EXPRESSION OF RECOMBINANT DENV NS2B/NS3 PROTEASE: *IN SILICO* SCREENING AND *IN VITRO* VALIDATION OF NOVEL ANTIVIRAL COMPOUNDS FOR DENGUE VIRUS INHIBITION

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by

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DEDICATION

Alhamdulillah

This thesis is dedicated to

MAMA, Hjh Fatimah bt Md Shah, my backbone, my strength,
PAPA, Hj Harun bin Hashim, you're gone but your words of wisdom are never forgotten,
MAK, Hjh Saibunnisa bt Ibraheem, my late mother-in law, your spirit lives within me,
HAMEETH SHAH BIN ABDUL WAHID, my other half, and my supporter,
LUQMAN, YUSUF, HUD, AISHAH, my children, my precious

Thank you for being a great source of inspiration and motivation.
I am so blessed to have all of you.

May Allah bless all of you.
Al Fatihah, Papa and Mak.

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LIST OF SYMBOLS

ºC	Degree Celsius
g	Gram
g	Gravity
L	Liter
mL	Mililiter
μL	Microliter
M	Molar
mM	Millimolar
μg	Microgram
mg	Miligram
μm	Micrometer
nm	Nanometer
rpm	Revolution per minute
v:v	Volume/volume
w:v	Weight/volume
1X	One time more concentrated than the working solution
5X	Five time more concentrated than the working solution
10X	Ten time more concentrated than the working solution

LIST OF ABBREVIATIONS

AMC	7-Amino-4-Methylcoumarin
ANOVA	A Two-Way Analysis Of Variance
ATCC	American Type Culture Collection
BCA	Bicinchoninic Acid
BLAST	Basic Local Alignment Search Tool
BPTI	Bovine Pancreatic Trypsin Inhibitor
BSA	Bovine Serum Albumin
CADD	Computer-Aided Drug Design
CC_{50}	50% Cytotoxic Concentration
CMC	Carboxymethyl Cellulose
CPE	Cytopathic Effect
DAA	Direct-Acting Antivirals
DENV	Dengue Virus
DENV	Dengue Virus
DMEM	Dulbecco’s Minimum Essential Medium
EC_{50}	50% Effective Concentration
ELISA	Enzyme-Linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum
FT	Flow Through
HRP	Horseradish Peroxidase
HTS	High Throughput Screening
IF	Immunofluorescence
IPTG	Isopropyl β- d-1-thiogalactopyranoside

LB	Luria-Bertani
LBDD	Ligand-Based Drug Design
MCA	Methylcoumaryl-7-Amide
MCS	Multiple Cloning Site
MD	Molecular Dynamic
MKA	Makmal Kesihatan Awam
MREC	Medical Research and Ethics Committee
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
Ni2+-NTA	Nickel Nitrilotriacetic Acid
NMRR	National Medical Research Register
NS	Non-Structural
ORF	Open Reading Frame
p-NA	Para-Nitroanilides
PBS	Phosphate Buffer Saline
PDB	Protein Data Bank
PFU	Plaque-Forming Unit
PR	Plaque Reduction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SBDD	Structure-Based Drug Design
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylmide Gel Electrophoresis
SEM	Standard Error Of The Mean
SI	Selectivity Index
TBS	Tris-Buffered Saline
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline Tween
TEMED	Tetramethylethylenediamine

VP-SFM	Virus Production Serum-Free Medium
WHO	World Health Organization
WR	Working Reagent

LIST OF APPENDICES

EKSPRESI PROTEASE REKOMBINAN DENV NS2B/NS3: SARINGAN IN SILICO DAN PENGESAHAN IN VITRO SEBATIAN ANTIVIRAL NOVEL UNTUK PERENCATAN VIRUS DENGGI

ABSTRAK

Di peringkat global, peningkatan tanpa henti dalam kes denggi di seluruh dunia telah menjadi semakin serius dan secara tidak sengaja diabaikan dalam latar belakang wabak COVID-19 yang berterusan. Walaupun kesan global jangkitan virus denggi (DENV) yang semakin meningkat, masih tiada rawatan yang berkesan. Oleh itu, penerokaan dan kemajuan rawatan antiviral denggi kekal sebagai fokus utama penyelidikan. Sasaran menarik untuk rawatan anti-DENV ialah protease serin seperti trypsin (NS3pro) dan kofaktornya (NS2B) yang dikodkan oleh virus. Kompleks protease DENV NS2B/NS3 tersebut bertanggungjawab meleraikan poliprotein virus kepada protein-protein virus yang mempunyai fungsi yang berbeza, menjadikannya penting untuk replikasi virus. Dalam 15 tahun yang lalu, penghasilan DENV NS2B/NS3 pro aktif kebanyakannya bergantung pada binaan yang menghubungkan terminal-C domain kofaktor hidrofilik NS2B kepada terminal-N NS3 pro menggunakan penghubung glisin yang fleksibel. Walaupun analisis menyeluruh telah dijalankan, tiada ubat perencatan yang berpotensi ditemui setakat ini. Tambahan pula, kesan penghubung sintetik yang diperkenalkan antara protease dan kofaktornya masih tidak pasti. Baru-baru ini, strategi alternatif untuk menghasilkan kompleks pro DENV NS2B/NS3 aktif secara katalitik yang tidak berhubung secara kovalen telah dilaporkan oleh Profesor Paul Young dari Universiti Queensland, Australia, dan pasukannya. Dengan menggunakan konstruk plasmid bukan kovalen, kajian ini berjaya

mengekspreskan dan menulenkan protein rekombinan keterlarutan tinggi DENV NS2B/NS3 pro, di bawah keadaan yang optimum dengan suplementasi betaine. Dalam pra-penyaringan dalam analisis *in-silico*, kompoun II, III, IV dan V memenuhi kriteria peraturan *Lipinski's rule of five*, menunjukkan sifat seperti ubat yang menggalakkan, dengan kompoun II menonjol secara positif berbanding kompoun hit lain dalam analisis ADMET. Untuk meramalkan aktiviti perencatan pengikatan lima calon perencat denggi (kompoun I hingga V), kaedah pengkomputeran docking telah diguna pakai. Dalam analisis ini, kompoun yang disaring daripada perpustakaan kimia RIKEN NPDepo, terutamanya untuk kompoun II, III, dan V, menghasilkan aktiviti yang memberangsangkan dengan pertalian mengikat rendah (masing-masing -7.18, -7.13, dan -6.93 kcal/mol) dan interaksi molekular dengan dua residu penting triad katalitik, His51 dan Ser135. Kompoun-kompoun hit ini, berserta dengan quercetin, menjana tenaga bebas yang setanding, menonjolkan potensi mereka sebagai perencat. Menggunakan protein DENV NS2B/NS3 pro yang telah dioptimumkan, ujian perencatan protease telah dijalankan, menghasilkan peratusan kepekatan perencatan yang ketara bagi kompoun II berbanding quercetin. Selain itu, kompoun ini juga menunjukkan kesan toksik yang rendah terhadap sel-sel mamalia, dengan nilai CC_{50} tertinggi iaitu 816.4 ± 0.03 μg/mL. Melalui ujian pembentukan plak, dos berjangkit virus denggi telah ditentukan dengan mengukur kepekatan virus. Tambahan lagi, hasil ujian pengurangan virus yang digunakan menunjukkan bahawa kompoun II menghasilkan perencatan yang kuat pada kepekatan rendah (EC_{50} 18.7 μg/mL) dan indeks selektiviti tinggi (nilai SI 43.6), dengan pengurangan ketoksikan pada kepekatan tertentu, justeru meningkatkan penilaian keselamatan dan potensi penggunaannya. Penemuan dalam kajian ini mencadangkan bahawa kompoun II

mempunyai potensi aktiviti antivirus terhadap DENV dan perlu diambil kira untuk penilaian *in vivo* dalam lanjutan kajian pembangunan antivirus denggi.

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ABSTRACT

Globally, the relentless rise in dengue cases worldwide has been further intensified and inadvertently neglected in the backdrop of the persistent COVID-19 pandemic. Despite the escalating global impact of dengue virus (DENV) infection, there remains a conspicuous absence of its effective treatment. Hence, the exploration and advancement of dengue antiviral treatments remain a primary focus of research. An appealing target for the anti-DENV drugs is the virally encoded trypsin-like serine protease (NS3pro) and its related cofactor (NS2B). The DENV NS2B/NS3 protease complex is responsible for cleaving the viral polyprotein into distinct functional viral proteins, making it crucial for virus replication. In the past 15 years, the production of an active DENV NS2B/NS3 pro has mostly relied on constructs that link the C-terminus of the hydrophilic cofactor domain of NS2B to the N-terminus of NS3 pro using a flexible glycine linker. Despite thorough analysis, no potential inhibitory drugs have been discovered thus far. Furthermore, the impact of the synthetic linker introduced between the protease and its cofactor remains uncertain. Recently, an alternative strategy for producing a catalytically active DENV NS2B/NS3 pro complex that is not covalently connected has been reported by Professor Paul Young from the University of Queensland, Australia, and his team. By employing the non-covalent plasmid construct, this study successfully expressed and purified a high-solubility protein of recombinant DENV NS2B/NS3 pro under optimal conditions, with betaine supplementation. In the pre-screening *in-silico* analysis, compounds II, III, IV, and V

met Lipinski's rule of five criteria, indicating favourable drug-like properties, with compound II standing out positively compared to other hit compounds in the ADMET analysis. To predict the binding inhibition activity of five dengue inhibitor candidates (compounds I to V), computational docking methods were adopted. In this analysis, the screened compounds from the RIKEN NPDepo chemical library, especially for compounds II, III, and V, resulted in promising activity with low binding affinity (-7.18, -7.13, and -6.93 kcal/mol, respectively) and molecular interactions with two crucial residues of the catalytic triad, His51 and Ser135. These hit compounds, along with quercetin, generated comparable free energies, highlighting their potential as inhibitors. Using the optimised DENV NS2B/NS3 pro protein, a protease inhibition assay was performed, resulting in a significant percentage inhibition concentration of compound II compared to the quercetin. Furthermore, this compound also exhibited low toxicity towards mammalian cells, with the highest CC_{50} value of 816.4 ± 0.03 µg/mL. Through plaque-forming assay, the dengue virus infectivity has been determined by measuring the virus concentration. Additionally, the outcome of the employed virus reduction assay demonstrated that compound II showed potent inhibition at low concentrations (EC_{50} 18.7 µg/mL) and a high selectivity index (SI value of 43.6), with reduced toxicity observed at specific concentrations, hence enhancing its safety assessment and potential utilisation. The findings in this study suggest that compound II exerts potent antiviral activity against DENV and should be taken into consideration for *in vivo* assessments in the dengue antiviral development pipeline.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Chapter 1 highlights the key issues of the dengue virus (DENV) and the infection that have attracted significant attention in this field of research. It provides a concise overview of the research background, states the research problem, clarifies the rationale of the study, points out the hypothesis and research question, formulates the research objectives to be addressed, identifies the research scope and highlights the study flowchart.

1.2 Research Background

Dengue infection, a mosquito-borne viral disease transmitted by female mosquitoes, primarily *Aedes aegypti* and, to a lesser extent, *A. albopictus* and *A. polynesiensis* (Leandro *et al*., 2022), is prevalent in tropical and sub-tropical regions globally, particularly in urban and semi-urban localities. The disease is caused by any of the DENV serotypes, which can result in a range of symptoms, varying from very mild (unnoticeable) to severe that may necessitate hospitalisation and medical interventions (Sahu *et al*., 2023). Moreover, in severe cases, fatalities can occur. Currently, other than supportive care and appropriate fluid therapy, there is no specific treatment for dengue.

Dengue, which has a captivating etymological history, is the most rapidly expanding viral disease transmitted by mosquitoes worldwide. The term "dengue" originates from the Swahili phrase "ka dinga pepo," which refers to an abrupt spasm resembling cramping ascribed to a malevolent spirit or plague. Its clinical description dates back to Benjamin Rush in 1780 and further to the Queen of Spain in 1801 (Saeed & Asif, 2020). These historical accounts depict an acute febrile illness marked by bone and joint pains, haemorrhage, jaundice, and distinctive patient behaviour. The etiologic of the virus was proposed in 1907, with the identification of DENV1 and DENV2 following World War II, and later, the recognition of additional serotypes when dengue haemorrhagic fever emerged in the Philippines and Thailand in 1954 (Morens, 2023). Factors contributing to the persistence of dengue include increased human population, urbanisation, and international migration (Rigau-Pérez, 1998). The DENV belongs to the *Flaviviridae* family, specifically within the *Flavivirus* genus, characterised by shared antigenic and genetic features. Other mosquito-borne viruses including yellow fever virus, Japanese encephalitis virus, West Nile virus, and tick-borne encephalitis virus are also found in this genus (Peinado *et al*., 2022). These viruses share commonalities in their

morphology, genome organisation, and replication processes. Additionally, within the *Flaviviridae* family, there are other recognised genera, *Pestivirus* and *Hepacivirus*, each with its own distinct properties, despite similarities in family-wide characteristics (Chase, 2022). This categorisation helps define the relationships and characteristics of various viruses in this taxonomic family.

Dengue has been recognised by the World Health Organisation (WHO) as a substantial worldwide threat, as evidenced by ongoing outbreaks in many nations. Approximately 390 million cases of DENV infections occur annually, with 96 million manifesting clinical symptoms (Sai *et al*., 2020). A recent study reveals that a staggering 3.9 billion individuals are susceptible to contracting dengue, with a significant concentration of cases in Asia(Tian *et al*., 2022). In addition, the European Centre for Disease Prevention and Control (ECDC) recently published an analysis stating that as of October 2, 2023, there have been a total of 4.2 million documented illnesses and 3,000 deaths related to dengue across 79 nations and territories globally **(Figure 1.1**). This growth is attributed to factors such as population increase, urbanisation, and international migration. In the context of the COVID-19 pandemic, the pressure on healthcare systems underscores the need to sustain efforts to combat vector-borne diseases like dengue. The dual effects of COVID-19 and dengue have resulted in catastrophic outcomes, emphasizing the urgency for coordinated efforts in disease prevention, control, and the development of antiviral agents and vaccines (Wilder-Smith *et al*., 2020).

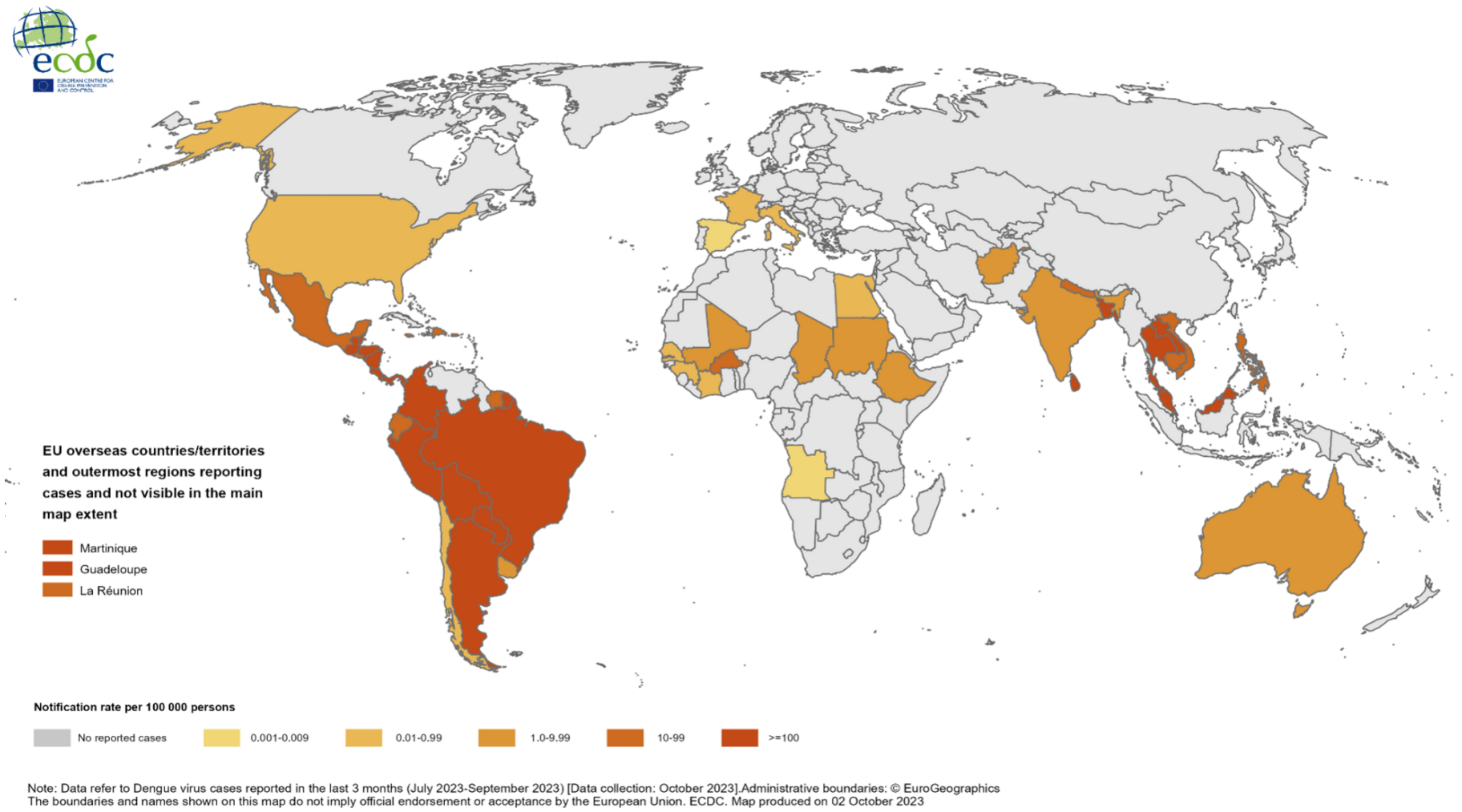

Figure 1.1 Three-month DENV disease case notification rate per 100, 000 population, August – October 2023. (Source: https://www.ecdc.europa.eu/en/dengue-monthly).

From a local perspective, Malaysia, a tropical Southeast Asian country with a population of around 32 million, has experienced a significant dengue outbreak. A counterfactual analysis revealed that dengue hyperendemicity significantly increases the severity of previous outbreaks (Lim, *et al*., 2021). In 1902, Malaysia experienced its first case of dengue and in 1973, the country faced its first major outbreak resulting in a surge of dengue cases. This has led Malaysia to be among the countries with the highest rates of DENV infection reported worldwide (Suppiah *et al*., 2018).Subsequently, there has been an upward trend in dengue occurrences in Malaysia, characterised by significant increases in both reported cases and fatality rates in recent years. The implementation of lockdown measures due to COVID-19 initially led to a reduction in dengue cases but was followed by a resurgence (Brady & Wilder-Smith, 2021). The intricate interaction of immunity specific to different dengue serotypes contributes to the challenge of predicting DENV outbreaks. Factors such as urbanisation, population movement, and poor environmental management have contributed to the increased number of dengue cases in Malaysia. Control strategies, including environmental cleanliness, vector control, and changes in human behaviour, are crucial for reducing dengue cases (Mahmud *et al*., 2023). Since 2011, the Ministry of Health, Malaysia has established an integrated management plan for the dengue preventive and control programme. This approach aims to control the various factors that contribute to the transmission of the disease. (Singh, 2019). However, it is crucial to prioritise the development of effective antiviral drugs and vaccines as it imperative to control the disease burden.

To develop effective interventions and treatments, it is essential to understand the transmission, life cycle, pathogenesis, clinical phases, and the current diagnosis and prevention of DENV infection. DENV is small, enveloped, and spherical and has a genome consisting of a positive sense single-stranded RNA that is approximately 11 kb in length (Akhtar, 2019). Its transmission primarily relies on *Aedes* mosquitoes, particularly *Aedes aegypti*. This mosquito

is well-suited for urban environments and sustains the DENV life cycle between humans and vectors. *Aedes albopictus* and *Aedes polynesiensis* can also act as vectors in certain locations. The virus is transmitted when infected female mosquitoes feed on human blood. Upon entering the mosquito, the virus undergoes replication in the midgut, haemolymphs, and salivary glands before being transmitted to the next host. Once the virus is introduced into humans through mosquito saliva during a bite, it replicates locally in a number of target organs, including the liver and nearby lymph nodes (Perveen *et al*., 2023). This entire life cycle, which is typical for flaviviruses, encompasses the attachment of virions to host cell surfaces, endocytosis, release of RNA into the cytoplasm, polyprotein translation, cleavage of polyprotein into both structural and non-structural viral proteins which lead to the replication of RNA, virus assembly, and, the release of new mature virions from the host cell via exocytosis, as depicted in **Figure 1.2**.

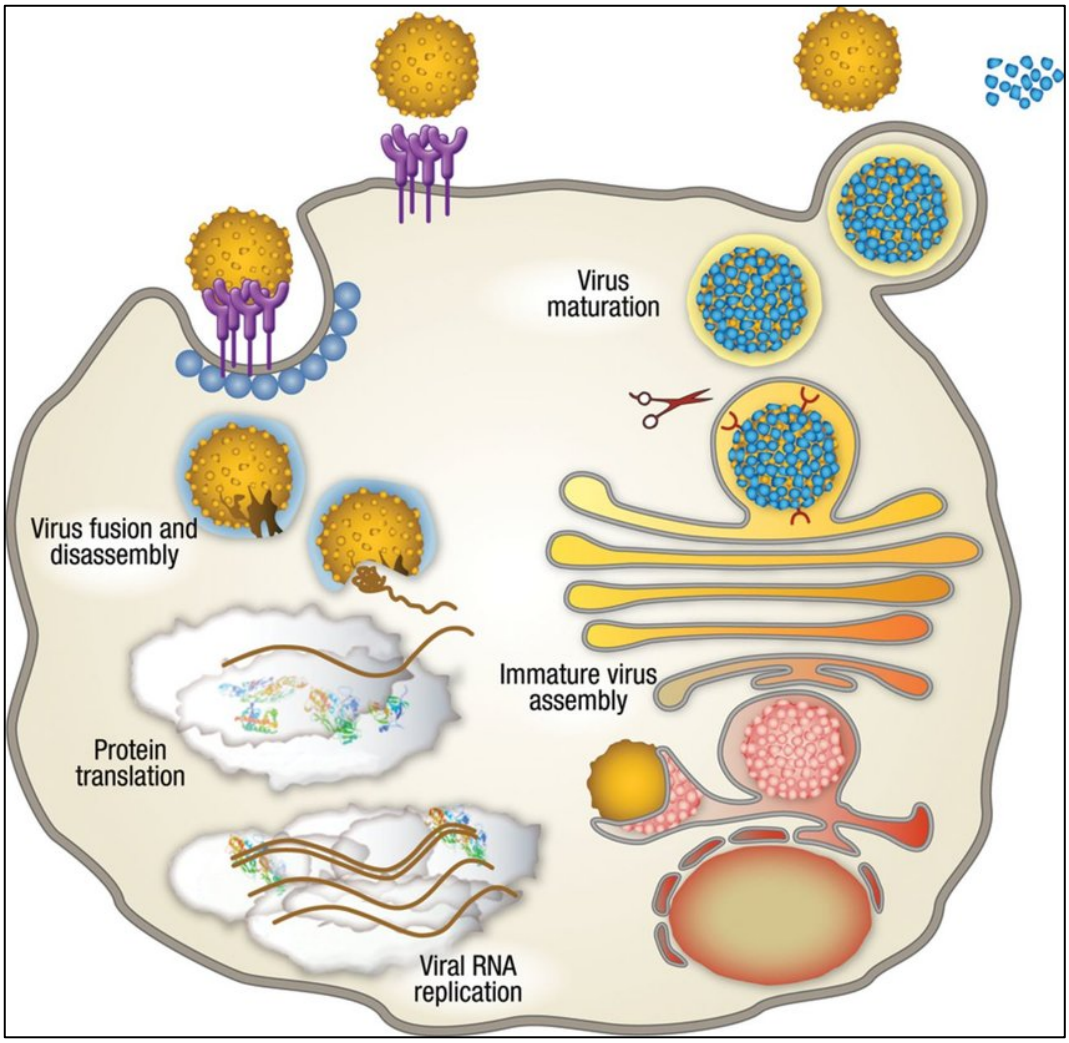

Figure 1.2 The viral life cycle of DENV (Source: Adapted from Laughlin *et al*., 2012)

DENV infection follows a specific pathogenesis within the human host. After being transmitted through a mosquito bite, the virus initially localises and replicates in the cells, specifically keratinocytes and Langerhans cells, prior to disseminating to lymph nodes, tissues, and organs. The virus exhibits tropism for various human cells and tissues, including retinal cells, endothelium (blood vessels), liver, and immune system (Bhatt *et al*., 2021). The onset of the second viremia phase is indicated by its presence in the bloodstream. During this phase, clinical symptoms can vary from a mild flu-like syndrome (dengue fever [DF]) to more severe manifestations such as coagulopathy, vascular fragility, and permeability (dengue haemorrhagic fever [DHF]). Occasionally, these severe symptoms might advance to a state of

hypovolemic shock (dengue shock syndrome [DSS]). These conditions are associated with an increase in vascular permeability due to endothelial dysfunction in the critical phase (Teo *et al*., 2021). Understanding the distinct clinical phases of dengue, from febrile to critical, is crucial for dengue antiviral development as it allows for the identification of specific points in the disease progression where interventions can effectively reduce viral replication and mitigate the severity of illness.

In addition, in the pursuit of antiviral development for dengue, the importance of accurate diagnosis and effective prevention cannot be overstated. These essential components not only enhance patient care but also collectively contribute to reducing the disease burden. Current diagnostic methods include molecular, biochemical, and serological tests, such as virus isolation, detection of nucleic acid, and antigen identification (e.g., DENV NS1 protein) which can distinguish between primary and secondary infections (Kabir *et al*., 2021; Parkash & Shueb, 2015). Virus isolation, while highly specific, is costly and time-consuming, leading to the adoption of more practical methods, like viral RNA detection or serological assays. However, dengue diagnosis can be challenging in tropical and sub-tropical regions with limited healthcare infrastructure, where clinical history often substitutes for laboratory confirmation (Silva *et al*., 2020). Inexpensive and specific diagnostic tools are essential, as serological tests may produce misleading results due to cross-reactivity with other flaviviruses. These challenges emphasise the need for rapid, accurate, and cost-effective diagnostics. Furthermore, dengue prevention at present, revolves around *Aedes* mosquito control through environmental, chemical, and biological approaches, with innovations like *Wolbachia*-infected mosquitoes showing promise (Chrostek *et al*., 2020). Another significant dengue vaccine development was the initial implementation of the inaugural authorised vaccine, CYD-TDV, also known as Dengvaxia®. Nevertheless, ongoing trials have uncovered several drawbacks and limitations of this vaccine, leading to the observation that it increases the probability of severe dengue

illness in certain individuals who receive the vaccination (Salje *et al*., 2021). Furthermore, while vaccine development is essential, it also faces challenges like antibody-dependent enhancement and limited animal models. As a result, researchers are putting a greater emphasis on the development of potent inhibitors that can halt the spread of infection. Hence, it is imperative to explore drugs that can specifically target viral components or essential host processes, which can serve as prophylaxis or treatment for the disease.

1.3 Problem Statement

In the pursuit of mitigating the global impact of dengue infection, various initiatives are actively underway. This includes the continuous development of dengue vaccines, a process marked by both progress and persistent challenges. To date, several vaccines are in different stages of clinical trials, aiming to provide broad protection against all DENV serotypes (Torres-Flores *et al*., 2022). However, concerns about vaccine safety, particularly regarding antibody-dependent enhancement, need careful consideration. Additionally, discussions centre around ensuring global accessibility to any developed vaccine, especially in regions with high dengue prevalence (Wong *et al*., 2022). Another issue that has become the main concern is the implications of severe dengue infection and its potential to lead to fatal outcomes, emphasising the critical need to comprehend and address this facet of the disease. Severe cases can rapidly escalate, posing a significant threat to life. Factors contributing to the severity include the complex interplay between the virus and the immune system, alongside underlying health conditions (Rathore *et al*., 2020). Recognizing the impact of dengue infection underscores the urgency for the development and implementation of effective dengue antivirals which can halt virus progression, hence playing a pivotal role in mitigating the severity of the disease and preventing potentially fatal outcomes.

In the existing studies, the investigation of dengue antivirals has primarily revolved around the expression of active NS2B/NS3 protease through linked constructs. However, the impact of the synthetic linker that was added to the protease-cofactor complex remains uncertain and it is notable that the current research does not yet indicate promising outcomes. This occurs either due to the scaffold being too labile (Shiryaev *et al*., 2007) or due to the weak binding of the inhibitors to the enzyme (Noble *et al*., 2010). Hence, for the purpose of expressing the catalytically active, non-covalently linked NS2B/NS3pro complex in bacteria, Shannon *et al*., (2016) developed two alternative (bivalent and polycistronic) methods. The non-covalent relationship between NS2B and NS3pro is expected to be more significant for exploring anti-DENV inhibitors that specifically focus on disrupting the connections between cofactors and proteases (Mushtaq *et al*., 2021), rather than targeting the active site of the protease. In addition, these technologies provide alternate methods for efficiently expressing additional protein assemblies with great productivity.

Despite extensive investigations into drug candidates, the drug development process faces a remarkably high attrition rate, with over 90% of candidates entering clinical trials ultimately being disapproved. Surveys indicate that a significant portion of these failures, particularly in Phases II and III, result from efficacy issues and safety concerns (Sun *et al*., 2022). In the present landscape of dengue research, the predominant approach centres on developing DENV inhibitors by utilising hits from biochemical assays and relying solely on *in-silico* exercises. (Lim, 2019). Focusing solely on one computational method in drug development can lead to biased perspectives and overlook critical issues in other domains, risking false positives or negatives and potentially advancing suboptimal candidates with undiscovered drawbacks (Raju *et al*., 2022). Additionally, the lack of experimental validation and the inherent uncertainty in predictive accuracy further underscore the limitations of depending solely on computational models (Viceconti *et al*., 2021). To mitigate these risks and

ensure a more comprehensive understanding, Agamah *et al*., 2020 and Jabalia *et al*., 2021 that an integrated approach that combines multiple *in-silico* exercises and incorporates experimental validation is preferred in the drug discovery process. By leveraging the strengths of each method, the strategy allows for early identification of drug-like compounds, optimisation of pharmacokinetics, and minimisation of late-stage failure risks. Moreover, the approach reduces experimental costs and time by facilitating the virtual screening of compound libraries. Overall, the integration of these *in-silico* techniques enhances decision-making by providing a holistic assessment of drug properties, supporting more informed and successful drug development endeavours.

1.4 Rationale of Study

Dengue fever, resulting from the DENV, remains a significant worldwide health issue, impacting millions of individuals annually. Despite its broad prevalence and high disease burden, no antiviral drugs are currently authorised for the treatment of dengue. Due to the scarcity of vaccines, such as Dengvaxia, which is approved for use only in seropositive individuals in some regions, a critical gap in the medical response to this disease remains (Dutta & Langenburg, 2023). Current approaches to dengue management primarily involve supportive care. Therefore, the development of effective antiviral therapies is imperative.

One of the key factors driving the urgency of this research is the significant link observed between the severity of dengue cases and the viremia level (Morsy *et al*., 2020). Severe manifestations of the disease, such as DHF and DSS, are often related with high viremia levels. Consequently, strategies aimed at reducing viremia through antiviral therapy have the potential to significantly alleviate the intensity of the illness.

Moreover, an ideal dengue antiviral should be capable of targeting all four serotypes of the DENV, acting rapidly, reducing viral loads significantly, and leveraging structural

information on dengue proteins for drug discovery (Anasir *et al*., 2020). The recent expansion of research into small-molecule inhibitors targeting dengue proteins, including the NS2B/NS3 protease complex, has generated considerable interest. This protease is essential for the maturation, replication, and viability of the DENV, making it an appealing candidate for drug development. The success of antiviral therapies against other viral diseases, such as HIV, HCV, HBV, and influenza, further underscores the potential impact of developing effective dengue antiviral agents.

In light of these factors, this study aims to contribute to the ongoing efforts to create antiviral therapies for dengue, addressing a critical unmet medical need. Through the exploration of diverse strategies, including the adaptation of an alternative strategy by utilizing an optimised and highly soluble unlinked protein construct, accurately predicting the physicochemical properties, drug-likeness of each hit compound and significant molecular interactions between the hit compounds and the protein through *in-silico* evaluation, confirming these interactions through *in vitro* assessment of inhibition activities and cytotoxic effects, and conducting comprehensive evaluations using plaque assays to examine their safety and effectiveness on cells. This work is not only of scientific interest but also holds substantial public health importance, particularly in regions where dengue is endemic and continues to pose a significant threat to human health.

1.5 Hypothesis of Study

We hypothesised that the exploration of diverse strategies, including the implementation of an optimised and highly soluble unlinked protein construct, along with the identification of potential compounds and their molecular interactions through *in- silico, in vitro,* and cell-based approaches, will result in the discovery of novel antiviral agents for dengue. These agents will demonstrate significant inhibitory effects on viral replication while maintaining a high degree

of safety and efficacy, thus addressing the critical unmet medical need for effective dengue antiviral therapies.

1.6 Objectives of Study

The objectives of this study were as follows:

Main Objective

To investigate the hits chemical compounds' ability to inhibit DENV NS2B/NS3 protease activity of the dengue virus.

Specific Objective 1

To optimise and analyse the unlinked construct of the recombinant DENV NS2B/NS3 protease.

Specific Objective 2

To identify the molecular interactions of the potential lead compounds through *in-silico* approaches.

Specific Objective 3

To evaluate the inhibition activities of the inhibitors against the optimised DENV NS2B/NS3 pro recombinant protein and its cytotoxicity effects

Specific Objective 4

To investigate the antiviral activity of various compounds against dengue virus using plaque assay as a screening tool for potential therapeutic agents.

1.7 Scope of Study

This study aims to investigate the hit compounds' ability to inhibit DENV NS2B/NS3 protease activity. To achieve this, this study focuses on optimizing an unlinked recombinant

protein construct, which is pivotal as it sets the stage for subsequent stages of hit compounds screening. Computer-based techniques are employed to understand the molecular interactions between these compounds and the target protein. Additionally, the hit compounds' ability to inhibit viral replication, coupled with an assessment of the cytotoxicity effects on mammalian cells is conducted. Furthermore, plaque assays were used as a screening tool to determine the efficacy of these compounds in impeding DENV NS2B/NS3 pro.

The study utilises a specifically designed unlinked recombinant protein construct of DENV NS2B/NS3 pro. Furthermore, the hit compounds are employed from the RIKEN Natural Products Depository (NPDepo) library. The selection of these compounds is based on natural product sources that have evidence of reduced adverse effects, low susceptibility to resistance, and strong antiviral properties. In the cell-based studies, two types of cells to be utilised are Vero cells (mammalian cells) and C6/36 cells (mosquito cells). Both cells are the common cell lines for viral isolation and replication in dengue studies.

The research is primarily conducted at the Institute for Research in Molecular Medicine (INFORMM) at Universiti Sains Malaysia, Penang and Makmal Kesihatan Awam (MKA) in Ipoh, Perak. The anticipated duration of the research is approximately thirty-six months. However, it is worth noting that the ongoing COVID-19 pandemic may introduce challenges that could impact laboratory accessibility and potentially extend the timeline.

1.8 Study Flowchart

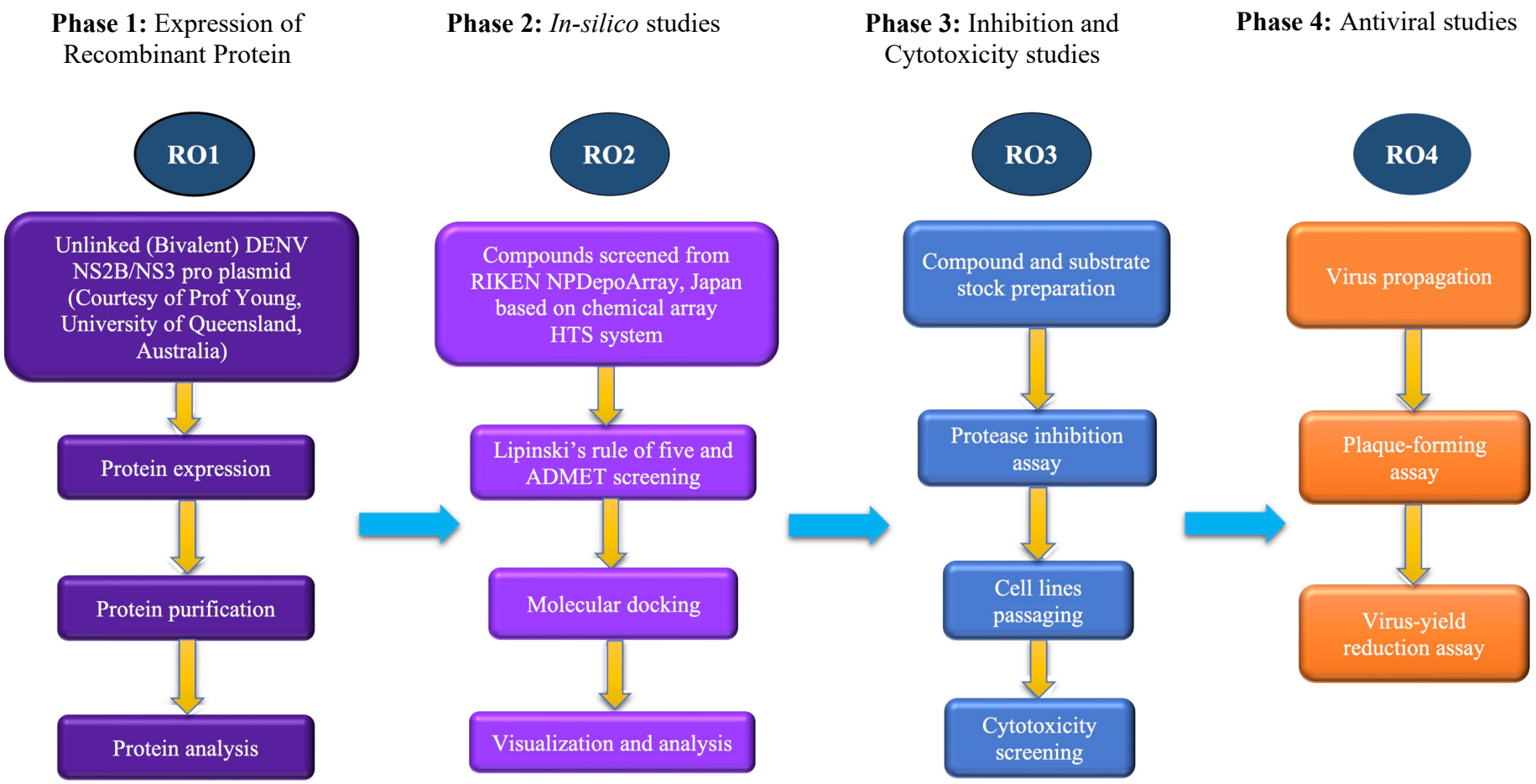

Figure 1.3 The study on exploration of dengue NS2B/NS3 protease activity using chemical biology approach flowchart.

1.9 Summary

To summarise, this chapter emphasises the critical issues surrounding the DENV and its infection, addressing key aspects that have garnered substantial attention in the field. This chapter also presents an overview of the research background, elucidates the research problem, articulates the rationale behind the study, and introduces the hypothesis and research question. Additionally, it outlines the specific research objectives to be explored, defines the scope of the study, and underscores the study's flowchart, setting the foundation for the subsequent exploration of dengue-related concerns and potential solutions.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter gives a comprehensive review of the targeted enzyme, the dengue NS2B/NS3 protease, and highlights the main experimental approaches of this study. This chapter begins by emphasizing the enzyme complex, followed by the structural biology of the pivotal enzyme. In this part, the active sites, crystal structures, and homology model of the enzyme are discussed to provide the structural basis that is relevant in identifying effective drug targets and designing therapies. Following that, this chapter provides an overview of ongoing clinical studies for dengue antiviral drugs and highlights recent pre-clinical candidates. In light of the recent efforts and the interesting target enzyme, we also highlight our experimental approaches in this study which involved *in vitro*, *in-silico*, and cell-based methods.

2.2 DENV NS2B/NS3 Protease

The DENV consists of particles with a diameter of around 50 nm and an open reading frame (ORF) with more than 10,000 bases. These particles have a central nucleocapsid, approximately 30 nm in size, and mostly consist of a single copy of DENV genomic RNA packed together with several copies of the highly basic capsid (C) protein. The viral prM and E proteins form a lipid bilayer that encloses the nucleocapsid. These immature particles enter the cellular secretory system, where prM is cleaved, leading to the formation of mature virus particles (Abduljalil & Abd Al Galil, 2022). The E protein changes structurally when the virion matures and fuses with the host membrane in the endosomal compartment. In the mature virions, the E protein forms homodimers, and these virions are internalised through receptor-mediated endocytosis. A new infection cycle is initiated when membrane fusion is triggered by a drop in pH in late endosomes, which releases the nucleocapsid into the cytoplasm (Nanaware *et al*., 2021). Upon entering the cytoplasm, the viral DNA undergoes translation and replication, resulting in polyproteins that are further cleaved into three structural and seven non-structural (NS) proteins (**Figure 2.1**). The mature infective virion integrates essential structural proteins—capsid, pre-membrane, and envelope—for viral particle replication. Simultaneously, NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) play a pivotal role in viral replication and assembly. In addition, these proteins are mostly present in all highly similar but antigenically distinct DENV serotypes, DENV1, DENV2, DENV3, and DENV4 with each serotype interacting differently with human antibodies (Chong & Khan, 2019). These serotypes, which have approximately 60%–70% sequence identity. A recently reported fifth serotype, DENV5, was discovered in 2013 in the Sarawak state of Malaysia. However, DENV5 is mostly a sylvatic strain that is now circulating among primates in Southeast Asia (Hussain *et al*., 2023). The pathogenic significance of this serotype and its potential emergence

as a novel serotype in the human transmission cycle remains undetermined, hence yet to be confirmed as one of DENV serotypes. Nevertheless, the four primary serotypes frequently circulate concurrently in tropical areas, thus leading to a growing focus on developing antiviral treatments for dengue that target the NS proteins.

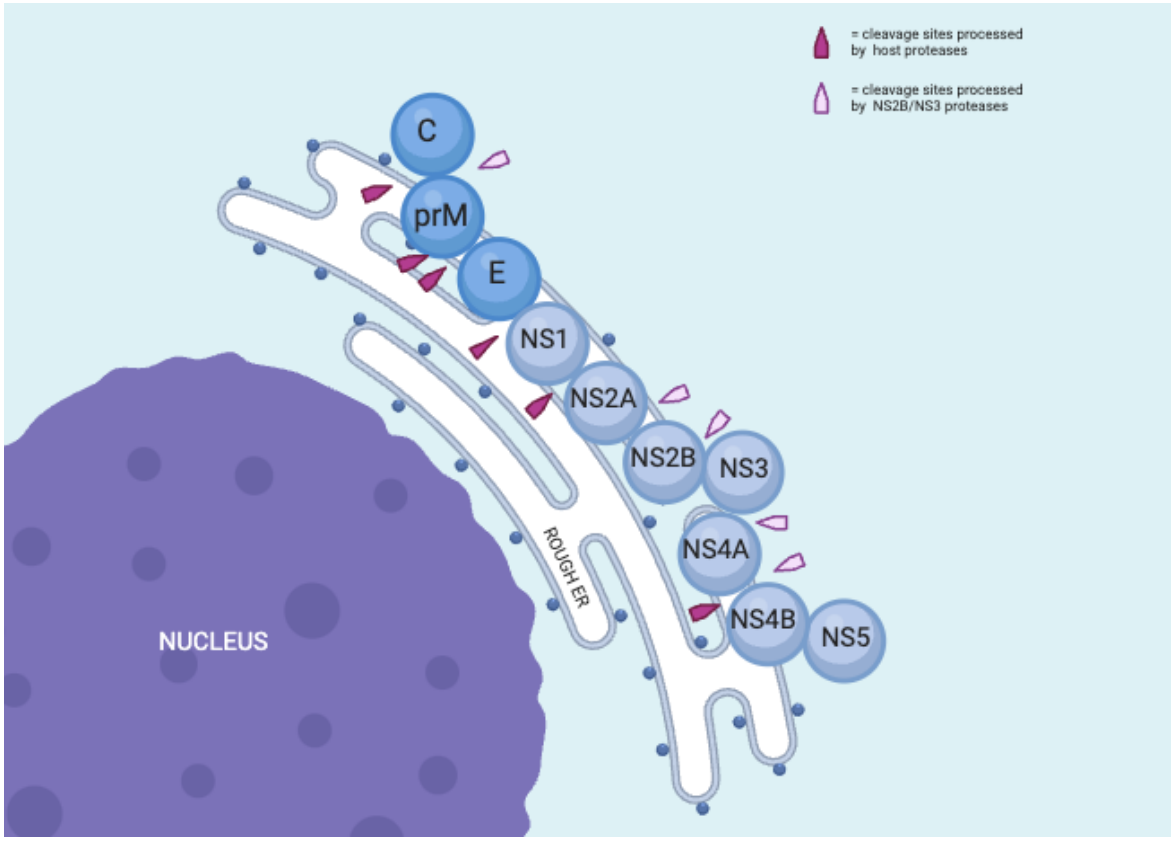

Figure 2.1 Illustration of DENV structural and NS proteins on the ER with its cleavage sites. (Modified from Uno & Ross, 2018).

2.2.1 The NS2B/NS3 pro complex

The NS3pro, or non-structural protein 3, contains the viral protease encoded in its first 180 amino acid residues at the N-terminus. This domain has a protease activity that helps with the processing of the DENV polyproteins. However, due to its insolubility and self-aggregation, NS3pro activity relies on the association with the virally encoded cofactor NS2B, which provides structural stability and solubility to NS3pro. The NS2B/NS3 pro complex is made up of N- and C-terminal hydrophobic domains that surround a core hydrophilic domain of roughly 40aa that wraps around the NS3 pro. The hydrophobic domains are required for anchoring NS3pro in close proximity to the ER membrane, where proteolytic processing occurs (Kumari *et al*., 2020). However, only the cofactor's core hydrophilic domain is essential for proteolytic action (**Figure 2.2**). Various *in vitro* expression and deletion studies of recombinant NS2B/NS3 pro complexes have shown this hydrophilic domain to be sufficient for the expression of a soluble and active protease (Obi *et al*., 2021).

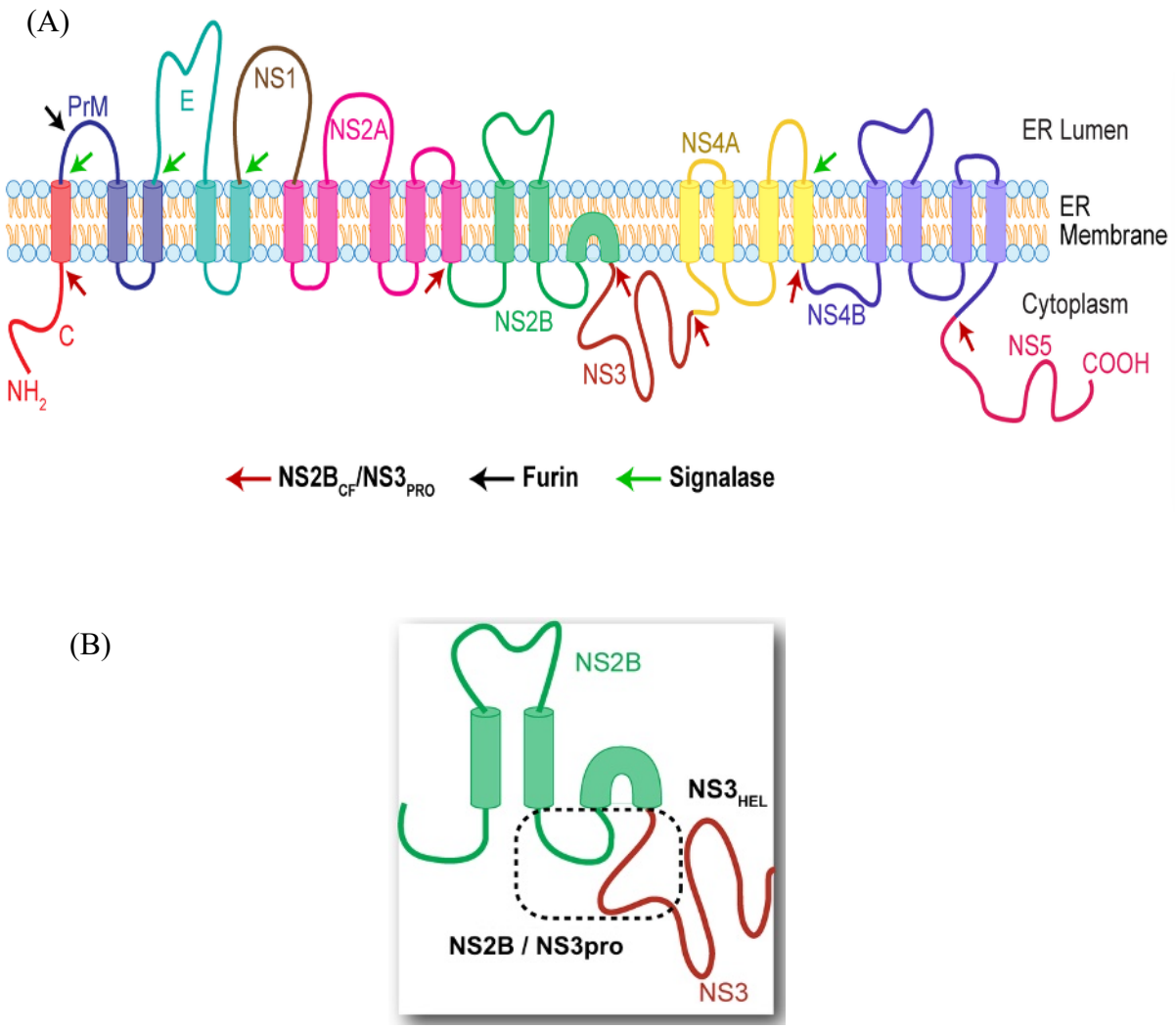

Figure 2.2 (A) Predicted membrane topology of the Dengue virus polyprotein and its cleavage sites. The processed site of the viral NS2B/NS3 protease is indicated by the red arrows. (B) The NS3 protease (NS3pro) domain, which is shown in the detailed box, requires the hydrophilic loop of NS2B, highlighted in green, as a cofactor to be fully active. Figure taken from de Almeida *et al*., 2013.

2.2.2 Structural Biology of DENV NS2B/NS3 pro

2.2.2(a) The active sites

The active sites of the dengue NS2B/NS3 protease are of paramount importance in the context of antiviral research. In the centre of the active site, the catalytic triad is crucial for the proteolytic activity of the enzyme. This conserved group of amino acid residues typically consists of serine, histidine, and aspartic acid. Serine acts as the nucleophile, attacking the peptide bond of the substrate, while histidine and aspartic acid serve to facilitate this reaction through their proton transfer capabilities (**Figure 2.3**). The charge relay system facilitates the conversion of serine into alkoxide, which is an ionised form of serine (Leppkes, 2022). Recognizing the specific arrangement and function of this catalytic triad is essential in order to develop inhibitors that can effectively impede the activity of the protease.

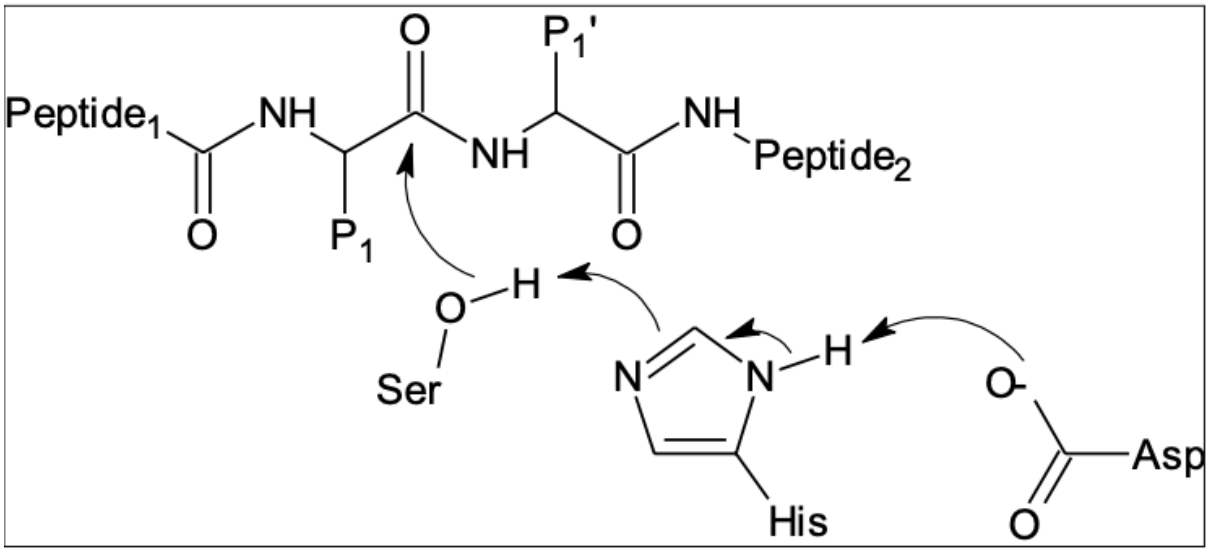

Figure 2.3 Charge relay system for activation of an active site serine residue

Another important component of the active site is the substrate binding site or substrate recognition site. This is where the protease identifies and binds to its substrate, typically a viral polyprotein that needs to be cleaved into individual functional proteins. The binding site demonstrates a high degree of specificity for substrates featuring a conserved motif rich in

positively charged amino acids, and this specificity arises from electrostatic interactions, which are further reinforced by hydrogen bonds and hydrophobic interactions. The selectivity and effectiveness of the enzyme depend on these particular interactions between the protease and substrate at this site (Zephyr *et al*., 2021). Additionally critical is the configuration of the binding pocket within the active site. The NS2B/NS3 pro enzyme's active site comprises four distinct pockets: S1, S2, S3, and S4. These pockets engage with specific substrate residues, namely P1, P2, P3, and P4, respectively. The sequence alignment of Dengue 2 Virus (PDB ID: 2FOM) and West Nile Virus (2PF7) in the NS2B and NS3 domains (Erbel *et al*., 2006) revealed that the residues in the pockets of these two viruses are either identical or comparable (Wichapong *et al*., 2009). This pocket accommodates the binding of the substrate and greatly influences the design of the inhibitor. Knowledge of the pocket's size, shape, and chemical properties is essential for creating small molecules or peptidomimetics that can effectively block the function of the protease, thereby inhibiting viral replication.

2.2.2(b) Crystal structures of NS2B/NS3 protease

X-ray crystallography has proven to be an invaluable tool for revealing the intricate connections between enzymes and their substrates or inhibitors. These structures provide a comprehensive, three-dimensional representation of the protease, exposing the specific locations of its active sites and substrate recognition sites (Parise, 2022). Researchers can utilise the understanding of the structure to develop antiviral drugs that specifically target the protease, impeding viral replication. Erbel *et al*. (2016) reported the first NS3 protease structure utilising a recombinant NS2B/NS3 construct with 47 NS2B cofactor residues connected to NS3 protease by an artificial glycine linker. The NS2B/NS3 structures from flaviviruses such as dengue, WNV, and ZIKV were constructed using this design. Currently, there have been