Biochemical and Biophysical Research Communications 440 (2013) 393–398

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

A small compound targeting the interaction between nonstructural proteins 2B and 3 inhibits dengue virus replication

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ARTICLE INFO

Article history:
Received 11 September 2013
Available online 23 September 2013

Keywords:
Dengue virus
NS2B/NS3 protease
Antiviral
Re-emerging infection
Infectious diseases

ABSTRACT

The non-structural protein NS2B/NS3 serine-protease complex of the dengue virus (DENV) is required for the maturation of the viral polyprotein. Dissociation of the NS2B cofactor from NS3 diminishes the enzymatic activity of the complex. In this study, we identified a small molecule inhibitor that interferes with the interaction between NS2B and NS3 using structure-based screening and a cell-based viral replication assay. A library containing 661,417 small compounds derived from the Molecular Operating Environment lead-like database was docked to the NS2B/NS3 structural model. Thirty-nine compounds with high scores were tested in a secondary screening using a cell-based viral replication assay. SK-12 was found to inhibit replication of all DENV serotypes (EC_{50} = 0.74–4.92 μM). *In silico* studies predicted that SK-12 pre-occupies the NS2B-binding site of NS3. Steady-state kinetics using a fluorogenic short peptide substrate demonstrated that SK-12 is a noncompetitive inhibitor against the NS2B/NS3 protease. Inhibition to Japanese encephalitis virus by SK-12 was relatively weak (EC_{50} = 29.81 μM), and this lower sensitivity was due to difference in amino acid at position 27 of NS3. SK-12 is the promising small-molecule inhibitor that targets the interaction between NS2B and NS3.

1. Introduction

The incidence of dengue fever, which results from a mosquito-borne virus, has grown dramatically around the world in recent decades. At least 2.5 billion people, representing 40% of the world's population, are now at risk. The World Health Organization estimates that there may be 50 million dengue virus (DENV) infections worldwide every year, resulting in approximately 25,000 deaths [1]. At present, no effective vaccines or drugs are available against DENV.

DENV belongs to the family *Flaviviridae*, genus *Flavivirus*, and comprises four serotypes, one to four (DENV-1 to -4). The genus *Flavivirus* consists of arthropod-borne disease viruses such as the yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus, and DENV [2]. The DENV genome consists of a 10.7-kb single-stranded RNA, which encodes a single precursor polyprotein that is co- and post-translationally processed by viral and cellular proteases into three structural proteins: the capsid, pre-membrane, and envelope proteins, and seven non-structural proteins (NSs), NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [2].

DENV possesses a protease complex composed of NS2B and NS3, both of which are necessary for viral protease activity [3,4]. The NS2B/NS3 protease complex cleaves the precursor polyprotein at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions [5]. The importance of this protease activity was demonstrated by mutational analysis using viruses carrying point mutations in NS3 [6]. The association of the NS3 protease with NS2B induces formation of the active serine-protease complex [3]. NS2B is also a molecular chaperone that helps fold NS3 into its active conformation [7,8]. Furthermore, NS2B is necessary to promote the membrane association of the protease complex [9] and regulates the interaction with the enzymatic substrate [8]. Recent structural studies have revealed the detailed functions of NS2B. The C-terminal region of NS2B wraps around NS3, and contacts between NS2B and NS3 stabilize binding of peptide substrates to the substrate-binding site [10,11]. Thus, the interaction between NS2B and NS3 is important for the composite function of active viral protease. These observations suggest that competitive drugs targeted to the NS2B-binding site of NS3 represent promising candidates for therapeutic agents that could inhibit the maturation of DENV

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http://dx.doi.org/10.1016/j.bbrc.2013.09.078

proteins [12] and thereby inhibit viral replication. In present study, we demonstrated that compound SK-12 inhibits DENV replication by targeting virus protease.

2. Materials and methods

2.1. Docking analysis

The MOE lead-like database was used for computational protein structure preparation and virtual screening. A crystal structure of the DENV-2 NS3/NS2B protease (PDB entry: 2FOM) was obtained from the Brookhaven Protein Data Bank (http://www.pdb.org/), and the docking site of the NS3-NS2B interaction region was identified using the MOE Site Finder. The identified docking site was used for the virtual screening, which consisted of two stages. In the first stage, default parameters were used for virtual screening. The second stage involved refinement, including a structure minimization by the force field MMFF94x, to reevaluate the candidates selected in the first stage of the screening.

2.2. Cells and viruses

DENV-1 (P03-08 strain), DENV-3 (P12-08 strain), and DENV-4 (P11-08 strain) were isolated at the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. DENV-2 (New Guinea C strain) was kindly provided by Dr. Okuno (Osaka Prefectural Institute of Public Health). These viruses were propagated in C6/36 cells for 5–7 days. The culture media were recovered and stored at −80 °C until use. The African green monkey kidney (Vero) cell line was cultured in Eagle's minimum essential medium (MEM) (Nacalai Tesque) supplemented with 10% FCS.

2.3. Screening by the cell-based viral replication assay

The compounds were initially dissolved in dimethyl sulfoxide to a concentration of 1 mM, and then diluted with serum-free MEM to their desired final concentrations. Solutions were centrifuged to remove precipitates. For the biological screening, Vero monolayers in 96-well plates were inoculated for 2 h with viruses at 100 focus-forming units (FFU)/well. The infected cells were further incubated for 72 h in MEM supplemented with 2% FCS (2% FCS-MEM) containing 2% carboxyl-methyl-cellulose in the presence of various concentration of compound ranging from 0.02 to 100 μM. Foci were visualized as previously reported [13]. Foci were counted using a light microscope. The percent inhibition of viral replication was calculated based on the average reduction in the number of foci of three different preparations containing virus. Half-maximal effective concentrations (EC_{50}) for each compound were calculated using GraphPad Prism 4 (GraphPad Software, Inc.).

2.4. Cytotoxicity assay

To evaluate the cytotoxicity of compounds, the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay was performed as previously described [14] with slight modifications. Vero cells plated on 96-well plates at a density of 2.0 × 104 cells/well were incubated in triplicate for 72 h with compounds at various concentrations ranging from 0.02 to 100 μM in 2% FCS-MEM. Vero cells were washed with PBS and incubated with 30 μl MTT solution (PBS containing 0.8 mg/ml MTT) for 2 h at 37 °C. The MTT solution was then replaced with 100 μl dimethyl sulfoxide. After gentle shaking for 10 min at room temperature, the absorbance at 560 nm was measured using a Corona Grating Microplate Reader SH-9000 (Corona, Electric Co., Ltd.). To determine cell viability, half-maximal cytotoxic concentrations (CC50) for each compound were calculated by nonlinear regression fitting using GraphPad Prism 4.

2.5. Construction of plasmids

Viral RNAs were extracted from the culture fluid of C6/36 cells using a QIAamp Viral RNA Mini Kit (Qiagen) in accordance with the manufacturer's instructions. For the synthesis of viral cDNA, reverse transcription was performed using SuperScript III reverse transcriptase with random hexamer primers (Invitrogen). The resulting cDNA was used as a template to amplify the viral genes. For the *in vitro* protease assay, the pGEX-6P-1-chNS2BglyNS3pro plasmid, which produces a glutathione *S*-transferase (GST)-fusion protein, was constructed. In this plasmid, sequences corresponding to the NS2B core hydrophilic (chNS2B) region and the NS3 protease (NS3pro) region were linked by a G4SG4 linker (Fig. 2), as previously reported [7,15]. The chNS2B region contained the 47-amino acid central portion of NS2B (residues 49–95), and the NS3pro region contained the 181-amino acid N-terminal portion of NS3pro (residues 1–181).

2.6. Site-directed mutagenesis

Single amino acid substitutions in plasmids were introduced by PCR. Complementary sense and antisense oligonucleotide primers were synthesized for PCR amplification of DENV-4, JEV cDNA clones (Phanthanawiboon et al., unpublished) and pGEX-6P-1-chNS2BglyNS3pro. The PCR products were digested with *Dpn*I and transformed into *E. coli* HB101, and then the plasmids were extracted and subjected to sequence analysis.

2.7. Steady-state kinetics analysis of DENV protease in vitro

The *in vitro* protease assay was performed according to previous report [16], with slight modifications. The fluorogenic short peptide substrate Boc–Gly–Arg–Arg–peptidyl-4-methylcoumaryl-7-amide (Boc–GRR–MCA) was purchased from Peptide Institute, Inc. (Osaka, Japan). The protease assay was performed in 96-well microtiter plates at 30 °C on a Corona Grating Microplate Reader SH-9000 at an excitation wavelength of 385 nm and an emission wavelength of 465 nm. Purified DENV-2 chNS2BglyNS3pro (50 nM) was pretreated with 25 μM SK-12 for 10 min at 37 °C and then mixed with various concentrations of Boc–GRR–MCA, ranging from 0 to 1.6 mM in cleavage buffer (10 mM Tris–HCl [pH 9.5], 20% glycerol). For each substrate concentratrion, initial reaction velocities were detemined and plotted using GraphPad Prism (GraphPad software). Reaction velocities at steady state were calculated from the slope of reaction progression by non-linear regression of initial velocities. Kinetics parameters, k_m and V_{max} were calculated assuming Michaelis–Menten kinetics equation. The double-reciprocal, known as the Lineweaver–Burk plot was created by plotting the inverse initial velocity ($1/V_0$) as a function of the inverse of the substrate concentration (1/[S]) using enzyme kinetics calculation of the GraphPad Prism.

3. Results

3.1. Structural study

To identify an inhibitor of DENV protease, we first performed a virtual screening to select candidates from a large number of compounds, based on the previously published structure of the NS2B/NS3 protease complex [17]. We screened 661,417 compounds from the Molecular Operating Environment (MOE) lead-like database (http://chemcomp.com/) against the NS2B-binding site of NS3 to

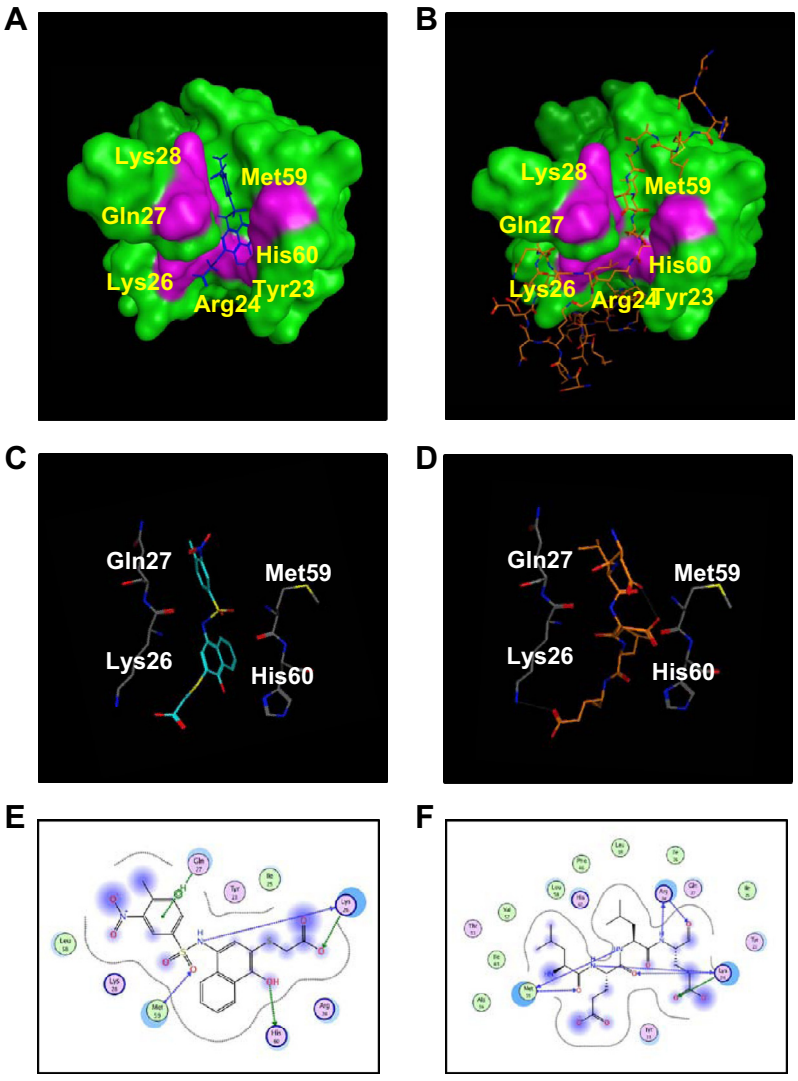


Fig. 1. Predicted model of the conformation of SK-12 and NS2B bound to NS3. (A) Stereo view of SK-12 (green) bound to NS3 (pink). NS3 is shown in a surface representation. SK-12 was predicted to interact with amino acids Lys26, Gln27, Met59, and His60 in the NS2B-binding site of NS3. (B) Stereo view of chNS2B binding to NS3. chNS2B is shown in a frame representation. The amino acids between Ala49 and Glu54 of NS2B are shown in orange. (C) Interactions of SK-12 with Lys26, Gln27, Met59, and His60 of NS3. The red, blue, yellow, and gray bars indicate oxygen, nitrogen, sulfur, and carbon hydrogen atoms, respectively. (D) Interactions of chNS2B with Lys26, Gln27, Met59, and His60 of NS3. (E) Interaction between the NS2B-binding site of NS3 and SK-12. The proximity contour indicates the amount of space that is available for substitutions on the ligand. The blue regions plotted onto the atoms indicate the solvent-accessible surface area of the ligand. Arrows with dotted lines indicate hydrogen bonds. (F) Interaction between NS2B and the NS2B-binding site of NS3. The ligand interaction diagrams were generated using MOE software. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

minimize the value of the interaction energy between NS2B and NS3 by docking analysis. Thirty-nine potential candidates with lower scores, from −90.7 to −30.0 at the second stage, were selected for testing in a cell-based viral replication assay. Among them, we identified SK-12 exhibiting highest inhibitory activity against DENV-2; its activity was similar to that of ARDP0006, a known inhibitor of the DENV protease (data not shown).

The conformation of SK-12 when bound to DENV-2 NS3 was analyzed using the MOE program to identify the intermolecular contacts that contribute to the interaction between SK-12 and NS3. SK-12 was predicted to interact with the NS2B-binding site of NS3 (Fig. 1A) via hydrogen bonds with the amino acid residues Lys26, Gln27, Met59, and His60 (Fig. 1C and E), whereas NS2B was predicted to interact with Arg24 (R24), Lys26 (K26), Gln27 (Q27), Met59 (M59), and His60 (H60) (Fig. 1B, D and F). The binding region of the pocket site for SK-12 overlaps with that of NS2B. We confirmed that SK-12 interferes with the interaction between NS2B and NS3 was interfered in the presence of 10 μM of SK-12 (Supplementary Fig. S1). We further analyzed SK-12 in the subsequent studies.

3.2. SK-12 inhibits all four DENV 1–4

An obstacle to developing therapeutics for DENV infection is the existence of four serotypes. Therapeutic agents ideally need to have a wide range of efficacy against each of these serotypes. Therefore, we next examined whether SK-12 broadly inhibits all four serotypes of DENV using a cell-based viral replication assay. SK-12 efficiently inhibited the replication of all DENV clinical isolates (EC_{50}: 0.97 ± 0.42 μM against DENV-1, 0.98 ± 0.39 μM against DENV-2, 2.43 ± 0.63 μM against DENV-3, and 0.74 ± 0.48 μM against DENV-4), and moderately inhibited that of JEV (EC_{50} = 29.81 ± 9.83 μM) (Table 1). ARDP0006 also efficiently inhibited the replication of all four DENVs (EC_{50}: 5.96 ± 1.86 μM against DENV-1, 0.44 ± 1.24 μM against DENV-2, 3.08 ± 1.16 μM against DENV-3, and 0.88 ± 1.22 μM against DENV-4), and moder-

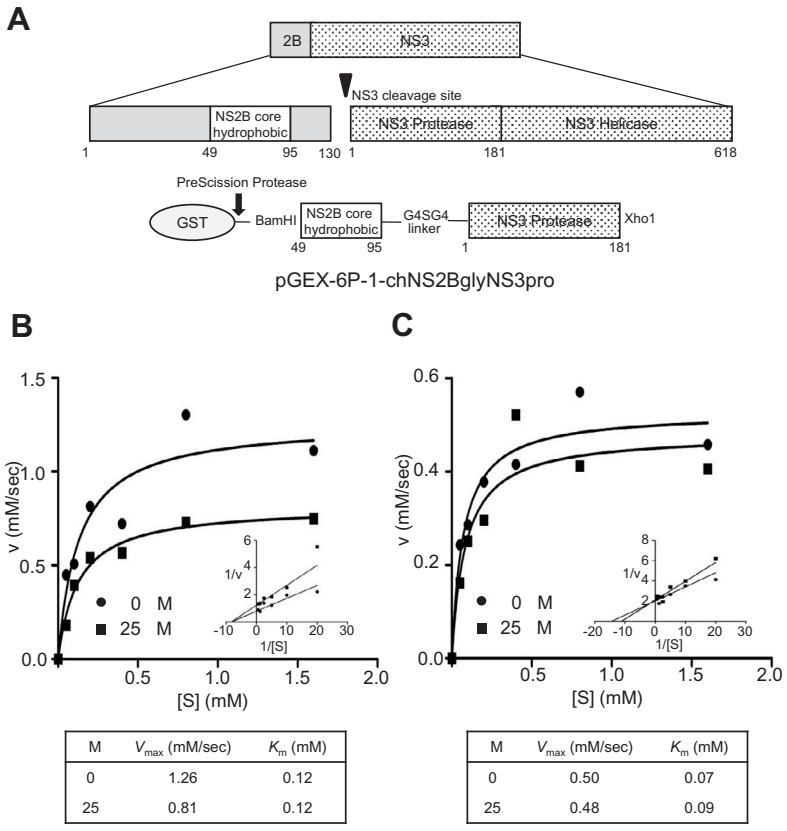
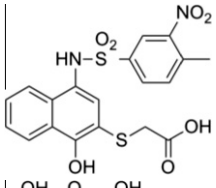
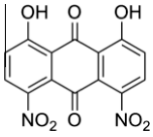


Fig. 2. Protease assay. (A) Diagram of pGEX-6P-1-chNS2BglyNS3pro plasmid. The chNS2B and NS3pro regions were connected by a 4GS4G linker and inserted into the plasmid downstream of the GST gene. The GST-chNS2BglyNS3pro protein was purified with Glutathione Sepharose 4B beads, and the purified protein was eluted by cleavage with the PreScission protease. The purified chNS2BglyNS3pro protein (50 nm) was pre-incubated with SK-12 and ARDP0006 for 10 min at room temperature, and then further incubated with the different concentrations of peptide substrate Boc–Gly–Arg–Arg–MCA for 30 min. Protease activities were determined by monitoring the fluorescence intensity from Boc–Gly–Arg–Arg–MCA (excitation at 385 nm, emission at 465 nm). (B) Analysis of inhibitory activities of SK-12 and ARDP0006 against DENV-2 chNS2BglyNS3pro protease were determined by varying the substrate concentration in the range of 0, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 mM at each inhibitor concentration fixed at 0 μM (circles) and 25 μM (squares). DENV-2 chNS2BglyNS3pro protease curves for SK-12 (B) and ARDP0006 (C). Reactions were performed in triplicate. Lineweaver–Burk plot of inhibition against protease activity (inset). Data were analyzed with the GraphPad Prism 4. The kinetics parameters were calculated as shown in below the graphs.

Table 1
Cytotoxicity and inhibitory effects of compounds to DENVs and JEV in cell-based replication assays.

IUPAC name	Abbreviation	Structure	CC_{50} (μM)[a]	Virus	EC_{50} (μM)[b]	SI[c]
2-{[1-hydroxy-4-(4-methyl-3-nitro benzenesulfon amido)naphthalen-2-yl]sulfanyl}acetic acid	SK-12		67.26 ± 7.00	DENV-1	0.97 ± 0.420	69.34
				DENV-2	0.98 ± 0.39	68.63
				DENV-3	2.43 ± 0.630	27.67
				DENV-4	0.74 ± 0.48	90.76
				JEV	29.81 ± 9.830	2.25
1,8-dihydroxy-4,5-dinitroanthracene-9,10-dione	ARDP0006		20.76 ± 4.63	DENV-1	5.96 ± 1.86	3.48
				DENV-2	0.44 ± 1.24	47.18
				DENV-3	3.08 ± 1.16	6.74
				DENV-4	0.88 ± 1.22	23.59
				JEV	30.11 ± 14.52	0.68

[a] CC_{50s} were determined by the MTT assay. Standard deviations were calculated from at least three independent experiments.
[b] EC_{50s} were determined by the cell-based viral replication assay.
[c] SIs (selectivity indexes) represent the CC_{50} divided by the EC_{50}.

ately inhibited that of JEV (EC_{50}: 30.11 ± 14.52 μM against JEV) (Table 1). MTT toxicity assays were performed to determine the cellular toxicity of candidates. The CC_{50s} of SK-12 and ARDP0006 were 67.26 ± 7.00 and 20.76 ± 4.63 μM, respectively (Table 1). Selectivity indexes ($SI = CC_{50}/EC_{50}$) of SK-12 against DENVs were better than those of ARDP0006 (SI: 69.34 vs. 3.48 against DENV-1, 68.63 vs. 47.18 against DENV-2, 27.67 vs. 6.74 against DENV-3, and 90.76 vs. 23.59 against DENV-4, 2.25 vs. 0.68 against JEV) (Table 1). SK-12, as well as ARDP0006, showed lower SI against JEV compared with their efficacy against DENVs (SI: 2.25 and 0.68).

3.3. SK-12 is a noncompetitive inhibitor against DENV

In the above experiments, we demonstrated that SK-12 inhibits the production of DENV progeny. To confirm whether SK-12 directly inhibits the activity of the NS2B/NS3 protease and deter-

mines the mode of actions, we performed an *in vitro* protease assay using the purified NS2BglyNS3 protease (Fig. 2A) and a fluorogenic short peptide substrate, Boc–GRR–MCA. Steady state enzyme kinetics were examined in the presence or absence of inhibitor. Experimental data were analyzed by using double reciprocal plots. Lineweaver–Burk plots had the nearly same abscissa intercept in the presence or absence of SK-12, and V_{max} value of the NS2BglyNS3 protease complex was decreased in the presence of SK-12 without changing K_m of the substrate (Fig. 2B). This result indicates that the mechanism was a noncompetitive. Contrarily, Lineweaver–Burk plots had the same ordinate intercept in the presence or absence of ARDP0006, and K_m value was increased with no change in V_{max} value (Fig. 2C). This result indicates that the mechanism was competitive, and corresponds to the result of previous prediction study [18]. The result indicates that SK-12 and ARDP0006 target different sites of the NS2B/NS3 protease.

3.4. Mutant analysis

SK-12 exhibited broad inhibitory activity against all DENV serotypes (1–4) but only moderate inhibition against JEV (Table 1). To understand the difference in sensitivities between DENVs and JEV, we performed sequence analysis. Among the interacting amino acids (R24, K26, Q27, M59, and H60), Q27 and H60 are highly conserved in DENV 1–4 but not in JEV (Supplementary Fig. S2), while K26 and M59 are not conserved among DENVs. Sequence variations at positions 26 and 59 of NS3 presumably do not affect the inhibitory activity of SK-12, because these amino acids contribute to the interaction of SK-12 with NS3 via their backbones (Fig. 1E). On the other hand, substitutions of Q27 and H60 may change the sensitivity of SK-12, because the side chains of these amino acids interact with SK-12 (Fig. 1E). Therefore, we hypothesized that Q27 and H60 are important for the association of NS3 with SK-12.

To determine the key residues responsible for the sensitivity to SK-12, we performed mutagenesis analysis using DENV-4 and JEV infectious cDNA clones. First, we investigated whether mutation of Q27 and H60 affect viral replication. The DENV-4 Q27A mutant exhibited virus replication kinetics similar to those of wild-type DENV-4 (Supplementary Fig. S3A). By contrast, the DENV-4 H60S mutant replicated slowly and yielded a peak titer slightly lower than that of wild-type DENV-4 (Supplementary Fig. S3A). The Q27A mutation significantly reduced the sensitivity of DENV-4 to SK-12 (EC_{50}: 10.77 ± 2.53 μM against DENV-4 Q27A mutant, 3.79 ± 2.54 μM against wild-type DENV-4), while the DENV-4 H60S mutant showed similar sensitivity to SK-12 (Table 2). On the other hand, wild-type JEV and two JEV mutants containing conserved DENV residues (A27Q and S60H) replicated similarly and reached similar peak titers (Supplementary Fig. S3B). The JEV A27Q mutant had an increased sensitivity to SK-12, and its EC_{50} was significantly lower than that of wild-type JEV (EC_{50}: 2.94 ± 2.55 μM against JEV A27Q mutant, 14.36 ± 2.79 μM against wild-type JEV) (Table 2). By contrast, the sensitivity of JEV S60H was similar to that of wild-type JEV (EC_{50}: 11.26 ± 7.49 μM). Taken together, these observations indicate that mutations at residue 27 (Q27A in DENV-4 and A27Q in JEV) drastically changed the sensitivities of these viruses to SK-12, suggesting that the amino acid 27 of NS3 underlies the differences in the sensitivities of DENVs and JEV to SK-12.

Table 2
Inhibition activity of SK-12 to DENV-4 and JEV mutants.

Virus	EC_{50} (μM) of SK-12
DENV4	
Wild type	3.79 ± 2.54
Q27A[a]	10.77 ± 2.53
H60S[b]	5.32 ± 1.14
JEV	
Wild type	14.36 ± 2.79
A27Q[c]	2.94 ± 2.55
S60H[d]	11.26 ± 7.49

Significance of differences was evaluated using Student's *t*-test.
[a] $P = 0.009$.
[b] $P = 0.121$.
[c] $P = 0.003$.
[d] $P = 0.105$ (versus wild types).

4. Discussion

Our study provides evidence that targeting the interaction between NS2B and NS3 is an effective antiviral strategy. By blocking the NS2B/NS3 protease, SK-12 significantly inhibited DENV replication. By combining a structural prediction study with a cell-based replication assay using mutant viruses, we elucidated the details of the interaction between NS3 and SK-12. Although SK-12 was less effective against JEV than against DENV, the A27Q mutation in JEV NS3 increased the sensitivity of JEV to SK-12. The presence of a Q residue at position 27 is one of the determinants of the interaction between SK-12 and NS3. Overall, these results not only contribute to the development of inhibitors but also to our understanding of the action of the NS2B/NS3 protease of flaviviruses.

Viral gene-encoded proteases are excellent targets for the development of antiviral agents because of their unique properties, which provide specificity toward the viruses that produce them. For example, small-molecules directed against proteases produced by human immunodeficiency virus type 1 and the hepatitis C virus are clinically effective drugs against these viruses [19–21]. Such drugs target protease active sites and effectively inhibit viral replication. Therefore, in the case of DENV infection, the most attractive target on the DENV protease is the protease catalytic site of NS3 [17]. However, it may be difficult to design inhibitors against DENV NS2B/NS3 protease complex because the catalytic site of DENV NS2B/NS3 protease complex is flat [22]. Alternative strategy is to target other sites forming pocket because small potent inhibitors can stably bind. Recent studies regarding the action of the NS2B/NS3 protease complex provide a potential alternative strategy for the development of DENV inhibitors (7, 8, 12). Indeed, a monoclonal antibody against NS2B blocks the activity of the protease by interfering with the interaction between NS2B and NS3 [23]. The results reported herein strongly support the hypothesis that the interaction between NS2B and NS3 is a valid therapeutic target for anti-DENV drugs. SK-12 and ARDP0006 target different regions of the NS2B/NS3 protease complex: SK-12 interacts with the NS2B-binding site of NS3 and competes for binding of NS2B (Fig. 1), whereas ARDP0006 interacts with the protease catalytic site of NS3 [18]. *In vitro* protease assay verified that SK-12 and ARDP0006 inhibit the protease activity in different manners (Fig. 2B and C), suggesting that two different sites of NS3 could be targeted for the development of drugs against DENV.

The structural study and mutant study demonstrated that the different sensitivities between DENVs and JEV are due to the difference in amino acid at position 27 of NS3. The substitution of Q27 in DENV NS3 with A27 caused a loss of interaction to SK-12 (Supplementary Fig. S4) because SK-12 interacts with the side chain of Q27 (Fig. 1C). DENV may have a chance to escape from SK-12 by introducing mutation to Q27, however, Q27 in DENV is completely conserved (Supplementary Table. S1). This suggests that Q27 is potentially important for virus life cycle and DENV has a lower chance to escape.

In conclusion, this study proved that a combination of structural docking and cell-based viral replication assays is a valuable method to identify a lead inhibitory molecule and the NS2B-bind-

ing site of NS3 could be a valid target for drug development against DENV.

Acknowledgments

We thank Dr. Takeshi Kobayashi, Dr. Wataru Kamitani, and Dr. Mitsuhiro Nishimura of the Research Institute for Microbial Diseases (RIMD), Osaka University, Japan for comments on this manuscript and helpful experimental advice. This work was supported by a Grant-in-aid (21790444) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan and by the JST/JICA, SATREPS projects. The manuscript was proofread by Bio-Edit (Manchester, UK).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.078.

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