Asia Pacific Journal of Molecular Biology and Biotechnology, 2007
Vol. 15 (2) : 53-59

Nonsubstrate Based Inhibitors of Dengue Virus Serine Protease: A Molecular Docking Approach to Study Binding Interactions between Protease and Inhibitors

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Received 8 February 2007 / Accepted 18 May 2007

Abstract. The protein-ligand binding interactions studies were carried out by performing dockings of the ligands that were found to be competitively inhibiting the activities of the DEN2 NS2B/NS3 serine protease onto the catalytic triad of a model of DEN2 NS2B/NS3 protease. Results indicate the importance of three out of the five residues reported to be essential for binding activities of the NS2B/NS3 serine protease. These residues are Tyr-150, Asn-152 and Gly-153. In addition, Ser-135 and Gly-151 were also found to be very important in forming hydrogen bonds with the inhibitors. Moreover, Ser-131, Pro-132, Tyr-150 and Asn-152 were found to be important for van der Waals interaction of the ligand, while Val-52, Leu-128, Pro-132 and Val-155 are involved in hydrophobic interaction with the inhibitors.

Keywords. Dengue virus, serine protease, NS2B/NS3 complex, ligand docking

INTRODUCTION

Dengue is a serious disease that is endemic in over 100 countries, with more than 2.5 billion people at risk for epidemic transmission. About 100 million cases of Dengue Fever (DF) and 500 000 cases of Dengue Haemorrhagic Fever (DHF) have been reported globally and this figure has been on the rise in the recent years. It was reported that 40 % of the world's populations to be at risk from dengue with no effective treatment, vaccine or drug (Kautner, *et al*, 1997, Monath, 1994). The Ministry of Health Malaysia, reported that in Malaysia dengue fever killed 44 people in the first four months of 2007 and a record number of dengue suspected cases were seen in 2007 with 900 suspected cases in the first week of June. This is an increase of more than 100 percent compared to the same period of 2006 (Sun Malaysia, 2007).

The main dengue vector is the female *Aedes aegypti* and *Aedes albopictus.* A mosquito feeding on a person during the first to fifth days of illness can transmit the virus to another person. Following the virus incubation period for 8-10 days in the vector, the virus can be transmitted by an infected mosquito to susceptible individuals through blood feeding (WHO, 2002).

DF and DHF are caused by the dengue virus which is a member of the Flaviviridae. There are four serotypes of dengue virus, DEN1, DEN2, DEN3 and DEN4, with DEN2 being the most prevalent. The RNA genome of DEN2 contains 10 723 nucleotides and encodes a large polyprotein precursor of 3 391 amino acid residues which consist of three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Irie *et al.*, 1989). Optimal activity of the NS3 serine protease is required for the maturation of the virus and the presence of the NS2B co-factor is a pre-requisite for the optimal catalytic activity of NS3 (Bianchi and Pessi, 2002 and references therein). Studies revealed that NS3, the second larger protein encoded by the virus, contains a serine proteinase catalytic triad within terminal region of 180 amino acid residues and that it requires the 40 amino acid residues of NS2B for protease activity (Chambers et al., 1993; Arias *et al.* 1993; Jan *et al.*, 1995). The processing of the polyprotein precursor occurs co-translationally as well as post-translationally and is performed by either the

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host signalase in association with the membranes of the endoplasmic reticulum or the viral protease. The NS2B/NS3 component of the protease activates the cleavage in the non-structural region of the viral polyprotein at NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions (Arias *et al*., 1993; Teo *et al*., 1997; Yusof *et al*., 2000). These sites have, in common, a pair of dibasic amino acids, Lys-Arg, Arg-Arg, Arg-Lys and occasionally, Gln-Arg at the P1 and P2 positions, followed by the short chain amino acid, Gly, Ser or Ala at the P1' position (see Schechter and Berger, 1967 for the nomenclature). In addition, the viral protease has been found to cleave internally within NS2A (Nestorowicz *et al.,* 1994) and NS3 (Falgout *et al*., 1991).

Mutational analyses revealed the importance of several amino acid residues that are highly conserved among the flaviviruses, where 5 putative substrate binding residues (Asp-129, Phe-130, Tyr-150, Asn-152 and Gly-153) were proposed (Valle and Falgout, 1998). Computer modelling and analysis of the substrate binding at the catalytic triad of the crystal structure of NS3 without its NS2B cofactor revealed that Gly-133 and Ser-135 to be the most likely to form the oxyanion hole (Murthy et al., 1999). Hydrogen bonding interactions have been observed between the main chain of P1 and P2 residues with appropriate main chain atoms of Gly-153 and Asn-152 to generate the short section of β-sheet common in serine protease-inhibitor interactions (Read and James, 1986). Three residues, Ser-131, Tyr-150, and Ser-163, are within the S1 pocket. A serine side chain at P1' fits into the S1' pocket formed by the catalytic His-51 and Ser-135 and residues Gly-35, Ile-36, and Val-52. The Oε1 atom of Asn-152 forms a salt bridge/hydrogen bond with Nε of the P2 Arg in the modelled complex (Murthy *et al*., 1999).

In this paper we describe the *in silico* study of the molecular binding interactions between the DEN2 NS2B/NS3 serine protease with competitive inhibitors that are observed *in vitro* (Tan *et al*, 2005). Although the crystal structure of DEN2 NS3 has been reported (Murthy *et al*., 1999), the absence of NS2B cofactor therein makes the mechanism of proteolytic process activation unclear. The orientation of the carboxyl side chain of Asp-75 away from His-51 in the catalytic triad of NS3 crystals formed an open conformation that may lead to the inefficiency of proteolytic activity. Previous study on the homology model generated from the HCV template have shown a better quality of protein folding and side chain conformation (Lee *et al*., 2006), as well as the orientation of the catalytic triad to enable efficient proton transfer, as compared to crystal structure of NS3 in absence of N2SB as cofactor. Hence, in this study, the crystal structure of the NS3 with its cofactor NS4A complex in HCV was chosen as the template to generate the DEN2 NS2B/NS3 model. Binding interactions between three competitive inhibitors (based on experimental studies) and dengue virus serine protease were studied by performing protein-ligand docking using AUTODOCK 3.05. It is hoped that the results obtained would provide some insights into the molecular interaction of these ligands and the NS2B/NS3 protease and into their mechanism of action.

MATERIAL AND METHODS

Homology model building. The Modeller software package (mod6v2) was used to build the NS2B/NS3 protease complex model (Sali and Blundell, 1993). The homology modelling of NS2B/NS3 of dengue virus type 2 was performed with HCV serine protease NS3/4A (pdb ID. 1jxp) as a template. The sequence alignment was carried out based on published results (Brinkworth *et al.,* 1999). The model generated was then submitted to the UCLA bioinformatics server for evaluation of its quality using PROCHECK (Morris et al., 1992), VERIFY3D (Bowie *et al*., 1991) and ERRAT (Colovos and Yeates, 1993).

Docking experiment using homology model. The docking of three competitive bioactive molecules, 4-hydroxypanduratin A (1), panduratin A (2) and ethyl 3-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxy)propanoate (3) (termed as "ester (3)" in later discussion), onto the catalytic triad of the serine protease were performed using AUTODOCK 3.05 software package (Morris et al., 1998). The homology model of DEN2 NS2B/NS3 protease molecule was added polar hydrogen atoms and its non-polar hydrogen atoms were merged. Kollman charges were assigned and solvation parameters were added to this enzyme molecule. For the ligands, non-polar hydrogen atoms were merged with Gasteiger charges assigned. All rotatable bonds of ligands were set to be rotatable. Docking was performed using genetic algorithm and local search methods. A population size of 150 and 10 millions energy evaluations were used for 100 times searches, with a 60 x 60 x 60 dimension of grid box size and 0.375 Å grid spacing around the catalytic triad. Clustering histogram analyses were performed after the docking searches. The best conformations were chosen from the lowest docked energy that populated in the highest number of molecules in a particular cluster with not more than 1.5 Å root-mean-square deviation (rmsd). The H-bond, van der Waals and other binding interactions were analysed using Viewerlite 4.2 (Accelrys Software Inc.).

RESULTS AND DISCUSSION

Homology model of DEN2 NS2B/NS3. Although the overall sequence identity between DEN2 NS2B/NS3 and HCV NS3/NS4A was only 14.8%, the region surrounding the catalytic triad of the protease and the residues involved in the substrate binding showed a high level of sequence identity and is conserved, as reported by Brinkworth and

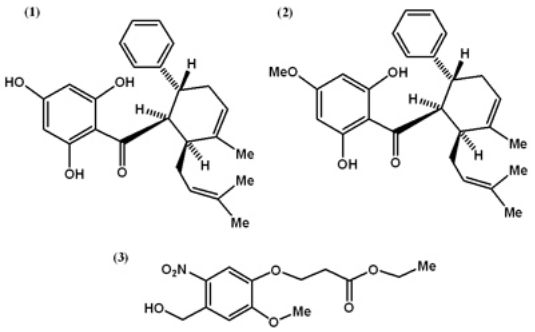

Figure 1. Structure of 4-hydroxypanduratin A (1), panduratin A (2) and ethyl 3-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxy)propanoate (3).

Table 1. Ki values of the found competitive inhibitors

Compound	K_i, μM
4-OH-panduratin A (1)	21
Panduratin A (2)	25
Ester (3)	59

co-workers (Brinkworth *et al.,* 1999). In addition, the results obtained from PROCHECK (all the non-glycine residue are in allowed region), VERIFY3D (90.4) and ERRAT (77.1) revealed the good quality of the model built as described by Lee and co-workers (Lee *et al.*, 2006).

Inhibition of bioactive compounds towards DEN2 NS2B/NS3. Several competitive inhibitors towards DEN2 serine protease activity have been discovered through a substrate-based approach by mimicking the polyprotein cleavage junctions (Chanprapaph *et al.*, 2005). In addition, α-keto peptidomimetic (Leung *et al.*, 2001) compounds and guanidine derivatives (Ganesh *et al.,* 2005) have also been targeted as competitive inhibitors towards the DEN2 serine protease. In our lab, the natural products, 4-hydroxpanduratin A (**1**) and panduratin A (**2**) as well as the synthesized compound, ethyl 3-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxy)propanoate, (**3**) were found to competitively inhibit the activity of the DEN2 serine protease. The structures of these compounds are shown in Figure 1. The K_i values for these compounds obtained from inhibition study using recombinant NS2B/NS3 enzyme showed that 4-hydroxypanduratin A (**1**) to be the most potent, followed by panduratin A (**2**) and ester (**3**) (Table 1).

Active site docking. The three inhibitors (Figure 1) were used as ligands in the binding interaction studies with the active site of the DEN2 serine protease. Docking of these compounds to the active sites revealed 4-hydroxypanduratin A (**1**) to have

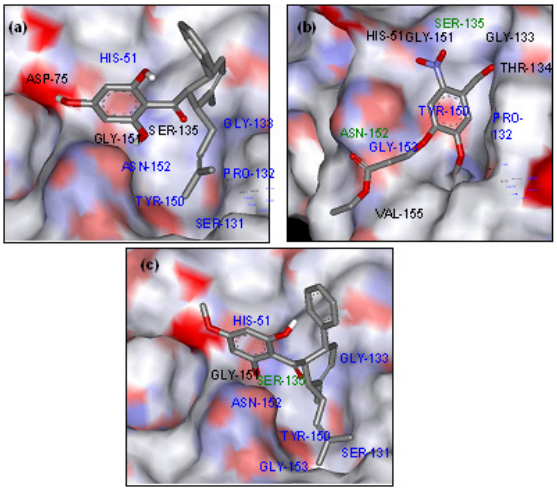

Figure 2. Connolly surface representations of the active site of serine protease domain of the NS2B/3 protease of DEN2 with the bound ligands, (a) 4-hydroxypanduratin A (1), (b) ester (3) and (c) panduratin A (2), which is shown in stick model. Connolly surface of the active site of the protease is coloured according to a charge spectrum: acidic groups are red, basic groups are blue and neutral groups are white. Residues labeled in black are those that may involve in H-bond/salt bridge interaction with ligand. Residues in labeled blue are involved in van der Waals interactions, while those in green are involved in both the van der Waals and H-bond interactions.

the lowest docked energy, followed by panduratin A (**2**) and the ester (**3**) (Table 2).

These results are parallel to the K_i value observed experimentally for these compounds. Although panduratin A (**2**) showed the best free energy of binding, estimated inhibition constant and intermolecular energy, it has a higher torsional free energy and internal energy values than 4-hydroxypanduratin A (Table 2). This may be the cause of its weaker binding to the enzyme when compared to 4-hydroxypanduratin A (**1**). The ester (3) with the most number of rotatable torsion points suffer a higher torsional free energy resulting in a lower binding affinity as compared to the other ligands.

All the ligands showed reasonably low internal energy indicating that the docked conformers were in their most favourable conformations. The spatial arrangement of the three ligands bound to the active site of DEN2 NS2B/NS3 serine protease is shown in Figure 2.

Interactions between inhibitors and residues in NS2B/NS3. Hydrogen bond analysis was performed on the docked NS2B/NS3 protein complex to determine the possibility of hydrogen bonding or salt bridge formation between the ligands and the active site of NS2B/NS3 protease. The criteria for hydrogen bond interaction used is when the distance between the hydrogen and the heteroatom is within

Table 2. Energies (in kcal/mol) calculated using AUTODOCK 3.05

Ligand	4-Hydroxypanduratin A (1)	Panduratin A (2)	Ester (3)
1. Estimated Free Energy of Binding	-7.4	-7.7	-6.1
2. Estimated Inhibition Constant, μM (K_i)	+3.9	+2.3	+33.6
3. Final Docked Energy	-10.2	-10.1	-9.2
4. Final Intermolecular Energy	-8.9	-9.6	-8.9
5. Final Internal Energy of Ligand	-1.3	-0.6	-0.3
6. Torsional Free Energy	+1.6	+1.9	+2.8

* Estimated Free Energy of Binging is derived from the Final Internal Energy (4) and Torsional Free Energy (6) while the Final Docked Energy is the combination of Final Internal Energy (4) and Final Internal Energy of Ligand (5).

Table 3. Residues in the active site of DEN2 NS2B/NS3 that are involved in hydrogen bonding with the various ligands

Residues	4-Hydroxypanduratin A (1)	Ester (3)	Panduratin A (2)
His-51		√	
Asp-75	√		
Ser-131		√	
Gly-133		√	
Thr-134		√	
Ser-135	√	√	√
Gly-151	√	√	√
Asn-152		√	
Val-155		√	

Table 4. Residues in the active site of DEN2 NS2B/NS3 that are involved in van der Waals interaction

Residues	4-Hydroxypanduratin A (1)	Panduratin A (2)	Ester (3)
His-51	√	√	
Ser-131	√	√	√
Pro-132	√	√	√
Gly-133	√	√	
Ser-135		√	√
Tyr-150	√	√	√
Asn-152	√	√	√
Gly-153		√	√

the range of 2.5-3.5 Å and the bond angle is at 109°-110°. Overall, these ligands exhibited binding interactions within the active site and the residues as suggested by Bazan and Fletterick (Bazan and Fletterick, 1989).

Oxyanion holes have been observed to interact with ligands within the active site of the serine protease at the residues Gly-133 and Ser-135 by Murthy *et al.* (Murthy *et al.,*1999). Interestingly, in our study, all the ligands were observed to form hydrogen bond with the carbonyl group of Gly-151 and the hydroxyl of Ser-135 side chain (Table 3). This seemed to suggest a different binding mode of the ligands to the enzyme than those observed by Murthy and his co-workers. The amino group of the residues backbone Gly-133 and Ser-135 were suggested by Murthy *et al.* to be involved in forming the oxyanion hole. However, in our study, the oxyanion holes formation by these residues was observed only in the binding interaction with the ester (**3**) but not with the 4-hydroxypanduratin (**1**) and panduratin A (**2**) (Table 3).

Substitution of the hydroxyl group in the 4-hydroxypanduratin A (**1**) with the methoxy group seemed to lead to a higher K_i value observed with panduratin A (**2**). This could be attributed to the loss of hydrogen bonding with the carboxyl of Asp-75 side chain in panduratin A (**2**) which was observed with 4-hydroxypanduratin A (**1**). Similarly, with the ester (**3**), a higher K_i value observed could be attributed to its structural flexibility. However, this flexibility was somewhat compensated by additional hydrogen bonds between the ester and Ser-131, Thr-134, Asn-152 and Val-155.

All the ligands exhibited van der Waals interactions with Ser-131, Pro-132 and Try-150, suggesting the importance of these three residues as part of the van der Waals specificity pocket. Nevertheless, His-51, Gly-133, Ser-135, Asn-152 and Gly-153 may also play important roles in such binding interactions (Table 4). The small hydrophobic specificity pocket for P1 which is formed by the residues Leu-135, Phe-154 and Ala-157 in HCV NS3/4A protease (Love *et al.*, 1996) is equivalent to the residues Ser-131, Tyr-150 and Gly-153 in the DEN2 NS2B/NS3 protease (Brinkworth *et al.*, 1999). These latter residues were found to be involved in the van der Waals interactions with the ligands (**1**), (**2**) and (**3**) suggesting a more hydrophilic interaction of the enzyme to the ligand as compared to the more hydrophobic interaction observed in HCV NS3/4A protease. In addition, Ser-131 which has been shown to not be important as a binding residue in previous study (Brinkworth *et al.*, 1999) is now observed to be involved in the van der Waals interaction with the ligands in our docking study.

Additional interactions were observed between the ligands (**1**) and (**2**) with the residues Val-52, Leu-128, Pro-132 and Val-155 in the hydrophobic site in the binding pocket of DEN2 serine protease whereby the orientation of these hydrophobic residues seemed to fit in with the ligands (**1**) and (**2**). Here, the phenyl group of the hexacyclic portion in ligands (**1**) and (**2**)

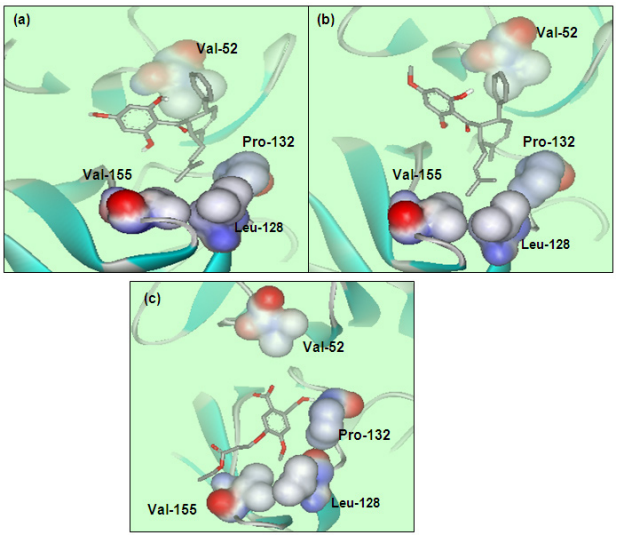

Figure 3. Hydrophobic interaction illustration between ligands (in stick) and NS2B/NS3 protease (in ribbon). Ligand in a: 4-hydroxypanduratin A; b: panduratin A; and c: ester(3). Residues that involved in hydrophobic interaction are shown as connolly surface. Non-polar hydrogen of ligands has been omitted for clarity.

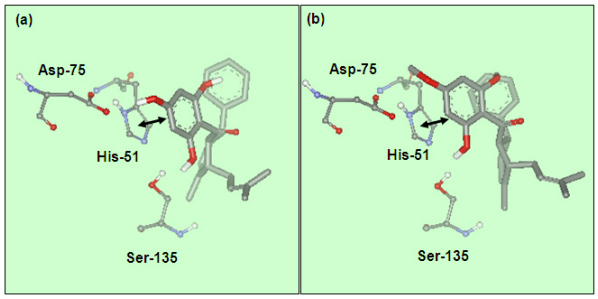

Figure 4. Molecular orientation of (a): 4-hydroxypanduratin A; and (b): panduratin A at the catalytic triad of NS2B/NS3. Docked ligand are shown in stick model, whereas catalytic triad residues are shown in ball and stick model. The double-ended arrow showed the suggested π-π type aromatic interaction between pentacyclodiazo side-chain of His-51 and the phenolic moiety of the ligands.

fitted into the hydrophobic pocket consisting of the residue Val-52, whilst the 2-methyl-2-butylene, another hydrophobic side-chain in the ligands, fitted into a second hydrophobic pocket formed by the residues Leu-128, Pro-132 and Val-155 (Figure 3, a and b). The ester group in (**3**) also fitted into the Leu-128, Pro-132 and Val-155 hydrophobic pocket but not with the residue Val-52 (Figure 3, c).

Another form of interaction observed with 4-hydroxypanduratin A (**1**) and panduratin A (**2**) but not observed in previous studies is the π-π type aromatic interaction (Figure 4). In this case, the position of the trisubstituted phenyl moiety in both the ligands (**1**) and (**2**) is located at about 3 - 4.2 Å and parallel to the pentacyclodiazo side-chain of His-51 which enabled a π-π type aromatic interaction to occur. However, such interaction was not been observed with the ester (**3**). The lack of both the hydrophobic group that could interact with Val-52 and aromatic interaction presumably has made the ester (**3**) less active as observed from its K_i value when compared to the two ligands.

CONCLUSIONS

The docking of various ligands with competitive activities to the model of DEN2 NS3 serine protease complexed with NS2B co-factor was carried out. Several modes of interactions such as H-bonding, van der Waals as well as π-π interaction were observed between these ligands and the DEN2 NS2B/NS3 active sites. These findings have provide further understanding on the binding interaction of the catalytic triad of the DEN2 NS2B/NS3 serine protease, thus giving input into the mode of action of the catalytic triad.

ACKNOWLEDGEMENT

The authors acknowledge financial support for this project provided by the Malaysian Ministry of Science, Technology and Innovation under the Top Down National Biotechnology Directory grant no 09-02-04-001BTK/TH/004 (UM 36-02-03-6008) and the Malaysian Academy of Science SAGA funds.

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