MOLECULAR MODELLING AND BIOLOGICAL EVALUATION OF PEPTIDE BASED INHIBITORS FOR DENGUE VIRUS

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by

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ABSTRAK

Denggi diakui sebagai salah satu penyakit virus arthropod yang paling kerap berlaku pada manusia. Dalam beberapa dekad ini, virus denggi (DENV) telah menjadi ancaman serius terhadap kesihatan global dan pangkalan data Pertubuhan Kesihatan Sedunia (WHO) menunjukkan peningkatan kes denggi yang dilaporkan sebanyak 800% dalam tempoh 2000 hingga 2019; dengan peningkatan yang ketara dari 505.430 kes pada tahun 2000 kepada lebih 2.4 juta kes pada tahun 2010 dan lebih daripada 4.2 juta kes pada tahun 2019. Oleh itu, vaksin yang sesuai mesti dibuat secepat mungkin. Penyelidikan ini akan merangukumi, interaksi antara tapak pengikat protease denggi NS2B / NS3 dan perencat peptidik menggunakan kaedah komputasi, penyelidikan panjang peptida yang sesuai untuk merancang perencat protease denggi NS2B/NS3pro dan menilai peptida terpilih menggunakan uji protease *in-vitro*. Substrat peptida, Arg-Arg-Arg-Arg-Ser-Ala-Gly-Met (RRRRSAGM) dari Kawasan kapsid telah dipilih sebagai ligan permulaan dan struktur tiga dimensi kompleks model homologi protease NS2B/NS3pro dari Wichapong et al., 2009 dipilih dan digunakan sebagai molekul sasaran untuk kajian ini. Program MOE telah digunakan untuk penyelidikan dok peptide. Tripeptide adalah panjang peptida yang paling sesuai untuk merancang perencat protease NS2B / NS3pro dengue protease. Tren graf menunjukkan, Tri 1.3 (Arg-Ser-Ala) dan Tri 3.1 (Arg-Ala-Arg) selalu berada di kedudukan lebih tinggi daripada Tetra 1.2 (Arg-Arg-Ser-Ala) walaupun Tetra 1.2 mempunyai urutan peptida yang hampir serupa dengan Tri 1.3. Hasil ini malah meningkatkan penemuan bahawa tripeptida adalah panjang peptida yang lebih baik untuk merancang antagonis NS2B / NS3pro.

Tripeptida 1.3 dengan nilai IC_{50} dengan 26.49 μM diikuti oleh kawalan untuk quercetin kajian ini dengan 48.13 μM, tripeptida 3.1 dengan 130.38 μM dan tetrapeptida 1.2 dengan 520.53 μM. Yang menunjukkan bahawa tripeptida 1.3 adalah perencat peptidik NS2B / NS3 denggi yang sesuai.

MOLECULAR MODELLING AND BIOLOGICAL EVALUATION OF PEPTIDE BASED INHIBITORS FOR DENGUE VIRUS

ABSTRACT

Dengue is recognized as one of the most prevalent arthropod-borne viral diseases in human. In these few decades, the pathogenic dengue virus (DENV) has become a serious threat to global health and the World Health Organization's (WHO) database shows an increase of reported dengue cases by 800% within the period of 2000 to 2019; with a significant jump from 505,430 cases in year 2000 to over 2.4 million cases in 2010 and to over 4.2 million cases in 2019. Hence a suitable vaccine must be developed as soon as possible. The possible interaction between dengue NS2B/NS3 protease binding site and the suggested peptidic inhibitors using computational method, investigate the suitable peptide length for designing NS2B/NS3 dengue protease inhibitor and evaluate the selected peptides using *in-vitro* protease assay was tested for this study. The peptide substrate, Arg-Arg-Arg-Arg-Ser- Ala-Gly-Met (RRRRSAGM) from the capsid's cleavage region was chosen as a starting ligand and the complex three-dimensional structure of the NS2B/NS3 protease homology model from Wichapong et al., 2009 was selected and used as the target molecule in for this study. MOE software was being used for molecular docking in this study. Tripeptide is the most suitable peptide length for designing a peptidic NS2B/NS3pro dengue protease inhibitor. The graph trend Tri 1.3 (Arg-Ser-Ala) and Tri 3.1 (Arg-Ala-Arg) always ranked higher than Tetra 1.2(Arg-Arg-Ser-Ala) eventhough Tetra 1.2 had similar peptide sequence with Tri 1.3. This result even enhances the findings that tripeptide is the more favourable peptide length for designing an NS2B/NS3pro antagonist. The tripeptide 1.3 with the

IC_{50} value with 26.49 μM followed by the control for this study quercetin with 48.13 μM, tripeptide 3.1 with 130.38 μM and tetrapeptide 1.2 with 520.53 μM. Which shows that tripeptide 1.3 is a suitable dengue NS2B/NS3 peptidic inhibitor.

CHAPTER ONE

INTRODUCTION

1.1 Problem statement

Until today there is no specific medication for dengue virus as usually the patient that being contacted by the viral infection will be treat by bed rest, and mild analgesic-antipyretic therapy. Hence, the general idea of this study is to find a suitable dengue virus NS2B-NS3 peptidic inhibitor for future drug development for dengue fever. Dengue virus is one of the most wide-spread arthropod-borne viral diseases among humans. The first case of dengue was recorded in the Asia, Africa and North America regions in the 1790s (Hadinegoro, 2012; Li et al., 2005). With increased cases trend observed in many tropical and subtropical regions, the pathogenic dengue virus poses a serious threat to world's health as well as socio-economy (Bhatt et al., 2013). For instance, it is estimated that dengue virus infects up to 290 million people per year (Bhatt et al., 2013) while 3.9 billion people are at risk of being infected (Brady et al., 2012). Moreover, the World Health Organization's (WHO) database shows an increase of reported dengue cases by 800% within the period of 2000 to 2019; with a significant jump from 505,430 cases in year 2000 to over 2.4 million cases in 2010 and to over 4.2 million cases in 2019. Dengue death cases also increases by fourfold between year 2000 and 2015; from 960 to 4032 cases (WHO, 2016a).

The 2016 dengue virus outbreak provides good example of the seriousness of the issue. According to WHO's data, the outbreak affected many regions; including America and Asian. In the region of America, from the total of 2.38 million dengue cases reported, 1.5 million cases were from Brazil alone. The 1.5 million cases in 2016 represents an increase of 300% from the 500,000 cases reported by this country in 2014 (WHO, 2016a). The 2016

outbreak also recorded 1032 reported dengue death cases in the region. As for the Asian region, two countries in particular, namely the Philippines and Malaysia were most affected with 176,411 cases and 100,028 dengue cases each totaling 375,000 cases (WHO, 2016a). Interestingly, both countries also experienced similar cases in 2015 (WHO, 2016a).

Recently, the spread of the dengue virus continues to affect many countries globally like Bangladesh, Brazil, Cook Islands, Ecuador, India, Indonesia, Maldives, Mauritania, Mayotte (Fr), Nepal, Singapore, Sri Lanka, Sudan, Thailand, Timor-Leste and Yemen recently (WHO, 2016a). Specifically, in the Asian region, more than 900,000 cases were recorded in 2019. Of the countries, Philippines shows the highest number of cases with 420,000 reported cases followed by Vietnam with 320,000 cases, Malaysia 131,000 cases and Bangladesh with 101,000 cases (WHO, 2016a).

The literature acknowledges vaccination as the well accepted solution to contain the dengue virus the world is dealing with. In theory, a person who gets him/herself vaccinated should be clear from the virus infection. This means that a dengue vaccine must be developed fast considering the global dengue scenario. However, researchers developing dengue vaccination are facing several obstacles. The main obstacle lies in the fact that the success in developing dengue vaccine depends very much on the diversity of virus types and the complexity nature of the virus the vaccine is fighting against. An individual who experienced been infected once by (any of the five) dengue virus primary serotypes is expected to have a life-long immunity against re-infection after s/he managed to overcome a range of illnesses during primary infection. To make it more difficult, an individual who experiences secondary heterotypic dengue virus infection will find him/herself in a situation whereby his/her body risked being infected with a severe dengue disease even though s/he has developed protective immunity from previous primary infection due to the antibody-dependent enhancement. As

Lin and Thomas (2013) argue, there is a need to design and provide an effective long-term dengue vaccine that can fight all the five of DENV serotypes at once (Lin and Thomas, 2014). In short, the obstacles described point to the urgent need for researchers to find ways on developing dengue vaccine that works and save lives.

There are several vaccine types being develop for combat the dengue virus which are that related to vaccines are live attenuated vaccine, in active vaccine, recombinant subunit vaccine and nucleic acid vaccine (Jasamai *et al.*, 2019). Live attenuated vaccine is the development of attenuated, live virus that mimics the viral infection to the body while not lead to significant illnesses hence the immune responses can be triggered. Sanofi using this vaccine method to introduce Dengvaxia to the market (Jasamai *et al.*, 2019). By propagating virus into Vero cells inactive vaccine were being introduce, and there is a study reported that elicit immunogenic response present from DENV-1 in *Aotus nancymaae* monkeys (Maves *et al.*, 2011). For recombinant subunit vaccine for dengue, the subunit primaly generated using R antigens from dengue virus (DE *et al.*, 2010). Nucleic acid vaccine is being made by expressing antigents from the DNS construct and introduce to the recipients directly. Invio Pharmaceuticals developed a possible dengue vaccine by expressing envelop domain III (EDIII) of DENV-1, DENV-2, DENV-3, DENV-4 separated by proteolytic cleavage sites and have high immunogenicity in mice and non-human primates models (Ramanathan *et al.*, 2009). Lastly, virus-like particle or known as (VLP) vaccines being made using the protein structures of the virus which have genetic information that allow the present of antigenic epitopes to the patient's immune systems. Cytos Biotechnology try to make dengue vaccine by coupling VLP carries to the recombinant dengue virus EDIII (Spohn *et al.*, 2008).

To date, Dengvaxia® (CYD-TDV) is the first and only dengue vaccine made available to the marketplace since it was introduced in December 2015 in Mexico. As the only current treatment of dengue is being treated by bed rest and mild analgesic-antipyretic

therapy. While this is good news for dengue patients, unfortunately, it has not been formally legalized and accepted by WHO yet (license still pending) (WHO, 2016a). The reason may be tied to the vaccine's efficiency performance which was reaches only 59.2% of overall efficiency. As WHO (2016) reported, the findings from pooled trials carried out in the year following the primary series (per protocol analysis), show Dengvaxia® performs better with higher effectiveness only against DENV serotypes 3 and 4 (71.6% and 76.9%, respectively) compared to DENV 1 and 2 (lower effectiveness at 54.7% and 43.0% respectively). The unsatisfactory findings from the pooled trials led WHO suggesting for the data to be fully reviewed by the Global Advisory Committee on Vaccine Safety and SAGE before it can be approved by the organization. Thus, as a precautionary and interim measure, WHO further suggested for the vaccine to be applied only to those who had been infected by dengue (the vaccine is not for those who have never been infected by dengue yet) while waiting for the Committee to come up with revised guidance on Dengvaxia®'s use (WHO, 2016a).

From the concerns raised by WHO about Dengvaxia®, the world urgently needed a safe and effective dengue vaccine against the DENV (all five) is very real and become the motivation of the current study. In this study, it is proposed that studying peptide-based inhibitors may provide researchers with an understanding on the development and discovery of a safe and effective drug against the DENV.

According to the literature, peptides compounds offers researchers with an alternative to small-molecule compound as currently practiced to be developed as therapeutic drugs. Peptides-based compounds offer several advantages; namely, specificity, selectivity and efficacy; and they are relatively safer than small-molecule drugs (Craik et al., 2013). In addition, they are also easily and widely available. For instance, prior to 2010, at least 140 peptides drugs were observed marketed around the globe while 400 peptides more were still in pre-clinical phases (Huther and Dietrich, 2007). The number is increasing today. As of

March 2017, more than 484 therapeutic peptides were available in the marketplace. Data from year 2018 shows three countries, namely, the United States, Europe, and Japan to have approved 60 peptides drugs; an additional 260 more have been tested in human clinical trials (Lau and Dunn, 2018). Enfurvitide (HIV fusion inhibitor), Tirofiban (platelet inhibitor), Exenatide (anti-diabetic), and Epifibatide (anti-coagulant) are examples of available peptide-based drugs in the market today (Craik et al., 2013; Huther and Dietrich, 2007). The literature has acknowledges the fact that amphibians are the source of a large number of peptides; in addition, the human peptide is also an effective antimicrobial dan viral activities (Uhlig et al., 2014).

For the lead for initial design of the peptidic dengue for this study Asn-Arg-Arg-Arg-Arg-Ser-Ala-Gly-Met-Ile (NRRRRSAGMI) from the capsid's cleavage region form were chosen as ths region form the highest number of hydrogen bonds with the protease (Yotmanee et al., 2015). Based on the substrate profiling experiment conducted by Li et al., 2005, they found strong preference for basic amino acid residues (Arg/Lys) at the P1, P2 and P3 sites and non-polar aliphatic residues at P4 site. Hence in this thesis, the location and substitution of arginine with alanine was also being tested for the development of the petidic dengue antagonist. The specific length of the amino acid for the NS2B/NS3pro peptide dengue inhibitor was also the main focus of the study as it is one of the crucial steps of designing a lead peptide drug. These justify reason for why peptide-based compounds are highly potential to be developed as therapeutic drugs for various medical conditions, in particular as effective anti-dengue viral agents.

1.2 Molecular Biology of Dengue Virus

DENV have four different serotypes which are DENV-1, DENV-2, DENV-3 and DENV-4 and it is belong to the *Flaviviridae* family specifically in *Flavivirus* genus (WHO, 2016a). Other serious virus and pathogens that are also related to the DENV are West Nile virus, Japanese encephalitis virus, and yellow fever virus as they are also in the *Flavivirus* genus.

DENV virus has an icosahedral shape and is surrounded by a lipid envelope. The icosahedral particle of the dengue virus has a diameter of ~ 50 nm (Kuhn et al., 2002). The virus consists of a positive single-stranded RNA with the length of ~11 kb and short non-coding regions at both 5' and 3' position. The ribosomes transcribe and translate the dengue virus genome into a single polypeptide chain before entering the host cell (Lindenbach and Rice, 2003).The envelope (E), capsid (C) and pre-membrane/membrand (prM/M) and the others seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) of the dengue virus are formed due to the proteolytic cleavage of the polypeptide by host cell proteases, i.e. furin and signalase, and virus-encoded protease, NS2B/NS3 (Falgout et al., 1991; Rodenhuis-Zybert et al., 2010). This is shown in Figure 1.1 from Sampath and Padmanabhan, 2009 paper which shows the molecular targets for flavivirus drug discovery (Sampath and Padmanabhan, 2009).

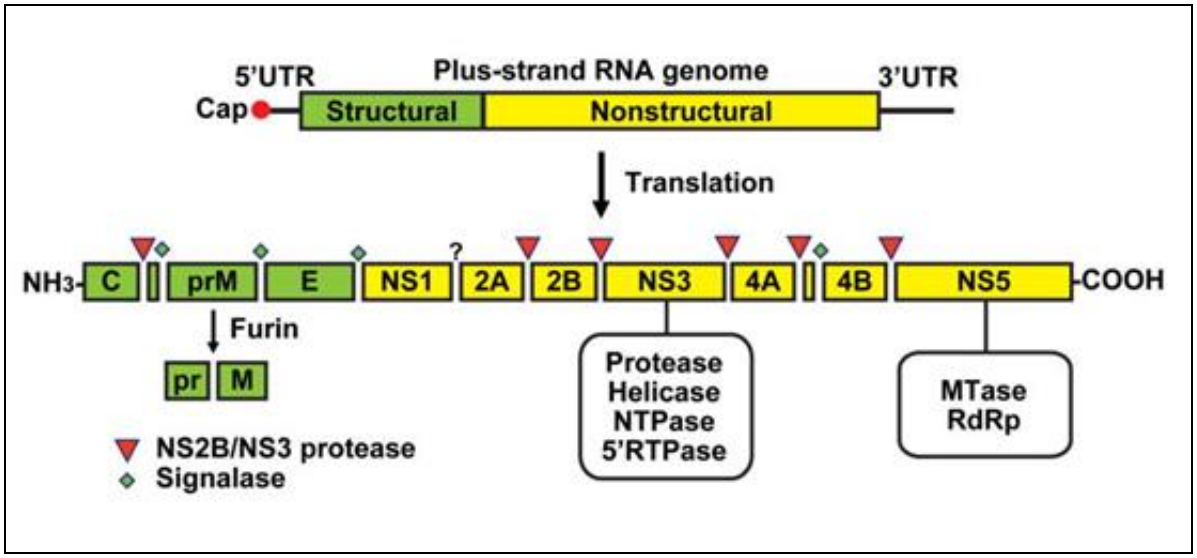


Figure1.1: Flavivirus RNA genome and polyprotein (Sampath and Padmanabhan, 2009)

These structural proteins are important and a necessity for the dengue virus; they are used by the dengue virus in assembling the viral genome for the interactions of viral receptors, virus entry, fusion, neutralization and encapsulation of the viral genome (Mairuhu et al., 2004). NS1 is a highly immunogenic protein and is required for flavivirus replication while the function of NS2A protein is to inhibit the interferon induction, one of the replication complex for virus assembly (Luo et al., 2008). NS3 - a non-structural protein with a size of 69 kDa plays a vital role in viral maturation using multiple enzyme activities, namely, i) serine protease when in complex with NS2B cofactor, ii) RNA triphosphatase (RTPase), iii) N triphosphatase (NTPase) and iv) helicase (Natarajan, 2010). The largest protein in the dengue virus that have two enzymatic domains which are S-adenosyl methyltransferase (SAM) at its N-terminal, and RNA-dependent RNA polymerase (RdRp) at the C-terminal is the NS5 (107 kDa). The S-adenosyl is used for required for RNA cap formation and RNA-dependent RNA polymerase is required for RNA genome synthesis (Potisopon et al., 2014).

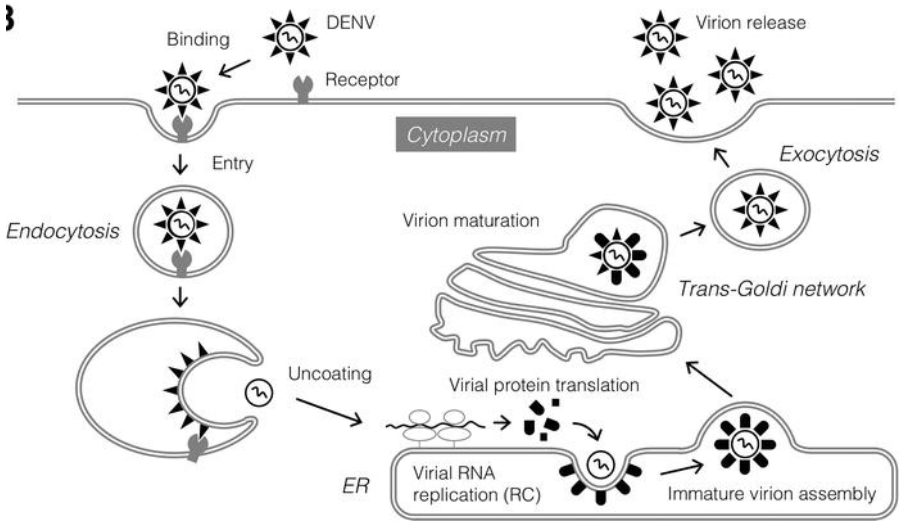


Figure 1.2: Steps of dengue virus replication and maturation in the host cells (modify from Hirotaka Takahashi and Youichi Suzuki, 2017)

Figure 1.2 shows the mechanism of the replication and infectious of the dengue virus. The replication of the dengue virus is initiated when the host cell receptor attaches itself with the virus resulting in the occurrence of receptor-mediated endocytosis. These processes occur when E protein forms glycoprotein shell to assist the internalization of dengue virus. Inside the cell, acidification of the endosomal compartment results in an irreversible trimerization of E protein which triggers the viral fusion between the dengue virus and the host cell allowing the viral genome to be released into the cytoplasm of the host cell; the dengue virus genome is then presented to the rough endoplasmic reticulum where it is translated into a single polyprotein product followed by a cleave by the host cell and virus proteases to form various important proteins needed for viral replication. The viral RdRp protein replicates the viral genome to form new virions; then it is packaged by the C protein to form immature viral particles in the Golgiu apparatus in which the virus maturation occurs before it eventually cause the secretion of fully matured dengue viral particles through the host secretory pathway (Mukhopadhyay et al., 2005; Rodenhuis-Zybert et al., 2010).

1.3 Epidemiology, Transmission and Pathogenesis

Dengue disease caused by dengue virus infection has emerged as an endemic and become clinically apparent in 128 countries, especially in the America, South-East Asia and the Western Pacific region as illustrated in Figure 1.3 (Brady et al., 2012; Tomlinson and Watowich, 2011) The earliest epidemics of dengue haemorrhagic fever (DHF) were reported in Manila, in the Phillipines (1953) and in Thailand (1958). In the 1980s and 1990s, the DHF epidemic has spread into other countries such as India, Pakistan, East China and Sri Lanka. Prior to 2010, there were approximately 250,000 - 500,000 cases of the DHF and dengue shock syndrome (DSS) reported annually, leading to around 25 000 death (Tomlinson and Watowich, 2011). Dengue has dramatically increased worldwide in recent decades. WHO estimates around 390 million infections per year with more than 3.9 billion people or more than 40% of the world's population predominantly in the tropical and sub-tropics have an increased risk of catching the disease (WHO, 2016a). Figure 1.3 displays worldwide distribution of dengue for year 2013. It shows that 128 countried have been at risk of DENV infection (Bhatt et al., 2013)

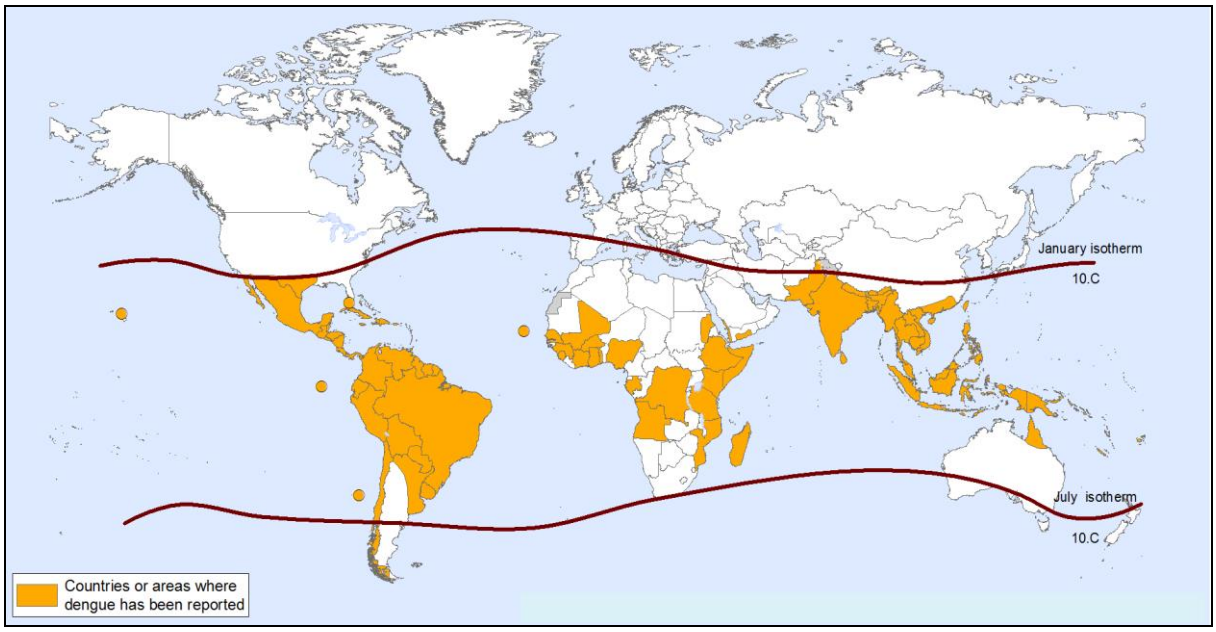


Figure 1.3: Worldwide distribution of dengue in 2013 (WHO, 2016a)

In terms of transmission of dengue, *Aedes aegypti* is the primary dengue vector, predominantly dwell in tropical and subtropical region, mostly between latitudes 35 °N and 35 °N (WHO, 2009). *Aedes aegypti* is a small, dark and an endophilic mosquito that can be identified by the white marking on its legs. The secondary dengue vector is *Aedes albopictus*, which is an aggressive, endophilic mosquito that inhabits numerous areas in Southern Europe; and in parts of Africa and America (Higa, 2011; Pham, 2015).

Humans are the main amplifying host of DENV and serve as a source of virus for uninfected dengue vector (Twiddy et al., 2003). The transmission of DENV from human host to the vectors, *Aedes* mosquitoes occurs in a human-to-mosquito-to-human cycle. It happens when *Aedes* mosquitoes becomes infected with the virus while feeding on the blood of an infected person. After a period of eight to ten days of virus incubation, the virus will infect and replicate at other secondary tissue, such as in the salivary gland. After this period, the mosquito will remain infected and is capable of transmitting the virus to new hosts for the rest of its life (Schmid et al., 2014). DENV can later be spread to healthy people through the bite of infected female vectors. The dendritic cells and other immune cells in humans will be

the primary host for the virus(Schmid et al., 2014). An infected person will develop viremia and other symptoms of dengue fever (DF) approximately after four to seven days of infection. Figure 1.4 illustrates the period of transmission of different DENV serotypes by *Aedes aegypti* mosquitoes to human hosts (Carrington and Simmons, 2014).

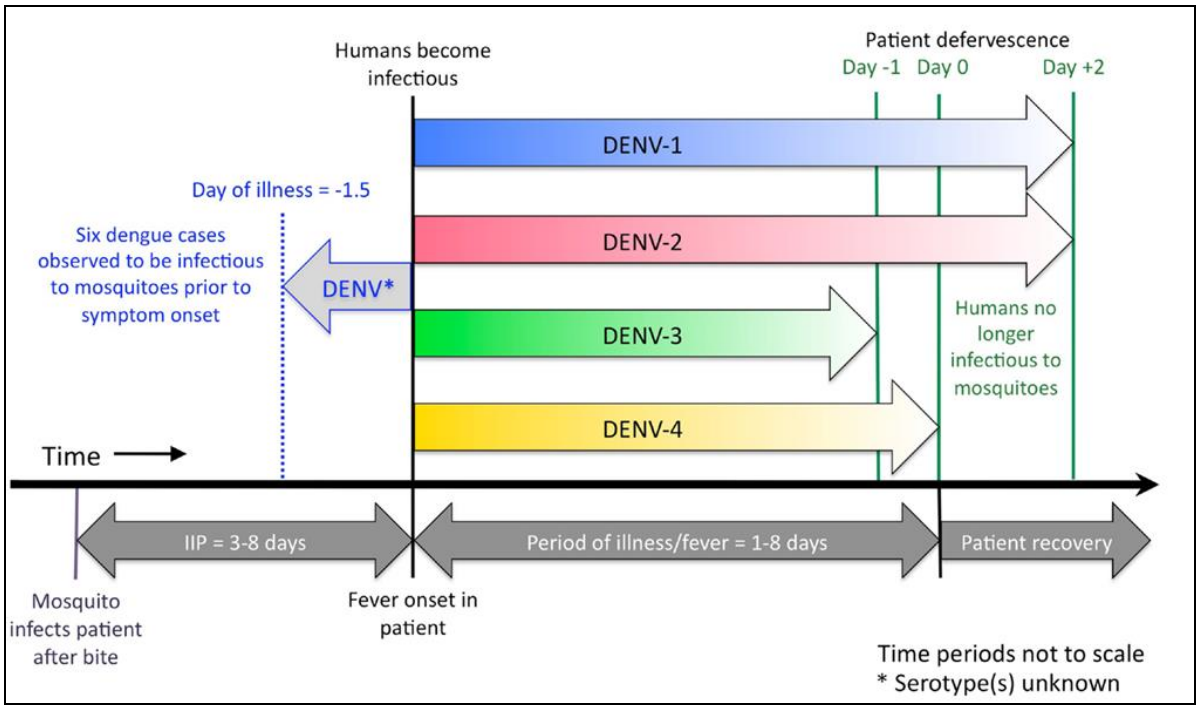


Figure 1.4: Period of human DENV infectiousness for each serotype (Carrington and Simmons, 2014)

1.4 Clinical Manifestations, Prevention and Treatment

Dengue virus can cause patients to experience many clinical symptoms from a mild DF (dengue fever), which is an acute febrile illness, to DHF (dengue hemorrhagic fever) and DSS (dengue shock syndrome), which are more severe. Most of the adolescents and adult's patients affected with acute febrile illness or DF will find themselves experiencing mild fever and two or more other symptoms, i.e. headache, retro-orbital pain, myalgia, leucopenia and facial flushing (Gubler, 1998; Ligon, 2005). During this period, some patients may also be experiencing minor haemorrhagic manifestation (such as petechiae, membrane bleeding and

epistaxis) as severe hemorrhage is uncommon during febrile phase (Hadinegoro, 2012). The acute febrile phase of DF usually lasts between five to seven days as patients will then show symptoms for onset recovery (Rigau-Pérez et al., 1998). This is when patients will show withdrawal symptoms from fever and other warning system including dengue virus removal from the patient's blood (Gubler, 1989). As for DHF, it usually affects dengue fever children/youngster patients who are in the age group younger than 15. Patients experiencing severe DHF will find themselves to have an extremely low platelets level in their blood vessel (thrombocytopenia (≤100,000 cells/mm3), an increase of permeability of the capillary, as well as experiencing of an abdominal pain (Funahara et al., 1987).

Lastly, DSS patients are in the critical phase of the illness (Whitehorn and Simmons, 2011). The critical phase commonly happens on the fourth day to the eight days of their illness as patients' fever reduces. Patients will show DF resembling symptoms, have severe failure of the circulation system with rapid reducing number of platelets in the blood stream, as well as high hematocrit level and high loss of plasma volume caused by plasma leakage. The severity of patients' plasma leakage can be detected when they show lower hematocrit level; and it is important to note that severity of plasma leakage varies among patients. Patients with excessive plasma leakage need an extra attention as the prolonged shock makes the leaked plasma to sip into the pleural and abdominal cavities which can lead to organ damage or can even cause death (Kalayanarooj, 2011). Figure 1.5 shows the schematic of the dengue case classification by severity based on WHO criteria in 2009.

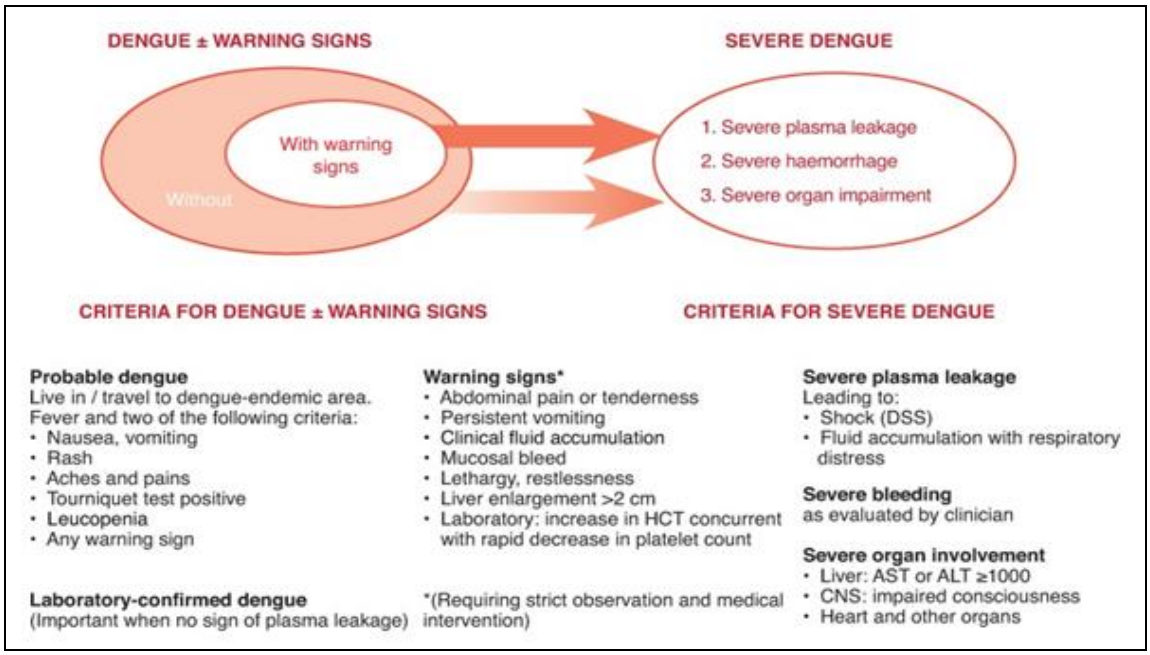


Figure 1.5: The 2009 WHO dengue case classification (WHO, 2009)

Below par of controlling *Aedes* populations, rapid and uncontrolled urbanization, increased number of population, as well as ease and rapid growth of global travel to dengue endemic are several factors that have been identified in the literature as possible causes for the increase of dengue virus spreading around the globe (Gubler, 1989; Gubler and Clark, 1995)

Eliminating natural and man-made dengue larvae habitat location is one of the easiest ways to control the *Aedes* population. According to WHO (2009), the community can implement simple practices like applying of insecticide and emptying stagnant water in their compounds to exterminate possible *Aedes* breeding sites, and to prevent *Aedes* propagation and population (WHO, 2009). Unclogging the drainage and roof gutters, proper removal of old tyres, and emptying of household containers are other effective practices that can help community to dispose breeding sites for the female *Aedes* mainly because these sources can retain water which is a favourable condition for the dengue larva to survive. In addition, community should be applying direct water supply system to household rather than using

water containers for storage as it reduces the access of *Aedes* vectors for oviposition (Mairuhu et al., 2004).

Adulticides and larvicides are examples of chemicals which are not harmful to humans and not contaminating the water source that can be applied to control the larval and adult dengue vectors population; WHO also identified space spray of suspected areas as the effective and well accepted method applied to reduce dengue cases globally (WHO, 2009).

Still, personal and household counter measures (including the use of long-sleeved cloths to reduce mosquito bites, use of materials that have been treated with insecticides and spraying mosquito repellent) are required to complement other environmental management preventive measures to control the epidemic and reduce the population contact with this vector-pathogen (Leontsini et al., 1993; Winch et al., 1994).

For the treatment of dengue infection, patients with mild infection need to have a good rest while being treated with oral rehydration therapy (to maintain the patient's blood circulation volume) and antipyretic (e.g. acetaminophen to reduce patients' fever)(WHO, 2016b). During treatment, it is crucial for physicians and health care workers to determine and monitor the warning signs shown by the patients; mainly to determine the stage of illness they are in, i.e. whether they are suffering from mild DF or are already experiencing severe DHF or DSS. While full blood count, blood pressure and hematocrit level are the main clinical tests required for this purpose, certain circumstances may require additional tests such as serum electrolytes, glucose, liver function and electrocardiogram to be carried out (WHO, 2009).

Unless patients are properly treated, patients suffering from DHF/DSS will experience loss of plasma that can cause severe bleeding or organ impairment. For these patients, they need to be treated using early and immediate volume replacement therapy with isotonic solution and expanders (Nguyen et al., 2006). As every patient may experiencing a different

degree of plasma leakage, physicians determine the amount of total fluid replacement that the patients need from the clinical test results (WHO, 2009).

As a general guide, patients are only discharged from hospital when they show onset recovery signs, namely, improved appetite, stable hematocrit level, normal value range of the white blood cell count and stable body temperature. However, they need to be retreated when they develop a recurrence or new warning signs after being discharged.

Even though dengue vaccine is really to reduce the dengue cases around the world, the complexity of the DENV structure causes the development of an effective vaccine against the disease challenging. Each of the 5 types of DENV serotypes can cause dengue illness to the population. This is why an effective vaccine must be found to provide a herd immunity for the general population from dengue. The solution is to find and develop a vaccine that is effective against all five serotypes of DENV as it can reduce antibody-dependent enhancement risk, which is a phenomenon that can cause serious dengue cases in human(Schwartz et al., 2015; Thisyakorn and Thisyakorn, 2014).

CYD-TDV is a weakened, live-attenuated vaccine developed by Sanofi Pasture and marketed under the brand name Dengvaxia®. It is the first dengue vaccine and was registered in Mexico in December 2015. The vaccine receives medical approval from 11 countries in 2016 and later also gets approval from the European Union in 2018 and the United States in 2019. Patients needed three doses of the vaccine over a year period (scheduled at month 0, 6, and 12) (WHO, 2016c); after which the patients' immune response would be triggered against four (4) types of dengue virus.

While it has been approved for use in many countries, it has not received formal approval from WHO itself. As explained by the organization, it is still evaluating the findings from two Phase 3 clinical trials of the CYD-TDV involving 35,00 participants from ages 2 to 16 years old from five Asian countries in Asia (used CYD14 vaccine; patients age between 2

to 14 years old) and five Latin America countries (used CYD15; patients age between 9 to 16 years old) administered in 2016. From the clinical trials that involved patients to be administered with randomized placebo and vaccine in 1:2 ratio, the overall result shows only 59.2% vaccine efficiency against both dengue pool. The vaccine was found to be more effective against serotypes 3 (71.6%) and 4 (76.9%) while the efficacy for serotype 1 and 2 were only 54.7% and 43.0% respectively (Pasteur, 2015). Table 1.1 shows the status of dengue vaccine candidates in development as in 2021.

Table 1.1: Status of dengue vaccines in development

Vaccine candidate	Vaccine type	Developer(s)	Status
DENVax	Live attenuated tetravalent vaccine	Inviragen (Takeda pharmaceutical)	Commercially available in 2021 (Dengvaxia)
TV003/TV005	Live attenuated tetravalent vaccine	NIAID and Butantan Institute	anticipated launch of TAK-003 in 2021/22
TDENV PIV	Purified inactivated tetravalent vaccine	GSK and WRAIR	I
V180	Sub-unit protein vaccine	Merck	I
D1ME100	Plasmid DNA vaccine	NMRC	I

1.5 Potential Anti-Dengue Medicinal Plants

According to WHO estimation in December 2008, around 80% of the population especially in Asian and African regions treat dengue using traditional medicine (Abd Kadir et

al., 2013)**.** To date, few natural products sourced from plants like seaweed, *Tephrosia* species and *H. cordata* (Chameleon plant) have been studied and found to be effective against dengue infections. For instance, fucoidan (a sulphated polysaccharide that was extracted from seaweed naturally found in Japan) has shown an antiviral activity against DENV-2. As reported by Hidari et al. (2008), at 10 µg/mL concentration, fucoidan compound managed to decrease DENV-2 activity by 80% into BHK-21 cells compared to untreated cells. Fucoidan compound recorded of IC_{50} values at 4.7 µg/mL for DENV-2, 500 µg/mL for DENV-3 and 365 µg/mL for DENV-4 respectively (Hidari et al., 2008).

In another study, Sanchez et al. (2000) reported how DENV-2 that has been tested with several flavonoids from *Tephrosia* species was found to show a 30% reduction of DENV-2 activity by two flavonoids, namely, glabranine and 7-O-methylglabranine (at concentration 25 µmol/L via *in vitro* testing) although the other flavonoids from *Tephrosia* species did not show any DENV-2 antagonist activity. The tests were carried out using LLC-MK2 cell via viral plaque assay (Sánchez et al., 2000).

A more recent study by Leardkamolkarn et al. (2012) also reported anti-dengue activity against DENV-2 by other native plants from Southeast Asian and Japan. In this research, the aqueous extract of *H. cordata* was tested as an DENV-2 antagonist by *in vitro* experiment with DENV-2 in LLC-MK_2 cell structure; the study found that *H. cordata* extract at concentration of 10-40 µg/mL to show an antiviral effect against the dengue virus (LEARDKAMOLKARN et al., 2012). In short, there is potential to develop and produce anti-dengue treatment using medicinal plants.

1.6 Structures of NS2B/NS3 Protease

1.6.1 NS2B Cofactor for Optimal Activity of Dengue Protease

14kDa NS2B protein acts as a co-factor for the optimal NS3 protease proteolytic activity (Luo et al., 2008; Nitsche et al., 2014) as this is the cause of the presence of hydrophilic segment of NS2B. The hyodrophilic segment increases solubility of the NS2B/NS3 complex (Phong et al., 2011). From the central hydrophilic segment of NS2B, 40 amino acid residues have been identified as important for the NS2B/NS3 complex structural stability and enzymic activity (Yusof et al., 2000). Yusof et al.'s (2000) *in vitro* experiment shows the absence of arginine reduces NS3pro cleavage efficiency of chromogenic substance from 3300 to 7600fold (Yusof et al., 2000). Thus, to promote membrane association of NS2B/NS3 with endoplasmic reticulum membrane, three transmembrane helices from NS2B are used for anchoring. (Aleshin et al., 2007; Bollati et al., 2010). The N-terminal of the NS2B fragment wraps around the NS3 protease and contributes to the formation of substrate recognition sites of the protease at the S_2 and S_3 pockets (Nitsche et al., 2014; Noble et al., 2012). NS2B cofactor is connected to the N-terminal of NS3 protease via a flexible and non-cleavable glycine linker (Erbel et al., 2006).

1.6.2 Expression of NS2B/NS3 Protease

NS2B/NS3 protease complex are expressed using a histidine tag at N-terminus of NS2B/NS3 protease complex and NS3 protease domain in *Escherichia coli (E. coli)* (Leung et al., 2001; Yusof et al., 2000). The NS2B/NS3pro polymerase chain reaction products are cloned into PQE9 and PQE30 vectors before they are transformed into *E. coli* hosts prior induction with isopropyl-ß-D-thiogalactopyranose to overexpress the NS2B/NS3 protease. To

yield pure NS2B/NS3pro and NS3pro product, Size-exclusion chromatography and ion-exchange chromatography are used.

To determine the purity of the express protease, two methods are used; namely, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Several research groups performed fluorometric and spectrophotometric assays to assess the catalytic activity and kinetic parameters of the proteases (Arakaki et al., 2002; Yusof et al., 2000).

As shown in Yusof et al.'s (2000) study, NS2B cofactor does not interact with the chromogenic substrates at S_1 pocket of the protease active site as NS3 protease had better cleavage activity than the NS2B/NS3 protease complex towards a single Arg residue at P_1 (Yusof et al., 2000). However, NS2B cofactor is found to be the important factor to optimise protease activity of NS3pro (Yusof et al., 2000). Leung et al (2001) modified the *in vitro* protease assay published previously to screen for potential inhibitors of pure NS2B/NS3 protease (Leung et al., 2001).

1.6.3 Nomenclature: NS2B/NS3 Protease

In 1967, Schechter and Berger made a nomenclature system that specifies the substrate of the protease (Schechter and Berger, 1967). As shown in Figure 1.6, the active site residues of the protease are divided into subsites (Sn,….,S_2,S_1,S_1',S_2',……Sn') while the amino acid residues of the substrates occupying the corresponding subsites are numbered as Pn,…..,P_2,P_1,P_1',P_2',…..Pn'; in addition, the scissile bond or the substrate cleavage site is located between the P1 and the P1' positions. The part to the N-terminus and to the C-terminus of the scissile bond are called prime and nonprime sites, respectively. The peptide substrate labelled P3-P1 on the N-terminal side of the scissile bond and P1'-P3' on the C-terminal side of the bond.

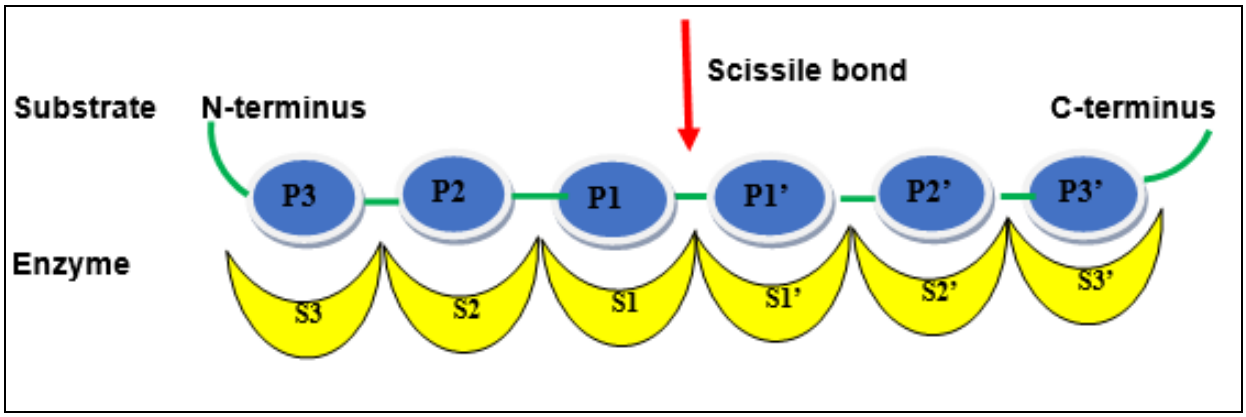
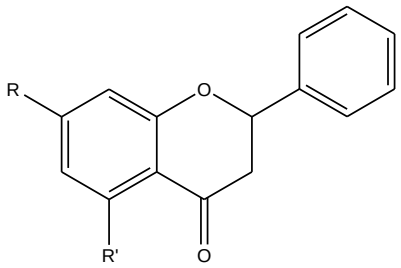


Figure 1.6: Schechter and Berger System of Nomenclature

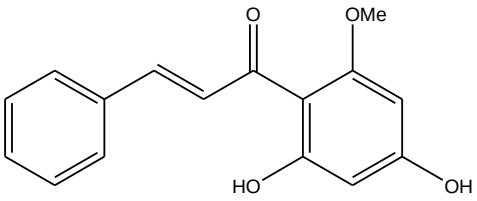
1.7 Current NS2B/NS3 Protease Inhibitors

1.7.1 Inhibitory Activity of Natural Compounds on DENV Protease

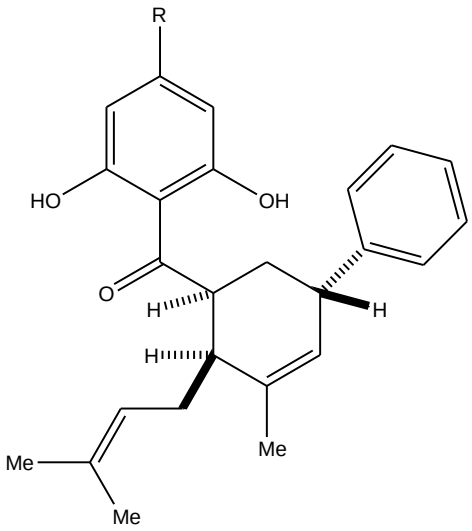
An inhibition activity of natural compounds on dengue protease conducted by Kiat et al. (2006) identified pinostrobin, pinocembrin, alpinetin, cardamonin, panduratin A and 4-hydroxypanduratin A as the six compounds that were extracted from *Boesenbergia rotunda*. (Figure 1.7) had exhibited more than 50% inhibition of DENV-2 NS2B/NS3pro at concentration of 400 ppm. Panduratin A and 4-hydroxypanduratin have Ki values of 25 µM and 21 µM, respectively; these values indicate a desirable inhibition activity of NS2B/NS3pro. Pinocembrin has the lowest inhibition to NS2B/NS3pro with the inhibitory activity around 30 %, 47 % and 56 % inhibition at three different concentrations tested, 120, 240 and 400 ppm, respectively. From these findings, one can conclude that Panduratin A and 4-hydroxypanduratin are good candidates for DENV-2 NS2B/NS3pro antagonist (Kiat et al., 2006). Dengue virus protease activity can be decreased by using methyl gallate that is extracted from *Quercus lusitanica.* As at 0.3 mg/mL of methyl gallete shows over 98% inhibition of NS2B/Ns3 activity (Rahman et al., 2006).



R = OMe, R’ = OH : Pinostrobin
R = OH, R’ = OH : Pinocembrin
R = OH, R’ = OMe : Alpinetin



Cardamonin



R = OMe : Panduratin A
R = OH : 4-hydroxypanduratin A

Figure 1.7: The structures of flavonone and chalcone derivatives extracted from *Boesenbergia rotunda*

Other flavonoids that are tested for DENV-2 and DENV-3 protease assay are agathisflavone, quercitrin, isoquercitrin, myricetin, querctin hydrate and kaempferol. The flavonoids are present when there are antagonist effects on DENV-2 and DENV-3 with K_i values ranging from 4.7 to 25.9 µM. Agathisflavone exhibited lowest IC_{50} values, 15.1 and 17.5 µM, while isoquercitrin was the less active compound of all flavonoids tested with IC_{50} values of 44.0 and 42.4 µM against DENV-2 and DENV-3 NS2B/NS3pro, respectively (de Sousa et al., 2015).

1.7.2 Inhibitory Activity of Small Molecules on DENV Protease

Small molecules are also tested for inhibition activity of NS2B/NS3 protease of DENV-2. Using *in vitro* enzyme assay, several acrylamides are synthesized and tested against DENV NS2B/NS3 protease as these acrylamides are expected to have an antagonist activity as at S_1 and S_2 pockets of NS2B/NS3 protease. Nitsche et al.'s (2014) study found one of the arylcyanoacrylamide derivative had decreases the dengue virus protease activity with the K_i value of 35.7 µM; this indicates the potential of this compound to be developed into a permissible DENV protease inhibitor (Nitsche et al., 2014).

In another study by Tomlinson and Watowich (2011), four out of the ten of 23 analouges of parent compound anthracene were found to have activity ranging from K_i values of 15 – 215 µM compared to the parent compound (432 µM) as shown in Figure1.8 (Tomlinson and Watowich, 2011).

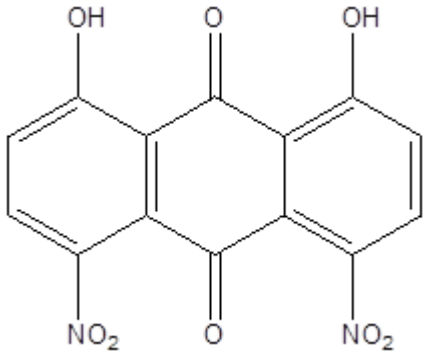


Figure 1.8: The structure of anthracene, potential lead compound as DENV-2 protease inhibitor.

A new lead for a potent NS2B/NS3 protease inhibitor small-molecule-based scaffold has been carried out by Deng et al. (2012) whereby 21 new compounds with quinolone scaffold were synthesized and characterized as antagonist activity against DENV NS2B/NS3 protease. Out of all 21 compounds, only three compounds were found to have significant inhibitory effects against the NS2B/NS3 proteas (IC_{50} values of 7.8, 7.5 and 9.5 µM) (Deng et al., 2012). In another study that searched for an effective DENV protease inhibitors, several Rhodanine- and Thiazolidinedione-Based Peptide Hybrids were synthesized and tested in protease inhibition assay. The synthesized compounds showed impactful effect on the protease activity with more than 85 % of inhibition NS2B/NS3 activity at 50 µM concentration. The study found thiazolidinedione-based peptide hybrids with hydrophobic groups as the most active compounds with low K_i values between 1.5 and 1.8 µM (Nitsche et al., 2013).

1.7.3 Inhibitory Activity of Peptide-Based Compounds on DENV Protease

Today, the literature shares a promising viewpoint in the development of substrates-based peptides drugs. As explained by Tambunan and Alamudi (2010), although the stability of peptides drugs are lower than normal conventional synthetic type ones, these peptide-

based drug are more preferred for a new drug development since they appeared to be less toxic and have higher specificity and selectivity compared to small molecule therapeutic counterparts (Tambunan and Alamudi, 2010).

The successful discovery and development of peptidic inhibitors of Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV) proteases, which are now in clinical use has also led to the development of peptide-based compounds as clinical candidates against dengue virus protease (Xu et al., 2012). Saquinavir is the first peptidomimetic HIV protease inhibitor that is effective against both HIV-1 and HIV-2. This drug has been approved by US Food and Drug Administration (FDA) and reached the market in 1996.

It was reported by Yusof et al. (2000) that a dibasic sequence (primarily with two arginine residues) of a peptide substrate positions next to the cleavage site resulted in increased affinity and potency against the NS2B/NS3 protease (Yusof et al., 2000) . In addition, peptidic inhibitors; Bz-Nle-Lys-Arg-Arg-H (K_i = 5.8 μM) and Bz-Ala-Lys-Arg-Arg-H (IC_{50} = 5.3 μM) have low K_i values and thus, are promising leads for the development of small peptidic inhibitor against NSB2/NS3 protease. A tripeptide aldehyde inhibitor, phenylacetyl-Lys-Arg-Arg-H demonstrated better potency towards DENV2 protease and exhibited inhibitory concentration, IC_{50} in the micromolar range, 6.7 ± 1.1 μM (Schüller et al., 2011) . Several cyclopeptides were synthesized and screened for their inhibition potential towards protease activity. Some of them showed significant inhibitory activities with calculated K_i value as low as 2.2 ± 0.2 μM (Nitsche et al., 2012; Xu et al., 2012).

Protegrin-1 (PG-1) peptide, an eighteen amino-acids with high content of basic residues linked together by two disulphide bonds were synthesized and tested in the dengue protease inhibition assay. At a concentration of 40 μM, this compound was observed to inhibit 95.7% of NS2B/NS3pro activity. Furthermore, the PG-1 was identified as inhibitors with IC_{50} value of 11.7 μM A disulphide cyclic peptide, retrocylin -1 expressed in *E. coli* by