾95% inhibition of WNV protease with the tri-peptide substrate (Ezgimen et al., 2012), were in the range of 48–69% at 10 μM and 60–85% at 25 μM against the DENV2 NS2B-NS3 protease with the tetrapeptide substrate (Table 1). All four compounds (compounds **1**–**4** in Fig. 1C) showed good inhibition (79–93%) against DENV2 protease. The activities of the four compounds were compared in the absence and presence of 0.1% CHAPS (Table 2). Meanwhile, the top four compounds **1**–**4** also showed good inhibition against WNV NS2B-NS3 protease in the presence of 0.1% CHAPS (Table 3). The results reveal that compounds **1** and **2** both having the benzyloxy (–OBn) phenyl moiety in the *meta* position in common (**R2** ring in

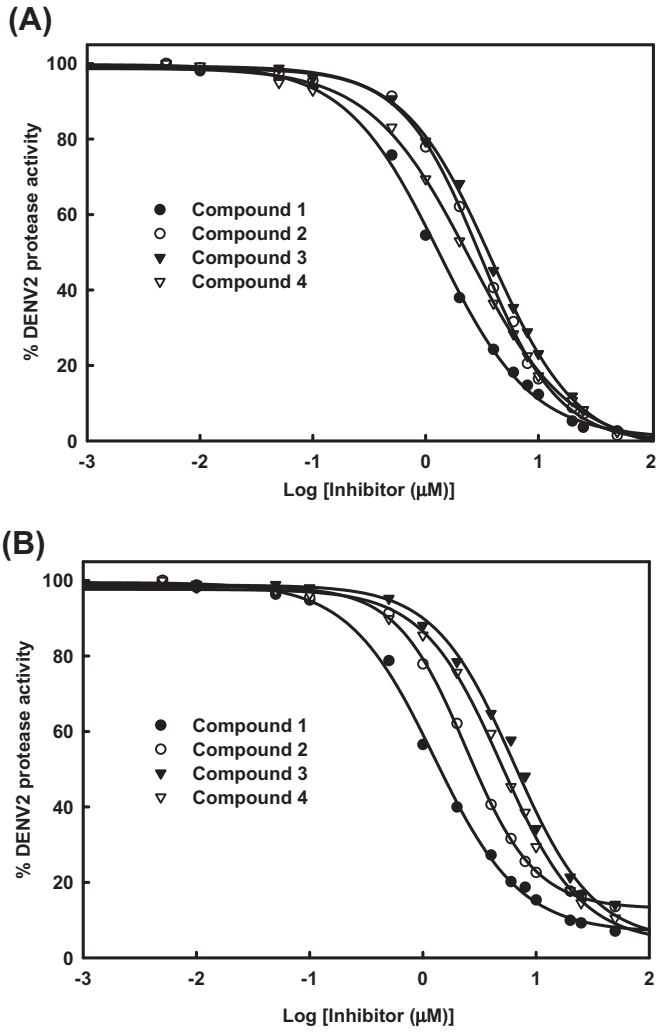

Fig. 2. Determination of IC_{50} values of inhibitor **1**–**4** against DENV2 protease in the presence (A) and absence (B) of 0.1% CHAPS. The IC_{50} values were determined as described under Section 2. The *x*-values are displayed as the log of compound concentration (0–50 μM). The solid lines are fitted lines using the four-parameter sigmoidal model equation. Inhibitor **1**–**4** were incubated with DENV2 NS2B/NS3pro (25 nM) in buffer (200 mM Tris HCl, 6 mM NaCl and 30% glycerol, pH 9.5) for 15 min. Bz-Nle-Lys-Arg-Arg-AMC (5.0 μM) was added to the mixture in a final volume of 100 μL. The fluorescence intensity was measured at 460 nm with excitation at 380 nm and converted to the percentage of protease activity in the absence and presence of inhibitors. The solid line is the theoretical fitting curve based on the Sigmoidal equation. The apparent IC_{50} values in the presence of 0.1% CHAPS for compounds **1**, **2**, **3** and **4** were 0.91 ± 0.02, 2.93 ± 0.07, 3.67 ± 0.08, and 2.34 ± 0.14 μM, respectively. The apparent IC_{50} values in the absence of 0.1% CHAPS for compounds **1**, **2**, **3** and **4** were 1.29 ± 0.03, 2.99 ± 0.06, 4.88 ± 0.15, and 3.25 ± 0.12 μM, respectively.

the general structure shown in Fig. 1A), the benzothiazole moiety (**R3** ring in Fig. 1A) in compound **1** contributed to better inhibition of the protease than the thiazole moiety in compound **2** (Table 2). This difference in potency of compounds **1** and **2** is not significant when compounds **3** and **4** are compared in which the –OBn substitution is in the *ortho* position which could be attributed to steric considerations. Yet, compound **4** is a more potent inhibitor than compound **3** indicating that benzothiazole is again preferred over thiazole moiety as an **R3** ring substitution.

3.2. Determination of IC_{50} values

Next, the IC_{50} value was determined for each of the four compounds using concentrations of the inhibitor between 5 nM and

50 μM in the presence and absence of 0.1% CHAPS (Table 2 and Fig. 2A and B, respectively). The RFU values were converted into% inhibition and plotted versus the Log_{10} of compound concentrations resulting in sigmoid concentration–response curves. The apparent IC_{50} values for compounds **1**, **2**, **3** and **4** were 0.91 ± 0.02, 2.93 ± 0.05, 3.67 ± 0.08, and 2.34 ± 0.14 μM, respectively. The results suggest that while all four compounds are good inhibitors of DENV2 NS2B-NS3pro, compound **1** showed the maximum inhibition among the four compounds with an IC_{50} in the ⩽1 μM range. Compound **1** was also the best of the four compounds in inhibition of WNV protease in the presence of 0.1% CHAPS (Table 3).

3.3. Kinetics of inhibition of DENV2 NS2B/NS3pro by compound 1

Since compound **1** is the best inhibitor in this series, we sought to analyze the mode of inhibition of the DENV2 NS2B/NS3pro-catalyzed cleavage of the *tetra*-peptide substrate by compound **1** in the presence of 0.1% CHAPS. We performed kinetic analysis to determine the K_m, k_{cat} and V_{max} values in the presence and absence of the inhibitor (compound **1**) at four different concentrations. As shown in Table 4 and Fig. 3, the apparent Michaelis–Menten constants (K_m(app)) increased and the k_{cat}/K_m decreased proportional to the increase in inhibitor concentrations in the range of 0, 1.0, 3.0 and 5.0 μM. The corresponding V_{max} values were essentially the same. The results indicate that compound **1** at increasing concentrations reduces the affinity of the enzyme for the substrate as evident from the increase of K_m values. The obtained K_i values was 2.36 ± 0.13 μM. This type of inhibition is a characteristic feature of a competitive mode of inhibition.

To test whether the 8-HQ moiety of the compound **1** has a potential to bind Zn^{2+} ions and thereby affecting its inhibitory activity or kinetic properties, we performed the steady state kinetic experiments in the presence and absence of 40 μM $ZnCl_2$. The obtained K_m, k_{cat} and V_{max} values are summarized in Table 4 and plotted as Fig. 3. The K_m values of DENV2 protease in the absence and presence of Zn^{2+} are 12.78 ± 0.49 and 12.82 ± 1.14 μM, respectively. The results suggest that Zn^{2+} ions do not affect DENV2 protease affinity with the corresponding *tetra*-peptide substrate. Meanwhile, the k_{cat} values of DENV2 protease in the absence and presence of Zn^{2+} are 0.0974 ± 0.0014 and 0.1000 ± 0.0014 s^{-1}, respectively. The results suggest that Zn^{2+} ions do not affect DENV2 protease activity with the corresponding *tetra*-peptide substrate. In the presence of inhibitors, the K_m and k_{cat} values are not affected in the presence of Zn^{2+} ions. The results suggest that the inhibitor **1** binds to the DENV2 protease specifically and the zinc chelation does not affect the binding affinity and activity of the inhibitor **1** against the DENV2 protease.

3.4. Molecular modeling of compounds 1–4 into DENV3 NS2B-NS3pro structure

The primary amino acid sequences of NS2B cofactor peptides and the NS3 protease domains of DENV2 and DENV3 exhibit overall sequence identity of ~73%. Therefore, the availability of crystal structure of the substrate-based inhibitor bound DENV3 NS2B-NS3pro in the biologically active conformation should facilitate lead optimization through SAR. To understand the SAR of the compounds **1–4**, we performed molecular docking using the coordinates of the crystal structure of DENV3 NS2B-NS3pro structure (PDB ID: 3U1I) (Noble et al., 2012), the only one available for any DENV serotype in a catalytically active closed conformation in which the hydrophilic beta-hairpin region of NS2B wraps around the NS3 protease core. For molecular docking, we used the software, Molegro virtual docker (Fig. 4A and B).

Overall, all the four inhibitors bind into the active site of the NS3pro in the similar location as that of the peptide based inhibitor observed in the crystal structure of the DENV3 NS2B-NS3pro:peptide complex (Fig. 4B and (Noble et al., 2012)). However, there are differences in the mode of binding and interaction of different moieties of the compounds **1–4** with the amino residues belonging to the subsites S1–S4 of DENV2 NS2B-NS3 pro. In detail, the S1 subsite formed by amino acid residues, D129, F130, K131, P132,

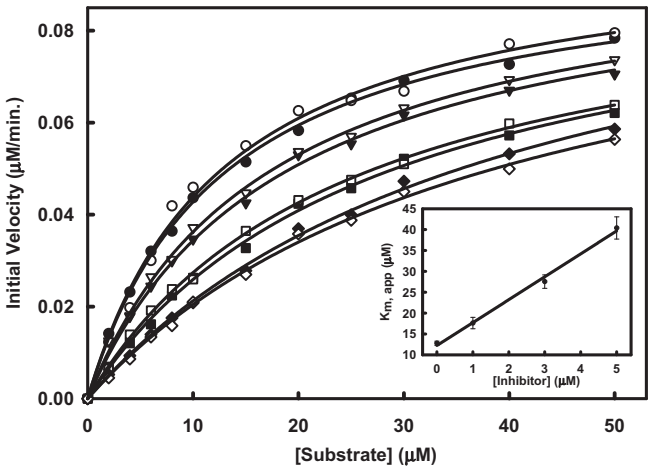

Fig. 3. Inhibition of DENV2 NS2B/NS3pro protease activity by compound **1**. Initial reaction rates of the *tetra*-peptide substrate (Bz-Nle-Lys-Arg-Arg-AMC) cleavage catalyzed by DENV2 NS2B/NS3pro protease (25 nM) in 200 mM Tris·HCl (pH 9.5), 6.0 mM NaCl, 30% glycerol and 0.1% CHAPS at 37 °C in the presence and absence of 40 μM $ZnCl_2$ were determined by varying the substrate concentrations in the range of 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40 and 50 μM at each concentration of inhibitor fixed at 0 (solid circle), 0 with 40 μM $ZnCl_2$ (open circle), 1.0 μM (solid triangle) and 1.0 μM with 40 μM $ZnCl_2$ (open triangle), 3.0 μM (solid square), 3.0 μM and 40 μM $ZnCl_2$ (open square), 5.0 μM (solid diamond) and 5.0 μM with 40 μM $ZnCl_2$ (open diamond). The reactions were initiated by the addition of DENV-2 NS2B/NS3pro protease and the fluorescence intensity at 460 nm was monitored with an excitation at 380 nm. Reactions were less than 5% completion in all cases to maintain valid steady-state measurements. The solid lines are fitted lines using the Michaelis–Menten equation. Inset: Secondary plot of $K_{m(app)}$ against the concentration of selected compound **1** in the absence of Zn^{2+}. Kinetic studies were carried out as described utilizing substrate concentrations of 0–50 μM Bz-Nle-Lys-Arg-Arg-AMC. Each experiment was performed in duplicate and repeated three times. Data were analyzed using SigmaPlot 2001 v7.0 software to determine values for apparent K_m and k_{cat}.

Table 4
Kinetic parameters for the *tetra*-peptide substrate and compound **1** against DENV2 NS2BH-NS3pro at 37 °C in the absence (−) and presence (+) of 40 μM $ZnCl_2$.

Inhibitors (μM)	$[Zn^{2+}]$ (40 μM)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
0	−	12.78 ± 0.49	0.0974 ± 0.0014	5082 ± 271
	+	12.82 ± 1.14	0.1000 ± 0.0014	5067 ± 567
1	−	17.61 ± 1.38	0.0952 ± 0.0032	3604 ± 423
	+	17.23 ± 0.86	0.0987 ± 0.0021	3683 ± 270
3	−	27.54 ± 1.61	0.0972 ± 0.0029	2353 ± 212
	+	26.89 ± 1.16	0.0956 ± 0.0022	2409 ± 159
5	−	40.39 ± 2.66	0.1071 ± 0.0041	1767 ± 189
	+	39.32 ± 2.92	0.1009 ± 0.0043	1816 ± 216

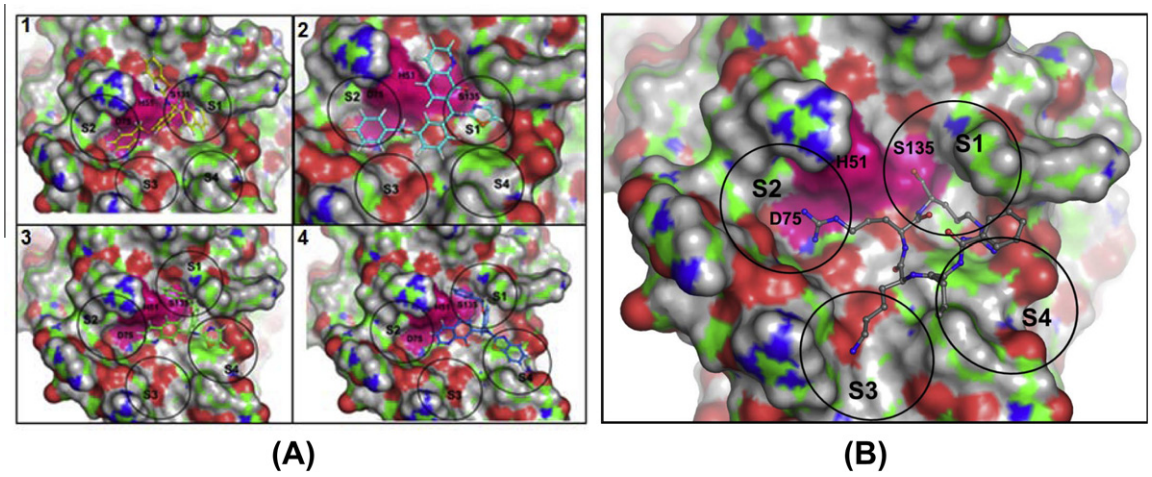

Fig. 4. (A) Putative binding modes of the docked poses of compounds 1–4 into the binding site of DENV3 NS2B-NS3 protease obtained by computational docking using Molegro virtual docker. Fig. 4 (A and B) were prepared using PyMol (http://pymol.sourceforge.net/). The ligands are shown as ball and stick, the protein is shown as molecular surface and the catalytic triad residues: His51, Asp75 and Ser135 are shown in pink. The sub-sites are labeled as S1, S2, S3, and S4. The S1 subsite is formed by residues D129, F130, K131 (S131), P132, G133, T134, S135, Y150 and Y161; S2 subsite is formed by residues D80 (E81), D81 (D82), G82 (G83), T83 (S84) of N2SB and V72, K73, K74, D75 of NS3 pro; S3 subsite formed by residues M84 (M85), R85 (S86), I86 (I87) of NS2B and S4 subsite formed by V154, V155. Where the corresponding amino acid residues or the numbers are different for DENV2 NS2B-NS3 pro are shown in parentheses. (B) Binding of the benzoyl-norleucine-Lys-Arg-Arg-H into the active site of DENV3 NS2B-NS3 pro as observed in the three-dimensional crystal structure of DENV3 NS2B NS3 pro:ligand complex (PDB ID: 3U1I). The subsites S1 to S4 and the catalytic triad residues are also shown (labeled in A). The peptide is shown as ball and stick and the protein as surface presentation.

G133, T134, S135, Y150 and Y161, is occupied by the 8-OH quinoline moiety of compound **1**, aminothiazole of compound **2** and phenylmethyl moiety of compounds **3** and **4**. The S2 subsite (D80, D81, G82, T83 of N2SB and V72, K73, K74, D75 of NS3 pro) is occupied by the phenylmethyl moiety of compounds **1** and **2** but there are no interactions between residues of this subsite and the compounds **3** and **4**. Similarly, there are no interactions between the residues of subsite S3 (M84, R85, I86 of NS2B) and any of the compounds, whereas in the crystal structure of DENV3 NS2B-NS3pro:peptide complex, the S3 subsite is occupied by the side-chain of the Lys residue of the peptide inhibitor (Fig. 4B). The S4 subsite (V154, V155) is occupied by amino-thiazole and amino-benzothiazole of compounds **3** and **4**, respectively.

It is worth noting, given the similarity between the structures of compounds **1** and **2**, and compounds **3** and **4**, compounds **1** and **2** interact with residues of subsites S1 and S2, whereas compounds **3** and **4** interact with subsites S1 and S4. The observed potency 0.91 ± 0.02 μM (Table 2) of compound **1** could be attributed due to the favorable hydrophobic interaction of the bulkier 8-HQ with three aromatic residues F130, Y150 and Y161 of the subsite S1, whereas, in compounds **2** and **3** a relatively smaller aromatic moiety occupies this S4 subsite. Interestingly, compound **4** also contains a bulkier benzothiazole moiety, however, this moiety occupies the S2 subsite formed by V154 and V155, a small aliphatic side chain residue and is also exposed towards the solvent as compared to the large hydrophobic pocket of S1 subsite occupied by the 8-HQ moiety of compound **1**. The docking of the compounds **1**–**4** using the catalytically active conformation crystal structure of the NS2B-NS3pro:ligand complex (Fig. 4A) and its comparison to the binding of the peptide based inhibitor bound to NS2B-NS3pro (Fig. 4B) provides valuable information to understand the structure activity relationship of the compounds and ideas to improve the biochemical and cellular potencies for the design and testing for this new class of non-peptide based 8-HQ as DENV protease inhibitors.

Many DENV proteins have been targeted for drug discovery (for a review, see (Noble et al., 2010). Aminothiazole and aminobenzothiazole moieties are core units in several naturally occurring alkaloids or synthetic compounds (Borzilleri et al., 2006; Das et al., 2006). These moieties elicit diverse physiological and chemotherapeutic activities. The interest in these chemical entities stems also from their importance as key intermediates in organic synthesis namely for the construction of various drugs and biologically active compounds. Many aminothiazole and aminobenzothiazole-containing compounds have so far exhibited very promising antiviral (Shipps et al., 2005; Spector et al., 1998), antiprion (Gallardo-Godoy et al., 2011; Ghaemmaghami et al., 2010; Heal et al., 2007), antitumor (Manjula et al., 2009; Oanh et al., 2011), and anticancer (Heiser et al., 2002; Sagi et al., 2005) and other biological properties (Patman et al., 2007; van Muijlwijk-Koezen et al., 2001). The 8-HQ moiety have been found in many drugs and showed broad spectrum of biological activities (Ezgimen et al., 2012; King et al., 2010; Lai et al., 2009; Lu et al., 2006).

In conclusion, our results reveal an inhibitor of DENV2 NS2B-NS3 protease by a compound bearing an 8-HQ scaffold with two aryl substitutions at 7-position, 3-(benzyloxy)phenyl and an amino-benzothiazole with an IC_{50} value in the $\leqslant 1$ μM range. It inhibits the DENV2 NS2B-NS3 protease by competing with the substrate binding in the vicinity of the active site which is supported by molecular modeling. Further work to solve the co-crystal structure of the protease in complex with the inhibitor is necessary to gain insight into the precise orientation of the compound in the active site.

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