RATIONAL DESIGN AND SYNTHESIS OF PEPTIDE INHIBITORS OF NS2B/NS3 PROTEASE AS THERAPEUTIC AGENTS FOR DENGUE VIRUS TYPE 2

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UNIVERSITI SAINS MALAYSIA

2021

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by

NADEEM ABDALSATAR ABDALRAZAQ

Thesis submitted in fulfilment of the requirement
for the degree of
Doctor of Philosophy

October 2021

ACKNOWLEDGEMENT

Praise is to our Almighty Allah gracious for enabling me to finish what I started and for helping me to present this work.

I would like to express grateful gratitude to my main supervisor Dr. Ezatul Ezleen Binti Kamarulzaman, for her advice, guidance, and support through the course of this work. Her understanding, kindness, fantastic personality, and expertise in guiding students helped me greatly in overcoming the difficulties encountered during the course of my study and in completing the thesis.

I would like to express my great thanks and appreciations to my Co-advisors: Dr. Yap Beow Keat and Dr. Suriani Mohamad, for their suggestions, assistance, and valuable advice. I wish to express grateful thanks to Prof. Dr. Habibah A. Wahab for allowing me to use her lab to perform the *in vitro* protease assay. I am sincerely grateful and would like to express my deepest thanks to the School of Pharmaceutical Sciences (Dean, academic and technical staffs) for allowing me to utilize all the necessary facilities to enable in completing this study. Great thanks to Prof. Dr. Nazalan Najimuddin and a Ph.D student Ahmad Faisal from the School of Biological Sciences, USM for valuable help in performing dengue protease expression and purification.

My deepest thanks and appreciations also go to all my friends postgraduate students, Fadi G., Mohammed T., Mohammad D., Bilal , Abdulsalam Q., Ahmed Y., Ghazi J., and Ahmed G. for their valuable help and support. My deepest thank and genuine gratitude extended also to the chairman and dean of URUK University, College of pharmacy, Iraq to give me the opportunity to complete my PhD study. My deepest thanks extended to my wife who providing me support, encouragement, and the power

to continue my postgraduate study with my warmest love and appreciation. Genuine thanks to my children, Ranya, Mustafa and Rand with greatest Love.

Last but not least, I would like to express my appreciation to my mother, my brothers and sisters who always give me a prayer and blessing to finish my study. Finally, my sincere thanks to all others who helped me in any way.

الحمد لله رب العالمين

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ΔG_{bind}	Binding free energy
A	Alanine (Ala)
ADE	Antibody dependent enhancement
AMC	7-aminomethyl-4-coumarin
Boc	Tertiary butyloxy carbonyl
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
C protein	Capsid protein
CADD	Computer aided drug design
C	Cysteine (Cys)
DCM	Dichloromethane
DENV	Dengue virus
DENV-1	Dengue virus serotype 1
DENV-2	Dengue virus serotype 2
DENV-3	Dengue virus serotype 3
DENV-4	Dengue virus serotype 4
DENV-5	Dengue virus serotype 5
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DIPEA	*N*,*N*-Diisopropylethylamine
DMSO	Dimethyl sulfoxide
DMF	*N*, *N*-dimethylformamide
DODT	2,2'-(ethylendioxy) diethanethiol
DSS	Dengue shock syndrome
EDS	Expanded dengue syndrome

E protein	Envelope protein
Equiv.	Equivalents
ER	Endoplasmic reticulum
Fmoc	Fluorenyl methoxycarbonyl
JEV	Japanese Encephalitis Virus
G	Glycine (Gly)
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HOAt/HOBt	1-hydroxybenzotrizole
HBTU/TBTU	(2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCTU	O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
I	Isoleucine (Ile)
IC_{50}	Half maximal inhibitory concentration
IPTG	Isopropyl-β-D-thiogalactopyranoside
K	Lysine (Lys)
Ki	Inhibition constant
L	Leucine (Leu)
LB	Luria Bertani
LGA	Lamarckian Genetic Algorithm
M	Membrane
MD	Molecular dynamic
MOE	Molecular Operating Environment
mRNA	Messenger RNA
N	Asparagine (Asn)
N_2	Nitrogen
NaOH	Sodium hydroxide
NS	Non-structural protein
NSAIDs	Nonsteroidal anti-inflammatory drugs
ORF	Open reading frame
OD	Optical density

PDB	Protein Data Bank
prM/M	Pre-membrane/ membrane protein
P	Proline (Pro)
Q	Glutamine (Gln)
R	Arginine (Arg)
RFU	Relative fluorescent unit
RP-HPLC	Reverse-phase high performance liquid chromatography
RNA	Ribonucleic acid
S	Serine (Ser)
ssRNA	Single stranded RNA
SBVS	Structure-based virtual screening
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel Electrophoresis
SEM	Standard error of mean
SPPS	Solid phase peptide synthesis
T	Threonine (Thr)
TIPS	Triisoprpylsilane
WHO	World Health Organization
WNV	West Nile virus
Xaa	Any amino acid
YFV	Yellow Fever Virus

LIST OF SYMBOLS AND UNITS

Å	Angstrom
α	Alpha
β	Beta
-	Dash
=	Equals
>	More than
<	Less than
^{0}C	Degree Celsius
KDa	Kilo dalton
M	molar
Min	Minute
ml	Millilitre
mM	Millimolar
µg	Microgram
µl	Microliter
µM	Micromolar
%	Percentage
®	Registered trademark
TM	Trademark

LIST OF APPENDICES

REKA BENTUK RASIONAL DAN SINTESIS PERENCAT PEPTIDA BAGI NS2B/NS3 PROTEASE SEBAGAI AGEN TERAPUTIK VIRUS DENGGI JENIS 2

ABSTRAK

Pada masa ini, jangkitan virus denggi merupakan salah satu masalah kesihatan penting di dunia, terutamanya di negara tropika dan subtropika. Objektif utama kajian ini ialah mereka bentuk perencat peptida berpotensi dan terpilih dengan mengkaji interaksi diantara rekaan peptida dan protease denggi NS2B/NS3 dengan menggunakan dua program berbeza; Molecular Operating Environment (MOE) dan AutoDock4.2, seterusnya, untuk menilai keputusan pendokan secara kuantitatif dan kualititatif dari kedua-dua program. Objektif lain ialah untuk mensintesis peptida yang berpotensi dengan menggunakan teknik Fmoc SPPS, untuk melakukan ekspresi dan penulenan protease denggi NS2B/NS3, dan akhir sekali untuk mengkaji penilaian biologikal peptida yang terpilih menggunakan asai *in vitro* perencatan aktiviti protease denggi NS2B/NS3. Empat kumpulan urutan novel peptida linier dan siklik dirancang berdasarkan kekhususan substrat dengan protease denggi NS2B / NS3. Ligan kawalan, Benzoyl-Nle-Lys-Arg-Arg-H, dan sejumlah 78 rekaan peptide terdokan ke atas protease denggi NS2B/NS3. Didapati bahawa keputusan kuantitatif dan kualitatif daripada kedua-dua program pendokan hampir sama dan selari. Tiga belas ligan peptida telah dipilih sebagai peptida berpotensi berdasarkan hasil pendokan molekul. Lima peptida linier dan empat peptida siklik telah disintesis, ketulenannya diperiksa dengan fasa berbalik kromatografi cecair bertekanan tinggi (RP-HPLC), dan jisim molekul dicirikan oleh spektometri jisim, sementara empat peptida lain diperolehi secara komersial.

Protease denggi NS2B/NS3 telah diekspres dan dianalisa menggunakan SDS-PAGE dan seterusnya ditulenkan menggunakan Resin Kolum Berafiniti TALON®. Asai perencatan protease NS2B/NS3 secara *in vitro* dilakukan terhadap tiga belas peptida yang berpotensi pada kepekatan 100 µM, ia menunjukkan bahawa tujuh peptida mencapai peratusan perencatan lebih daripada 50% dan seterusnya diuji untuk nilai IC_{50}. IC_{50} terbaik ditunjukkan oleh Cys-Arg-Lys-Arg-Cys dengan nilai 17.94 µM dan diikuti oleh Cys-Lys-Arg-Cys (25.41 µM) dari peptida siklik, manakala peptida linear Lys-Lys-Arg -Arg mempamerkan IC_{50} dengan 30.38 µM berbanding dengan aprotinin (kawalan) dengan IC_{50} 18.29 µM. Keupayaan peptida berpotensi dengan nilai IC_{50} yang baik memberikan harapan bahawa peptida ini dapat dibangunkan lebih lanjut sebagai perencat protease denggi NS2B/NS3 yang berpotensi. Secara umum, penemuan potensi perencat protease denggi berasaskan peptida telah berhasil dilakukan dengan menggabungkan teknik pengkomputeran dan asai perencatan protease secara *in vitro*, seterusnya eksperimen seperti asai virus denggi dan kajian *in vivo* adalah diperlukan untuk dijalankan sebelum peptida tersebut boleh berfungsi sebagai perencat virus denggi yang sesuai.

RATIONAL DESIGN AND SYNTHESIS OF PEPTIDE INHIBITORS OF NS2B/NS3 PROTEASE AS THERAPEUTIC AGENTS FOR DENGUE VIRUS TYPE 2

ABSTRACT

Nowadays, dengue virus infection is one of the world's significant health problems, particularly in tropical and subtropical countries. The main objectives of this study are to design potent and selective peptide inhibitors by studying the interactions between the designed peptides and the NS2B/NS3 dengue protease using two independent docking programs (MOE and AutoDock4.2), thus, to evaluate the quantitative and qualitative docking results from both programs. The other objectives are to synthesize and characterize the potential peptides by using Fmoc Solid Phase Peptide Synthesis (SPPS) technique, to perform NS2B/NS3 dengue protease expression and purification, and finally to investigate the biological evaluation of the selected peptides using *in vitro* NS2B/NS3 dengue protease inhibition assay. Four groups of novel sequence of linear and cyclic peptides were designed based on the substrate specificity with the NS2B/NS3 dengue protease. The control ligand, Bz-Nle-Lys-Arg-Arg-H, and a total of 78 designed peptides were docked onto NS2B/NS3 dengue protease. It was found that the quantitative and qualitative results from both docking programs could give almost the same and consistent results. Thirteen peptide ligands have been chosen as potential peptides based on molecular docking results. Five linear and four cyclic peptides have been synthesized, their purity was checked by Reverse Phase – High Performance Liquid Chromatography (RP-HPLC), and the molecular mass was characterized by mass spectrometry, while the other four peptides were

obtained commercially. The NS2B/NS3 dengue protease was expressed and analysed by using SDS-PAGE and further purified using TALON® Affinity Column Resin. The *in vitro* NS2B/NS3 protease inhibition assay was performed for the thirteen potential peptides at a concentration of 100 µM. Seven peptides exhibited percentage of inhibition more than 50% and were further tested for IC_{50} value. The best IC_{50} is shown by Cys-Arg-Lys-Arg-Cys with a value of 17.94 µM and followed by Cys-Lys-Arg-Cys (25.41 µM) from the cyclic peptides, while the linear peptide of Lys-Lys-Arg-Arg exhibited IC_{50} with 30.38 µM compared to aprotinin (a control) with IC_{50} of 18.29 µM. The ability of potential peptides with a good IC_{50} values gives a hope that these peptides can be further developed as potential NS2B/NS3 dengue protease inhibitors. In general, the findings of the potential peptide-based dengue protease inhibitor have been successfully done by combining computational technique and *in vitro* inhibition protease assay, thus further experiments such as dengue virus assay and *in vivo* study are necessary to be carried out before these peptides could serve as suitable inhibitors for dengue virus.

CHAPTER 1

INTRODUCTION

1.1 Background

Arthropod borne infection like dengue is a major concern to the world and a leading cause of illness and death in the tropical and subtropical regions of the world. Countries with subtropical and tropical climates face one of the major health complications, which is dengue virus infection. It is also considered a health issue in Southeast Asia, including Malaysia, where infection and fatality cases related to dengue are rising.

Dengue virus infection (DENV) caused by the dengue virus and transmitted by mosquitoes: *Aedes aegypti* and *Aedes albopictus*. An approximate 390 million dengue infections occur annually, of which 96 million are represented clinically (Bhatt, 2013). In 2017, WHO reported that this disease had put most people at risk, and the incidence of dengue has risen rapidly in recent times (WHO, 2017). Dengue became the most rapidly spreading, infectious vector-borne virus disease in 2012 and a very remarkably higher number of disease in the world, including Malaysia (G. M. Thomas, A. , 2013).

Viral proteases have been shown to serve as good inhibitory targets. For instance, protease inhibition was shown to be a successful strategy in treating human immunodeficiency virus (HIV) infection. The NS5, NS3 and NS2B (co-factor) proteins were known to play a major role in enzymatic activities for DENV infection, thus making them ideal antiviral targets (Egloff et al., 2002; Stocks et al., 1998).

The role of the computational method in discovering drugs and developing target identification that is leading towards the discovery, also towards the optimization from preclinical to clinical trials, has been undeniably significant since the 1980s (Y. Wang

et al., 2015). The resources needed for the trial experiment are decreased by the *in silico* methods, where the working environment is stimulated, and this will help improve the whole process of discovering the drug's efficacy and efficiency. The inhibition activity towards dengue NS2B/NS3 protease can be seen through several attempts for discovering an alternative treatment that includes a few peptides (Yin et al., 2006), non-peptides (Ganesh et al., 2005), small molecules (Deng et al., 2012) and natural compounds (de Sousa et al., 2015).

1.2 Problem statement

Dengue is an old disease, yet unfortunately, dengue infections are untreatable following the absence of a licensed vaccine or effective, relevant drugs. Until now, the sole treatment for dengue disease is a symptomatic treatment or intensive supportive therapy for saving patients' lives. However, as undeveloped countries do not have sufficient healthcare facilities, the fatality rates due to dengue fever (DV) epidemics have steadily become worrying, especially when its primary victims are children. According to the data recorded by Word Health Organization (WHO), approximately 3.9 billion people have been at risk of infection in 128 countries, or about (40 %) of the world's population, and the incidence of dengue has overgrown in recent years (WHO, 2019). To date, we still have no news about an efficient marketed drug that can treat or cure dengue. In the absence of the right kind of animal model, and we still have to accept the fact that the dengue virus infection still has an unclear mechanism. Thus the discovery of a drug for dengue slows down (Coronel-Ruiz et al., 2020; Zompi et al., 2012).

It has been found that NS3 protein of dengue virus is very important non-structural protein having a trypsin like serine protease domain at the N-terminal region is known as NS3pro and its activity depends on its interaction with cofactor (NS2B) and form a complex named NS2B/NS3 protease complex. This complex is important because of its ability to cleave at different parts of viral proteins. Thus, the NS2B/NS3 complex is considered as an important target for drug as antiviral agents.

The population rise, changes in environmental factors, host–pathogen interactions, population immunological factors and inadequate vector control measures; all of these factors consider as a favourable condition for dengue virus transmission and its mosquito vectors. The dengue cases dramatically increased over the last 50 years. Therefore, adequate studies, efforts, and effective medicines that are safe and provide long-term defence against dengue viruses are necessary to develop.

1.3 Objectives of the research

1) To design, predict, and discover the potent and selective peptide inhibitors of dengue NS2B/NS3 protease based on the quantitative and qualitative results from the two independent computational docking programs (MOE and AutoDock 4.2).

2) To synthesize the potential peptides by using Fmoc solid phase peptide synthesis (SPPS) and perform characterization of the peptides using mass spectrometry.

3) To express and purify the NS2B/NS3 dengue protease.

4) To evaluate the inhibitory activity of the selected peptides using *in vitro* NS2B/NS3 dengue protease inhibition assay.

The flowchart of overall study is demonstrated in Figure 1.1.

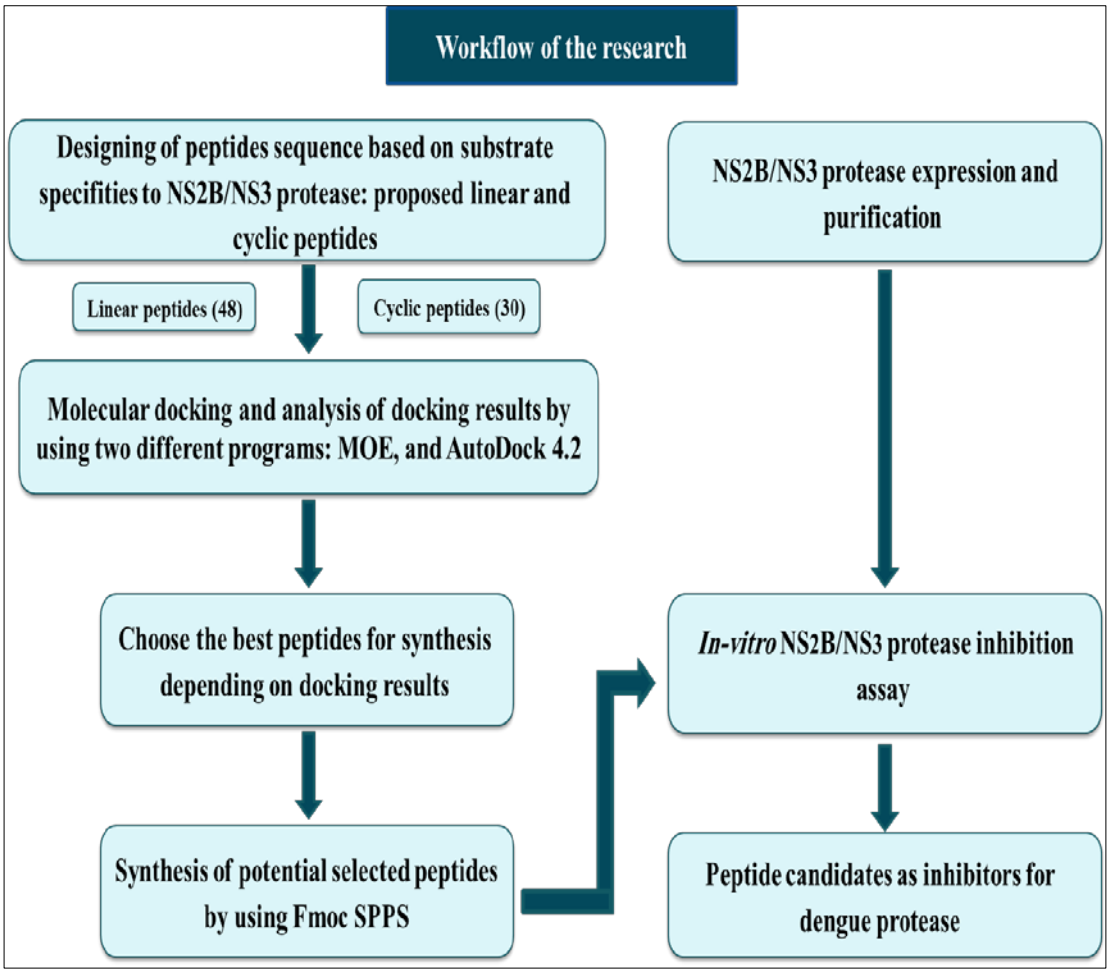

Figure 1.1 The flowchart of overall study.

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of the dengue virus

In many parts of the world, dengue has become a common endemic infectious disease. The disease was first reported in the 18th century, when a major dengue epidemic was reported to have occurred at intervals of 10 to 40 years in North America, Africa, and Asia (Guzman et al., 2016; Moi et al., 2010).

Since 1980, Malaysia has reported an increasing number of dengue infections. For example, a large number of dengue cases have been reported, which have increased seven-fold between 2000 and 2010 (Mohd-Zaki et al., 2014). In the year 2020, the Health Ministry of Malaysia recorded that the cumulative number of dengue cases reported was 86,406 until November 2020. In the same period of 2019, this was down from 119,524 cases. In 2020, 140 deaths were reported, compared to 164 in 2019 in the same period. Dengue activity generally decreases by the number of cases reported in 2020 as compared to 2019 (Figure 2.1) (WHO, 2020) .

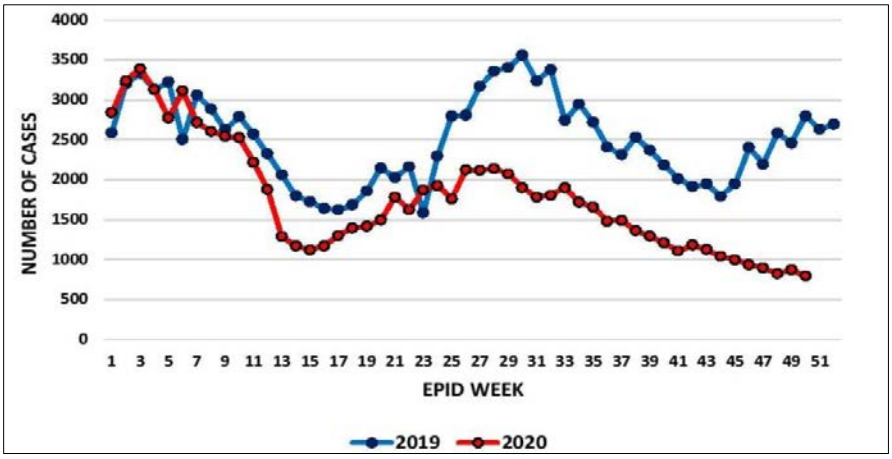

Figure 2.1 The dengue cases reported weekly in the year 2019 and 2020, Department of Health, Malaysia (WHO, 2020).

2.2 The structure and genome of Dengue Virus

DENV is an enveloped, positive single-stranded (ss) RNA Flavivirus of the Flaviviridae family; under the same genus, there are also West Nile virus (WNV), Yellow Fever Virus (YFV), Japanese Encephalitis Virus (JEV), and Zika virus (Kuno et al., 1998). The virus particle (virion) is a sphere-shaped virus that has a diameter of about 50 nm, and it is covered with structural proteins; envelope (E), membrane (M) located on its surface, and nucleocapsids; which is composed of capsid (C) proteins and genomic RNA, located within the inner region of the virion. The genome contains a single open reading frame (ORF) with untranslated regions (UTRs) at both ends. The 5- terminal is capped, but the 3- terminal is non-polyadenylated. A large precursor polyprotein of more than 3000 amino acids is encoded by the ORF and is cleaved by host proteases and virus NS3 protease DENV into three structural proteins (C, prM/M, and E proteins) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A NS4B, and NS5) (Figures 2.2 and 2.3) (Lindenbach, 2007).

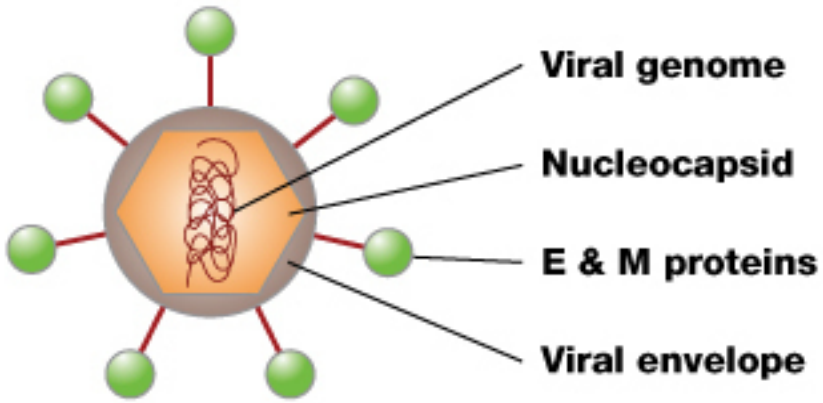

Figure 2. 2 Dengue virus structure (Guzman et al., 2010).

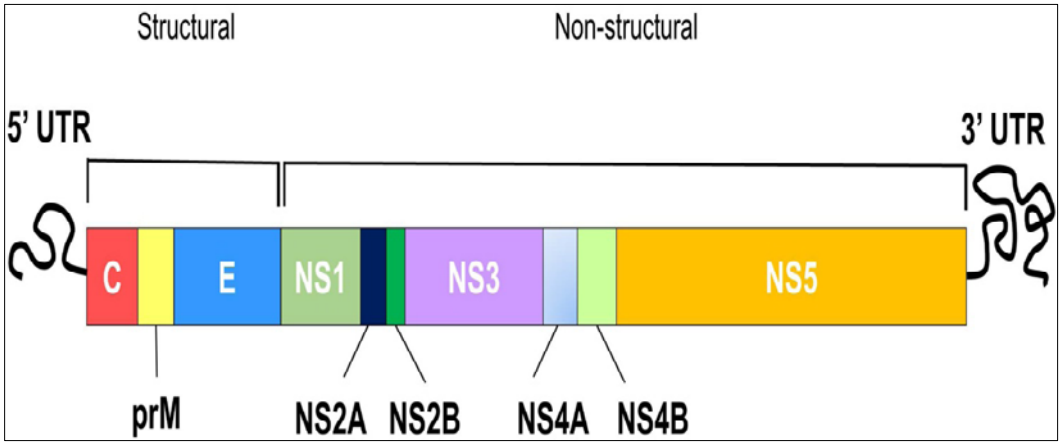

Figure 2.3 Schematic diagram of the DENV genome showing structural and non-structural polyproteins that are encoded by the DENV genome (Lindenbach et al., 2003)

2.3 Dengue virus serotypes

Currently, there are five dengue virus serotypes that are considered unique in terms of their genetics and antigenic properties, but they show a similar range of disease manifestations in the phase of infection (Mustafa et al., 2015). These serotypes (DENV1-5) have similar genomes and morphology, but their antigens are different. Due to this, the body will not be able to guard itself against another serotype's infection as it increases pathogenicity (Rajamanonmani et al., 2009). The genetic serotypes of the DENV share similarity about 65% of their genomes (Guzman et al., 2010).

2.3.1 Dengue virus serotype 1

DENV-1 is one of strains of dengue-borne arthropod viruses (Karabatsos, 1978). Its genotypes are dependent according to regions; for example, genotypes 1 and 3 are commonplace in Asia, genotype 2 is common in Thailand, genotype 3 was found in Asia, including the sylvatic strain collected in Malaysia, while genotype 4 is common in Asia, Pacific region, the Indian Ocean, Australia, and America (Villabona-Arenas et al., 2013). The Southeast Asian countries have been named the source countries of dengue epidemics, and the entire topology of DENV-1 genotypes is marked by the oldest strains' basal areas and new isolates (R. Chen et al., 2011).

The rapid spreading of DENV-1 all over the world was evident when the first outbreak was recorded in Brazil in 1981 and later propagated to many other parts of the country (Schatzmayr et al., 1986).Vietnam and Myanmar were the first Asian countries that reported the dengue outbreak (R. Chen et al., 2011). Next, the epidemic outbreak had caused dengue fever in Japan (Nukui et al., 2006). The disease outbreak caused dengue fever (DF). However, there were no fatality, dengue hemorrhagic fever (DHF), or dengue shock syndrom (DSS) cases were reported during the same year (Martin, 2005). Several tests were carried out on symptomatic passengers, and scholars found

that DENV-1 strains came from South-East Asia, most of them in Japan and Korea (Ito et al., 2007). From this discovery, dengue infection caused by DENV-1 struck as an important health issue, especially since there is a steady frequency of people traveling internationally in developing countries (Chen and Vasilakis, 2011; Villabona-Arenas and Zanotto, 2013).

2.3.2 Dengue virus serotype 2

DENV-2 has recognised the five unique genotypes; the first hypothesis surrounds DENV, which is said to originate from sylvatic areas in the western parts of Africa. This is shown through the phylogenetic studies of DENV-2 strains, in which it is proposed that the strains are somewhat different from all other strains (Rico-Hesse, 1990). The evolution of DENV serotypes is detached from a sylvatic ancestral lineage in areas in Malaysia (E. Wang et al., 2000). This lineage primarily spread among animals before humans (Rico-Hesse, 1990; Wang et al., 2000). The Southeast Asia has been identified as the regional source of DENV-2 (Rico-Hesse et al., 1997). Less than five decades ago, scientists had reported the presence of DENV-1 and DENV-2 in both Central America and Africa and that all the other four serotypes have been found in Southeast Asia (Guzman et al., 2010).

For more than three decades, DENV-2 has been found in Malaysia, and this disease is considered an endemic, with approximately 400 to 7000 dengue fever cases annually. Although DENV-2 became a dominant serotype in the late 1960s to the early 1970s, it then continued and was stated responsible for significant outbreaks in the late 1980 to early 2000s (Chee et al., 2003). In 2003, Santos and colleagues found that DENV-2 was more infectious than DENV-1 and these findings are consistent with

immunological tests (Santos et al., 2003). In another study, Vaughn and his colleagues revealed that DENV-2 patients appeared to have the highest secondary antibody responses and that DHF is more likely compared to other serotypes. A higher DENV-2 viremia label is linked to more severe diseases in conjunction with DENV-2 replicating capacity (Vaughn et al., 2000). DENV-2 is more common and predominant in dengue diseases than those of other serotypes (Frimayanti et al., 2011; Suppiah et al., 2018).

2.3.3 Dengue virus serotypes 3 and 4

Latin America reported its first outbreak of DENV-3 in the 1960s, and after being no dengue cases for more than two decades, it appeared back in 2003 (Uzcátegui et al., 2003). Pathogenic research found that DENV-3 was a genotype 5 virus belonging to Malaysia's sylvatic strains, which was prevalent during the Latin American epidemic in the 1960s. Lanciotti and his colleagues identified a geographical location independent evolution of DENV-3. All of the genotype 1 has been identified in Indonesia, Malaysia, Southern Pacific, and the Philippines, genotype 2 has been reported in Thailand, genotype 3 has been discovered in Sri Lanka, India, and Africa, genotype 4 has been found in Puerto Rico and Tahiti and genotype 5 detected in the Malaysian sylvatic strain (Lanciotti et al., 1994).

There are few comprehensive genetic diversity studies, especially for Southeast Asia, performed on DENV-4. Since it has been the first dengue serotype to differ in the phylogenetic study of the Flavivirus group (Gaunt et al., 2001). As it is, the DENV-4 has identified three main genotypes, and all of them have been classified by area (Lanciotti et al., 1997). In 1967, DENV-4 was reported in Malaysia as the prevalent serotype for a considerable number of dengue cases. However, looking into all these

dengue infections in a decade, only 5 % of total dengue fever are DENV-4 related disease (AbuBakar et al., 2002). DENV-4 was first identified in America in 1981 and triggered DF epidemics throughout the region (Carrington et al., 2005).

2.3.4 Dengue virus serotypes 5

Until recently, the cause of dengue infections was assumed to be in four separate antigenic serotypes (DENV 1-4), with each of the serotypes have its own distinctive immune reaction from the infection. Such four genetic serotypes share about 65% of their genomes. Dengue virus is transferred by the mosquito vector of the Aedes genus to non-primates (sylvatic form) and humans (Holmes, 1998). DENV-5 was discovered and announced in 2013. It was detected after a viral screening was done on a patient who was admitted to a hospital in Sarawak (Malaysia). Initially, it was considered to be a common cause of DENV-4, a complete genetic analysis was performed, and the results showed that the virus varied significantly from the other three types of DENV-4 and showed some similarity with DENV-2 (Normile, 2013). Since no new serotype viruses have been detected worldwide over the past five decades, the new virus was initially considered a form of DENV-4. However, when pre-infected with 4 other serotypes, a macaque group of monkeys developed various kinds of antibodies and recovered from an infection. This lead to the discovery of the new serotype (DENV-5) (Mustafa et al., 2015).

2.4 Life cycle of the dengue virus

The dengue virus life cycle begins when the virus attaches to the susceptible host cell receptors (viral attachment), DENV like other flaviviruses, use cell surface receptor-mediated endocytosis for entrance (viral entry), then catalysed by acidic pH of the environment, induces a membrane fusion of the viral envelope (viral infusion) to uncover the nucleocapsid and release the viral genome into the cytosol host cell. The next step involved the translation of the RNA genome into a single polyprotein through the host ribosomes and translocated through the endoplasmic reticulum membrane (ER). Finally, the polyprotein is converted into three structural proteins and seven non-structural proteins utilizing the cellular (host) and virus-derived proteases. The NS3 virus protease has an autocatalytic feature and plays an essential role in this process (Chambers et al., 1990).

The newly synthesized viral RNA is enclosed in the C proteins, forming a nucleocapsid. The nucleocapsid enters the rough ER and is surrounded by M and E proteins in the ER membrane. The created viral proteins and replicated genome are packaged into virions in the endoplasmic reticulum (ER) during viral maturation. The prM is clustered in membrane (M) protein by host furin after the virions are passed into the Golgi vesicles and induce viral maturation. Finally, by the exocytosis process, the maturated viruses are released and can go on to infect other cells (Mazeaud et al., 2018; Rodenhuis-Zybert et al., 2010). The life cycle of DENV is illustrated in (Figure 2.4).

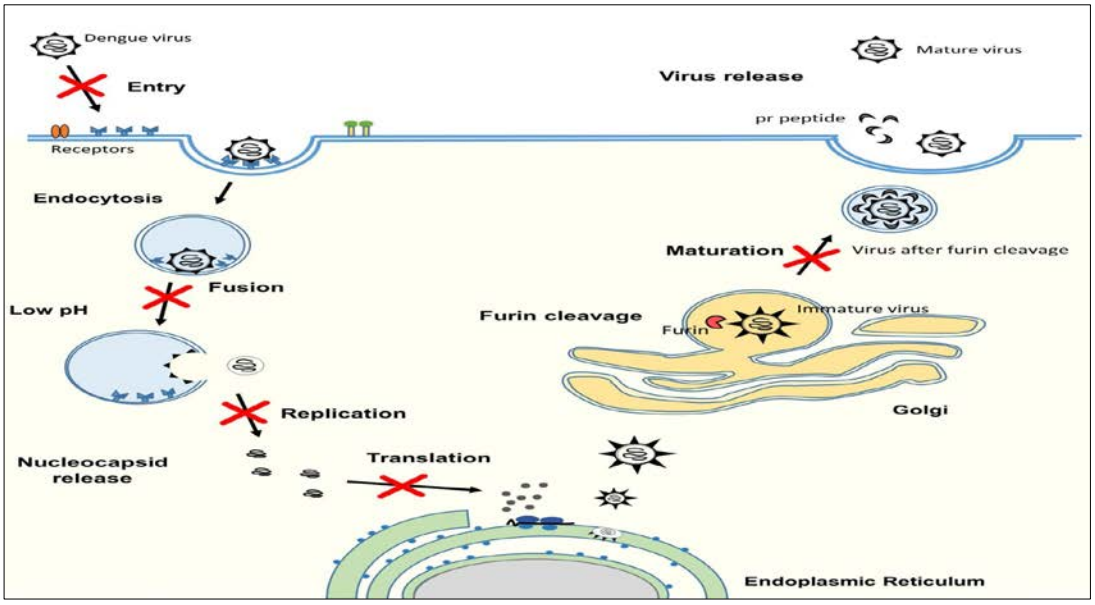

Figure 2.4 An overview of the DENV life cycle (Rodenhuis-Zybert et al., 2010).

2.5 Infection by the dengue virus

Within the five days of viremia, the mosquitoes get infected after they have sucked in human blood (Liu et al., 2017). Following ten days of the incubation period, the virus starts circulating as it is then transferred from the intestinal tract into the salivary glands. Thus the salivary proteins of the mosquito could confirm the infection caused by mosquito bites (Schneider et al., 2004). Salivary proteins of the mosquito consist of D7 proteins with various functions to facilitate blood-feeding. These proteins are known to be anti-haemostatic, contain platelet aggregation inhibition, anti-vasoconstriction activity, and comprise allergens and immune-modulatory compounds (Sim et al., 2012). Subsequently, DENV infection happens in the skin's immature dendritic cells. The matured cells will then be transferred to the lymph nodes, presenting the viral antigens to T cells. Therefore, it initiates the immune responses that are characteristically cellular and humoral (Jessie et al., 2004). Infection by DENV is illustrated in Figure 1.6.

In a given population, the co-circulation of dengue serotypes might be improved by the antibody-dependent enhancement (ADE) phenomenon, with macrophages and monocytes serving as the mediator (Sun et al., 2018). When the enzyme-antibody complex enters monocytic cells, ADE greatly enhances the titre of the virus by increasing the number of ADE infection pathway to suppress the antiviral molecules, so this aids the production of virus (Sim et al., 2012).

Humans are susceptible to several illnesses caused by dengue infection ranging from rash, joint pain to dengue haemorrhagic fever and shock syndrome. DENV infection will further be the cause of either dengue fever (DF), life-threatening dengue hemorrahgic fever, or dengue shock syndrome (DSS) (Moi et al., 2010; Muller et al., 2017). People having asthma, diabetes, or other chronic diseases may endanger life when infected with the virus (Kouri et al., 1987). Secondary Dengue infections typically cause classical DSS or severe haemorrhage-complicated disease (Guzman et al., 2010). There are several syndromes produced by DENV infection that are conditioned by age and the person's immune system status. Most children develop subclinical or moderate undifferentiated febrile syndromes at the initial stage of DENV infection (Guldahl, 2017). Subsequent infections then bring about dramatic changes in the disease's pathophysiology, especially in sequence infections. This secondary infection causes chronic vascular permeability syndrome called DSS (Guzman et al., 2016).

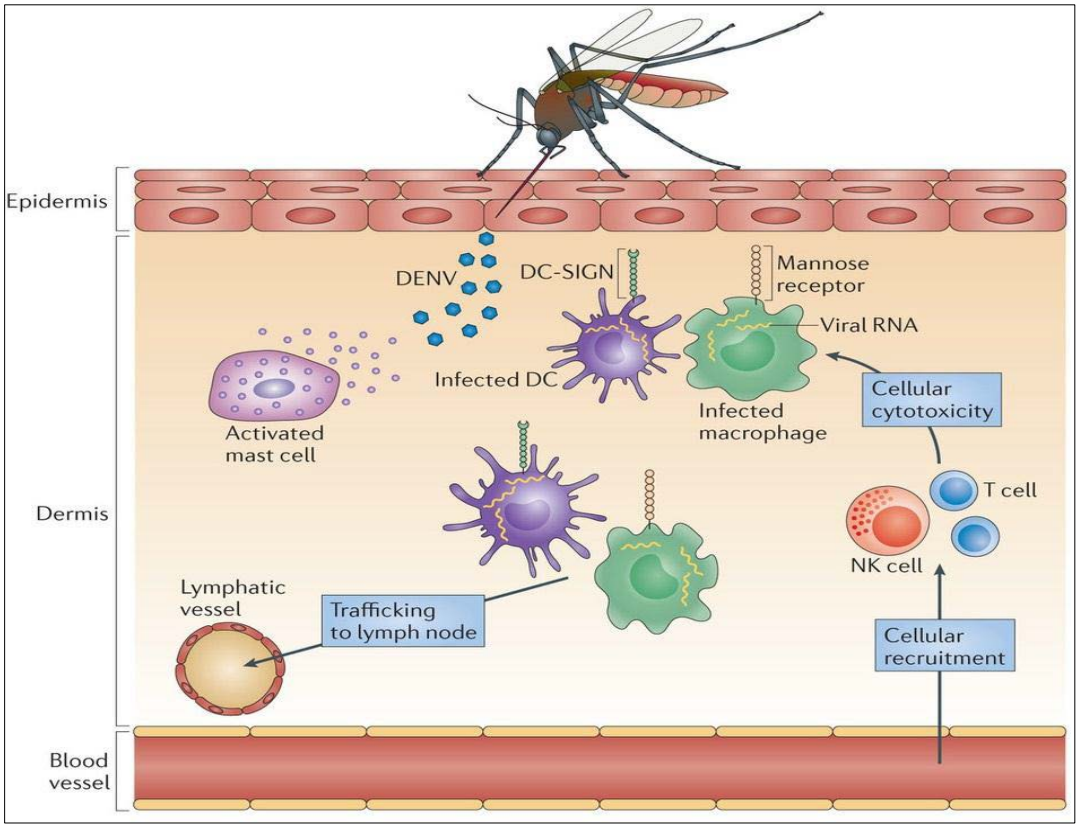

Figure 2.5 An overview DENV infection occurring after subcutaneous injection of the virus into the skin by the vector (St John et al., 2013).

2.6 Clinical manifestations of DENV infection

Clinical signs of DENV infection range from asymptomatic to symptomatic cases with life-threatening diseases such as dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). It affects infants, young children, and adults in a way that inflicts an illness like flu, but fortunately, it is seldom leading to death. In young children, dengue infections in most cases are mild and distinct from other common causes of febrile diseases where UF and DF are predominant cases. According to the severity, DHF is graded into four severity grades, with grades III and IV identified as DSS (Kalayanarooj, 2011; WHO, 2019).

DF is a disease that resembles flu, and it shows various signs and symptoms. It should be the first to expect when a person is down with a high fever (40 $^{\circ}$C) that

happens concurrently and demonstrates any of the following signs such as feeling nauseous, having a severe headache, feeling muscular and joint pain (Kurosu et al., 2020; Rodenhuis-Zybert et al., 2010). Such symptoms typically occur 3 to 14 days incubation period after the virus is infected. The effect of DHF, on the other hand, is even worse. It is fatal, caused by high fever, and may lead to dengue hemorrhagic manifestations, which leads to DSS. Health warning signs, such as intense abdominal pain, rapid breathing, bleeding gums, fatigue, frequent vomiting, and/or bloody vomiting, usually appear three to seven days after first symptoms. Shock patients can die within 8 to 24 hours but typically recover after anti-shock therapy (Gubler, 1998; WHO, 2019).

DHF is primarily seen in young children. The host factors that help increases the risk of progression to the severe disease include gender (female), the person has blood type AB, and single-nucleotide polymorphism in the tumor necrosis factor gene (Fernandez-Mestre et al., 2004; Sakuntabhai et al., 2005; Stephens et al., 2002). Initially, DHF looks like an early DENV infection and is characterized by sudden onset of high temperature for up to 2-7 days without DHF symptoms. However, a clinical symptom indicative of DHF following fever eliminates plasma leakage (Gubler, 1998). Hemorrhagic manifestations are seen through symptoms of skin hemorrhages such as purpuric lesions at the site of intravenous access, epistaxis, gastrointestinal hemorrhage, bleeding gums, and hematuria with ground-coffee looking vomit and melena (black tarry feces). The melena is related to iron oxidation from increased hemoglobin in the gastrointestinal tract. Some patients experience moderate to extreme shock from decreased blood flow and loss of blood in the absence of an early diagnosis (Gubler, 1998; Sumarmo et al., 1983). The patient’s condition begins to deteriorate in dengue shock syndrome (DSS), where he/she will start having fever and show other signs and

symptoms. Post-fever, the patient is still suffering, as his or her skin may become blotchy and congested. Their pulses may weaken, they may become restless, they may suffer from abdominal pain, and at this point, they may go into a critical stage of shock as their plasma leaks (Sumarmo et al., 1983). Other symptoms of DSS are similar to those of DHF and can result in death. The anti-shock therapy may be able to save lives if it is used right after signs of DSS are noticeable (Gubler, 1998; Gubler et al., 1995).

2.7 Diagnosis of dengue infection

Efficient and reliable dengue diagnosis is essential in the clinical field (such as early identification of extreme cases, confirmed and differential diagnosis of other infectious conditions), surveillance, disease management, pathogenesis, medical research, production of vaccines, and clinical trials. Laboratory diagnostic methods include virus identification, viral nucleic acid, antigens or antibodies, or a combination of these techniques to confirm dengue virus infection. The virus can be identified in serum, plasma, blood cells circulating, and other tissues for 4–5 days after the initiation of the disease. Viral isolation, nucleic acid, or antigen detection may be used in the early stages of the disease for diagnosis. while serology is the diagnostic tool of choice at the end of the acute infection stage (Calderón-Peláez et al., 2019; WHO, 2017)

Dengue is currently diagnosed in the laboratory by the detection of DENV-markers in patient serum. These include viral components and antibodies present in the patient's serum at different time intervals of the infection. This makes the diagnostics of acute dengue infection complicated, and often several test types or paired samples are needed for a reliable diagnosis. Furthermore, information on the timing of sampling in regard to the disease onset is needed for choosing the adequate diagnostic method. A

patient is reported to have DENV infection confirmed in the laboratory if the patient shows signs of suggestive for dengue in the presence of one of the following: DENV IgM seroconversion or high levels of DENV-reactive IgM/IgG in plasma samples, DENV viremia and DENV isolated in cell culture (Muller et al., 2017; WHO, 2009).

2.8 Managements of dengue virus infection

2.8.1 Therapeutic management of dengue virus infection

It is heartbreaking to say that we have yet to discover a specific treatment for dengue fever in this modern era. Dengue fever is typically a self-limited disease. There is no specific antiviral treatment currently available for dengue fever. Supporting treatment is usually satisfactory with analgesics, fluid replacement, and bed rest. Acetaminophen can be used for the treatment of fever and other symptoms. Nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and Aspirin should be avoided. Patients with severe dengue need physicians' and nurses' expertise, those who are very familiar and well-versed with the disease's effects and progression. Such expert knowledge and actions can decrease mortality rates drastically. It is worthy of note that patient's body fluid volume is critical for their survival (Beber et al., 2018; WHO, 2019).

Febrile phase management entails reducing high fever by means of consuming paracetamol, oral rehydration solution and promote oral feeding (Kalayanarooj, 2011). Once the disease can be detected early, patients needed to be monitored consistently. Patients with critical illness need to be cared intensively where their blood pressure, urinary output and some other medical aspects need to be very closely observed (Baker et al., 2017).

2.8.2 Dengue vaccine

Vaccination provides a safe alternative and an appropriate option to reduce the frequency of dengue without the presence of successful antiviral drugs. A safe and efficient vaccine is the most effective way of managing this infection (Wilder-Smith et al., 2019). There are several obstacles to overcome when developing an efficient and safe dengue vaccine, such as an ineffective and insufficient animal disease model for all four serotypes, the difficulty to produce a vaccine against all four antigenically distinct DENV serotypes, and lack of proper understanding of the pathogenesis of severe dengue disease (Durbin et al., 2010).

In December 2015, Sanofi Pasteur was developed and licensed for the first dengue vaccine, Dengvaxia ® (CYD-TDV). It was approved for use in an endemic area by people between the ages of 9-45 years by regulatory authorities in various countries. WHO subsequently recommended vaccination in areas with high endemic levels (70% or higher seroprevalence). Clinical studies have shown that the vaccine works and is safe in individuals who have never suffered from the dengue virus (seropositive people) but are also more vulnerable to serious dengue in contrast with those who have first naturally undergone post-vaccination dengue infection (seronegative). There were also clinical reports stating that children less than nine years old were subject to higher risks of hospitalization after they are vaccinated with CYD-TDV. Thus, this leads to its use only in countries with high rates of dengue cases, based on the reference on the epidemiological data (WHO, 2019). The third phase of the clinical trials has shown that there has been a successful reduction of dengue hospitalizations through the use of the vaccine. However, all in all, its efficacy against DENV is still low, especially against DENV-1 and DENV-2. But it gets higher for DENV-3 and DENV-4 (Capeding et al., 2014).

Failure to implement efficient vector management strategies and feel unsure of CYD-TDV's long-term protecting effectiveness against all four DENV serotypes needs dengue therapy, particularly in countries known to have poor resource conditions. At present, antiviral medications are not readily available. However, supportable fluid treatment and clinical monitoring for crucial phases of disease tend to be the only response to dengue disease (Chew et al., 2017).

2.9 Peptides development as therapeutic agents

Peptides are biologically active compounds, which incorporate a peptide bond with two or more amino acids. They are smaller than large proteins in size and contain fewer than 100 residues of amino acids. Because of their high selectivity and relatively safe properties, peptides have appealing pharmacological features. Peptides are useful for drug discovery in the creation of antiviral therapeutic drugs. Peptides with decreased toxicity, low cumulative in the tissue, and high selectivity against organ targets, all of these properties make it a perfect drug development candidate (Brunetti et al., 2016; M. Wang et al., 2018).

In the past, peptides have been limited in drug research processes because of their unstable conditions (degradation), where a minimum of 569 proteases in the human body could easily degrade them (Inamdar et al., 2021). Current technologies have made it possible to alter peptide to generate more stable versions and overcome pharmacodynamics flaws. Improvements with the automated peptide synthesis, spectroscopy, and drug design via computer simulation (*in silico*), all of these examples have allowed high-level drug screening. Moreover, the bioavailability of peptides has

been enhanced by developments in peptide technology like *D*-amino acid synthesis, cyclic peptide, chemical integration, and nanocarriers (Gentilucci et al., 2010).

Currently, there are numerous examples in the markets for effective and safe peptide medicines. Approximately, more than 140 peptide-based compounds were used as commercial drugs with more than 400 peptides are now in pre-clinical phase trial (Huther et al., 2007). The peptide drug Fuzeon^{TM} (enfuvirtide) was developed with great success, and it is the only antiviral peptide marketed which is a synthesized peptide that prevents human immunodeficiency virus (HIV) from infecting healthy cells, and it is used together with other medications to treat HIV preventing the viral fusion by binding protein type GP41 (polypeptide chain) (Chew et al., 2017). Some antimicrobial peptide candidates were evaluated in preclinical and clinical trials, such as MU1140, Arenicin, IMX924, Novexatin and Lytixar (Fjell et al., 2012; Fox, 2013). In a Phase II trial, also a clinical study is being performed in Myrcludex B, which is antihepatitis peptide targeting liver cell sodium taurocholate co-transportation polypeptide (Bogomolov et al., 2016) .

2.10 Mode of action of peptides as antiviral agents

Viruses are one of the major causes of infectious diseases, primarily because it is typically challenging and time-consuming to diagnose and produce new vaccines. Despite this cause, antiviral medication is the most commonly used and alternative effective option for vial control. Overall, the most popular modes of action for antivirals drugs are antivirals targeting the virus itself (virus-targeting antivirals) and host-targeting antivirals (Boas et al., 2019a; Lou et al., 2014).

Virus-targeting antivirals rely on replication enzymes inhibition, such as proteases and polymerases (Kiser et al., 2013; McDonald et al., 1997), or the direct

inactivation of viral structural proteins (Yu et al., 2013). In comparison, antivirals targeting the host are focus on the inhibition of an essential cellular factors which are shielded by some of the viruses during the replication cycle (Lou et al., 2014)

Antiviral peptides are often called virucidal when they act specifically by inhibiting the viral particle or competing for the site of protein link on the host cell membrane, interfering in their interaction and consequent adsorption (Galdiero et al., 2013) . Although they may also be active in other phases of the viral cycle, for instance, inducing the inhibition the viral gene (Zapata et al., 2016).

Figure 2.6 shows the mechanism of action of antiviral peptide inhibition sites on viral replication cycle. The mechanism of action starts with peptides which are called virucidal when they act directly by inhibiting the viral particle; or by competing for the protein link site in the host cell membrane, interfering in their interaction and consequent adsorption. However, they may also act in other stages of the viral cycle, causing, for example, the suppression of viral gene expression (Boas et al., 2019b).

Antiviral peptide inhibition sites on viral replication cycle are demonstrated in Figure 2.6 where the antiviral peptides with a described mechanism of action were placed in their inhibition sites as follows: 1, virion inhibition; 2, adsorption; 3, viral penetration; 4, endosomal escape; 5, viral uncoating; 6, viral genome replication and 7, release of mature virions.

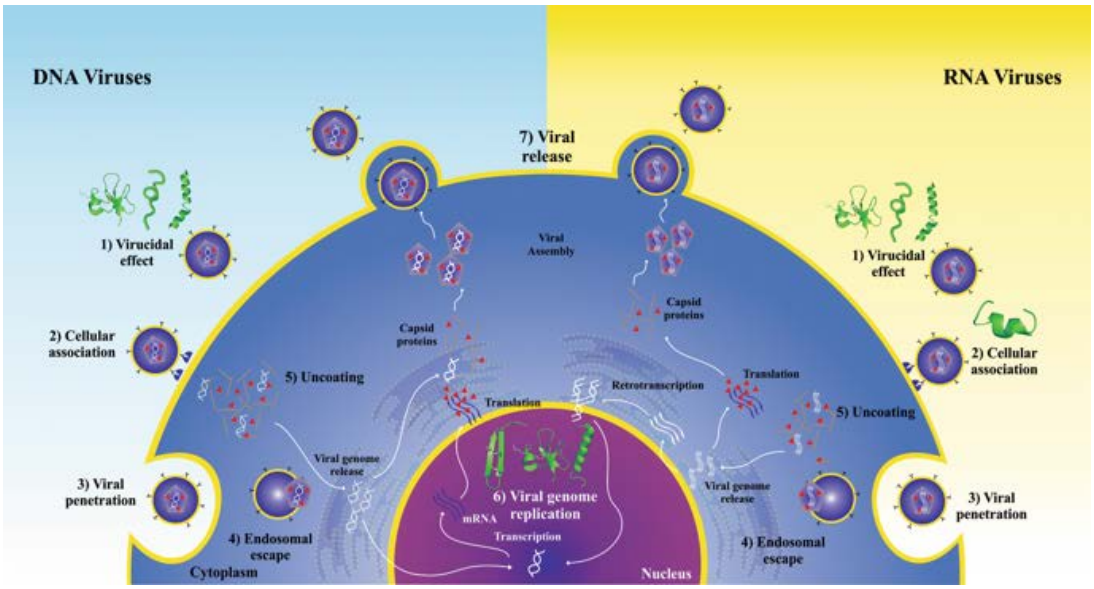

Figure 2.6 Antiviral peptide inhibition sites on viral replication cycle (Boas et al., 2019b).

2.11 Mode of action of peptides as antiviral agents for dengue infection

Antiviral peptides that come into contact with virus particles or aim critical life-cycle replication steps may be used as therapy or for dengue prophylaxis (Rothan et al., 2019). Several mechanisms have been investigated for the inhibition of dengue virus infection, either targeting the attachment factors for host cell receptors, the structural proteins and non-structural (NS) protein of the virus; hence the drugs design to act against dengue virus should address one of these three significant targets. The binding of the protein of the virus to the cell of the host is prevented by targeting cell receptors or attachment factors, thus blocking subsequent DENV inputs (Chew et al., 2017). In comparison, pharmaceutical candidates ultimately led to the structural proteins of virus capsid (C), pre-membrane (prM/M) and envelope (E), which could compromise the linking with the host cells, thus inhibiting viral fusion/attachments and a viral input. Finally, the design of NS-proteins drug candidates will manipulate cycles of viral replication effectively because non-structural proteins are the most critical components of the mechanism for replication (Chew et al., 2017).

2.12 Peptide targeting non-structural proteins (viral protease)

The inhibitor goals of viral proteases have been shown to be excellent, for example proteases inhibition was a successful strategic approach to human immunodeficiency virus (HIV) treatment (De Clercq, 2009). The proteins NS5, NS3, and NS2B (co-factor) play a crucial role in enzyme activity for DENV infection and therefore can be used as potential antiviral targets (Egloff et al., 2002).

Protease classes of enzymes accelerating protein hydrolyses at an absolute rate and specificity prevent uncontrolled proteolysis of any proteins. Proteases can also be divided into serine, cysteine, aspartic and threonine proteases. In the site of serine proteases, the hydroxyl serine catalytic nucleophile helps to degrade proteins. Protease links to the substrate in a manner distinctive to the Schechter and Berger nomenclature scheme (Abramowitz et al., 1967) (Figure. 2.7).

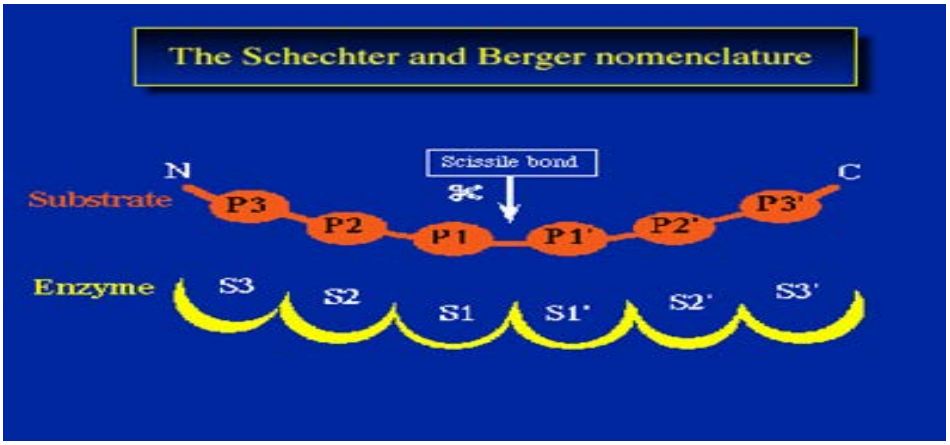

Figure 2.7 Schechter and Berger system of nomenclature (Papain et al., 1967).

In this system of nomenclature, it is considered that the amino acid residues of the polypeptide substrate bind in enzyme sub-sites of the active site. By convention, these sub-sites on the protease are called S (for sub-sites), and the substrate amino acid residues are called P (for peptide). The P1 or P1' residues are those residues located near the scissile bond. The protease sub-sites that complement the substrate binding residues