CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

7.1 General discussion

This thesis describes the structural investigation of the interactions between inhibitors and the binding site of NS2B-NS3 serine protease of DEN-2. The inhibitors were reported to be either non-competitive or competitive in their activities (Tan, 2005; Tan *et al.*, 2006). For competitive inhibitor, the hypothesis of the study was that the inhibitor would interact with the catalytic triad of the active site and thus compete with the substrate for this site. For non-competitive inhibitors, the study reported herein was performed on the premise that different modes of interactions with the binding site could explain the ranking of the inhibition activities observed in the experimental setup.

In the earlier part of this project, predicting and building of the secondary structure profile of NS3 protease (Chapter 4) work was carried out. The secondary structure profile, however, was not conclusive in generating the DEN-2 structural model. This new method comprises heuristic, probabilistic and more sophisticated overlaying techniques, which can be very useful, especially for cases where there are low homology sequence relationships between query and template proteins. Following the validation studies of the methods applied using the DEN-2 crystal data (D'Arcy *et al.*, 2006; Erbel *et al.*, 2006), it was concluded that the approach adopted in this study is able to yield good prediction results. The work described in this chapter has been published (Othman *et al.*, 2007).

Structural information is the key point for the progress of this study. To obtain the structural data for the inhibitors was easy, since the data were available from crystallographic database, or if they were not available, structural models could be built and optimized, and served as the starting or the input files for the *in silico* experiments.

However, in the case of proteins, generally, structural data could be obtained experimentally in two ways; from an NMR study, and from crystallographic study. Both approaches demand high quality purified protein. At the start of this project, the crystal data for DEN-2 protease complex was not available, hence, our attempts to crystallize the protease (Chapter 3). This involved laborious experimental techniques, which started with protein expression procedures, followed by protein extraction and protein purification. Optimization of the methods from previously reported work was performed and discussed in Chapter 3. Inspite of the pure protein solutions from our recombinant and purification processes, the protein did not crystallize. This is attributed to the dynamic nature of the dengue protease which underwent auto-cleavage upon folding into its active conformation and produced degenerative products. The crystallized protease of DEN-2, however, was subsequently reported in 2006 by D'Arcy *et al.* This structural data was then used as input for the subsequent computational simulations since it should be more accurate than the homology model.

The latter part of this thesis discussed the computational docking of non-competitive and competitive inhibitors into the DEN-2 protease complex. For the study on the non-competitive inhibitors, the results discussed in Chapter 5 on the computational studies are in agreement with the previously reported experimental studies (Tan, 2005; Tan *et al.*, 2006) which provided some insights into how these non-competitive ligands inhibited proteolytic activities. The *in silico* visualization of the inhibitor bound to the binding site of the protease indicated the most important residue in the binding site for interaction with the non-competitive inhibitors to be Lys74. Other residues are also involved in the interactions with the inhibitors via H-bonds, van der Waals and hydrophobic interactions. SAR studies conducted yielded the important features of the inhibitors that are responsible for activities. The work described in this

chapter has been published (Othman *et al.*, 2008).

Chapter 6 discussed the computational docking of the competitive inhibitor (4-hydroxypanduratin A) to DEN-2 protease complex. Docking results showed two possible binding modes; the first being H-bonding of the inhibitor to Ser135, and the second being H-bonding of the inhibitor to Asp75. Both Asp75 and Ser135 comprise the catalytic triad in the active site. Garrett & Grisham (1997b) described the substrate binding mechanism to serine protease active site through Ser135. To confirm the most possible binding mechanism, energy calculation at a higher level was conducted using QM/MM method (by applying ONIOM2 protocols). Whilst it was not viable with AutoDock, ONIOM2 was able to yield energy values of significant difference between the two systems under investigation. This reflects the capability of quantum mechanics (here is in hybrid with molecular mechanics) to facilitate the elucidation of reaction mechanism in a complex system such as protein. Accordingly, the binding of the inhibitor to the active site was proposed to be mediated by H-bond to Ser135. Structural investigation of the protein-inhibitor complex simulation indicated that the inhibitor was able to block the S1 pocket of the binding site. This seems to hinder the recognition of the pocket for two basic amino acids at the P1 position of a substrate (Chapter 2). This blockade seemed to disable the substrate from entering the S1 pocket.

In general, this work is able to demonstrate how the interactions with ligands affect the structure of the protein which consequently affect its activities. Experimental results (Tan, 2005; Tan *et al.*, 2006) showed the competitive inhibitors to be more potent in its activity compared to the non-competitive inhibitors. Logically, this is to be expected since competitive inhibitors would bind directly to the active site and block the substrate from the site. On the other hand, the non-competitive inhibitors bind to sites

which are not the active site. Often, this would only infer conformational change to the protease and to the nearby active site. Thus, from the conformational aspect, the substrate would not be able to fit perfectly into the active site and would not form strong interactions to yield a stable enzyme-substrate complex.

7.2 General conclusion

The present work was also able to give structural information essential for future studies towards the design of potential anti-dengue therapeutics. Modifications of the present lead compounds, pinostrobin (non-competitive inhibitor) and 4-hydroxypanduratin A (competitive inhibitor), can be performed based on the suggestions proposed in Chapter 5 and Chapter 6, respectively. It would seem that the development of a potent non-competitive inhibitor is more preferable since these inhibitors will not have to compete with the substrate for the same active site to perform its inhibitory activity. But, the challenges foreseen are the inhibitor-protein interactions would have to be strong enough to maintain a stable inhibitor-enzyme complex, and the inhibitor should be specific in its binding interactions.

7.3 Suggestions for future work

In further work on the development of anti-dengue drugs, the information obtained from these studies are useful for designing new drug candidates against dengue virus infections. Other areas which can be studied include, firstly, performing co-crystallization of the dengue protease with the competitive and non-competitive ligands. This will serve to validate the computational results reported in this thesis. Secondly, looking at the other dengue serotypes and studying their differences and similarities, and

subsequently, determining whether there will be a need for different sets of inhibitors for the different serotypes. Finally, investigation on how the NS2B cofactor acts in optimizing the function of NS3pro could also be carried out.