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Identification and characterization of nonsubstrate based inhibitors of the essential dengue and West Nile virus proteases

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Abstract—The 72 known members of the *flavivirus* genus include lethal human pathogens such as Yellow Fever, West Nile, and Dengue viruses. There is at present no known chemotherapy for any *flavivirus* and no effective vaccines for most. A common genomic organization and molecular mechanisms of replication in hosts are shared by flaviviruses with a viral serine protease playing a pivotal role in processing the viral polyprotein into component polypeptides, an obligatory step in viral replication. Using the structure of the dengue serine protease complexed with a protein inhibitor as a template, we have identified five compounds, which inhibit the enzyme. We also describe parallel inhibitory activity of these compounds against the West Nile virus Protease. A few of the compounds appear to provide a template for design of more potent and specific inhibitors of the dengue and West Nile virus proteases. Sequence similarities among flaviviral proteases suggests that such compounds might also possibly inhibit other flaviviral proteases.

1. Introduction

The mosquito-borne human pathogens, dengue, and West Nile viruses (WNV) are two of the 72 currently known members of the *flavivirus* genus of flaviviridae. Dengue is endemic to most tropical and sub-tropical regions where the mosquito vectors, *Aedes aegypti* and *Aedes albopictus* are abundant, placing over 40% of the world's population at risk for infection. The self-limiting dengue fever and the more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), caused by four serotypes of dengue (DEN**1**–**4**), together cause thousands of fatalities annually.[1] The WNV is currently re-emerging in North America and Europe.[1,2] At present there is no known chemotherapy for flaviviral infections and effective vaccines are available only against yellow fever,[3] Japanese encephalitis,[4] and tick-borne encephalitis viruses.[5] Recently, therapeutic strategies for use against dengue[6] and WNV[7] has been reported. Although no vaccines licensed for human use against dengue or WNV exist, the efficacy of a recently developed ChimeriVax, candidate WNV vaccine,[8] is currently being evaluated. There is thus a need to explore antiviral chemotherapeutic approaches against both dengue and WN viral infections as a supplement to ongoing vaccine efforts.

Studies on flaviviruses[9–11] have revealed that genomic organization and key molecular events in flaviviral lifecycles are highly conserved (for reviews, see Refs. 12,13). The viral polyprotein is processed co-translationally and post-translationally by cellular signal peptidase,[14,15] a novel host protease[16] and the viral NS3 protease.[17–22] Thus, the viral NS3 protease, NS3-pro, is an attractive potential target for chemotherapy.[17–22] NS3-pro, in association with NS2B, cleaves the viral polyprotein at several polypeptide junctions for both dengue[2,12,21,23,24] and WNV.[19,25] Mutational analyses have established that a 40 residue hydrophilic domain NS2B(H) in NS2B (130 aa) is sufficient for activation

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of NS3-pro in vitro and in cultured mammalian cells.[24,26] All the NS2B:NS3 cleavage sites share an invariant Arg or Lys residue at P1 (nomenclature for the description of protease subsites,[27]) with a second basic residue occupying P2 at most cleavage sites.[20] An amino acid with a short side chain (Ala, Gly, or Ser) frequents the P1′ position. Mutations that inactivate the NS3 protease are lethal for viral replication, underscoring an indispensable role for the protease in the viral life-cycle.[17,28,29] The strongly hydrophilic nature of NS2B(H) indicates that NS2B:NS3-pro interaction is likely to be dominated by hydrophilic contacts, some evidence for which has been obtained from mutagenesis studies on the yellow fever virus protease.[30] The structure of dengue2 (DEN2) NS3-pro has shown it to be a quintessential serine protease, closely resembling the hepatitis C virus (HCV) NS4A:NS3 protease.[31] The structure of the complex of DEN2 NS3-pro with the mung bean Bowman–Birk Inhibitor (MbBBI) indicated that the enzyme was inhibited by classical serine protease inhibitors by the standard mechanism.[32,33] Using the NS2B(H):NS3-pro mimic of the dengue protease, and NS3-pro domain alone, we have demonstrated that NS3-pro can hydrolyze the chromogenic substrate, Arg-*p*-nitrophenylanilide, efficiently but was essentially inactive on the fluorogenic tripeptide substrate, Boc-Gly-Arg-Arg-AMC (7-amido-4-methylcoumarin).[34] Furthermore, NS2B(H) activates NS3-pro by approximately four orders of magnitude in hydrolysis of tripeptide substrates, while it has little effect on hydrolysis of substrates that possess only a P1 side chain, suggesting that interactions between the P1 residue and the enzyme are not strongly dependent on NS2B.[34] The structure of DEN2 NS3-pro:MbBBI identified a bifurcated, redundant recognition mode for an Arg at P1 by NS3-pro (Fig. 1). Although a recent study[35] has proposed a model for NS2B:NS3-pro interaction, in the absence of supportive experimental data the molecular mechanism of activation of NS3-pro by NS2B is currently obscure.

The observation of a bifurcated S1 pocket is unique to DEN2 NS3-pro among trypsin-like proteases. Although its significance, in the absence of NS2B in the structure is unclear, because of the current unavailability of additional structural clues, and in light of the kinetic observation implying a weak effect of NS2B binding on the P1 side chain, it appeared reasonable to search for compounds that could potentially mimic this interaction. While the structure of the corresponding WNV enzyme is unknown, all flaviviral NS2B:NS3 proteases share significant sequence similarities, including among those residues expected to interact with side chains of substrates and inhibitors. It is thus potentially possible for inhibitors of one flaviviral protease to be active against others, although to different extents. We report the identification of five small molecular compounds with inhibitory activity against DEN2 NS2B(H):NS3-pro. In addition, a modeled structure for the WNV protease as well as the inhibitory activities of these compounds against this enzyme are also reported.

2. Results and discussions

Although the structure of the inhibited DEN2 protease has been determined in the absence of the activator NS2B,[32] it was apparent, from our kinetic studies, using DEN2 NS2B(H):NS3-pro, that while NS2B(H) enhances the hydrolysis of tripeptide substrates by about 10^4, it has negligible effect on substrates that only have a P1 side chain.[34] This observation is similar to those on structurally closely related proteases, such as the HCV NS3 protease, that the effects of the cognate activator on the P1 residue are rather small.[36] Undoubtedly, the most appropriate target for a structure assisted search for inhibitors would be a ternary complex in which NS2B, NS3-pro, and a suitable inhibitor are all present. However, despite significant effort by us, and perhaps by others, no such structure has yet been reported. Thus, given the wide spread nature of dengue infections and the current re-emergence of the WNV, it appeared reasonable to explore whether currently existing data might permit a limited search for inhibitory compounds based on the NS3-pro:MbBBI structure. We

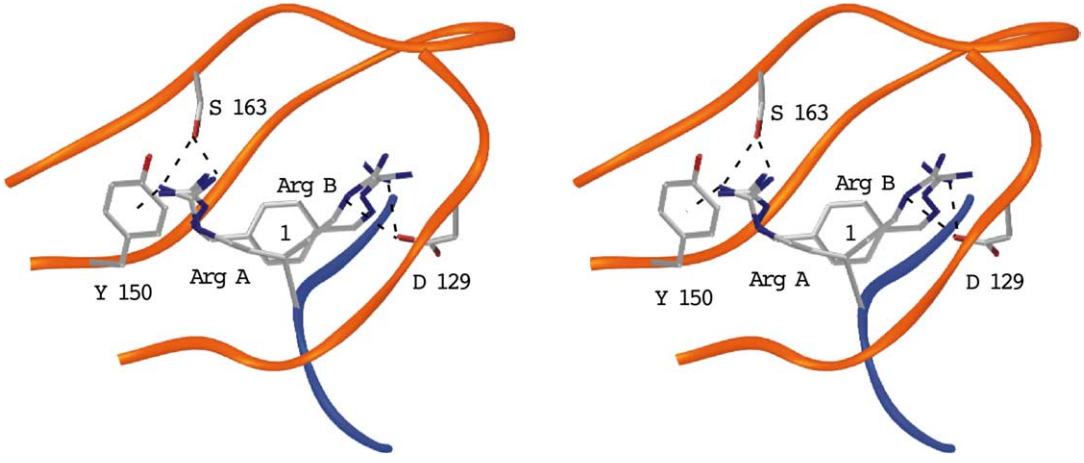


Figure 1. Structural similarity of inhibitor **1** to P1 Arg. Stereo view of a ribbon drawing of a portion of dengue NS3 protease is shown in orange with a section of the main chain of MbBBI that carries the P1 Arg residue shown in blue. Residues that make electrostatic interactions with the P1 Arg side chain are shown as stick objects. Atoms involved in electrostatic interactions are shown connected by dotted lines. The minimum energy conformation of compound **1** is superimposed. Carbon atoms are colored silver, oxygen atoms red, and nitrogen atoms blue.

reasoned that if the bifurcated recognition mode of the P1 Arg seen in the structure represented a satisfactory structural approximation (see Fig. 1), then compounds with terminal guanidino groups, which could be superimposed on the guanidino groups of the bifurcated Arg side chain, might be possible candidates as selective inhibitors of DEN2 NS2B:NS3-pro. Inhibitors of DEN2 protease, derived from peptide substrates with the scissile bond modified to produce α keto-amide transition isosteres, have recently been reported.[37] However, it is equally important to characterize potential nonsubstrate based compounds to obtain information of possible value for design of specific inhibitors. We have restricted our focus to P1 interactions as only these, currently, can be related directly to the NS3-pro:MbBBI structure. However, the NS2B activated form of the dengue enzyme has been used for all kinetic experiments, so that the inhibitors could be evaluated against the more realistic tripeptide substrate.

2.1. Identification of potential inhibitory compounds

None of the 25 compounds identified as potential mimics of bifurcated Arg side chain in NS3-Pro–MbBBI complex in the CSD search were commercially available for further experimental studies, prompting us to search for compounds with guanidino groups in the Available Chemical Directory (ACD, MDL Systems, Foster City, CA). Six compounds containing two terminal guanidino groups were obtained as candidates, of which three compounds **1**–**3** (Fig. 2a) were commercially available. These three biguanidines as well as 17 commercially available compounds with single terminal guanidine group (Fig. 2b) were used for initial inhibition studies. Of these compounds **1**–**5** (Fig. 2a,b) had detectable inhibition ability toward DEN2 NS2B(H):NS3-pro and were chosen for detailed characterization. Modeling studies showed that compound **1** can adopt two conformations, due to possible rotation about the bonds that connect the phenyl ring and the two arms with terminal guanidino groups (Fig. 2a). Two minimum energy conformations are possible; one that is approximately U shaped and a second that has an approximate Z shape. Compound **2** has a single minimum energy conformation, which is nearly planar and rigid, due to the conjugated double bond system extending over it. Compound **3** is more flexible than compound **2**, by virtue of the puckering possible at its central cyclohexyl moiety (Fig. 2a).

2.2. Inhibition of dengue protease

The K_i values for inhibition by biguanidines **1**–**3** (Table 1) show that **1** has the best inhibition constant against DEN2 NS2B(H):NS3-pro, while compound **3** is barely inhibitory, as can be anticipated from geometric fits (Fig. 2a, Table 1). Based on the rms deviation and the centroid-to-centroid (C···C) distance between the guanidino groups in the potential inhibitory compounds (**1**–**3**) (Table 1), the best likely inhibitor for DEN2 NS2B(H):NS3-Pro is expected to be compound **1** in the U conformation (Fig. 3). Although the C···C distance for **2** does not appear to be much different from that in the NS3-Pro–MbBBI complex (Table 1), **2** is too rigid for appropriate orientation of the guanidino groups, to interact with both subpockets, within the NS3-Pro S1 site. Compound 3 has a C···C distance that is significantly larger than the template (Table 1, Fig. 2a) and the flexibility due to the puckering is not sufficient to overcome this and permit it to interact simultaneously with both S1 sub-pockets. Preliminary inhibition studies showed that only two of the 17 compounds with single terminal guanidino group (**4** and **5**) showed good inhibition. K_i values were determined for compounds **4** and **5** and are listed in Table 1. Structural comparison of all the 17 compounds with single terminal guanidino groups showed that only compounds **4** and **5**, that showed better inhibition than compound **1** (Fig. 3), possesses electronegative oxygen that could mimic the carbonyl oxygen of the P1 residue of a protein/peptide inhibitor. Modeling studies on **4** and **5** showed that the amide oxygen at the indolin ring in compound **4** and oxygen of the phosphonic acid group in compound **5** (Fig. 2b) could make hydrogen bond with active site Ser 135 in DEN2 NS3-pro. Such an inhibitor–protein interaction is observed in all serine protease–peptide/protein inhibitor complexes. Out of the 17 compounds with single guanidino arm, one other compound, compound **6**, (Fig. 2) is similar to compound **1**, but with one guanidino arm attached to the phenyl ring, instead of two found in compound **1**, shows poor inhibition when compared to compound **1**. Comparison of the chemical structures of compounds **6** indicate that the second guanidino arm plays a role toward the activity, possibly toward specificity to dengue protease based on the two S1 subsites observed in the crystal structure of DEN2-pro. Similarly comparing compound **5** and **6** (Fig. 3) indicate that the substitution of a phosphonic acid group greatly increases the potency.

2.3. West Nile virus protease: homology model and inhibition

There are significant sequence similarities among flaviviral proteases that have led to expectation of functional similarity. It was thus reasonable expect that the WNV NS2B(H):NS3-pro would behave in a functionally analogous manner to its DEN2 counterpart. Sequence alignment of NS3-Pro of WNV and dengue is shown in Figure 4a and alignment of the 40 residue hydrophilic region of WNV and dengue virus NS2B (NS2B(H)) is shown in Figure 4b. From the sequence alignment (Fig. 4a) 97 of the 180 residues are identical and 24 are similar for an approximately 67% similarity between DEN2 and WN NS3-pro domains; implying a three-dimensional structure for WNV protease that would be largely similar to that of its DEN2 counterpart. We have thus been able to build a three-dimensional model for the WN NS3-pro (Fig. 4c) quite readily from the DEN2 NS3-pro structure.[31] In particular all of the residues involved in binding the P1 residue in the DEN2 NS3-pro–MbBBI complex are strongly conserved in the WNV enzyme (Fig. 5a), prompting us to assay the inhibitors for DEN2, compounds **1**–**5**, against the WNV NS2B(H)–NS3-pro. Kinetic parameters obtained for these compounds are listed in Table 1. As in the case of DEN2, among the biguanidines, **1** shows the best

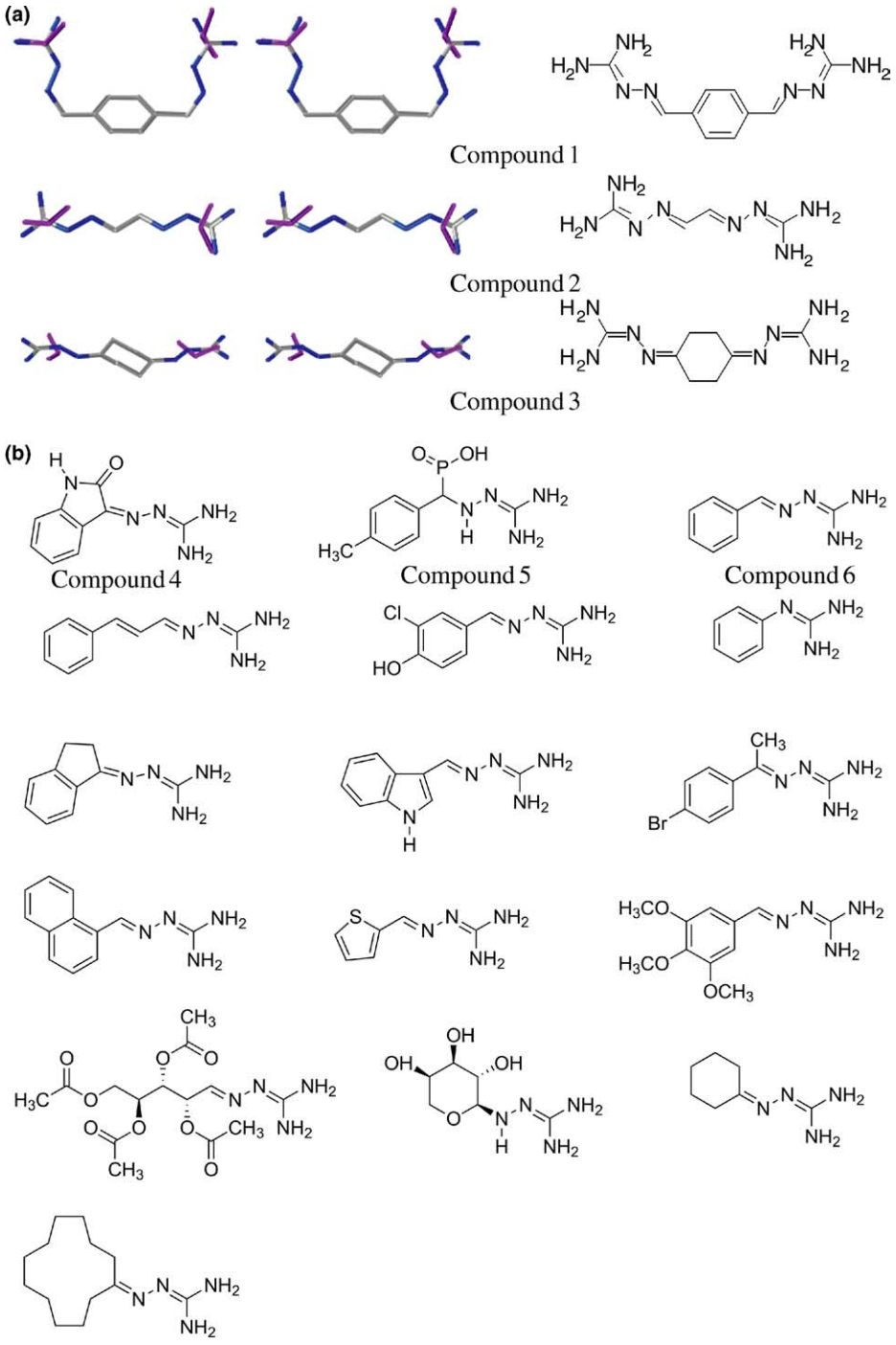


Figure 2. Structures of potential inhibitory compounds. (a) Stereo plots of the minimum energy conformations of compounds **1** (U conformation), **2**, and **3** are superimposed over the two guanidino groups (N–C–N) of NS3–MbBBI complex (pink). Carbon atoms are colored silver and nitrogen atoms blue, for compounds **1**, **2**, and **3**. Corresponding schematic diagrams of compounds **1**, **2**, and **3** are shown beside each of the stereo pairs. (b) Schematic diagrams of compounds **4**–**20**.

activity toward WNV protease. Compounds **4** and **5** also show activities similar to that against the dengue enzyme.

2.4. Inhibition of other proteases

All three biguanidine compounds (**1**–**3**) are clearly selective toward trypsin-like proteases, as they inhibited bovine α-chymotrypsin very poorly. Only inhibition by compound **2** could be measured for α-chymotrypsin ($K_i = 1346 \pm 57\,\mu M$). Bovine trypsin was inhibited with lower potency by both compounds **1** ($87 \pm 13\,\mu M$) and **2** ($155 \pm 20\,\mu M$), while **3** ($51 \pm 5\,\mu M$) was the best trypsin inhibitor. The nearly two-fold greater potency shown by compound **1** toward dengue and WNV NS2B(H):NS3-pro, although experimentally significant, is less than that desired in a potent, specific inhibitor. These observations could, to a large extent, be rational-

Table 1. Kinetic and modeling parameters

Compound	K_i (μM)		RMS (Å)	Cζ–Cζ[a] Distance (Å)
	Dengue 2 NS2B(H):NS3	WNV NS2B(H):NS3		
1	44 ± 5	35 ± 5	0.68	7.7 (10.3)[b]
2	423 ± 50	337 ± 56	0.88	8.3
3	1783 ± 113	1088 ± 162	1.50	9.6
4	23 ± 2	16 ± 2	—	—
5	14 ± 2	13 ± 1	—	—

[a] Refers to the analog, in inhibitors, of the distance between Cζ atoms of the two conformations of the P1 Arg residue (7.5 Å).
[b] Distance for the compound **1** in the U conformation is shown (that for the Z conformation is in parentheses). All values are given as three measurements ± standard error.

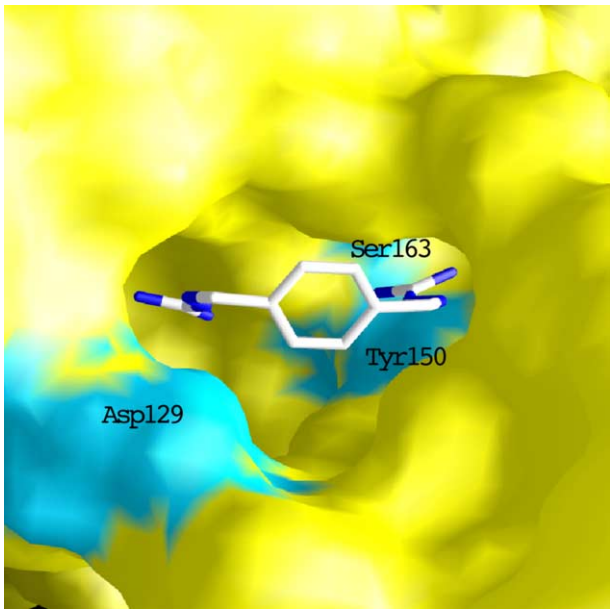


Figure 3. Putative mechanisms of inhibition. Molecular surface of DEN2 NS3-pro, colored yellow, with binding of compound **1** is shown. Contribution to the surface from residues that provide primary electrostatic stabilization for the P1 Arg in the S1 pockets of NS3-pro (Asp129, Tyr150, and Ser163) are colored turquoise and labeled.

ized by the modeling. Although the presence of the guanidino groups make **1**–**3** likely trypsin inhibitors, trypsin has a S1 pocket designed to accommodate a single Arg/Lys conformation, equivalent to the B conformation in NS3-pro:MbBBI (Fig. 1), with Asp189 playing the role of Asp129 in the latter. Thus, **1** in U conformation could not be docked in the S1 pocket without serious steric clashes involving the second guanidino arm in the trypsin S1 pocket. The Z conformation of **1** has a better fit, with no main chain short contacts. Modeling of **2**:trypsin complex showed that the **2** either occupies a position with good hydrogen bonding geometry with Asp189 of trypsin with attendant severe steric clashes, or a position with fewer clashes but with a sub-optimal interaction geometry toward Asp189. Compound **3** is similar to some cyclohexyl ring containing trypsin inhibitors. With one guanidino arm in the pocket, the only steric clashes of **3** are with Ser195 O^{γ}, easily relieved by the puckering of the cyclohexyl ring or by a small change in the Ser195 $\chi 1$. Crystal structure of **3**:trypsin complex has now been determined and will be published elsewhere. Since compounds **4** and **5** has single guanidino group, it is expected from the structures that these compounds could be more potent inhibitors of trypsin. Compounds **4** and **5**, respectively, showed inhibition constants of 4.0 ± 0.4 and 3.2 ± 0.5.

2.5. Potential for inhibition of other flaviviral proteases

There are significant sequence, and consequent structural similarities among flaviviral proteins, including NS3-pro.[9,12,38] The similarity is evident among protease residues that are likely to contact substrate side chains during catalysis. There is also a remarkable conservation of the P1 side chain among flaviviral polyprotein cleavage sites, with nearly 80% of these being Arg residues.[32] These two observations together suggest that compounds that are inhibitory against the DEN2 protease might also inhibit proteases from other flaviviruses, as we have observed for the WNV enzyme. The DEN2 NS3-pro:MbBBI structure permits a comparison of residues in flaviviral proteases that are likely to be involved in substrate and inhibitor interactions. A multiple sequence alignment of flaviviral NS3-pro sequences (Fig. 5a), shows residues that make van der Waals or electrostatic contacts with the P1 residue in MbBBI in DEN2 NS3-pro display remarkable conservation. Figure 5b shows these residues highlighted in the NS3-pro:MbBBI structure. It can be seen that residues Asp129, Gly133, Thr134, Ser135, Tyr150, Gly151, Asn152, Ser163, and Ile165 are absolutely conserved (green in Fig. 5a,b); all except the last make specific, polar interactions with either a P1 Lys or Arg residue. Residues that vary, Thr115 (interacting through its methyl group), Ala127, Leu128, Pro132, and Gly160 in DEN2, make nonpolar interactions in the NS3-pro:MbBBI structure and do not contribute to binding specificity (blue in Fig. 5a,b). Conservation of the S1 pockets and the dominance of Arg at P1 sites, suggest that compounds, such as **1**, **4**, and **5**, that inhibit DEN2 NS2B(H):NS3-pro and its WNV counterpart, might also inhibit other flaviviral NS3 proteases.

3. Conclusions

In summary, compound **1** shows reasonable inhibitory potency toward Dengue and WNV NS2B/NS3-pro and is a possible potential selective inhibitor based on the structure. Replacing the central phenyl ring in compound **1** with other heterocyclic rings and/or substituting

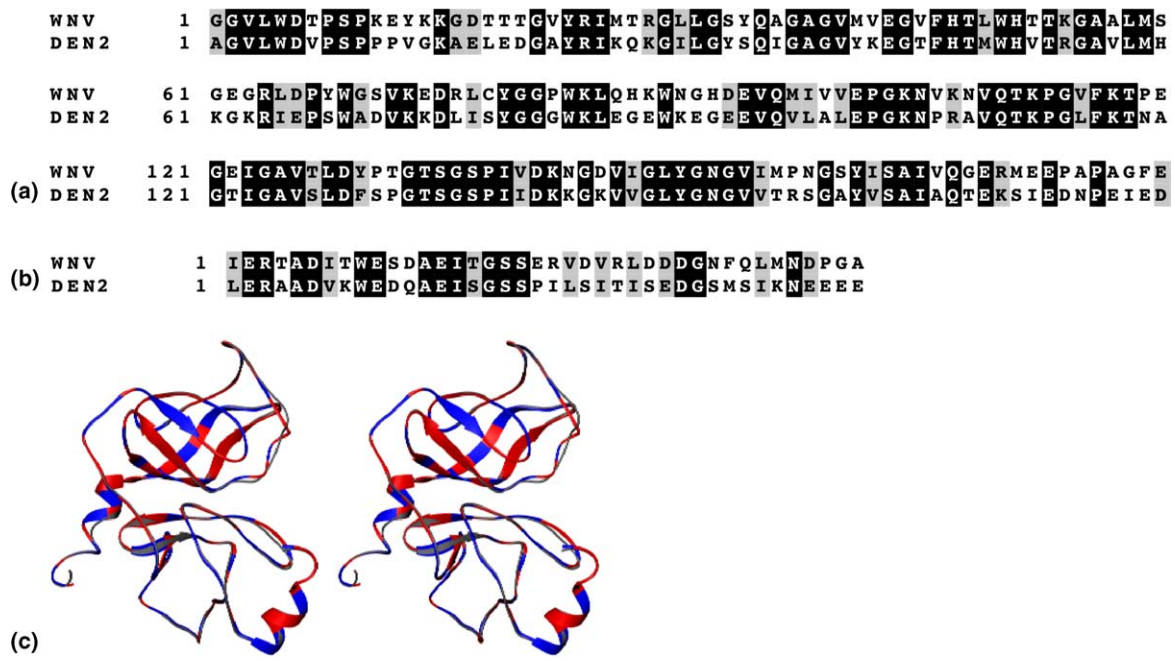


Figure 4. Sequence alignment and homology modeling. (a) Sequence alignment of the NS3 protease domain (NS3-pro) of WNV and dengue virus protease. (b) Sequence alignment of the 40 residue hydrophilic region of WNV and dengue virus NS2B (NS2B(H)). Identical residues are shaded black. (c) Ribbon representation of the superposition of the homology model of WNV NS3-pro on the crystal structure of dengue2 NS3-pro (gray). Identical residues between dengue and WNV NS3-pro are colored red and nonidentical residues are colored blue.

the biguanidine arm at various positions in the ring could improve the potency of the compounds. Substitution of second guanidine group in compounds **4** and **5** could also help to achieve possible specificity toward dengue and WNV. It thus appears that compounds **1**, **4**, and **5** are reasonable compounds for further development of lead compounds. Because there currently are no antiviral compounds in use against dengue, these results are the first step toward possible chemotherapy of dengue infections. In the larger context, our results suggest the possibility of designing pan-flaviviral protease inhibitors. Because of the progressive spread of flaviviruses from their native habitats, it is hard to anticipate appearance of a new *flavivirus* into a given geographical area as shown by the current appearance of WNV in areas where flaviviruses had not been seen before or had been eradicated. Inhibitors that are likely to be effective against several flaviviral proteases, although optimally against the intended target, could form the initial line of defense against a novel infection. The sub-optimal, but reasonable, level of protection provided against new flaviviruses could prevent fatalities and provide time for optimization of extant inhibitors against the new target. Because the new target is likely to have significant structural complementarity against already available inhibitors these latter could form excellent starting points for the process of optimization.

4. Experimental

4.1. Reagents

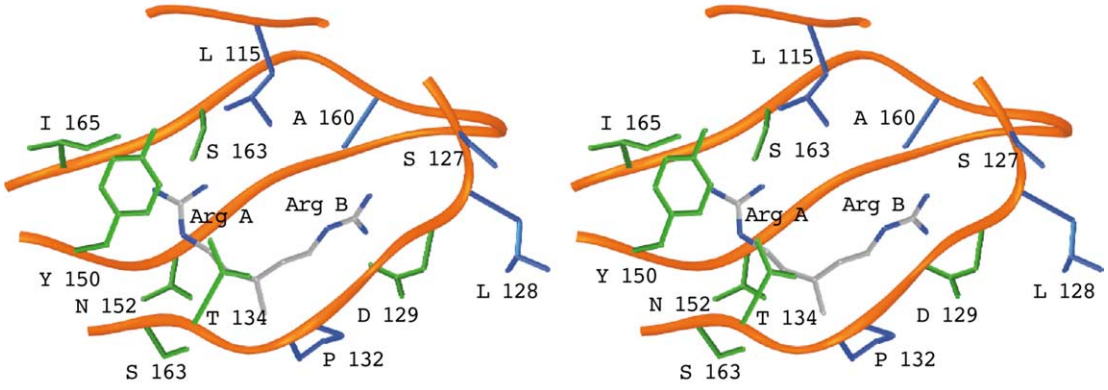
All potential inhibitory compounds for testing were purchased from Ryan Scientific (Isle of Palms, SC). Peptide substrates with fluorescent reporter 7-amido-4-methylcoumarin (AMC) were used for the protease assays. Boc-Gly-Arg-Arg-AMC, substrate for DEN2 and WNV NS2B(H)–NS3-pro, was purchased from Peninsula laboratories (Belmont, CA).

4.2. Molecular modeling

Since there were no three-dimensional coordinates available for the compounds identified from ACD, minimum energy conformations were computed to understand their conformational flexibility and restrictions by performing molecular mechanics (MM) and molecular dynamics (MD) simulations. Computations were performed using the BUILDER and DISCOVER modules in Insight II (Accelrys Inc., San Diego, CA). After building the model for each compound, it was subjected to energy minimization using the steepest descent method. Subsequently dynamics simulation was performed for 200 ps with a step size of 1 fs and an initial equilibration for 1 ps. A distance dependent dielectric constant and the CFF91 force field[39] were used for all MM and MD calculations. The five lowest energy snapshots of the simulation were energy minimized by the steepest descent method to arrive at an energetically favorable conformation for each of the compounds. The two guanidino groups of **1**, **2**, and **3** were then superimposed on the guanidino groups of the two conformations of arginine in the crystal structure to obtain the RMS deviation between $(N–C–N)_2$. Modeling of the complexes of the two compounds **4** and **5**, with single guanidino groups (Fig. 2b), with DEN2 NS3-pro, was done by manually docking the compounds in the S1 site in DEN2 NS3-pro.[32]

Virus	115	127.129	132..135	150.152	160	163	165
Langat	E	P I D	K G T S	Y G N	T	S	I
TBEV	E	P I D	K G T S	Y G N	T	S	I
Powassan	K	P I D	R G T S	Y G N	V	S	I
JEV	V	S L D	R G T S	Y G N	S	S	I
MVEV	I	S L D	I G T S	Y G N	A	S	I
Kunjin	V	T L D	T G T S	Y G N	S	S	I
WNV	V	T L D	T G T S	Y G N	S	S	I
Den 1	T	A L D	P G T S	Y G N	T	S	I
Den 2	I	A L D	P G T S	Y G N	G	S	I
Den 3	L	S L D	P G T S	Y G N	A	S	I
Den 4	L	T L D	P G T S	Y G N	D	S	I
YFV	L	A L D	S G T S	Y G N	S	S	I

(a)



(b)

Figure 5. Similarity in flaviviral sequences of residues that interact with P1 Arg. (a) Sequence concordance of residues binding P1-Arg: Residues that interact with the P1 side chain in the dengue NS3-pro–MbBBI complex aligned with 12 other flaviviruses is shown. Sequence positions (dengue numbering) are shown with dots showing breaks. Residues that are conserved are colored green while others are colored blue. The abbreviations (GenBank accession No.) are: Langat, Langat virus (AAF75259); TBEV, Tick Borne Encephalitis Virus (P14336); Powassan, Powassan virus (Q04538); JEV, Japanese Encephalitis Virus (GNWVJS); MVEV, Murray Valley Encephalitis Virus (GNWVMV); Kunjin, Kunjin Virus (GNWVKV); WNV, West Nile Virus (P06935); DEN1-4, four serotypes of the Dengue Virus (A42551, JS0219, GNWVD3, AAK01233); YFV, Yellow Fever Virus (P19901). (b) Conservation of residues binding P1-Arg: Stereo pair of a ribbon drawing of sections of the main chains of NS3-pro (orange). Carbon atoms are colored silver and nitrogen atoms blue, for the bifurcated P1 Arg side chain of MbBBI. All residues that are conserved (panel A) are shown in green. Residues that are different from those in dengue NS3-pro in any other *flavivirus* are shown in blue.

Homology modeling of the WNV NS3-pro was performed using MODELLER 4.0,[40] using the crystal structure of DEN2 NS3-Pro (PDB # 1BEF) as the template. The template and the target sequences were aligned automatically, without gaps, using CLUSTALW.[41] The aligned sequences were used to construct a model of WNV NS3-Pro using the 'Model' ROUTINE in the MODELLER 4.0. The constructed model was then optimized using conjugate gradient (CG) method for 100 cycles and further refined by simulated annealing with molecular dynamics in MODELLER. The resultant model was finally optimized for 100 CG cycles. Figures 1, 2a, 4c and 5b were made using RIBBONS[42] and Figure 3 was made using GRASP.[43]

4.3. Protease assays

DEN2 NS2B(H):NS3-pro was purified as reported earlier.[34] The expression and purification of WNV NS2B(H):NS3-pro was essentially as described for the DEN2 enzyme with minor modifications, which will be described elsewhere. All proteases were assayed at room temperature with a microtiter plate spectrofluorometer under conditions optimal for each. The assay conditions were, DEN2 and WNV NS2B(H):NS3-pro:100 mM Tris pH 8.5, 50 mM NaCl, 5 nM protein; trypsin:100 mM Tris pH 7.6, 1 nM protein. Three different concentrations of inhibitors were used for calculating K_i values. Kinetic data were measured with an excitation wavelength of 390 nm and emission wavelength of 460 nm. From the initial velocities of the reactions K_i values were calculated by nonlinear regression method using the program EZ-fit (Perella Scientific).

4.4. Multiple sequence alignment

The known NS3 protein sequences for 12 flaviviruses were extracted from GenBank. Multiple sequence alignment was done using CLUSTALW.[41]

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