Delving into Dengue Virus Drug Discovery- Insights into the Structural Characteristics of the RNA-Dependent RNA Polymerase.

Miss Nomagugu B. Ncube

2017

A thesis submitted to the School of Health Sciences, University of KwaZulu

Natal, Westville, in fulfilment for the degree of Master in Medical Sciences (Pharmaceutical Chemistry)

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A thesis submitted to the School of Pharmacy and Pharmacology, Faculty of Health Sciences, University of KwaZulu-Natal, Westville, for the degree of Master in Medical Sciences (Pharmaceutical Chemistry).

This is the thesis in which the chapters are written as a set of discrete research publications, with an overall introduction and final summary. Typically, these chapters will have been published in internationally recognized, peer-reviewed journals.

This is to certify that the contents of this thesis are the original research work of Miss N.B. Ncube.

As the candidate's supervisor, I have approved this thesis for submission.

Supervisor:

Signed: Mahmoud E. Soliman Name: Prof Mahmoud Soliman

Date: 29 November 2017

ABSTRACT

A precipitous increase in the number of *flaviviral* infections has been noted over the last five years. The present study sought to investigate a notorious *flavivirus* that has been in circulation for over 30 years. Over the last few decades, DENV has re-emerged in various serotypes and is causing mayhem in the lives of many. Dengue is dreaded for the severe fever it causes in its advanced stage. Dengue has the reputation of what is known as Dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS).

Dengue remains an unmet medical need that demands prompt attention. There remains no cure or preventative therapy due to the intransigence nature of this *flavivirus*. Its tenacity to resist antiviral therapy has left the scientific community with the burden of finding new and accelerated techniques to curb this virus. The onus is on scientists to probe further into understanding the Dengue virus by the use of cheminformatics and bioinformatics tools in the pursuit for an inhibitor against this pernicious virus. Of the Dengue structural and non-structural enzymes, the NS5 RNA-dependent RNA polymerase has been established as a promising target due to its conserved structure amongst all serotypes and its lack of an enzymatic counterpart in mammalian cells.

Attempts have been made to design vaccines and small drug molecules as potential inhibitors against DENV. The virus however is resilient, and exists in 5 serotypes with numerous strains under them, thwarting the efforts of researchers to curb its spread. This prompted us to design a study that would address the above challenges by use of CADD tools, which elaborated on the design of target-specific inhibitors of DENV from an atomistic perspective. This included a pharmacophoric approach, which utilized computational software to map out a pharmacophore model against multiple *flaviviruses,* as well as a focused review on DENV serotype 2 and 3, which included a route map toward the design of target-specific DENV RdRp inhibitors.

We believe that these findings will aid in mitigating the effects of the DENV in the lives of compromised individuals, as well as prevent the transmission of DENV from patients to healthy individuals.

PREFACE

This thesis is divided into **six chapters**, including this one:

Chapter 1:

This is an introductory chapter that addresses the background, rationale and relevance of the study as well as the proposed aim and objectives. The general outline and structure of the thesis concludes this chapter.

Chapter 2:

This chapter provides a comprehensive literature review on the DENV epidemic and the urgent research taking place toward the development of FDA approved inhibitors of the virus. Included in this chapter is the epidemiology, historical background, life cycle, viral diagnostics, modes of transmission, DENV-linked neurological diseases, viral characteristics (mechanistic and structural), viral/host drug targets, specifically the RdRp protein and the design of potential inhibitors in DENV rational drug design and discovery.

Chapter 3:

This chapter conceptualizes computer-aided drug design by discussing a various molecular modeling and molecular dynamic techniques and applications. The computational tools needed to investigate comparative enzymatic structural/conformational characteristics as well as methods used to analyze binding affinity are elucidated upon.

Chapter 4: (Accepted Manuscript- this chapter is presented in the required format of the journal and is the final version of the submitted manuscript)

This chapter demonstrates a unique route map entitled "An "All-in-one" pharmacophoric architecture for the discovery of potential broad spectrum anti-*flavivirus* drugs", demonstrating potential drug targets, strategies for design and computational software available to map out a pharmacophore model against *flaviviruses*. This article has been accepted in *Applied Biochemistry and Biotechnology* (Manuscript ID: ABAB-D-17-01159) (IF =1.75).

Chapter 5: (Accepted Manuscript- this chapter is presented in the required format of the journal and is the final version of the submitted manuscript)

This chapter investigates the second objective of the thesis and is entitled "Using bioinformatics tools for the discovery of Dengue RNA-dependent RNA Polymerase Inhibitors."

Narrowing down from the first study, this study focused on one *flavivirus* that is the basis for the rest of the *flavivirus* in terms of drug design and intervention methods. The most pathogenic serotypes were selected and discussed. Popular inhibitors were identified and docked to DENV and their binding affinities were discussed. This article has been accepted in *PeerJ* (Manuscript ID: 2018:01:23118:0:1) (IF = 2.35).

Chapter 6:

This is the final chapter that proposes future work and concluding remarks.

DECLARATION I -PLAGIARISM

I, Nomagugu B. Ncube, declare that

1. The research reported in this thesis, except where otherwise indicated, and is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a. Their words have been re-written but the general information attributed to them has been referenced.

    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

A detailed contribution to publications that form part and/or include research presented in this thesis is stated (include publications submitted, accepted, in press and published).

Signed: **N.B. Ncube**

DECLARATION II- LIST OF PUBLICATIONS

1. Nomagugu B. Ncube, Pritika Ramharack and Mahmoud E.S. Soliman (2017). An "All-in-one" Pharmacophoric Architecture for the Discovery of Potential Broad Spectrum Anti-*flavivirus* drugs**.** *Journal of Applied Biotechnology and Biochemistry (Accepted Manuscript).*

Contribution:

Nomagugu B. Ncube: contributed to the project by performing all the experimental work and manuscript preparation and writing.

Dr. Pritika Ramaharack contributed to manuscript editing and providing technical support.

Mahmoud E.S. Soliman: Supervisor

2. Nomagugu B. Ncube, Pritika Ramharack and Mahmoud E.S. Soliman (2017). Using bioinformatics tools for the discovery of Dengue RNA-dependent RNA Polymerase Inhibitors. *PeerJ Journal (Accepted Manuscript).*

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RESEARCH OUTPUT

A- LIST OF PUBLICATIONS

1. Nomagugu B. Ncube, Pritika Ramharack and Mahmoud E.S. Soliman (2017). An "All-in-one" Pharmacophoric Architecture for the Discovery of Potential Broad Spectrum Anti-*flavivirus* drugs**.** *Journal of Applied Biotechnology and Biochemistry (Accepted Manuscript).*

2. Nomagugu B. Ncube, Pritika Ramharack and Mahmoud E.S. Soliman (2017) Using bioinformatics tools for the discovery of Dengue RNA-dependent RNA Polymerase Inhibitors. *PeerJ Journal (Accepted Manuscript).*

B- CONFERENCES

1. CHPC National Conference, 3-7 December, 2017, Velmore Hotel Estate, Pretoria
Role: Poster presentation on An "All-in-one" Pharmacophoric Architecture for the Discovery of Potential Broad Spectrum Anti-*flavivirus* drugs**.**

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To my brother Terrence B.Z “Stokononzi” Ncube, I do regret breaking your tooth when we were young, I was literally “God’s tool” in your life. I will forever cherish your companionship. Also, I still don’t know what “Stokononzi” means. However that name came about, it ought to be added onto your birth certificate. Even though you don’t say much, you are still loved. Maybe one day, you will decide to join the chatter-box squad. A kind reminder that my dollies and I are still waiting for you to play with us, we’ve been waiting fifteen years now, give us a ring when you are ready to play house! I strongly believe if you have a daughter she can be named after me, to avoid inheriting the “one-word-answer” gene.

University of KwaZulu-Natal College of Health Sciences and National Research Foundation

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LIST OF ABREVIATIONS

Å	Amperes
α	Alpha
ATP	Adenosine Triphosphate
β	Beta
CADD	Computer-Aided Drug Design
DENV	Dengue Virus
FDA	Food and Drug Administration
ΔG	Free Binding energy
GAFF	General amber Force Field
HCV	Hepatitis C Virus
JEV	Japanese Encephalitis Virus
MD	Molecular Dynamics
MM	Molecular Mechanics
MM/GBSA	Molecular Mechanics/Generalized Born Surface Area
MM/PBSA	Molecular Mechanics/Poisson-Boltzmann Surface Area
ns	nanoseconds
NS	Non-structural
ORF	Open Reading Frame
PDB	Protein data bank
QM	Quantum Mechanics
RdRp	RNA Dependent RNA polymerase
RESP	Restrained Electrostatic Potential
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
WNV	West Nile Virus
WHO	World Health Organization
ZIKV	Zika Virus
3D	Three-Dimensional

LIST OF AMINO ACIDS

Three Letter Code	Amino Acid
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic Acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

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CHAPTER 1

Introduction

1.1 Background and Rational

The *Flaviviridae* family is named from the Latin word *flavus* due to the jaundice caused by infection with the Yellow fever virus. It constitutes of the species *Flavivirus*, *Pestivirus*, *Pegivirus* and *Hepacivirus* [1]. Of the four species, *Flaviviruses* have become the most conspicuous in the medical field [2,3]. The yellow fever virus is the prototype for the *Flavivirus* genus as *Flavivruses* were discovered during yellow fever epidemic investigations. The genus contains over 90 viruses with West Nile, Zika, Dengue, Japanese encephalitis and Yellow fever being viruses to note [4,5].

Flavivirus case reports have been alarming over the last decade. The Zika virus has been reported in 42 countries, and there is an estimate of 1.5 million reported cases [6,7]. Dengue virus (DENV) case reports have been noted in 128 countries [7,8]. In 2002, 142 cases of West Nile were reported in the USA alone [9], and Japanese encephalitis has about 30-50 000 case reports annually in Asia [10]. These two *flaviviruses* share the same antigenic complex, and though they were once dominant in specific locations, they have spread across the continents at alarming rates [11,12]. The Yellow fever virus has claimed 117 lives in Angola, and continues to cause distress in West and East Africa. It is worthy to note that of the mentioned *flaviviruses*, Yellow fever is the only one with a vaccine against it. The vaccination of travellers entering and leaving the borders of East and West Africa has caused a blockade in the spread of Yellow fever to unaffected countries [13,14]. The international science and research community has summoned researchers to accelerate *flavivirus* investigations due to the unprecedented outbreaks of great magnitude [15].

From the aforementioned *flaviviruses*, DENV stands out as the “black sheep” of the *flaviviridae* family. This statement can be justified by its unyielding nature to antiviral therapy and rapid mutation rates [16–19]. The first DENV vaccine trial left scientists with mixed feelings. Some welcomed the vaccine that provided immunity against 3 of the 4 serotypes. Others were disappointed that overall it was ineffective and showed no protection against the common strain

that was circulating at that moment, serotype 2 [20,21]. In order for a vaccine to be deemed effective, it must provide immunity against all 4 serotypes. Should an individual be vaccinated against 1 serotype, they will never be infected with that serotype again. If the individual however is subsequently infected with a different serotype, he or she will have an increased risk of developing severe DENV illness [22]. The obstinate nature of DENV is caused by its recurring mutations. Its periodic appearances in 1986 eventually led to its major debut in 2004 were it presented itself with 4 serotypes in an outbreak [23,24]. These 4 serotypes, DENV1-4, are reported to have been in co-circulation for as long as 30 years [25]. Among these 4 serotypes, each serotype presents further challenges of various strains and mutation of strains within the serotype. Studies have shown that DENV viral etiology and serotype is constantly changing [25–27]. Clade replacements have also been noted within the DENV serotypes. An increase in one serotype leads to an abundance of the same serotype, simultaneously decreasing the prevalence of another serotype[28–30]. These never-ending fluctuations have made antiviral therapy onerous. In the midst of this mayhem to find a vaccine and antiviral drug that is effective against all 4 serotypes, and account for the potential serotype mutations, a 5th serotype emerged thus perpetuating DENV's stubborn nature to respond to treatment [31–33]. Research that has been channelled towards finding inhibitors against the 4 serotypes has to be readjusted to accommodate the 5th serotype, further complicating the current state of affairs in finding a vaccine and antiviral drugs for DENV. The battle remains to find antiviral therapy against DENV.

A divergence from conventional methods of drug design and discovery to novel techniques has been warranted by the ever-mutating DENV. Computer-Aided Drug design is a useful strategy to by-pass many hurdles encountered during drug discovery and design, with the major ones being time and money. Certain computational tools can be employed to identify and landscape inhibitors against *flaviviruses* [34].

Due to the paucity of fundamental research in the previously neglected tropical disease, we have utilized key computational techniques to fill the gap in drug design research against *flaviviruses*. In this thesis we will be looking at identifying and designing possible inhibitors, based on pharmacophoric features of popular FDA approved drugs that have better potency thus giving insights toward novel viral drug targets against this new epidemic.

1.2 Aim and objectives

The principal purpose of this thesis is to identify, characterize and map out inhibitors which are more efficient and effective for DENV virus treatment by the use of Computer-Aided Drug Design techniques.

In order to accomplish this, the following objectives were outlined:

1. To design an "All-in-one" pharmacophoric architecture for the discovery of potential broad spectrum anti-*flavivirus* drugs by:

1.1. Identifying an experimentally validated inhibitor and assess its physicochemical properties for all 5 *flaviviruses.*
1.2. Performing MD simulations to create MD ensembles of flavivirus RdRp inhibitor systems.
1.3. To landscape a pharmacophore model based on free energy calculations (MMGBSA) and per residue decomposition analysis.

2. To provide a comprehensive cheminformatics based review for the discovery of DENV serotypes 2 and 3 inhibitors. This may be achieved by:

2.1 Sequence and structural analysis of the RdRp of DENV.
2.2 Active site classification of the DENV RdRp.
2.3 Identification of potential RdRp inhibitors against DENV.

1.3 *Novelty and Significance of study*

Dengue has been declared a global threat by the World Health Organisation(WHO) [7,17]. It has savaged the lives of many, and continues to do so at alarming rates [8]. The Dengue virus has been in circulation for over 30 years, and only recently has it become a nuisance to the health community [25]. One of the most devastating effects of DENV is its teratogenic properties. Vertical transmission leads to babies born with neurological disorders and thus experience impaired growth and retardation [35–37].

Notable progress has been made in mapping out inhibitors against DENV [38–41]. Despite these efforts, there still remains no approved drug or vaccine for Dengue therapy. This is due to DENV's serotypes. Mutations have led to the co-existence of 5 serotypes of DENV which in turn have numerous strains under them. The constant evolution of DENV has led to some inhibitors being nullified as potential treatment [23,42]. In addition, the inhibitors so far have elevated toxicity issues and have not been validated for use in humans [15,43–45]. There is therefore a need of constant development in drug design. This has prompted us to investigate potential inhibitors, based on pharmacophoric features of popular FDA approved drugs, which are more efficient and can address all serotypes of the DENV.

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CHAPTER 2

2. Background on Dengue virus

2.1 Introduction

Members of the *flaviviridae* family have caused a widespread morbidity and mortality throughout the world. There are several species under the *flavivirus* genus that have caused, and continue to cause detrimental effects in people's health. Viruses of concern include the Zika virus, West Nile virus, Dengue virus, Yellow fever virus and Japanese encephalitis virus. These viruses are the causal agents of the most important emerging diseases that have no available treatment and prevention measurements to date [1,2].

Dengue is an arbovirus whose neuro invasive effects have set the alarm bells ringing over the last decade. Dengue is one of species under the *flavivirus* genus. Dengue virus is the causative agent of Dengue fever (DF) and Dengue haemorrhagic fever/Dengue shock syndrome (DHF/DSS). It affects people of all ages from neonates to geriatrics. It is closely related to other mosquito borne viruses including Zika, Yellow fever and West Nile [2]. The primary vector for DENV and viruses of its nature is the *Aedes Aegypti* mosquito found in tropical and sub-tropical areas [3].

This chapter contextualizes the current research on DENV, including previous outbreaks, pathogenesis and life cycle of the virus. The structural characteristics of DENV will also be reviewed, thus distinguishing possible viral targets in the design of effective and non-toxic therapeutics.

2.2 Epidemiology and Transmission

The first probable record case of Dengue fever is found in the Chinese medical encyclopedia from the Jin Dynasty (265-420AD)[4]. It referred to a water poison related to flying insects [5]. Benjamin Rush confirmed the first case report in 1789. He coined the term break bone fever because of the myalgia and arthralgia symptoms that accompany Dengue fever [6,7]. Severe Dengue was first recognized in the 1950s in the Philippines and Thailand. Before 1970, DENV was only experienced in 9 countries. Over the years it has spread to most Asian and Latin American countries, and has become the leading cause of hospitalization among children. Dengue is now a pandemic in over 100 countries in Africa, America, South-east Asia, the Eastern Mediterranean and the Western Pacific. The most affected areas are south-east Asia and the Western Pacific [8,9].

Dengue has shown a 30-fold increase globally over the past five decades. It is estimated that 50 to 100 million new infections occur annually in more than 100 countries. Death tolls reach an astonishing figure of 20 000 a year [10]. In some Asian and Latin American countries, severe DENV is a leading cause of serious illness and death among children. Across the Americas, South-East Asia and Western Pacific, cases exceeded 1.2 million in 2008. This number surged to over 2.2 million in the year 2010, and has continued to increase in the subsequent years. The number of case reports is not only increasing, but outbreaks are being noted. Dengue has also manifested itself in different serotypes. Besides the already mention areas affected by DENV, Europe is under threat for a potential outbreak. Local transmission of Dengue was reported for the first time in France and Croatia in 2010 [9,11,12]. Statistics show that over 2.5 billion people, being over 40% of the total world's population, are vulnerable to DENV. This has made Dengue the most important acute systematic arthropod-borne viral infection in humans [8].

This rampant transmission of DENV has been due to the increase in travel across the continents. Disease carriers travel to regions with no cases of DENV and infect the people around them. In addition, climate changes have allowed the spread of mosquitoes across continents, allowing for increased infection rates [13,14]. Another reason for the continental dissemination of the virus is the identification of new modes of viral transmission. Studies have evidenced DENV to be transmitted through perinatal transmission, blood transfusion and sexual transmission [3,15,16]. The co-existence

of 5 DENV serotypes further exacerbates the situation as when DENV spreads, a new strain emerges which is more virulent than the previous one, making it more difficult to eradicate [17–19].

2.3 Characterizing DENV

With the upsurge of DENV case reports, much research has been allocated into providing a better understanding of the major physiological and molecular mechanisms underlying this infectious disease. It is therefore imperative to understand the DENV structure and life cycle so as to design anti-therapeutic agents and delve into the structural implications of DENV drug resistance mutations. The Dengue virus is highly mutable given the presence of its 5 serotypes and vast number of strains [20]. This fortifies the challenge of designing efficient inhibitors against the virus.

2.3.1 Life cycle of DENV

The Dengue virus must exploit cell machinery in order to survive and complete its infectious cycle. Subsequent to viral entry into host, the DENV virion attaches to the surface of target cells by interactions between the envelope protein and the host cell surface receptors. The host cell receptors that have been evidenced to mediate virion endocytosis include phosphotydylserine receptor, AXL, as well as DC-SIGN, TIM-1 and Tyro3. Virions undergo this receptor-mediated endocytosis and are internalized to the cell cytoplasm. The viral envelope is then uncoated and the viral RNA is released into the cell cytoplasm. The viral RNA is then translated produce a large polyprotein at the endoplasmic reticulum and is subsequently cleaved into the individual viral proteins, leading to the replication of the viral genome. The viral RNA as well as the structural and non-structural proteins, and some host proteins are involved in the packaging of the viral complex into vesicles and assemble by budding into the endoplasmic reticulum, whereas immature viral particles utilize the host secretory pathway, where virion maturation occurs followed by release from the cell. [21–24]

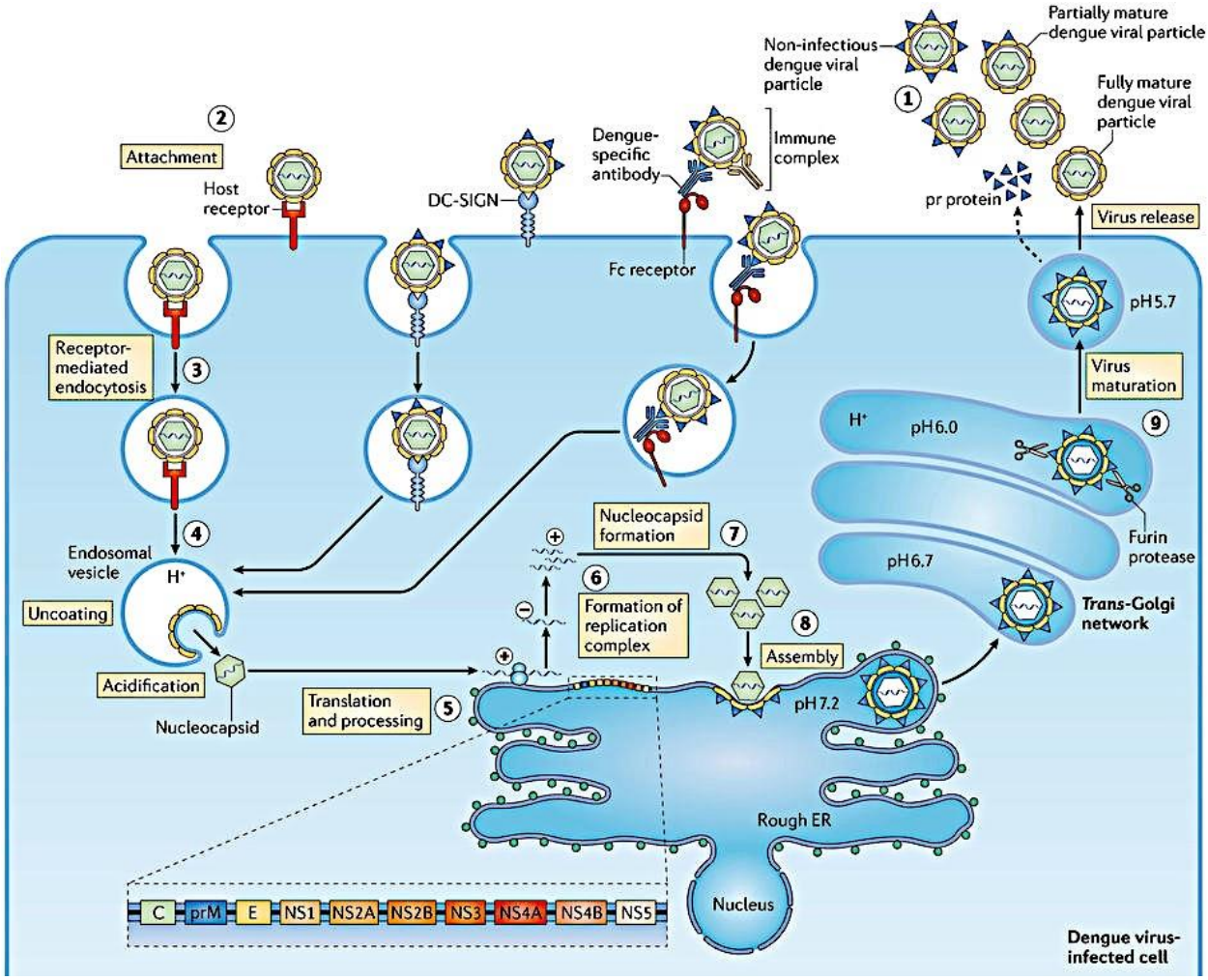


Figure 2.1: Life cycle of the DENV virus [24].

2.3.2 Structural Characteristics of DENV

Dengue is an enveloped, non-polyadenylated positive-strand RNA virus whose viral genome comprises of a positive-sense RNA virus approximately 11kb in size [25]. This genome encodes a single open reading frame flanked by highly structured 5'- and 3' UTRs. There are key RNA elements in the viral genome that are essential for DENV replication; the stem-loop(SLA), the cyclization elements present on both sides of the genome, the capsid-coding region hairpin element (cHP) and the highly conserved 3' stem-loop(3'SL) [23]. At the 5'end is found the SLA which functions as the promoter for RNA synthesis. This promoter is also responsible for binding and activating the polymerase NS5 that will in turn initiate RNA synthesis at the 3' end of a circulized viral genome [26]. The cHP is located in the capsid protein-coding sequence. It is required for

efficiency in the viral replication process. The terminal of 3' end presents genomes that fold into a highly conserved 3'SL. Upstream of this loop is the conserved sequences CS1 which contain the cyclization sequence also needed for RNA replication. The RNA genome also encodes 3 structural proteins that form the components of the virion: the capsid (C), precursor membrane (prM) and the envelope (E). In addition to this assembly are seven non-structural (NS) proteins namely NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5 [26–29]. These non-structural proteins are involved in virus assembly, viral RNA replication and modulation of the host cell responses.

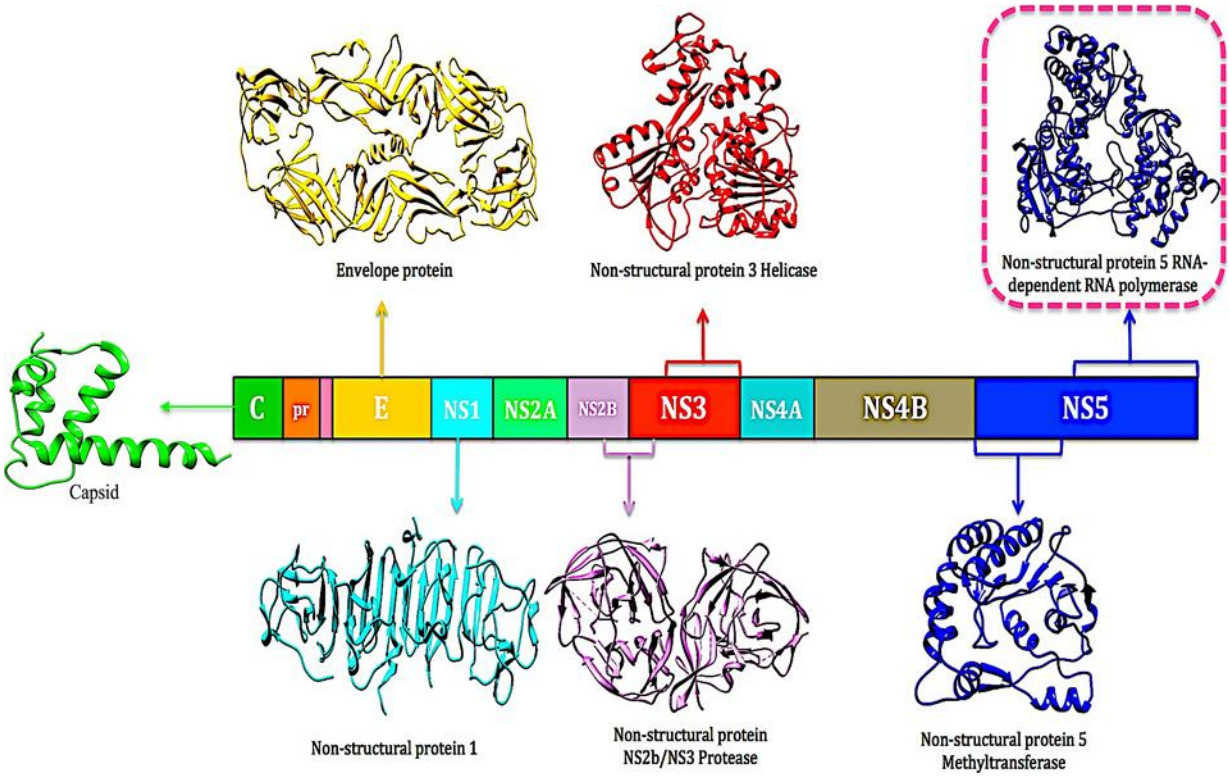


Figure 2.2: Dengue polyprotein demonstrating available protein crystal structures (PDB codes: 1R6R, 4UTC, 4O6B, 2FOM, 2VBC, 1L9K, 5K5M) (Prepared by Authors).

2.3.3 DENV Pathogenesis and Clinical Features

The mosquito borne viral infection comes with severe flu-like illness when initially acquired. A high fever of 40°C/104°F accompanied by either severe headache, pain behind the eyes, muscle and joint pains, nausea, vomiting, swollen glands or rash confirms Dengue infection. This fever is known as break bone fever as it is accompanied by arthralgia and myalgia. Symptoms usually last for 2–7 days, after an incubation period of 4–10 days after being bitten by an infected mosquito. Dengue fever seldom causes death. Severe Dengue (Dengue haemorrhagic fever/Dengue shock syndrome (DHF/DSS) is, however, a potentially deadly complication due to plasma leaking, fluid accumulation, respiratory distress, severe bleeding, or organ impairment. Caution signs crop up 3–7 days after the first symptoms in conjunction with a decrease in temperature (below 38°C/100°F). They also encompass severe abdominal pain, persistent vomiting, and rapid breathing, bleeding gums, fatigue, restlessness and blood in vomit. The subsequent 24–48 hours of the critical stage can be fatal [30–32]. Adequate medical care is therefore imperative to evade complications and death.

The simultaneous presence of two or more infections which increases the graveness and duration of a disease is defined as co-infection. Studies have reported incidence of DENV and CHIKV co-infections in humans and vectors [33,34]. Both infections pose overlapping clinical presentation and diagnosis can be challenging. In order to differentiate the two, there are major symptoms to look out for. Dengue fever classically is accompanied by a severe headache, eye pain, and prominent myalgia and enlarged lymph nodes. Chikugunya(CHIKV) is also characterized by a severe headache and enlarged lymph nodes, but instead of general myalgia, it is marked by joint pain and arthritis [35–38]. It is imperative to distinguish these two so as to manage the symptoms accordingly.

2.4 Rationale of DENV RdRp as Potential Therapeutics Targets

2.4.1 NS5-RdRp

The NS5 is the largest viral protein that has caught the eye of numerous researchers [39,40]. It is the most conserved non-structural protein amongst the viral proteins. The NS5 is a bi-functional protein that contains 900 amino acids. The S-adenosyl methionine transferase is found on the N-terminal. On the C-terminal, residues 270 to 900 make up the RNA-dependent RNA polymerase (RdRp).

The RNA-dependent RNA polymerase (RdRp) domain of the NS5 protein will be explored in this study by the use of cheminformatic tools in the search for an inhibitor to alleviate the DENV. The RdRp plays a significant role in RNA synthesis by catalyzing the replication of RNA synthesis via a two-step mechanism, thus validating it as a target for antiviral therapy [41,42].

The high genetic diversity of DENV virus serotypes is a result of its high mutation rate caused by error-prone RdRp, which lacks proofreading activity and generates approximately one mutation per round of genome replication [43,44]. Genetic recombination is also known to cause intra-serotype genetic variation in DENV [45–48].

The architecture of the DENV RdRp adopts the canonical right-hand conformation comprising of fingers, palm and thumb surrounding its polymerase active site [49]. This applies to most polymerases [50,51]. Dengue however has the nuclear localized structure (NLS) playing a major role in its structural formation [40,52]. The NLS domain signatures are distributed between the fingers and thumb subdomains from residues 316-415. They are the hotspots for interactions with other viral and host proteins [53,54 55]. Alterations within the NLS region lead to structural destabilization [52,56]. The RdRp can be divided into three subdomains: the fingers, palm and thumb [57].

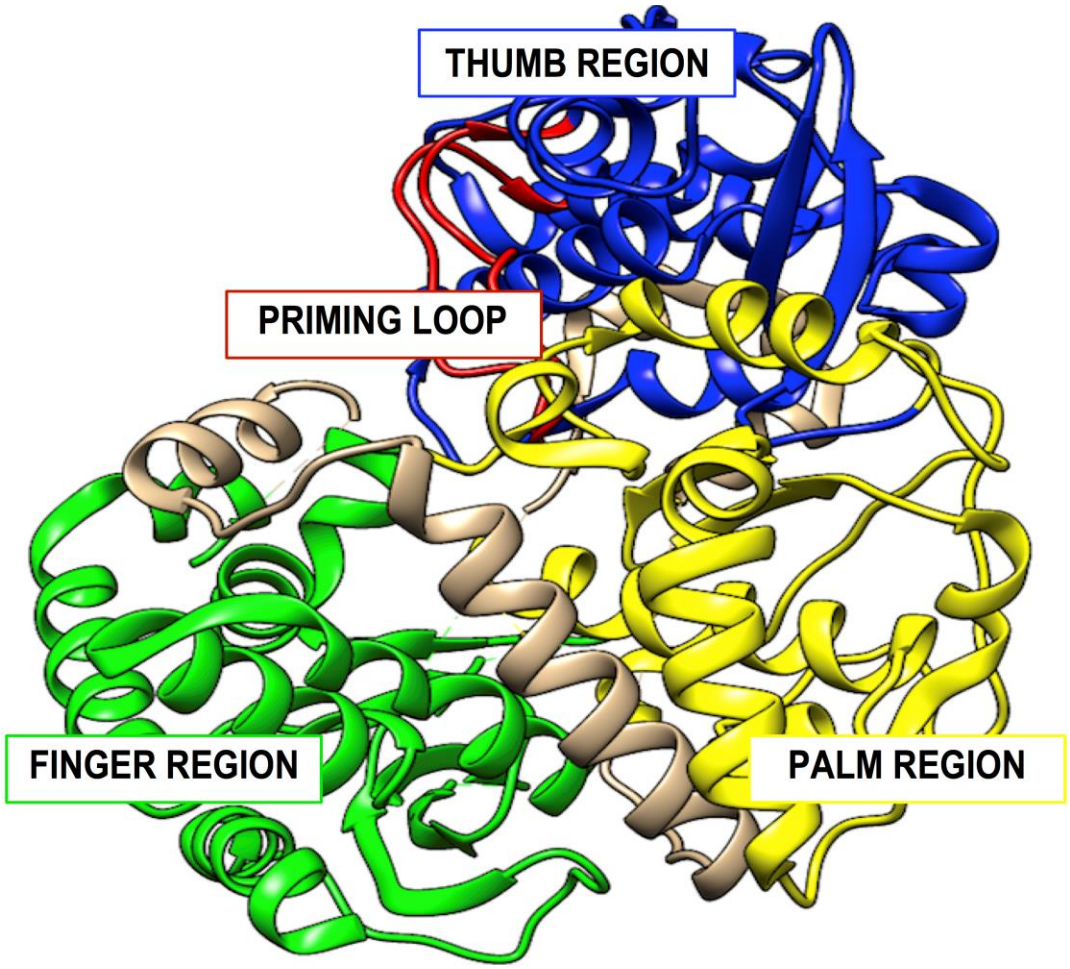


Figure 2.3: Complete Structure of DENV RdRp (Prepared by Author).

Finger domain

The finger domain is divided into two subdomains. The first strand is a beta-strand-rich (β) subdomain and the fingers found on this strand are termed beta fingers [50]. The other strand is rich in alpha-helices (α) and therefore the fingers found in this region are alpha fingers. In addition, the finger region has four flexible loops, β1-α2 loop, α3-α4loop, α6-α7 loop and α7- α8loop. Overall, the residues in the finger region are from 273-600 [51,58,59]. The α1-9 residues are from 273 to 496, and α 12 to β 3 residues are from 543 to 600 [51,60]. The fingers subdomain is located at the top of the RdRp molecule and appears to be more mobile than the palm and thumb domain [54,61,62].

Palm domain

The palm domain is the catalytic domain and encompasses a folding motif that is highly conserved among polymerases [52,57]. The palm constitutes of 2 antiparallel β-strands, β4 and β5, and is surrounded by eight helices which are α11 to α13 and α16 to α20. The residues found on the palm

domain are 497 to 542 on α 10, α 11 and 310 helix g1, and residues 601 to 705 on α 14-to 310-helix g2. There are six conserved sequence motifs located in the palm region [51,58,63–67].

Thumb domain

The thumb domain molds the C-terminal end of the RdRp, [68] and is composed of residues 706 to 900 on the β 6 to α 23 [52,58]. It has portrayed itself to be the most structurally variable among known polymerase structures [41,69]. The thumb domain contains two conserved sequence motifs. Motif E forms an antiparallel β-sheet wedged between the palm domain and several α-helices of the thumb domain [61,70]. In conjunction with the finger tips, the path chosen by this loop greatly contributes to the shaping of the RNA template tunnel [52,71,72].

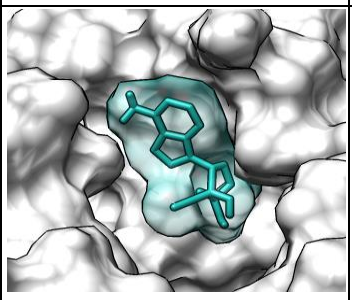
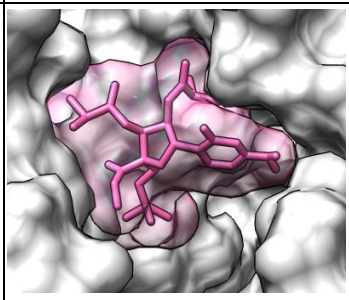
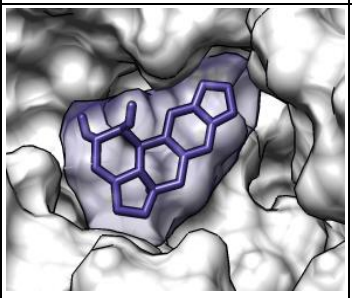
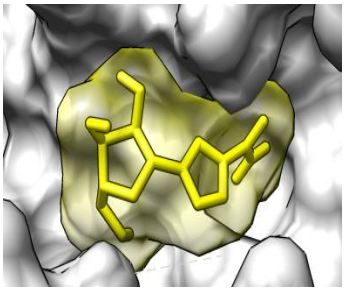
Priming loop

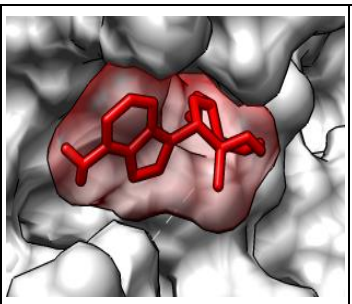
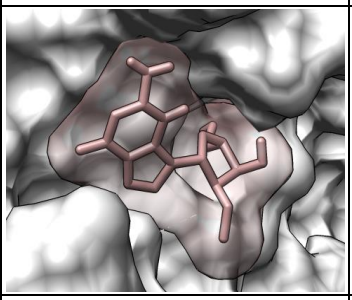
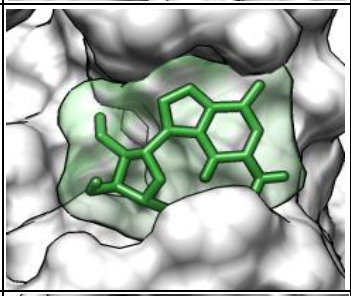
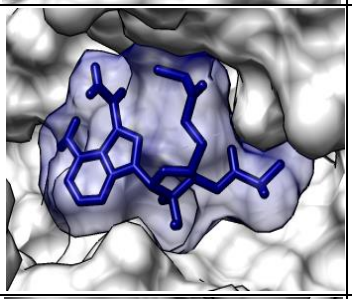
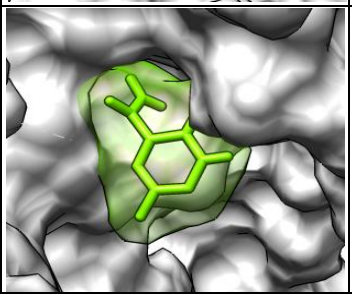
A second loop consisting of amino acids 782-809 forms the priming loop which partially blocks the active site. The priming loop plays a key role in initiating the RdRp complex [73–75]. This path is stabilized by internal interactions that include hydrogen bonds. This assists in maintaining its orientation in relation to the protein structure [61,76]. It is also called the G-loop because it corresponds to motif G in primer dependant RdRps. It's tip is partially disordered in the *flavivirus* RdRp structures thus suggesting conformational flexibility [60,77].

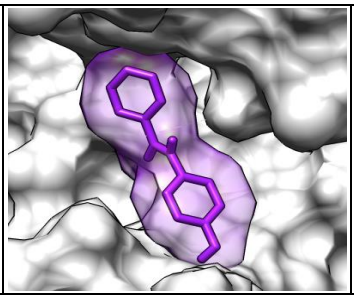
2.4.2 Potential Inhibitors of DENV

Identification of small molecules that inhibit a major step in the viral cycle demands a detailed biochemical and structural characterization of the RdRp protein essential for replication [78]. The development of an antiviral drug for DENV is complicated due to the presence of its five serotypes. This is due to the fact that when protection against one serotype is being generated, protection against other serotypes lasts only a few months [79]. Over the years, inhibitors that have shown potential antiviral activity against the RdRp region have been discovered. These inhibitors, however, come with multiple challenges including elevated toxicity levels. Scientists are therefore still battling to find an inhibitor that is potent, efficacious and non-toxic for the treatment of *DENV* virus [58,78,80].

Table 2.1: Popular *flavivirus* inhibitors bound to DENV RdRp.

Compound	Mechanism of Action	Binding Energy (kcal/mol)	Pose	Amino acid residues
NITD008	Inhibitor of all 4 DENV serotypes [93–95].	-5.5		PHE 127 ARG 210 LYS 130 VAL 131 ASN 134 ALA 135
Balapiravir	A cytidine nucleoside that is a domain RdRp inhibitor [94].	-6.1		TYR451 ASN452 VAL577 ARG594 VAL576 LYS575 SER593
Lycorine	A potent *flavivirus* inhibitor in cell culture through suppression of viral RNA replication	-5.8		TRP 474 TRP 477 ILE 473 SER 470 VAL 579 LYS 578
Ribavirin	Significant *in vitro* activity against *flaviviruses* [98,99].	-5.0		LYS 578 ASN 452 GLN 580 VAL 579 VAL 310 ILE 473

7-deaza-2’methyladenosine	Potent ZIKV inhibitor that reduces viremia [100]	-5.5		ILE 283 GLU 286 ILE 473 MET 453 MET 589 TYR 451
3’Dgtp	GTP analogue that inhibits DENV-2 RdRp but has a low IC50 value [67]	-5.0		MET 589 VAL 579 LYS 578 ASN 452 MET 453 VAL 310
2’O-metil GTP	Showed low IC in vitro DENV-2 [67].	-5.3		THR 343 THR 313 SER 470 TRP 477 GLY 349 ASN 452
NITD 203	Inhibitor of all 4 DENV serotypes [93].	-6.4		VAL 358 GLY 599 TYR 451 ILE 591 GLY 601 SER 600
Favipiravir	A pyrazine-substitute compound that has shown to inhibit *flavivirus* mortality in	-4.0		ASN 452 TYR 451 VAL 579 LYS 578

Ivermectin	Pretreatment of ivermectin with N-(-4-hydroxyphenyl) inhibits the nuclear	-5.3		ASN 452 LYS 578 MET 589 VAL 579 VAL 310 MET 453

2.5 The Scientific Advancements of DENV Anti-viral Therapy

Dengue infection has been found to be associated with severe and fatal hemorrhagic fever conditions as mentioned above. The concerns of the scientific and clinical community are the consequences of DENV viral mutations, thus suggesting the urgent need for viral inhibitors. There have been large strides in vaccine development against the virus. The first registered vaccine was Dengvaxia by Sanofi Pasteur in Mexico 2016 [81–83]. The vaccine however does not cover immunity against all DENV serotypes, thus getting the vaccine and being exposed to other serotypes not covered by the vaccine leaves one at risk of acquiring severe Dengue infection [81]. Vaccines against DENV are still under evaluation in clinical trials [84,85]. Rapid rational drug design and discovery research is fundamental in the production of potent inhibitors against the virus to annihilate it. There are no FDA approved drugs against DENV [5,8,86]. There are potential inhibitors that have shown antiviral activity against DENV. They, however, come with a plethora of side effects including high toxicity levels [87–90]. Therefore, there remains a need for the pursuit of antiviral therapy that will ameliorate the current situation with DENV, and put an end to its dynasty.

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CHAPTER 3

3. Molecular Modeling and Bio-Computational Strategies to Drug Discovery and Design

3.1 Introduction

There has been a paradigm shift in the design of drugs and other biological compounds in the medical world today. Novel methods of drug design are continuously being exploited in an endeavour to improve efficiency and encounter challenges faced during the drug design process [1,2]. Molecular modeling, also known as computational chemistry is a rapidly growing discipline of science in the research community. It encompasses theoretical and computational methods used to simulate the behaviour of molecules [3]. According to Pensak, molecular modeling is defined as "anything that requires the use of a computer to paint, describe or evaluate any aspect of the properties of the structure of a molecule" (Jensen, 2002,)[5]. They are various disciplines within molecular modeling. These include computational chemistry, drug design, cheminformatics, bioinformatics and computational biology amongst many others [6]. For the purpose of this study, the methods focused on included computational chemistry, cheminformatics tools.

Computational chemistry has become the mainstream of drug design and discovery in the medical domain. It entails the use of algorithms that perform calculations, data processing and automated reasoning tasks to solve a class of chemical problems [7]. By definition, computational chemistry is a subsidiary of chemistry that entails the use of computer simulation to aid in the solution of chemical problems. It uses equations encapsulating the behaviour of matter on an atomic scale and uses computers to solve these equations, calculate structures and properties of molecules, gases, liquids and solids and to explain and predict chemical phenomena [7–9]. It provides a platform for scientists to predict chemical reactions and their outputs using computer software. This by-passes empirical processes performed in the laboratory thus saving time and enormous costs that follow practical laboratory experiments [10–12]. Computational chemistry covers an extensive range of topics including quantum chemistry, molecular mechanics and molecular dynamics [13]. This chapter concentrates on the computational tools and theoretical tools germane to this study. There are two basic components used to describe computational chemistry:

1) Quantum Mechanics

2) Molecular Mechanics

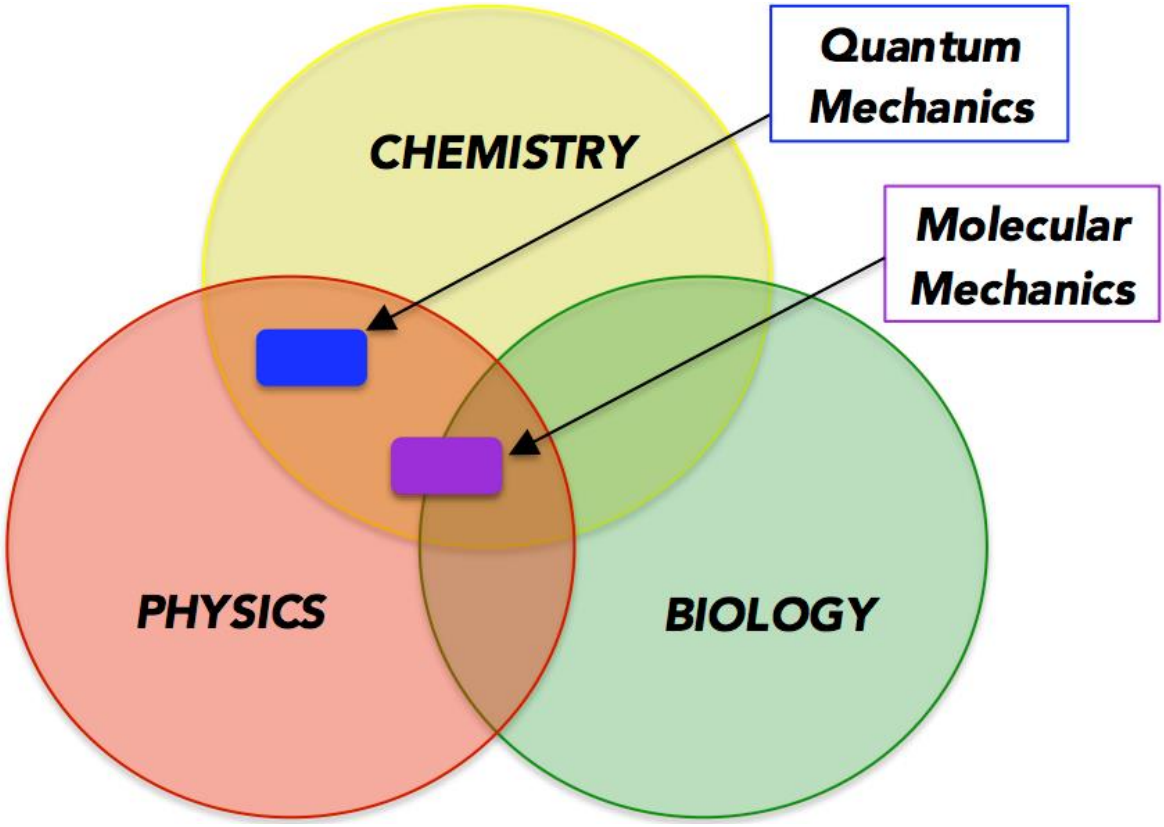

Figure 3.1: Schematic representation of the two components of computational chemistry (Prepared by Author).

3.2 Quantum Mechanics

Quantum mechanics is the study of the behaviour of matter and energy at the molecular, atomic, nuclear and smaller microscopic levels. Quantum mechanics(QM) is also known as quantum physics or quantum field theory [14,15]. It is the most successful theory in the history of science. The quantum hypothesis was suggested by Max Planck in 1900 [16]. It states that any energy radiating atomic system can be theoretically divided into a number of discrete energy elements such that each of the energy elements is proportional to the frequency with which each of them radiate energy individually [17]. Planck theorized that energy was transferred in portions called quanta, hence the name quantum hypothesis. It is illustrated by the equation below:

$$\mathbf{E=hv} \qquad \text{(Eq 3.2.1)}$$

where E is energy, h is Planck's constant, equal to 6.626068×10^{-34} Joule-second (J-s) and the Greek letter v is the photon's frequency [18–20].

The quantum phenomena can be best explained by the Copenhagen interpretation. Danish physicist Niels Bohr and German physicist Werner Heisenberg came up with a set of statements which attempt to explain how QM explains our understanding of nature [21]. The Copenhagen interpretation states that physical systems normally do not have distinct properties prior to being measured, and quantum mechanics can only predict the probabilities that measurements will produce certain results. It states that a quantum particle does not exist in one state or another, but it exists in all of its possible states concurrently. Only upon observation is a quantum particle compelled to choose one probability, and this is the state we observe. This explains why a quantum particle is said to behave erratically. This state of a quantum particle existing in all possible states at once is called an object's coherent superposition. The sum of all possible states which an object can exist forms the object's wave function. When an object is observed, its superposition collapses and the object is forced into one of the states of its wave function [22–24].

Although there have been many objections to the Copenhagen interpretation, it still remains the frequently taught explanations of quantum mechanics [25]. The quantum hypothesis laid a foundation for many physicists like Albert Einstein, Niels Bohr, Werner Heisenberg, Erwin Schrödinger and many others to further develop the field of quantum physics [14,26].

3.2.1 The Schrödinger Wave Function

The Viennese physicist Erwin Schrödinger vetted the Copenhagen theory by the famous thought experiment involving a cat and a box. Schrödinger placed a cat in a sealed box and added a flask with poison inside the box. Schrödinger proposed that if an internal monitor detects radioactivity, the flask will be shattered, releasing the poison thus killing the cat. This challenged the Copenhagen interpretation which implies that the cat can be simultaneously alive and dead, a state known as coherent/quantum superposition. Schrödinger went on to say that upon observation of the cat, the superposition of the cat would collapse into either the cat is alive or dead, not both [27–30].

This paradox created by Schrödinger led him to his next theory, which had a great impact in the world of quantum physics. At the dawning of the twentieth century, scientific evidence implied that atomic particles were wave-like in nature [31]. It was feasible to assume that a wave equation could explain the behaviour of atomic particles. Schrödinger was the first person to write down a wave equation and earned a Nobel prize in 1933 [32]. Schrödinger argued that Newton's laws do not apply to miniscule things. In other words, the rules used to govern the motion of a car cannot be used to explain how an electron or atom works. After a lot of discussion and debate, the wave equation was accepted to be a probability distribution [33,34].

The Schrödinger equation, often called the Schrödinger wave function describes all of the possible states particles can have including properties like energy, momentum and position. Schrödinger utilized mathematical equations to describe the probability of locating an electron on an exact path (Schrödinger, 1961).

The Schrödinger wave equation [36]:

$$\mathbf{H\psi = E\psi} \qquad \text{(Eq 3.2.2)}$$

$$\mathbf{H = T + V} \qquad \text{(Eq 3.2.3)}$$

$$\mathbf{H} = \left[-\frac{\mathbf{h^2}}{\mathbf{8\pi^2}} \sum_{\mathbf{i}} \frac{\mathbf{1}}{\mathbf{m_j}} \left(\frac{\mathbf{\partial^2}}{\mathbf{\partial x^2}} + \frac{\mathbf{\partial^2}}{\mathbf{\partial y^2}} + \frac{\mathbf{\partial^2}}{\mathbf{\partial z^2}} \right) \right] + \sum_{\mathbf{i}} \sum_{\mathbf{<j}} \left(\frac{\mathbf{e_i e_j}}{\mathbf{r_{ij}}} \right) \qquad \text{(Eq 3.2.4)}$$

The Schrödinger wave function is an integral equation of physics for illustrating mechanical behavior. It is a partial differential equation that describes how wave function of a physical system evolves over time. When quantum mechanical systems are observed from the Schrödinger equation perspective, it is known as the Schrödinger picture, as opposed to viewing quantum mechanical systems from the matrix mechanical view point, known as the Heisenberg picture [37–39].

3.2.2 The Born-Oppenheimer Approximation Theory

Max Born and J. Robert Oppenheimer proposed the Born- Oppenheimer approximation in 1927 [40]. This theory assumes that the motion of atomic nuclei and electrons in a molecule can be separated. In mathematical terminology, it allows for the wave function of a molecule to broken into its electronic and nuclear components. The Born-Oppenheimer theory allows for the Schrödinger equation to be solved for the kinetic energy of the electrons alone since the kinetic energy for the nuclei remains constant. Electrons are considered to be of lighter weight than the nuclei. This leads to the electrons having a greater velocity and moving instantaneously to nuclei movement. The distribution of electrons within a molecule is therefore defined by the location of the nuclei [41–44].

The disparity in velocities of the nuclei and electrons allow for the application of the Born-Oppenheimer approximation, thus minimizing the complexity of the wave function of the Hamiltonian equation. The simplified wave function is as follows:

$$\boldsymbol{\psi}(\mathbf{r_{elec}}) = \boldsymbol{\psi}(\mathbf{r_{elec}})\,(\boldsymbol{\psi}(\mathbf{r_{nucl}}) \qquad \text{(Eq 3.2.5)}$$

Eq 3.2.2 is converted:

$$\boldsymbol{H_{EN}}\boldsymbol{\psi}(\mathbf{r_{elec}}) = \boldsymbol{E_{EN}}\boldsymbol{\psi}(\mathbf{r_{elec}}) \qquad \text{(Eq 3.2.6)}$$

Where H_{EN} denotes a difference between terms based activity to fixed nuclear positions (V_{NN}) or their activity to the non-fixed electron positions. Eq. 3.2.5 shows $E_{EN,}$ which is derived from 2 sources being the fluctuating electron co-ordinates and fixed nuclear co-ordinates.

$$(\mathbf{H_{el}} + \mathbf{V_{NN}})\,\boldsymbol{\psi}(\mathbf{r_{el}}) = \mathbf{E_{EN}}\boldsymbol{\psi}(\mathbf{r_{el}}) \qquad \text{(Eq 3.2.7)}$$

Electronic motion is best described by the electronic Schrödinger equation. This approximation is more accurate when it is applied to ground electronic states. Assessment of equilibrated states is

only possible once the equation has been solved. The potential energy surface curve also can be now constructed [45,46].

3.2.3 Potential Energy Surface

The potential energy surface is an effective mathematical representation between molecular vibrational motions of a molecule, along with its geometry and its nuclear probability distribution by finding solutions to the time-dependent Schrödinger equation. This concept is birthed from the Born-Oppenheimer approximation elucidated above, where electrons differ according to the positional states of the nuclei in a manner such that the potential energy surface is taken as the potential of atoms to collide with each other in a molecule [47,48]. A potential energy surface displays high potential energy regions, indicating high-energy nuclear arrangements or molecular conformations and low energy regions indicating low nuclear energy conformations. The potential energy surface is utilized in computational chemistry to analyse the lowest energy state and the positional geometry of a molecule at this state [49,50].

The potential energy function is thus described as follows:

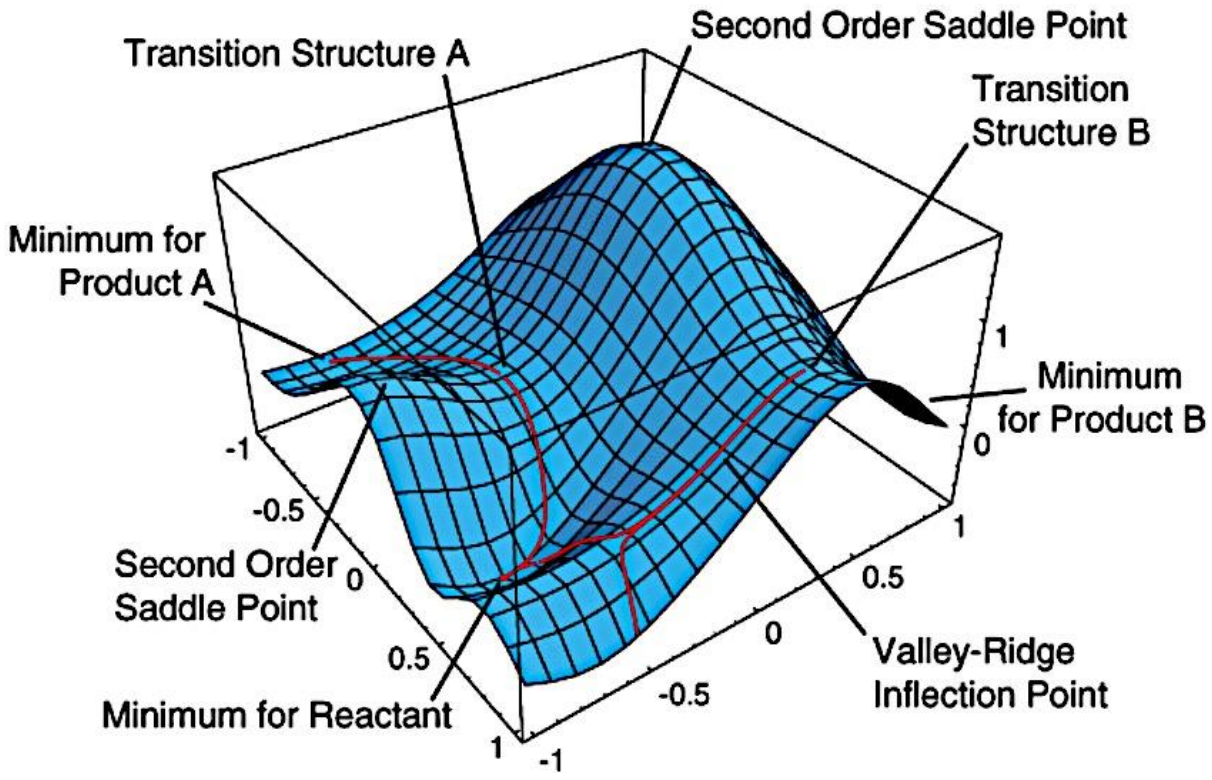

Figure 3.2: Graphical representation of a 2-D potential energy surface (PES)

3.3 Molecular Mechanics

Molecular mechanics (MM) or force-field methods use classical type models to predict the energy of a molecule as a function of its confirmation. This involves predictions of equilibrium geometries and transition states, relative energies between conformers or between different molecules [51,52].

Molecular mechanics is a computational method that computes the potential energy surface for a specific order of atoms by the use of potential functions that are obtained using classical physics [53,54]. It is based on the following assumptions:

1) Electrons are treated around a nucleus that is a perfect sphere.
2) The bonds between molecules are treated as springs.

3) Potential functions are dependent on experimental parameters such as force constants and equilibrium values.

4) The potential energy function is the sum of individual functions for bond stretching, angle bending, torsional energies, and non-bonding interactions.

Molecular mechanics requires less of a computer than QM methods. It is however restricted by certain parameters of equations such as the different force-field for different types of atoms. Moreover, it is inapplicable for electronic properties [5556].

The potential functions generated by molecular mechanics have no absolute meaning. They are merely for comparing different configurations of a molecule. Molecular mechanics can be beneficial for large molecules such as proteins. Its prime use is mainly in the field molecular dynamics [57]. MM calculations are often referred to as force field calculations [58].

3.3.1 Potential Energy Function

Atoms are known as the monads in force field methods whereas electrons are not considered as single entities. This implies that rather than finding solutions to the Schrödinger equation, explicit bonding information must be provided [59]. Molecules are described by a "ball and spring" analogy, with atoms of different sizes and bonds of different lengths. It was detected that different molecules might have structural similarity due to the atoms they are made up of. The notion atom types was then coined, and is dependent on the atomic number and chemical bonding fixating it in place [6061]. The potential energy function (PEF)/force field of a molecular system may be assembled in terms of a set of force field energy equations that are solely based on Newtonian classical physics. These equations calculate the energy of the system as well as the atom types that construct the molecule [62].

The following equations best describe the sum of all individual molecular components that make up the total potential energy:

1. Bond stretching (between directly bonded atoms)

$$\mathbf{E_r} = \sum \mathbf{K_r}(\mathbf{r} - \mathbf{r_0})^2 \qquad \text{(Eq 3.3.1)}$$

2. Angle bending (atoms bounded to same central atoms)

$$\mathbf{E_\theta} = \sum \mathbf{K_\theta}(\boldsymbol{\theta} - \boldsymbol{\theta_0})^2 \qquad \text{(Eq 3.3.2)}$$

3. Bond torsion

$$\mathbf{E_\phi} = \sum \mathbf{K_\phi}[\mathbf{1} + \mathbf{cos}(\mathbf{n}\boldsymbol{\phi} - \boldsymbol{\phi_0})] \qquad \text{(Eq 3.3.3)}$$

4. Non-bonded interactions (van der Waals and electrostatic)

$$\mathbf{E_{nb}} = \left[\sum\sum\left(\frac{\mathbf{A_{ij}}}{\mathbf{r_{ij}}^{12}} - \frac{\mathbf{B_{ij}}}{\mathbf{r_{ij}}^{6}}\right)\right] + \left[\sum\sum\left(\frac{\mathbf{q_i q_j}}{\mathbf{D r_{ij}}}\right)\right] \qquad \text{(Eq 3.3.4)}$$

Where: K_r, K_θ, K_ϕ are force constants for bond, angle, and dihedral angle and r_o, θ_o, ϕ_o are the equilibrium distance, angle and phase angle. Parameter r_{ij} is distance, while A_{ij} and B_{ij} are van der Waal parameters. D is the molecular dielectric constant; q_i and q_j are charge points.

3.3.2 Force Field

In order to describe the time progression of bond lengths, bond angles and torsions, including the non-bonding van der Waals and electrostatic interactions between atoms, one uses a force field. The force field is a collection of equations and associated constants designed to reproduce molecular geometry and selected properties of tested structures [63].

The MM force field was first formulated during the 1970s [59]. A force field is a mathematical function with a delineated set of parameters. These parameters describe the rapport of molecular energy systems to specific particle co-ordinates [64]. Commonly used biomolecular force fields include: AMBER [65], CHARM [66], NAMD [67], GROMOS [68] and OPLS-AA [69]. All force field parameters are obtained from either experimental data sources, ab initio or semi-empirical QM [59].

Force field functions are assumed to be wholly dependent on atomic orientations. They have been used as representatives for describing potential energy surface for different types of molecular systems with varying degrees of freedom [70]. This force field function must bring depth into understanding the specific forces acting within the molecular system. In this thesis, the AMBER14 force field was implemented to characterise the enzymes, whilst GAFF was used to interpret the ligands [71]. The AMBER14 force field provides a favourable balance in energy between the helical and extended regions of the peptide and protein backbones with improved dihedral torsions [72].

3.4 Molecular Dynamics

Molecular dynamics (MD) is a computer simulation process for analysing the kinetics of atoms and molecules. Properties of assemblies of molecules in terms of their structure and the microscopic can be expounded using MD simulations. In MD, complex systems are modelled at atomic level and the equations of motion are mathematically solved to indicate the time of evolution of a specific system, thus allowing a derivation of its kinetic energy and thermodynamic properties through the application of computational tools [49,73,74]. Atomic trajectories are generated through the integration of Newton's equations of motion for atoms on an energy surface. This can be illustrated by the equation below:

$$\mathbf{F_i} = \mathbf{m_i}\frac{\mathbf{d^2 r_i(t)}}{\mathbf{dt^2}} \qquad \text{(Eq 3.4.1)}$$

Where r_i (t) is the particle position vector, t is time-evolution, m is the mass of the particle and F_i depicts the interacting force on the particle.

Molecular dynamics can be categorized into 4 continuous technical steps that are repeated numerously to generate a trajectory. The categories can be summarized by:

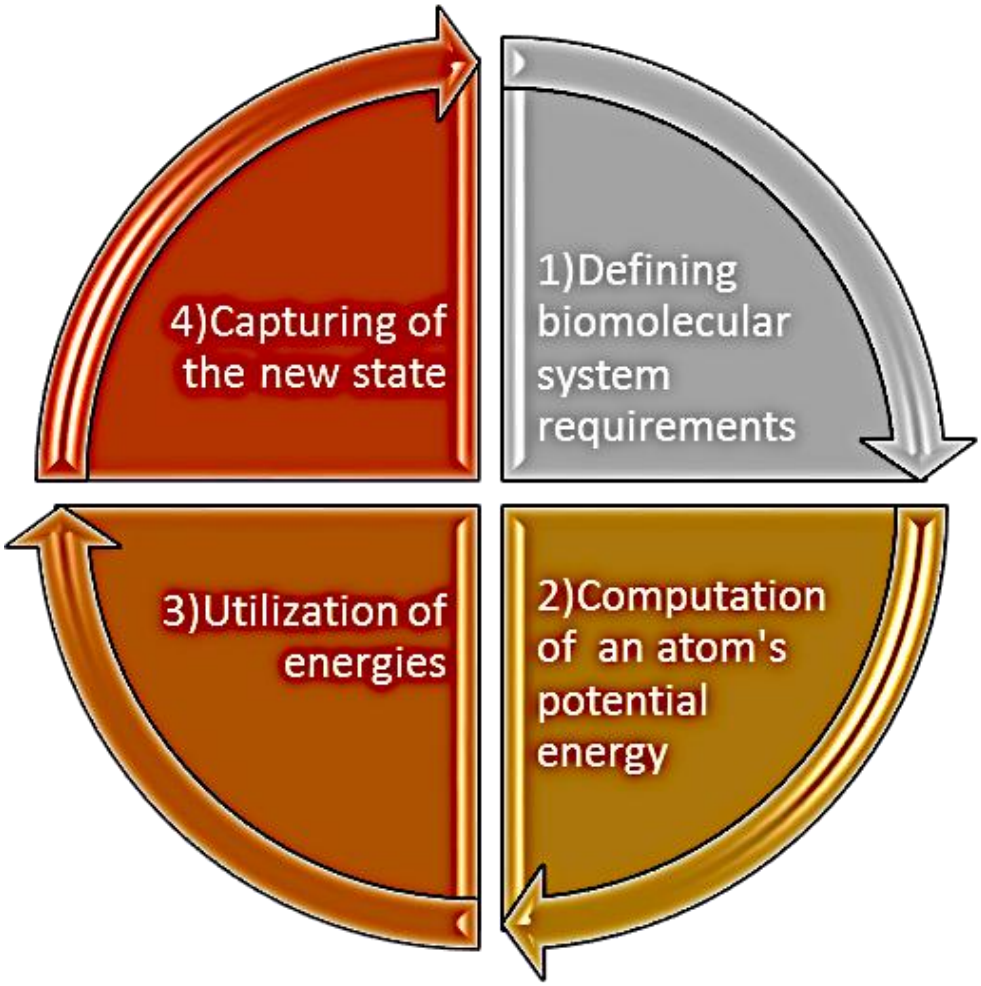

Figure 3.3. Cycle of molecular dynamics steps

The steps are outlined below:

1) The fundamental requirements of the biomolecular system are defined:
    - The co-ordinates of each atom
    - The bond characteristics between each atom
    - The acceleration of atoms

2) Each atom's potential energy is computed.
3) The energies from step are then utilized to solve the equation of motion.
4) The new state of the system needs to be saved, and the atoms' co-ordinates changed, ad step forward in the simulation taken. The cycle then restarts from step 1.

3.4.1 Molecular Dynamics Post-Analysis

Molecular post-dynamic techniques and calculations have been used to describe the protein features in studies conducted in this thesis. Molecular dynamic trajectories are a product of the run molecular dynamic simulation. They can be defined as sequential snapshots characterized by both positional co-ordinates and velocity vectors [74,75]. In this study, post dynamic analysis of the trajectories is critical for determining the:

1) Energetic and conformational stability of the biomolecular system.
2) The characteristics of the system's small molecule binding landscape and the thermodynamic energy fluctuations along the system's clustered trajectory.
3) Dynamic conformational features or variability of the biomolecular system.

3.4.1.1 System Stability

Convergence:

Convergence is an empirical description of protein dynamics. It specifically describes protein dynamics based on bond types and bond angle vibrations during the unfolding of a protein. This fusion toward equilibrium and portrayal of a conclusive plateau is impertinent for a MD trajectory to be accurate and reproducible. At this plateau, the protein-ligand system displays energetically stable conformations [76].

Root Mean Square Deviation (RMSD):

Spatial difference between two static structures of the same trajectory measure the deviation of a complex [77]. The RMSD of a trajectory is defined as:

$$\mathbf{RMSD} = \left(\frac{\sum_{\mathbf{N}}(\mathbf{R_i} - \mathbf{R_1^0})^2}{\mathbf{N}}\right)^{\frac{1}{2}} \qquad \text{(Eq 3.4.2)}$$

Root Mean Square Fluctuation (RMSF):

The root mean fluctuation (RMSF) of a protein measures residue's Cα atom fluctuations is based on the average protein structure along the system's trajectory. The RMSF captures the fluctuation for each atom around its average position [78,79]. It is calculated using the equation below:

$$\mathbf{sRMSF} = \frac{(\mathbf{RMSF_i} - \overline{\mathbf{RMSF}})}{\boldsymbol{\sigma}\,(\mathbf{RMSF})} \qquad \text{(Eq 3.4.3)}$$

Where: $RMSF_i$ is the RMSF of the i^{th} residue, from which the average RMSF is subtracted. This is then divided by the RMSF's standard deviation to yield the resultant standardized RMSF.

3.4.1.2 Binding Free Energy

Calculations of binding energy are crucial in computational chemistry studies as they are the end point method that accounts for the ligand-receptor interactions. Approximations of binding free energy leads to the development of various algorithms including energy perturbations, thermodynamic integration, molecular docking calculations amongst many.

The Molecular Mechanics/Generalized Born Surface Area method (MM/GBSA) methods have been regarded as the most accurate and efficient in estimating binding free energies for biological macromolecules [80]. The free binding energy () computed by these methods for a protein system which comprises of the complex, ligand and receptor can be represented as:

$$\Delta\mathbf{G_{bind}} = \mathbf{G_{complex}} - \mathbf{G_{receptor}} - \mathbf{G_{ligand}} \qquad \text{(Eq 3.4.4)}$$

$$\Delta\mathbf{G_{bind}} = \mathbf{E_{gas}} + \mathbf{G_{sol}} - \mathbf{TS} \qquad \text{(Eq 3.4.5)}$$

$$\mathbf{E_{gas}} = \mathbf{E_{int}} + \mathbf{E_{vdw}} + \mathbf{E_{ele}} \qquad \text{(Eq 3.4.6)}$$

$$\mathbf{G_{sol}} = \mathbf{G_{GB/PB}} + \mathbf{G_{SA}} \qquad \text{(Eq 3.4.7)}$$

$$\mathbf{G_{SA}} = \boldsymbol{\gamma}\mathbf{SASA} \qquad \text{(Eq 3.4.8)}$$

The term Egas denotes the gas-phase energy, which consist of the internal energy Eint; Coulomb energy Eele, and the van der Waals energies Evdw. The Egas was directly estimated from the FF14SB force field terms. Solvation free energy, Gsol, was estimated from the energy contribution from the polar states, GGB and non-polar states, G [81,82].

3.5 Pharmacophore generation

The principle behind computer-aided molecular design methods is to curtail the costs associated with discovery and development of potential drugs. One way this can be achieved is by the formation of a pharmacophore [83]. A pharmacophore model is a geometrical description of the chemical functionalities that are mandatory in order for a ligand to interact with the receptor. Paul Ehrlich birthed the idea of a pharmacophore in the 1800s. At that time, it was established that specific chemical groups were liable for certain biological effects, and that molecules with similar effects had similar functions in common. Schueler coined the word pharmacophore in 1960. He defined a pharmacophore as "a molecular framework that carries (*phoros*) the essential features responsible for a drug's (*pharmakon*) biological activity [84,85]. The definition shifted from chemical groups to patterns of abstract features. The International Union of Applied Chemistry decreed a definition that still stands today;

A pharmacophore is the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger or block biological response [86]. A pharmacophore therefore does not represent a real molecule or a set of chemical groups, but is rather an abstract concept [87]. The pharmacophore concept has become a vital tool in Computer-Aided Drug Design. Atoms or functional groups in a molecule that exhibit specific features that are related to molecular recognition can be reduced to a pharmacophore feature [88–90].

3.6 Cheminformatics Tools Utilized in this Study

The focal point of cheminformatics is to analyze, simulate and manipulate chemical information in order to accelerate decision making and reduce expenses in the areas of drug lead identification and optimization in the process of drug discovery [91]. Cheminformatics, therefore seeks to fill the void between surging DENV case reports and absence of antivirals. This study adopts cheminformatics and bioinformatics tools in a quest to map out an inhibitor against the most pathogenic DENV *flavivirus* serotypes 2 and 3. By the use of bio-computational techniques, comprehensive informational data will be provided, which will allow for the identification and design of inhibitors against DENV infections.

3.7 Other Molecular Modeling Tools Used in this Study

Molecular Docking

Molecular docking has become a fundamental tool in the drug discovery industry. It employs multiple methods to predict the binding affinity and configuration of a complex. Most general use of docking is in ligand-receptor complexes, although other uses are documented. There are two major steps in docking:

1. Sample conformations of a ligand in the active site of protein.

   Different algorithms may be used when sampling the numerous conformations of the docked complex. This can be the "lock and key" model which describes the ligand and receptor as rigid structures, or the ligand may reflect flexibility by random or simulation-based methods. The latter algorithm is the frequently used method as it permits a more realistic fit of the ligand to the protein [92].

2. Ranking the different conformations by scoring function.

   The scoring function may be based on statistically preferred contacts, MM force fields or pre-existing protein-ligand binding affinities [92].

Molecular docking comes with many inconsistencies. Docked compounds are often criticized due to incorrect binding sites or choice of docked complex. Due to these concerns, all docked complexes in this study were verified with MD simulations and the stability of the ligand at the active site was demonstrated.

3.7 References

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CHAPTER 4

An "All-in-one" Pharmacophoric Architecture for the Discovery of Potential Broad Spectrum Anti-flavivirus drugs

Abstract

Background: A precipitous increase in the number of *flaviviral* infections has been noted over the last five years. Despite these outbreaks, treatment protocols for infected individuals remain ambiguous. Numerous studies have identified NITD008 as a potent *flavivirus* inhibitor, however, clinical testing was dismissed due to undesirable toxic effects.

Methodology/Results: The binding landscape of NITD008 in complex with five detrimental *flaviviruses* at the RNA-dependent RNA polymerase active sites were explored. An "all-in-one" pharmacophore model was created for the design of small molecules that may inhibit a broad spectrum of *flaviviruses*.

Conclusions: This pharmacophore model approach serves as a robust cornerstone, thus assisting medicinal experts in the composition of multifunctional inhibitors that will eliminate cross-resistance and toxicity, and enhance patient adherence.

Keywords:

Flaviviridae, pharmacophore model, Free-binding energy, binding landscape, NITD008, RdRp

Introduction

Flaviviruses have triggered an abrupt global health concern in the medical field over recent years. *Flaviviruses* come from the family *Flaviviridae*[1, 2] and are characterized as RNA viruses[3, 4]

that are mostly spread by arthropods like mosquitoes and ticks, and are therefore termed arboviruses [5, 6]. Examples include the notorious Zika virus (ZIKV) that is transmitted by the *Aedes* mosquito, West Nile virus (WNV), Dengue virus (DENV), Japanese Encephalitis virus(JEV) and Hepatitis C virus (HCV) [7–9].

The lack of antiviral therapies amplifies the expanding threat of these *flaviviruses* in public health [10]. There are currently no established drugs for treating *flavivirus* diseases [11]. The treatment plans are often empiric and border around symptomatic management [12]. Scientists are faced with the mammoth task of finding chemical compounds that can not only cure, but also prevent transmission of *flaviviruses.*

Computer-aided drug discovery methods play a major role in the development of therapeutic molecules in the medical field [13, 14]. The computational method of designing drugs enables the binding properties of a drug to be predicted before it is synthesized thus saving enormous time and cost [15–17]. Computational methods will be employed in this study to identify a potential viral inhibitor that may aid in the production of a multipurpose antiviral agent against numerous *flaviviruses.*

Despite the efforts scientists have put into the research of *flaviviruses*, there is an absence of drugs that can cure infections caused by *Flaviviruses* [2, 18]. The dearth of antiviral therapy warrants the development of a treatment strategy against *flaviviruses*. It is therefore imperative that more researchers delve into finding antiviral treatments that cure *flavivirus* infections.

In this study, computer-assisted approaches will be used to design a pharmacophore model based on the binding landscape and structure activity relationship between potent inhibitor, NITD008 [11, 19, 20], in complex with five well established *flaviviruses* being WNV, DENV, JEV, HEPC and the ZIKV. This pharmacophore model approach will be able to assist medicinal chemists in the design of small molecules against a wide range of *flaviviruses*, including the re-emerging Zika virus, to which there are currently no available FDA approved inhibitors [21–23]. This approach may also eliminate common drug design challenges, including cross-resistance and toxicity.

Computational Methods

The computational approached adapted in this study was outlined in **Figure 4.1**

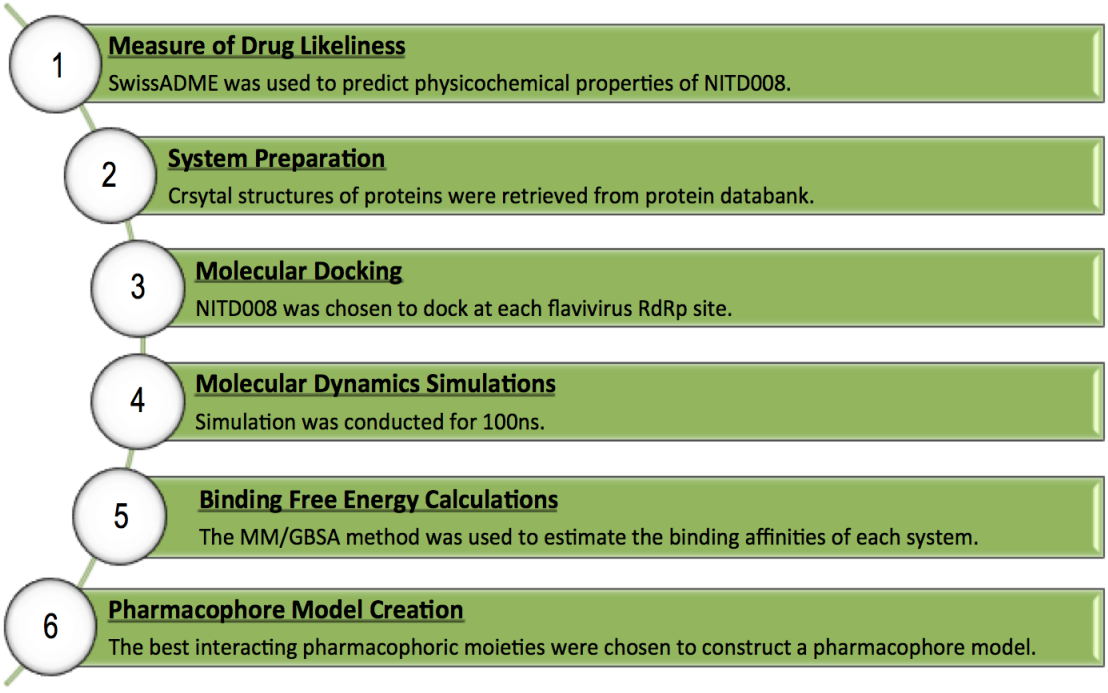


Figure 4.1: Computational Methods

Measure of Drug Likeliness

The online software SwissADME was used to determine the physicochemical descriptors and define the pharmacokinetic properties and drug-like nature of NITD008. The "Brain Or Intestinal Estimated permeation, (BOILED-Egg)" method was utilized as it computes the lipophilicity and polarity of small molecules [24].

System Preparation

The Crystal structures of the RNA-dependent RNA polymerase proteins of WNV (PDB code:2HFZ)[25], DENV (PDB code:3VWS)[26], JEV (PDB code:4K6M)[27], HCV (PDB code:4WTL)[28] and the ZIKV (PDB code:5WZ3)[29] were retrieved from Protein Data Bank[30]. The crystal structures were then super-imposed and all additional ligands were removed using the CHIMERA software [31].

Molecular Docking of Experimental Flaviviruses

The potent ATP analog, NITD008, was then chosen to dock at each of the *flavivirus* RdRp sites [19, 32, 33]. AutoDock Vina software was used for docking of the compound[34]. The software default settings were used to run the procedure. The grid box used to characterize the screening site was demonstrated using the AutoDock Vina functionality which is incorporated into Chimera [31]. The grid box size and center parameters of the RdRp grid box dimensions were x(18.09, 18), y(16.80, 52.9), z(13.75, 12.16). Results from AutoDock Vina were produced in the pdbqt format and the optimal geometric conformation having the best binding energy was selected[34] from the ViewDock feature and saved in complex with the reference enzymes. The enzyme and ligand for

each system was prepared using Chimera[31] and MMV molecular modeling suites[35] and subsequently subjected to molecular dynamic simulations [36].

Molecular Dynamic Simulations

The molecular dynamic(MD) simulation was carried out using the GPU version of the PMEMD engine provided with the AMBER 14 package [37]. The FF14SB force field which is a component of the AMBER package[38] was used to describe the complex.

The Restrained Electrostatic Potential (RESP) and the General AMBER Force Field (GAFF) systems were used by ANTECHAMBER[39] to generate the atomic partial charges for the ligands [40]. The Leap module of AMBER 14[41] facilitated the addition of hydrogen atoms to the systems, and counter ions (Na^+ and Cl^-)for neutralization.

Minimization was done in two stages. The first stage of minimization was carried out with 2000 steps with a restraint potential of 150 kcal/mol $Å^2$. A full minimization of 1000 steps was then carried out by conjugate gradients algorithm with no restrain.

The MD simulation heating was executed gradually from 0 K to 300 K for 50 ps thus allowing the system to maintain a fixed number of atoms and fixed volume. The solutes inside the system were set with a potential harmonic restraint of 10 kcal/mol Å 2 and collision frequency of 1.0 ps^{-1}. After heating, the system was equilibrated for an estimated time of 500 ps with the operating temperature being kept constant at 300K. An isobaric-isothermal ensemble (NPT) atmosphere was created by keeping constant features such as the number of atoms and pressure.

The MD simulation was conducted for 100ns. In each simulation, the SHAKE algorithm played a vital role in the constriction of the bonds of hydrogen atoms. Each simulation step took 2 fs and an SPFP precision model was used. The simulations coincided with isobaric-isothermal ensemble (NPT), with randomized seeding, constant pressure of 1 bar maintained by the Berendsen barostat, a pressure-coupling constant of 2 ps, a temperature of 300 K and Langevin thermostat with collision frequency of 1.0 ps^{-1}.

Coordinates were saved every 1 ps. The PTRAJ module employed in AMBER14 [37] was used to analyze the trajectories.

Binding Free energy Calculations

The Molecular Mechanics/GB Surface Area method (MM/GBSA) [42] was employed for the calculation of binding free energies. This was done to estimate the binding affinities of each system. Binding free energies were then averaged over 10 000 snapshots extracted from the 100ns trajectory. The following equations depict free binding energy (ΔG) which was computed by the MM/GBSA method for each molecular species (complex, ligand and receptor):

$$\Delta G_{bind} = G_{complex} - G_{receptor} - G_{ligand} \quad (1)$$

$$\Delta G_{bind} = E_{gas} + G_{sol} - TS \quad (2)$$

$$E_{gas} = E_{int} + E_{vdw} + E_{ele} \quad (3)$$

$$G_{sol} = G_{GB} + G_{SA} \quad (4)$$

$$G_{SA} = \gamma SASA \quad (5)$$

The term Egas denotes the gas-phase energy, which consist of the internal energy Eint; Coulomb energy Eele, and the van der Waals energies Evdw. The Egas was directly estimated from the FF14SB force field terms. Solvation free energy, Gsol, was estimated from the energy contribution from the polar states, GGB and non-polar states, G. The non-polar solvation energy, SA.

Pharmacophore Model Creation

Simulation of the inhibitor, NITD008, occurred at the active site of RdRp, for a period of 100ns. This allowed for the bound conformation of the ligand to be created. Per-residue energy decomposition analysis [43, 44] was then used to derive the amino acids that greatly participated towards the binding of the ligand. The pharmacophoric moieties that significantly interacted with the most contributing residues were selected to construct our model. The model was then added to ZincPharmer[45] and LigandScout[46] to generate and validate the pharmacophore model.

Results and Discussion

Assessment of NITD008's Drug-likeness

NITD008 has a plethora of cytotoxic and cardiovascular side effects due to its vasodilatory characteristics [32, 33]. This proves to be a challenge when trying to inhibit multiple *flaviviruses* without the manifestation of physiological adverse effects. The online software SwissADME was utilized to compute the physicochemical descriptors as well as predict the pharmacokinetic properties and drug-like nature of NITD008. SwissADME utilizes the "Brain Or Intestinal Estimated permeation, (BOILED-Egg)" method which computes the lipophilicity and polarity of small molecules [24].

NITD008								
Molecular Formula	***Molecular Weight (g/mol)***	***Lipophilicity (iLOGP)***	***Water Soluble***	***GIT Absorption***	***BBB Permeability***	***Bioavailability Score***	***Synthetic Accessibility***	***Druglikeness (Lipinski)***
C13H14N4O4	290.27	1.56	Yes	Low	No	0.55	4.09	Yes

"Boiled-Egg" Method Summary

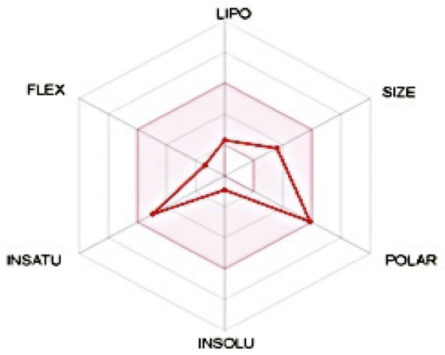


Figure 4.2: Swiss ADME profile of NITD008

Drugs designed for human use should have an ideal balance of pharmacokinetics and safety, as well as potency and selectivity. Human oral bioavailability is a paramount pharmacokinetic property [47]. Lipophilicity, or LogP, influences a compound's behavior in biological processes pertinent to drug discovery such as solubility, permeability or hepatic clearance. A LogP value of between 2 and 3 is more favourable to achieve permeability and first-pass clearance [48]. This optimal LogP value is not observed in NITD008 as it falls below the above-stipulated range. It may also be worthwhile to mention that although NITD008 is available as an oral drug, the gastro-intestinal absorption is low. This may decrease its inhibitory activity as a large quantity of the drug may be excreted.

During 2016, influxes of feotal ZIKV-related microcephaly cases were evidenced [6, 49]. Studies showed that the ZIKV could pass the blood-brain-barrier (BBB) and infect neuronal cells, thus

requiring an inhibitor to cross the BBB [50–52]. NITD008 has however, been predicted not to pass the BBB, warranting the need for its optimization.

Docking of NITD008 with RdRp of Multiple Flaviviruses

NITD008 is an adenosine analogue that has shown potent antiviral activity against *Flaviviruses* [11, 19, 33]. In this study, NITD008 was docked with five RdRp complexes: DENV, HEPC, JEV, WNV and ZIKV. Figure 4.3 illustrates the binding affinity of the five docked complexes.

Molecular docking is extensively utilized bioinformatics tool as a mode to predict the binding affinity and orientation of a ligand when it binds to a biological target. After docking, the complexes display various alignments of the ligand NITD008 within the active pocket. In as much as scoring functions endeavor to replicate experimental binding affinities, most software is not consistent in yielding the best prediction. To overcome this challenge, the five docked complexes were subjected to 100ns molecular dynamic simulations. This assisted in the validation of the binding affinity and allowed for further analysis of the enzyme's flexibility.

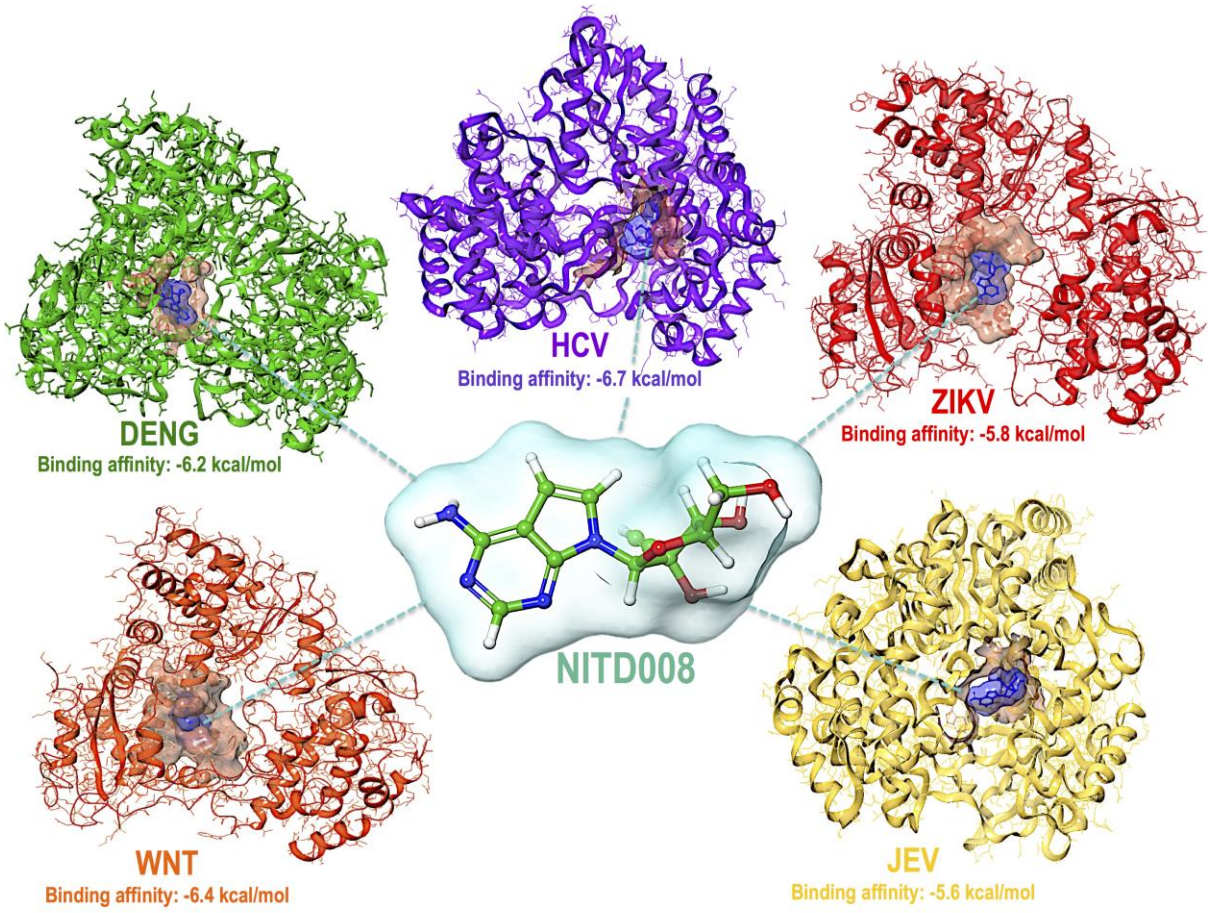


Figure 4.3: Binding affinity of NITD008-*Flavivirus* complex

System Stability and Thermodynamic binding free energy of the NITD008-RdRp Complexes

To assure the stability and thus convergence of each system, RMSD was monitored throughout the 100ns simulation and it was observed that there were no major fluctuations (stable RMSD < 2 Å) (Figure 4.4). The ligand also remained stable throughout the trajectory, thus validating the docking pose of NITD008 at the active site of the five *flaviviruses*.

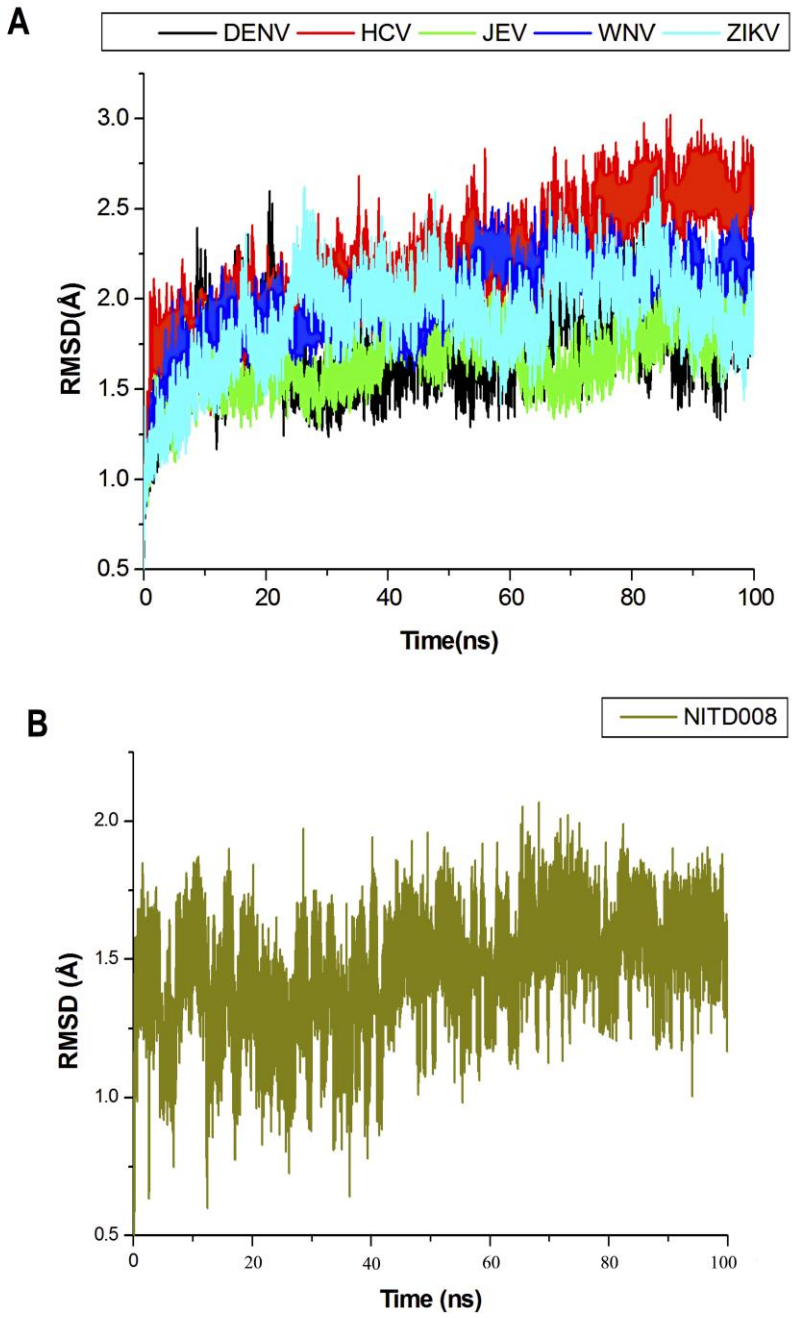


Figure 4.4: RMSD plot for the NITD008-*flavivirus* complex (A) and RMSD plot for the ligand NITD008 (B)

The RMSF plot demonstrated significant fluctuations for DENV, JEV and WNV with JEV being furthest from the reference HCV (7.830359Å). ZIKV had a more stable RMSF closer to HCV (Figure 4.5).

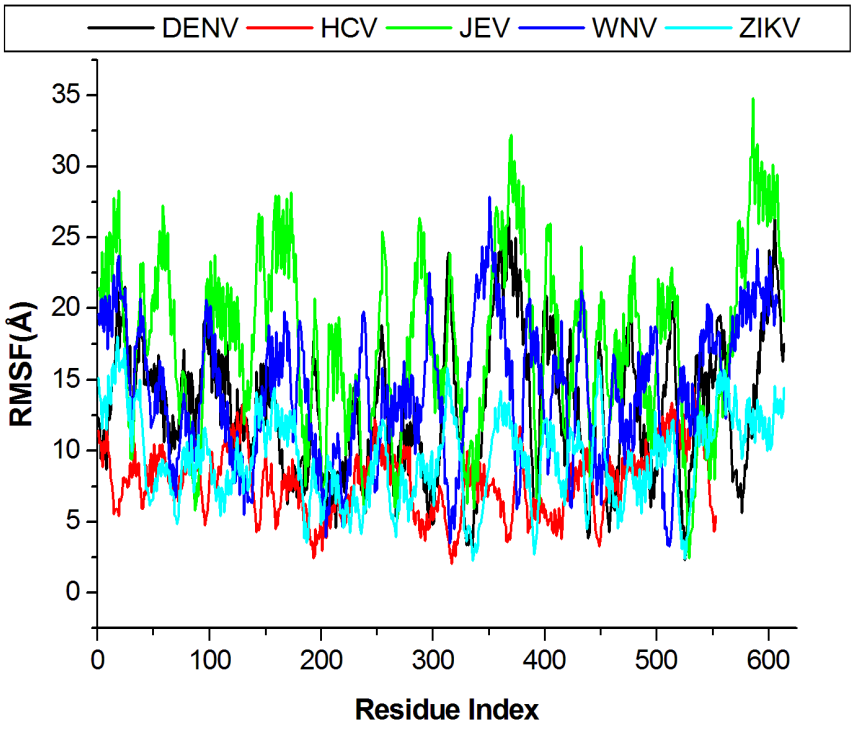


Figure 4.5: RMSF plot for the NITD008-*flavivirus* complex

The assessment of the free binding energy provided a thorough insight into the total energy contributions of each *flavivirus* complex. Table 4.1 demonstrates the binding energy of each complex when bound with NITD008. When compared to the reference complex, HCV, DENV and ZIKV shared similar trajectories. The NITD008-DENV complex had a total binding energy of -25.5361 kcal/mol, which was the closest to that of HCV (-25.2330 kcal/mol). Although ZIKV had a total binding energy of -22.2054 kcal/mol, it presented with a more stable RMSD and RMSF, exhibiting similar structural characteristics to that of HCV. The NITD008-JEV complex demonstrated the lowest binding energy (-18.8802 kcal/mol), thus portraying the least favourable

enzyme for NITD008 binding. The RMSD and RMSF of the NITD008-HEPC reference complex were stable as expected, presenting minimal residue fluctuation.

Table 4.1: Summary of free binding energy contributions of the NITD008-*flavivirus* systems

	Energy Components (kcal/mol)				
	ΔE_{vdW}	**ΔE_{elec}**	**ΔG_{gas}**	**ΔG_{solv}**	**ΔG_{bind}**
DENG	-27.1636 ± 3.8246	-39.6363 ± 15.3853	-66.7999 ± 16.5166	41.2638 ± 9.7511	-25.5361 ± 8.3310
HEP	-28.8569 ± 3.7748	-54.7969 ± 14.1195	-83.6538 ± 13.7680	58.4207± 10.0382	-25.2330± 5.3868
JEV	-31.7695 ± 5.2318	-25.1863 ± 11.3221	-56.9558 ± 12.2408	38.0757 ± 8.3342	-18.8802 ± 6.3868
WNT	-38.5150 ± 3.6551	-54.9122 ± 7.6237	-93.4273 ± 6.6148	45.6621 ± 3.9428	-47.7652 ± 4.3516
ZIKV	-23.5965 ± 4.0950	-48.3198 ± 23.0612	-71.9164± 22.3474	49.7110 ± 15.1079	-22.2054± 8.4532

Energy Contributions of the Highest Interacting Residues at the Active of NITD008

Based on these results ligand/residue interaction plots were designed to establish the influential functional groups of the ligand in each complex (Figure 4.6-4.10).

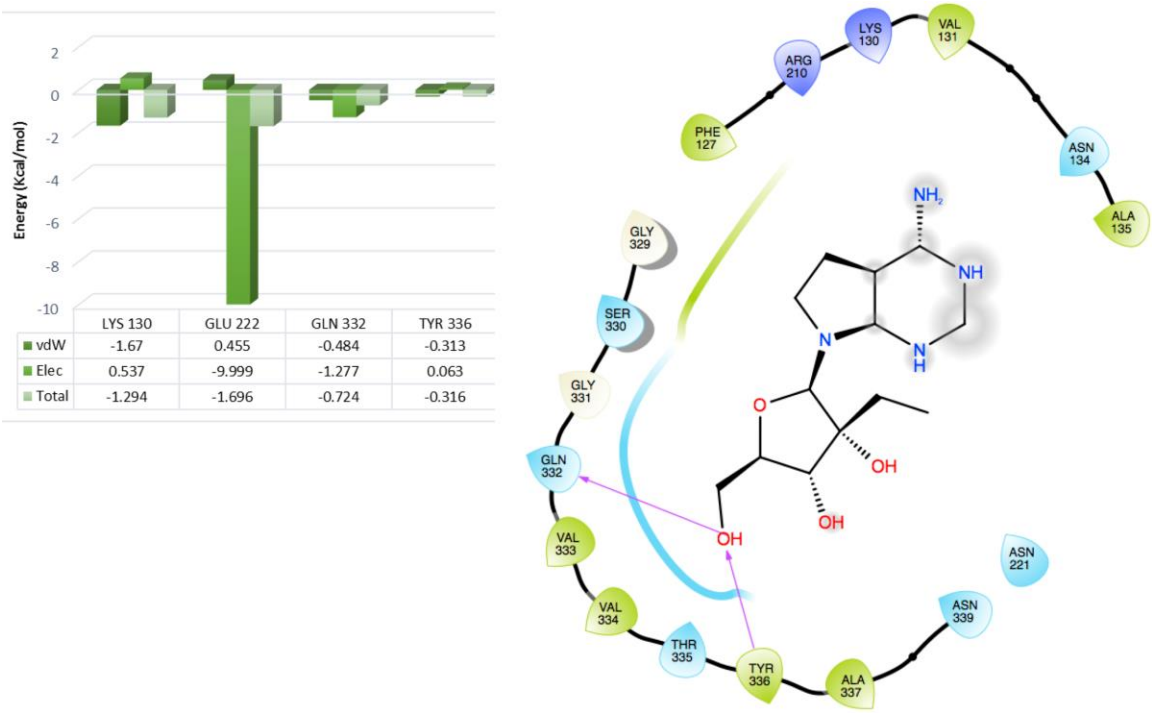


Figure 4.6: Ligand/residue interaction plot for the NITD008-DENV complex

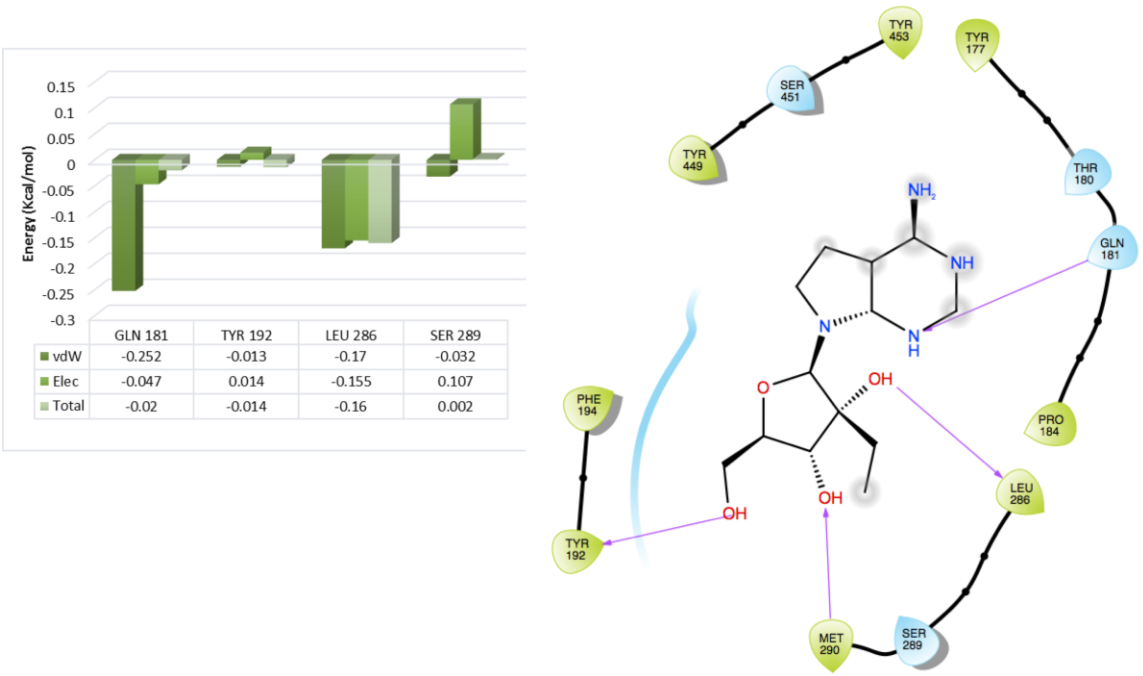


Figure 4.7: Ligand/residue interaction plot for the NITD008-HCV complex

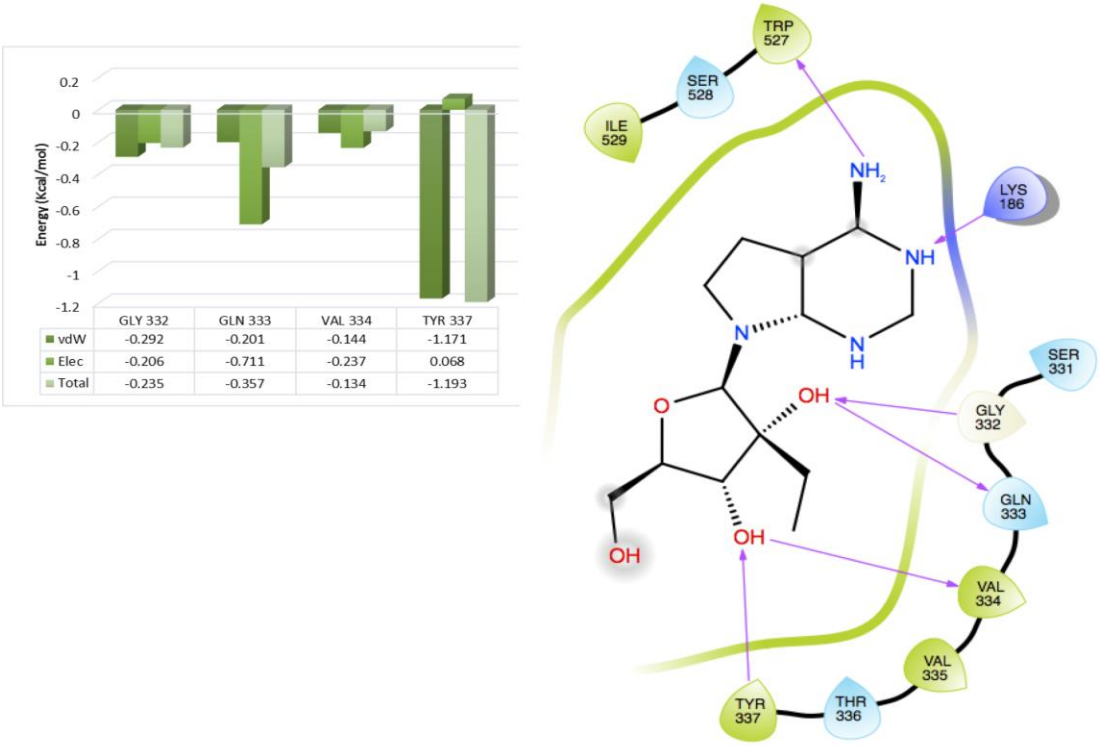


Figure 4.8: Ligand/residue interaction plot for the NITD008-JEV complex

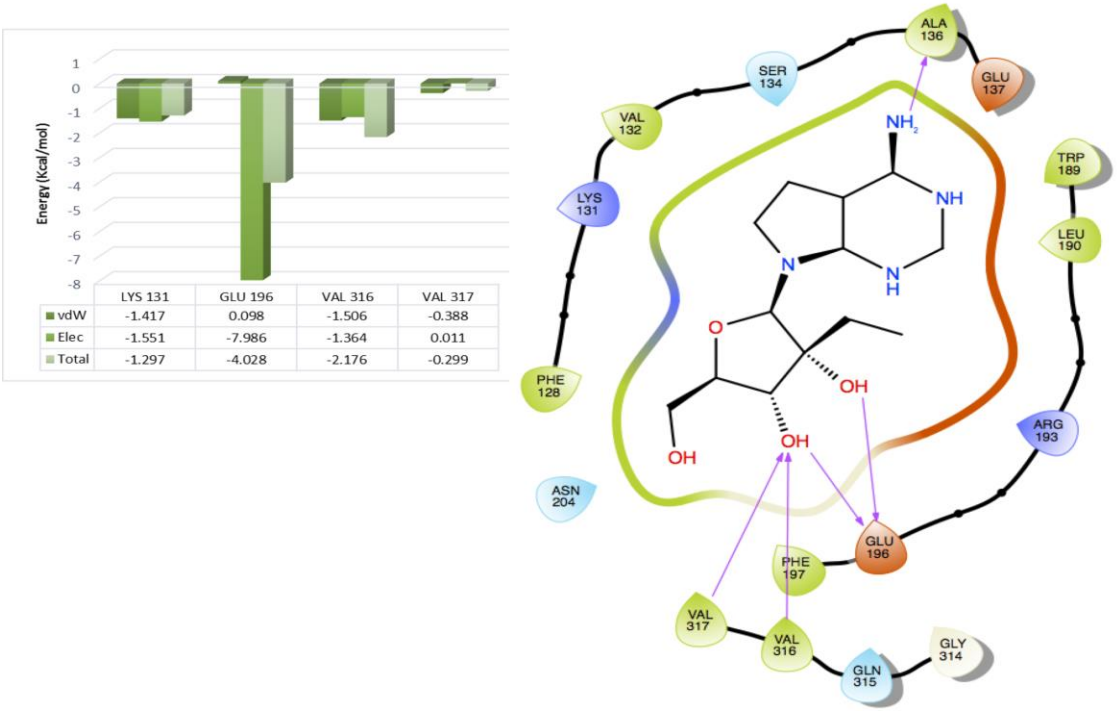


Figure 4.9: Ligand/residue interaction plot for the NITD008-WNV complex

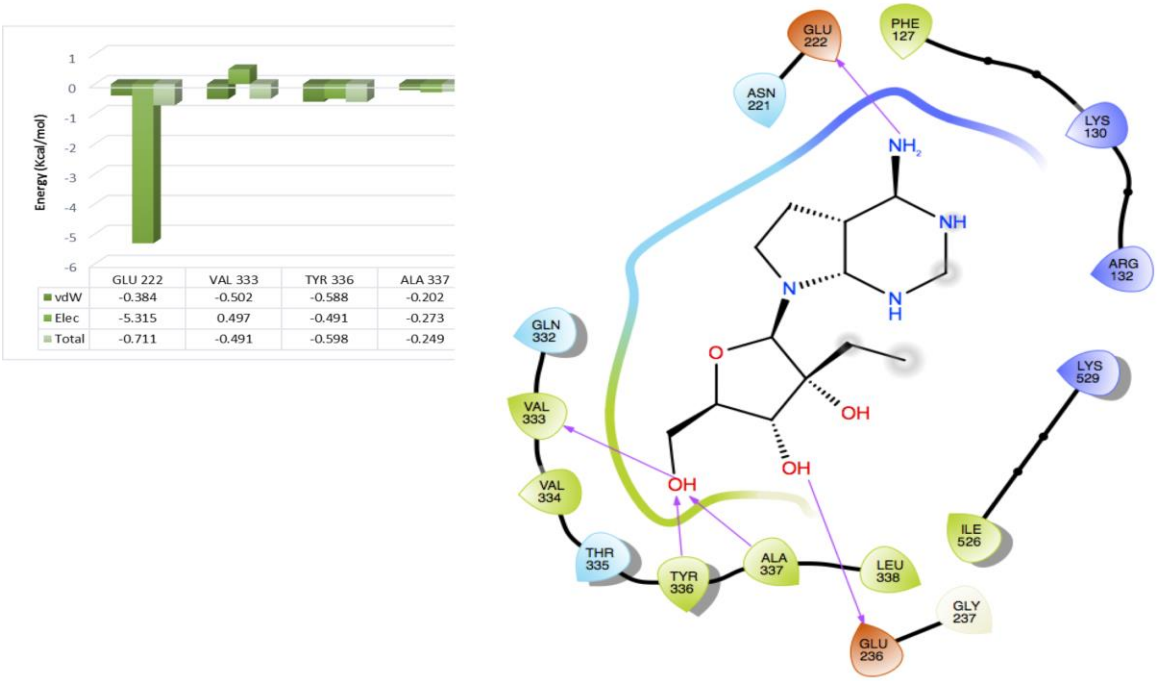


Figure 4.10: Ligand/residue interaction plot for the NITD008-ZIKV complex

Pharmacophore Model Generation

Based on the ligand-residue interaction plots as well as the active site residue energy contributions, an informative chemical structural map was developed to identify the crucial functional groups featured in each *flavivirus* system. These chemical features were then assembled to form a pharmacophore model of the ligand that could pave the road toward the design of one inhibitor to treat multiple *flaviviruses*.

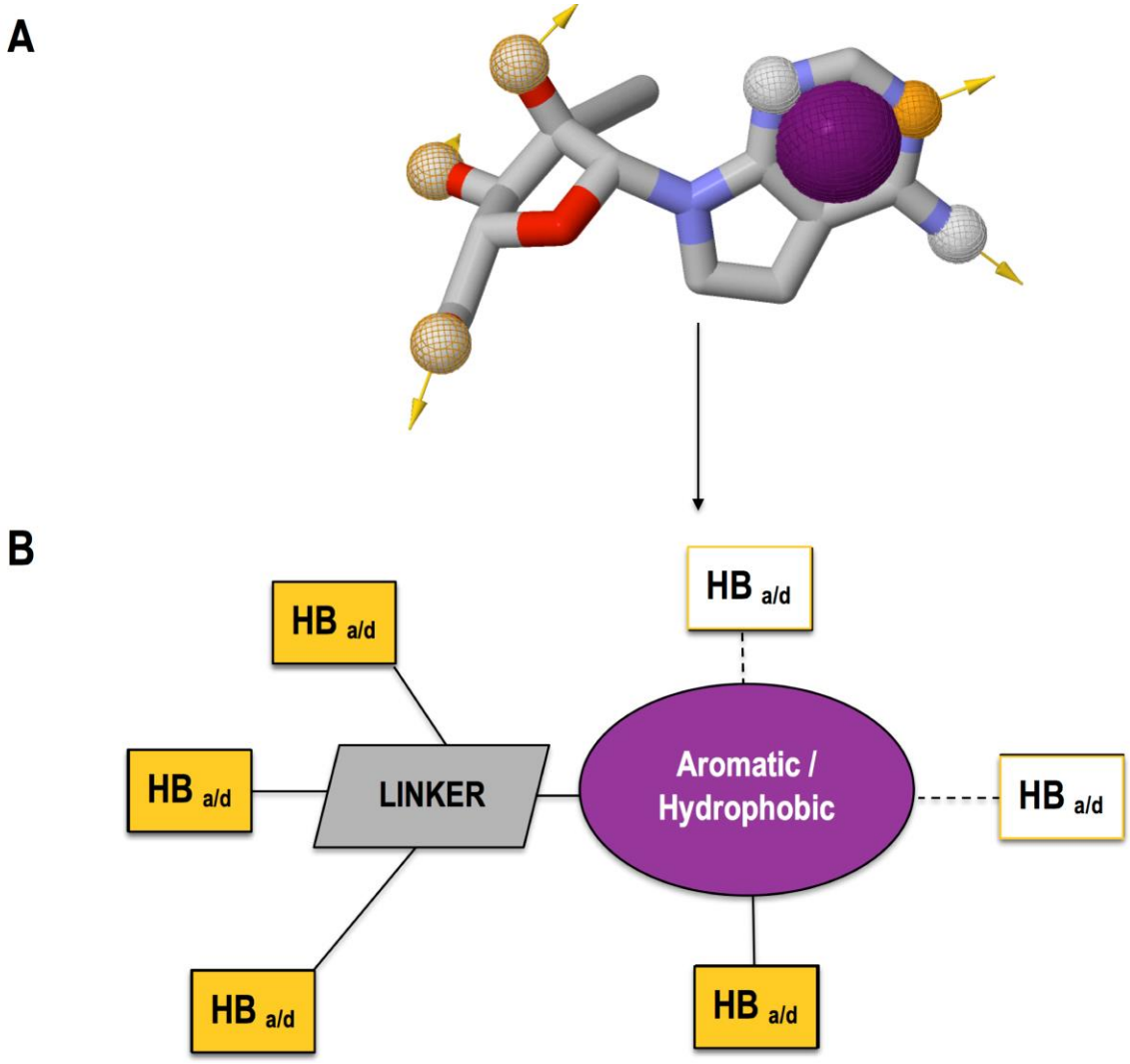
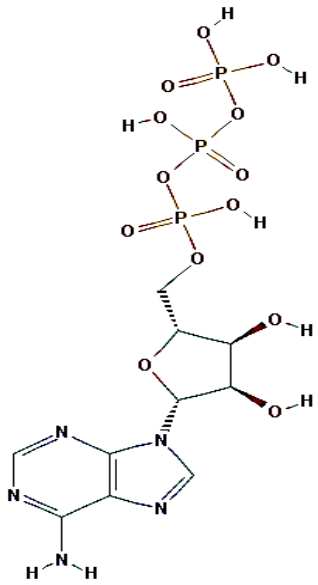
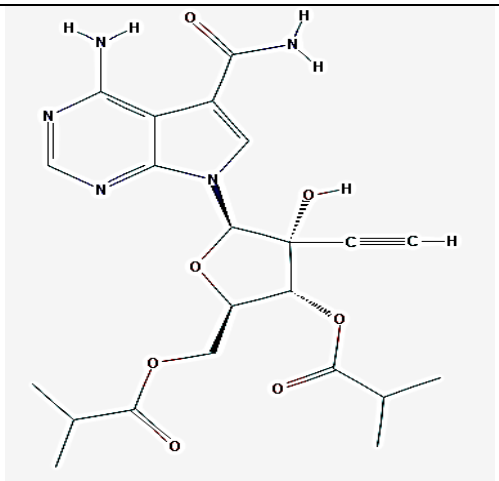


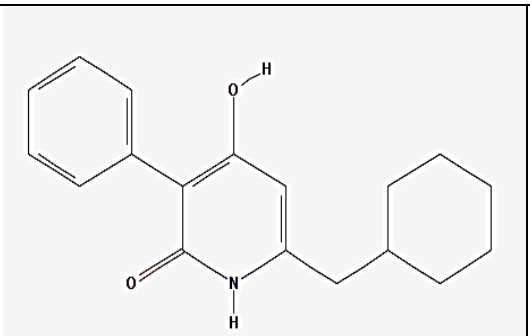
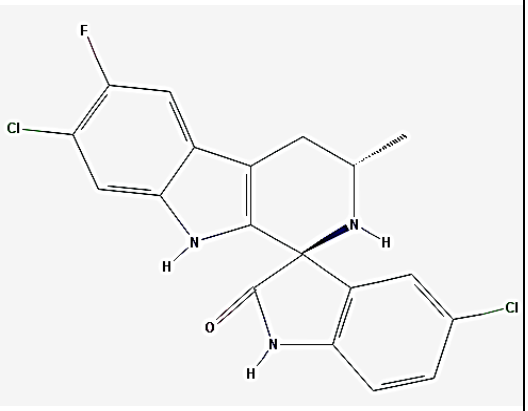
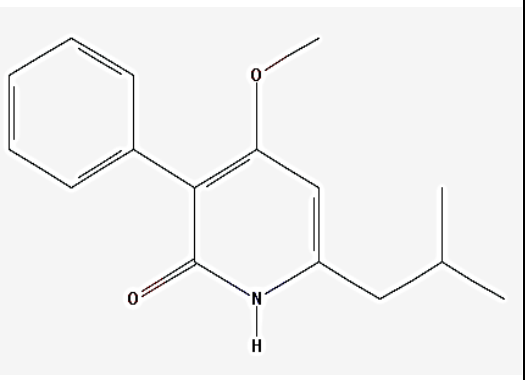
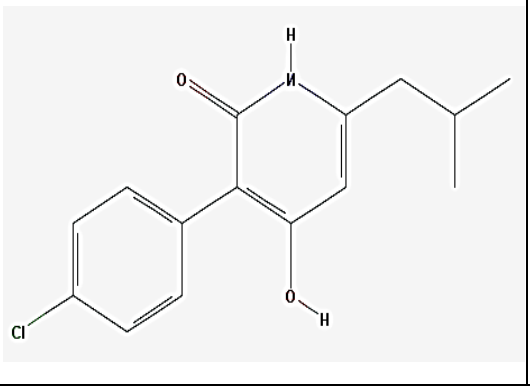
Figure 4.11: Common pharmacophoric features from alignment of the *flavivirus* ligand/residue interaction plots, (A) 3D pharmacophore model generated on ZINCPharmer (purple- aromatic moiety, gold and white- hydrogen bond donor/acceptor (B) 2D representation of the chemical features required for potential *flavivirus* inhibitors.

A pharmacophore model should not only predict the activity of the training set compounds, but it ought to be also capable of predicting the activities of other compounds as active or inactive. We validated the generated pharmacophore by docking ATP, for which NITD008 acts as a competitive inhibitor, and NITD analogs as true positives. The docking score is illustrated in the table below:

Table 4.2: Validation of Pharmacophore

TRUE NEGATIVE			
Compound Name	PubChem ID	2D structure	Docking score
ATP	5957		-5.1

TRUE POSITIVE			
Compound Name	PubChem ID	2D structure	Docking score
NITD-203	44633774		-6.4

NITD-564	76284487		-8.5
NITD-609	44469321		-6.0
NITD-560	127041113		-7.2
NITD-526	127038059		-8.6

These NITD analogs may thus be utilized as a starting point in the design of an "all in one" *flavivrus* inhibitor.

Conclusion

In the present study, a renowned *flaviviral* inhibitor (NITD008) was used to analyze the structural binding landscape of five *flavivirus* enzymes. The NITD008-*flavivirus* systems were subjected to 100ns MD simulations and the trajectories evaluated using free energy decomposition analysis (NITD008-HCV complex as a reference) [19]. Binding affinities were ranked and the results demonstrated similar binding interactions to the NITD008-HCV positive control, with the NITD008-ZIKV system portraying the greatest similarity. Furthermore, the study prompted us to map out a set of structural criteria that could be crucial for further optimization of novel *flavivirus* inhibitors. Chemical features that met the desired criteria were then assembled to form a pharmacophore model of the ligand that could pave the road toward the design of one inhibitor to treat multiple *flaviviruses*. This pharmacophore approach may assist medicinal chemists in the design of small molecules that may overcome cross-resistance and increase patient adherence.

Acknowledgements

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CHAPTER 5

Using bioinformatics Tools for the discovery of Dengue RNA-dependent RNA polymerase inhibitors

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Abstract

Background. Dengue fever has rapidly manifested into a serious global health concern. The emergence of various viral serotypes has prompted the urgent need for innovative drug design techniques. Of the viral non-structural enzymes, the NS5 RNA-dependent RNA polymerase has been established as a promising target due to its lack of an enzymatic counterpart in mammalian cells and its conserved structure amongst all serotypes. The onus is now on scientists to probe further into understanding this enzyme and its mechanism of action. The field of bioinformatics has evolved greatly over recent decades, with updated drug design tools now being publically available.

Methods. In this study, bioinformatics tools are used to provide a comprehensive sequence and structural analysis of the two most prominent serotypes of Dengue RNA-dependent RNA polymerase. A list of popular *flavivirus* inhibitors were also chosen to dock to the active site of the enzyme. The best docked compound was then used as a template to generate a pharmacophore model that may assist in the design of target-specific Dengue virus inhibitors.

Results. Comparative sequence alignment exhibited similarity between the domains of serotype 2 and 3. Sequence analysis revealed highly conserved regions between residues "401-441" and "782-809". Mapping of the active site demonstrated four highly conserved residues: lysine, glutamate, serine and tyrosine. Of the active site interacting residues, TYR 451 was common amongst all docked compounds, indicating its importance in the drug design process. Of the 10 docked *flavivirus* inhibitors, NITD-203 showed the best binding affinity to the active site. Further pharmacophore modeling of NITD-203 depicted significant pharmacophoric elements that are neccesary for stable binding to the active site.

Discussion. This study utilized publically available bioinformatics tools to provide a comprehensive framework on Dengue RNA-dependent RNA polymerase. Based on docking studies, a pharmacophore model was also designed to unveil the crucial pharmacophoric elements that are required when constructing an efficacious DENV inhibitor. We believe that this study will be a cornerstone in paving the road toward the design of target-specific inhibitors against DENV RdRp.

Introduction

There are several species under the *flavivirus* genus that continue to cause detrimental effects to infected individuals (King et al., 2007; Holbrook, 2017). One of these species is the Dengue virus (DENV), which is the causative agent of DENV fever (John, 2003; Guzman & Harris, 2015). The mosquito borne viral infection comes with severe flu-like illness when acquired (Guzman et al., 2010; Ross, 2010).

Studies have shown that approximately 3.9 billion people are prone to DENV infection (Murray, Quam & Wilder-Smith, 2013)(World Health Organization (WHO), 2016). To date, there are currently five DENV serotypes in circulation (Dar et al., 2006; Bharaj et al., 2008; Christenbury et al., 2010; Mustafa et al., 2015; Joob & Wiwanitkita, 2016). Of these serotypes, serotype 2 and 3 are the most common (Balmaseda et al., 2006; van Panhuis et al., 2010; Fatima et al., 2011). Despite the growing number of strains, the RNA-dependent RNA polymerase (RdRp) remains conserved. This RdRp non-structural enzyme also remains specific for viral replication and lacks an enzymatic counterpart in mammalian cells. This allows researchers to utilize this promising target in the design of DENV inhibitors. Although there is constant evolution in this area of research, there still remains no approved antiviral drug or vaccine specific to the RdRp region of DENV (Thomas & Endy, 2011; Lam, 2013; World Health Organization (WHO), 2016). It is therefore imperative to source RdRp-specific inhibitors that aim to put an end to the devastating effects of DENV infections.

The use of information technology has become a critical part of the drug discovery process (Hooft, Sander & Vriend, 1997; Huang et al., 2010). Bioinformatics is an emerging science that is being exploited to replace the old “hand-crafted” synthesis and testing of new

chemical entities approaches (Xu & Hagler, 2002; Chen, 2006). This has been profitable to the drug design and discovery industry as it has aided in the circumvention of bottle necks which entail time and costs of making new chemical entities in the design process (Firdaus Begam & Satheesh Kumar, 2012; Xu & Hagler, 2002). The focal point of bioinformatics is to analyze, simulate and manipulate chemical information in order to accelerate decision making and reduce expenses in the areas of drug lead identification and optimization in the process of drug discovery (Xu & Hagler, 2002; Krasky et al., 2007; Liu et al., 2014; Firdaus Begam & Satheesh Kumar, 2012). This study utlizes these bio-computational techniques to provide comprehensive informational data that will allow for the identification or design of inhibitors specific to DENV RdRp.

Methods

Bioinformatics tools were used in this study to analyze the structure of DENV RdRp, and map out a potential inhibitor specific to the enzyme.

Crystal Structure Acquisition and Alignment

The crystal structures of DENV RdRp serotype 2 and 3 were retrieved from the Protein Databank (Berman et al., 2000). Serotype 2 and 3 of DENV are represented by PDB codes 5K5M and 5I3Q, respectively (Lim et al., 2016). Comparitive sequence and structural investigations were then carried out using the Chimera software (Pettersen et al., 2004). The PDB structures were opened simultaneously in Chimera and superimposed using the Match maker function. The sequences were then aligned, and regions of similarity were highlighted. It is important to note that following the alignment of the two serotypes, amino

acids were renumbered. The correct numbering of the amino acids are depicted to the left of the alignment (residues: 266-900).

Sequence and Structure Analysis

After aligning the two sequences of DENV, the conserved regions between the two serotypes were identified. Important structural features of the RdRp, such as the priming loop, were then defined and elaborated on.

The active site residues were obtained from previous studies (Source et al., 2013; Klema et al., 2016). Comparitive analysis between the active site regions of the two serotypes were then investigated using the alignment tool available through Chimera.

Identification and Docking of Popular Flavivirus Inhibitors Specific to the RdRp Region

Various inhibitors of the RdRp region of DENV were selected from literature based on their compelling inhibitory characteristics (Garcia et al., 2017). The best 10 compounds were then docked to the active site of DENV RdRp using the Autodock plugin of Chimera (Morris et al., 2009). Of each of the docked complexes, the RdRp residues interacting with the compounds were identified. After evaluating the binding affinities, the inhibitor with the best docked pose was used as a modeltodemonstrate the pharmacophoric elements that are required when designing an efficient lead compound. Based on active site residue interactions with functional groups of NITD-203, the Ligandscout software (Wolber & Langer, 2005) was used to show these vital pharmacophoric elements.

Results and Discussion

Assembly of Structural and Non-structural DENV proteins

Dengue is an enveloped *flavivirus*, which consists of a positive-sense RNA virus approximately 11kb in size (Miller et al., 2008). This RNA encodes 3 structural proteins that form the components of the virion: the capsid (C), precursor membrane (prM) and the envelope (E). In addition to this assembly, there are seven non-structural (NS) proteins namely NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5 (Kuhn et al., 2002; Shu et al., 2004; Perera & Kuhn, 2008; Sahili & Lescar, 2017). Of the structural and non-structural proteins, crystal structures are available for the capsid (1R6R), envelope (4UTC), NS1 (4O6B), NS2 (2FOM), NS3 (2VBC) and NS5 (1L9K, 5K5M) (Figure 1).

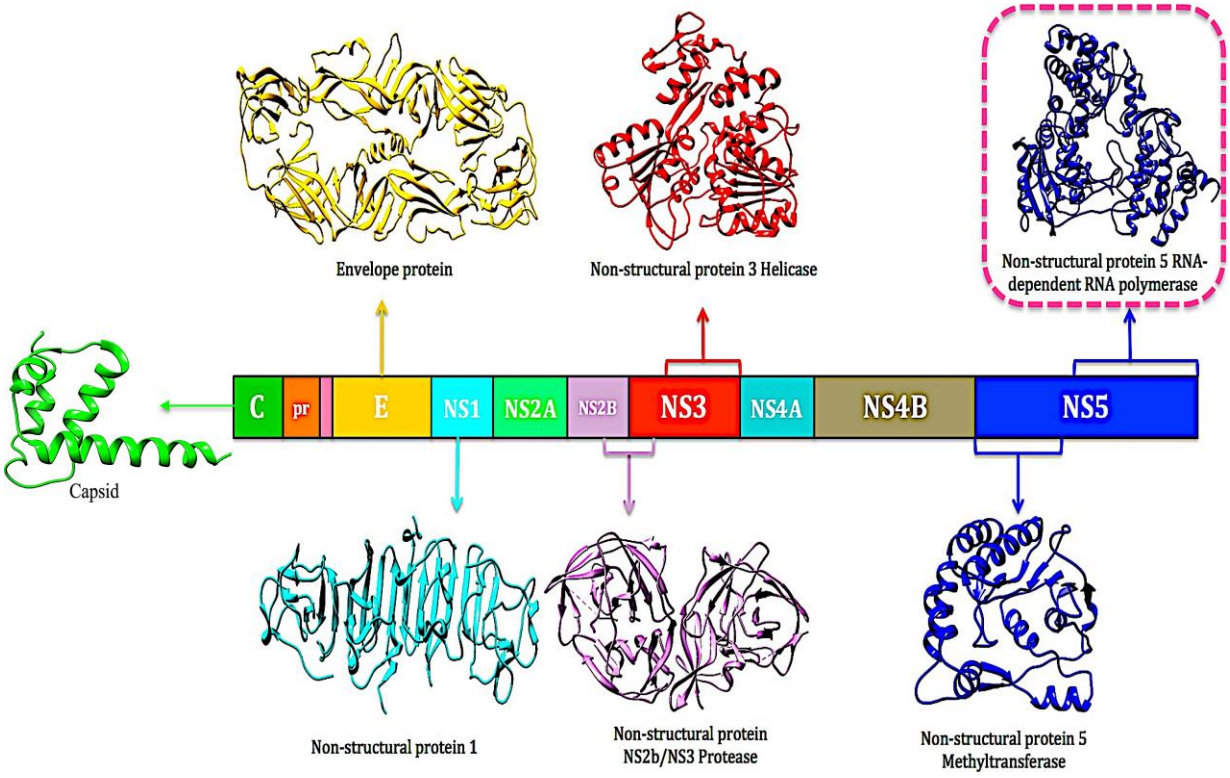


Figure 5.1: Dengue polyprotein demonstrating available crystal structures (PDB codes: 1R6R, 4UTC, 4O6B, 2FOM, 2VBC, 1L9K, 5K5M) (Prepared by Authors).

The NS5 is the largest non-structural protein and comprises of the methyltransferase and RNA-dependent RNA polymerase (RdRp) (Zhao et al., 2015; Klema et al., 2016). In the study, bioinformatics tools were utilized to explore the unique characteristics of the RdRp enzyme. The RdRp plays a significant role in RNA synthesis by catalyzing the replication of RNA synthesis via a two-step mechanism, thus validating it as a target for antiviral therapy (Ferron et al., 2005; Paschal et al., 2008).

Sequence and Structural analysis of DENV RdRp

The RdRp of DENV is located on the C terminus of the NS5 protein from residue number 266-900 (Appleby et al., 2005; Yap et al., 2007; Perera & Kuhn, 2008; Klema et al., 2016).

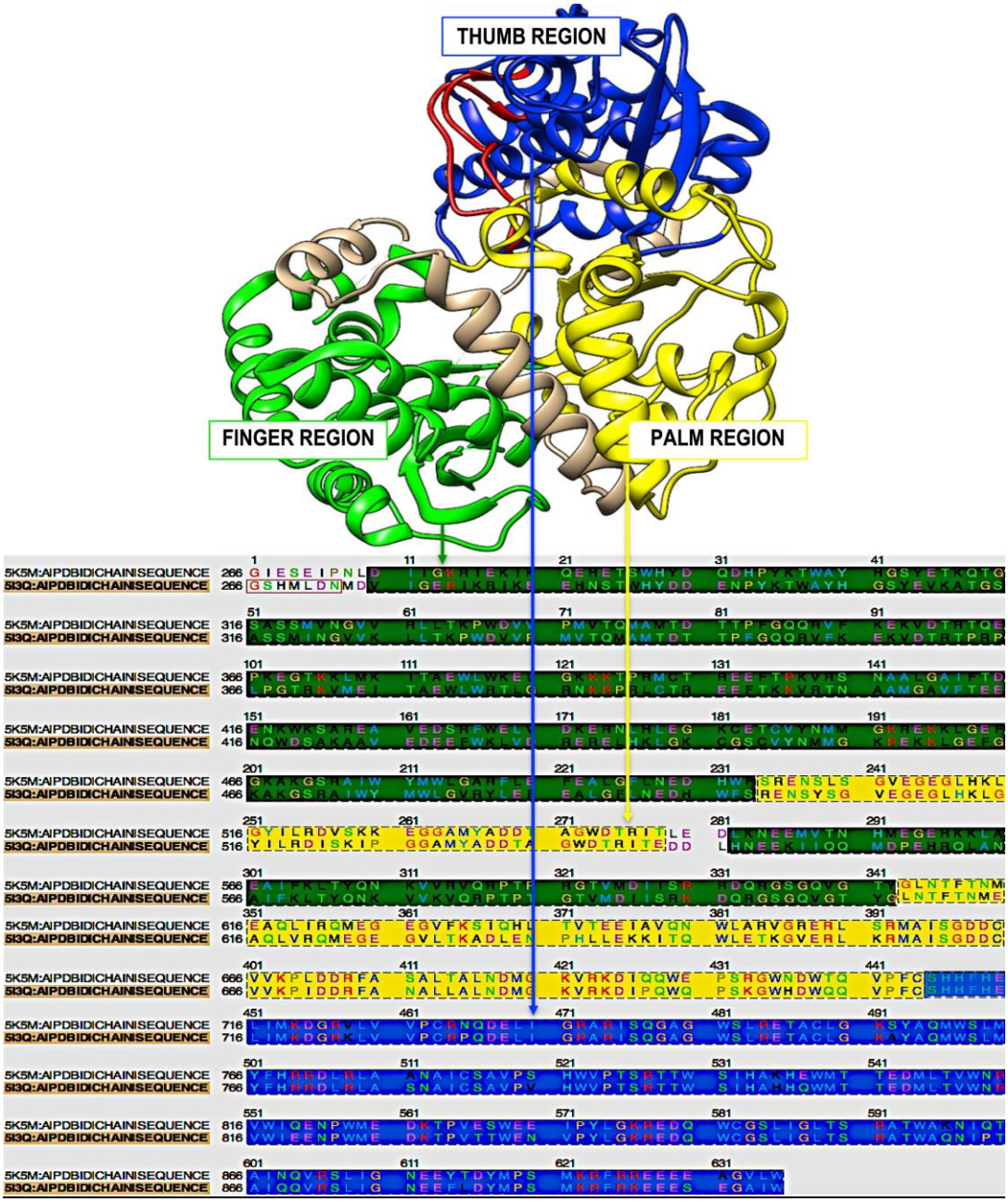


Figure 5.2: The overall structure and sequence analysis of the RdRp enzyme of DENV serotype 2 and 3. The green colour represents the finger domain (273-315,416-496 and 543-600); the yellow represents the palm domain (497-542 and 601-705) and the blue represents the thumb domain (706-900). The priming loop found in the thumb domain is represented by the red colour (782-809).

The highlighted regions of the sequence represent the three domains of the RdRp region (Lu & Gong, 2017). The green highlight represents the finger region, the yellow represents the palm and the blue represents the thumb (Yap et al., 2007; Klema et al., 2016) (Figure 2). A general resemblance of amino acids was noted between DENV serotypes 2 and 3 (King et al., 2007; Wu, Liu & Gong, 2015; Silva et al., 2008; Zhao et al., 2012; Manakkadan et al., 2013; Pitcher et al., 2015). Regions of maximum resemblance lie between 401 and 441 within the palm region. However, significant variations are prominent within the finger region. This is due to genetic alterations that have caused the DENV virus to mutate (Holmes & Burch, 2000; Holmes & Twiddy, 2003; Hellenthal & Stephens, 2006). This genetic variation is caused by error-prone RdRp, which lacks proofreading activity and generates approximately one mutation per round of genome replication (Elena SF, 2005; Sessions et al., 2015). Genetic recombination is also known to cause intra-serotype genetic variation in DENV (Uzcategui et al., 2001; Craig et al., 2003; Holmes & Twiddy, 2003; Perez-Ramirez et al., 2009).

The architecture of the DENV RdRp adopts a canonical right-hand conformation comprising of a finger, palm and thumb domain surrounding its active site (Lu & Gong, 2017). This applies to most polymerases (Ago et al., 1999; Duan et al., 2017). Dengue, however, has a nuclear localized structure (NLS) that plays a major role in its structural formation (Yap et al., 2007; Zhao et al., 2015). The NLS signatures are distributed between the finger and thumb domains from residues 316-415. This region forms the hotspot for interactions with other viral and host proteins (Johansson et al., 2001; Zou et al., 2011; Brooks et al., 2002). Alterations within the NLS region lead to structural destabilization (Pryor et al., 2007; Yap et al., 2007; Malet et al., 2007).

Finger domain

The finger domain is divided into two subdomains. The first strand is a beta-rich-strand (β) subdomain and the fingers found in this strand are termed beta-fingers (Ago et al., 1999). The other strand is rich in alpha-helices(α) and therefore the fingers found in this region are alpha-fingers. In addition, the finger region has four flexible loops, β1-α2, α3-α4, α6-α7 and α7- α8. Overall, the residues in the finger region are from 273-600 (Egloff et al., 2002; Galiano et al., 2016; Duan et al., 2017). The finger domain is located at the top of the RdRp enzyme and appears to be more mobile than the other two domains (Poch et al., 1989; Ng, Arnold & Cameron, 2008; Zou et al., 2011).

Palm domain

The palm domain is a catalytic domain that encompasses a highly conserved folding motif (Malet et al., 2007; Yap et al., 2007). The palm consists of two antiparallel β-strands, β4 and β5, and is surrounded by eight helices which are α11-α13 and α16-α20. Of the six conserved sequence motifs located in the palm region, four are specifically involved in nucleotide triphosphate (NTP) binding and catalysis (Bruenn, 1991; D'Haeseleer, 2006; Ferrer-Orta et al., 2006; Doolittle & Gomez, 2011; Asnet Mary, Paramasivan & Shenbagarathai, 2015; Galiano et al., 2016; Duan et al., 2017).

Thumb domain

The thumb domain stabilizes the C-terminal end of the RdRp, (Midgley et al., 2012) and is composed of residues 706-900 on the β6-α23 strands(Yap et al., 2007; Galiano et al., 2016). Of the known polymerase structures, the DENV thumb region shows the most

unique structural variation (Paschal et al., 2008; Cramer & Arnold, 2009). The thumb domain contains a conserved sequence motif that forms an antiparallel β-sheet wedged between the palm domain and several α-helices of the thumb domain (Ng, Arnold & Cameron, 2008; Pierson & Diamond, 2012). This unique structure contributes to the shaping of the RNA template tunnel (Benarroch et al., 2004; Yap et al., 2007; Welsch et al., 2009).

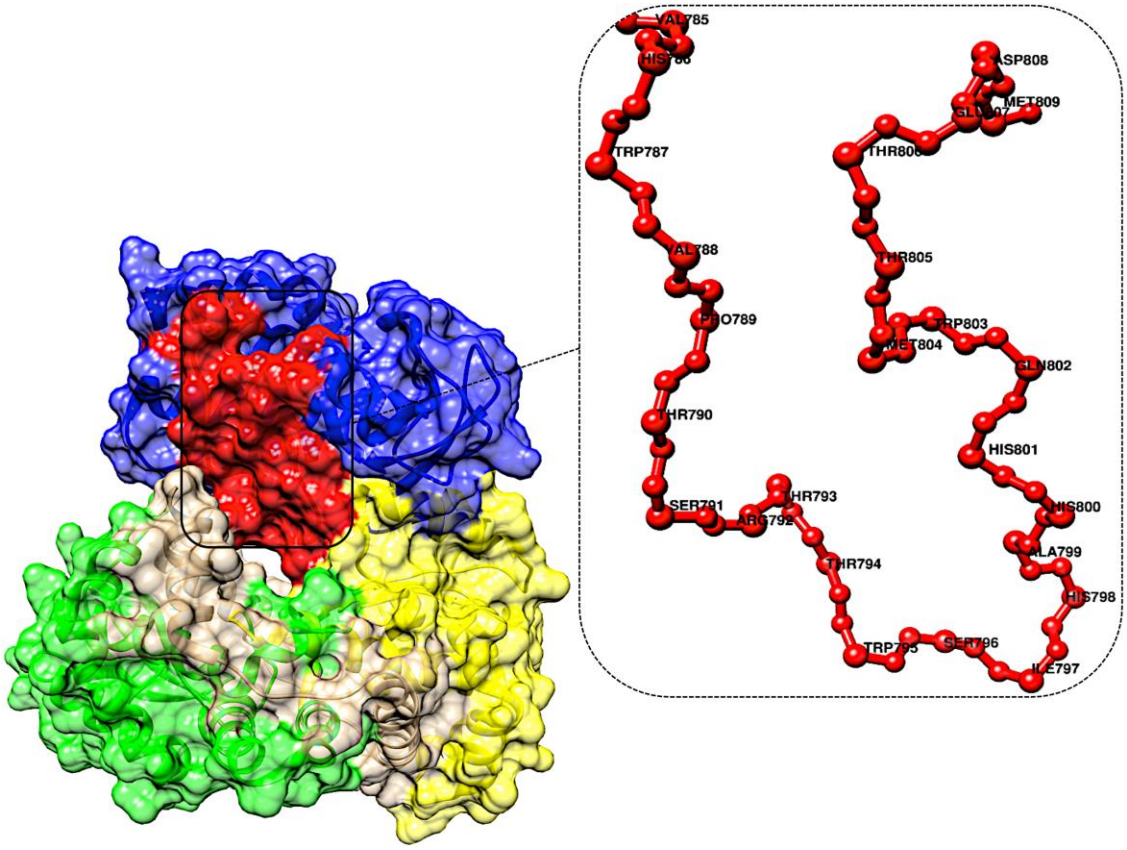


Figure 5.3: A closer look into the priming loop region highlighted in red in the DENV RdRp region.

Priming loop

A second loop consisting of amino acids 782-809 forms the priming loop, which partially blocks the active site (Figure 3). The priming loop plays a key role in initiating the enzymatic activity of the RdRp (Gebhard, Filomatori & Gamarnik, 2011; Selisko et al., 2012; te Velthuis et al., 2016). Internal interactions, including hydrogen bonds, act to stabilize the priming loop, thus maintaining the orientation of the protein structure (Ng, Arnold & Cameron, 2008; Campagnola et al., 2015). The priming loop is also known as the G-loop because it corresponds to motif G in primer-dependant RdRps. The characteristic "hairpin" structure of the loop is partially disordered in *flavivirus* RdRp structures, suggesting conformational flexibility (Malet et al., 2008; Source et al., 2013).

Comparative Mapping of DENV RdRp Active Site

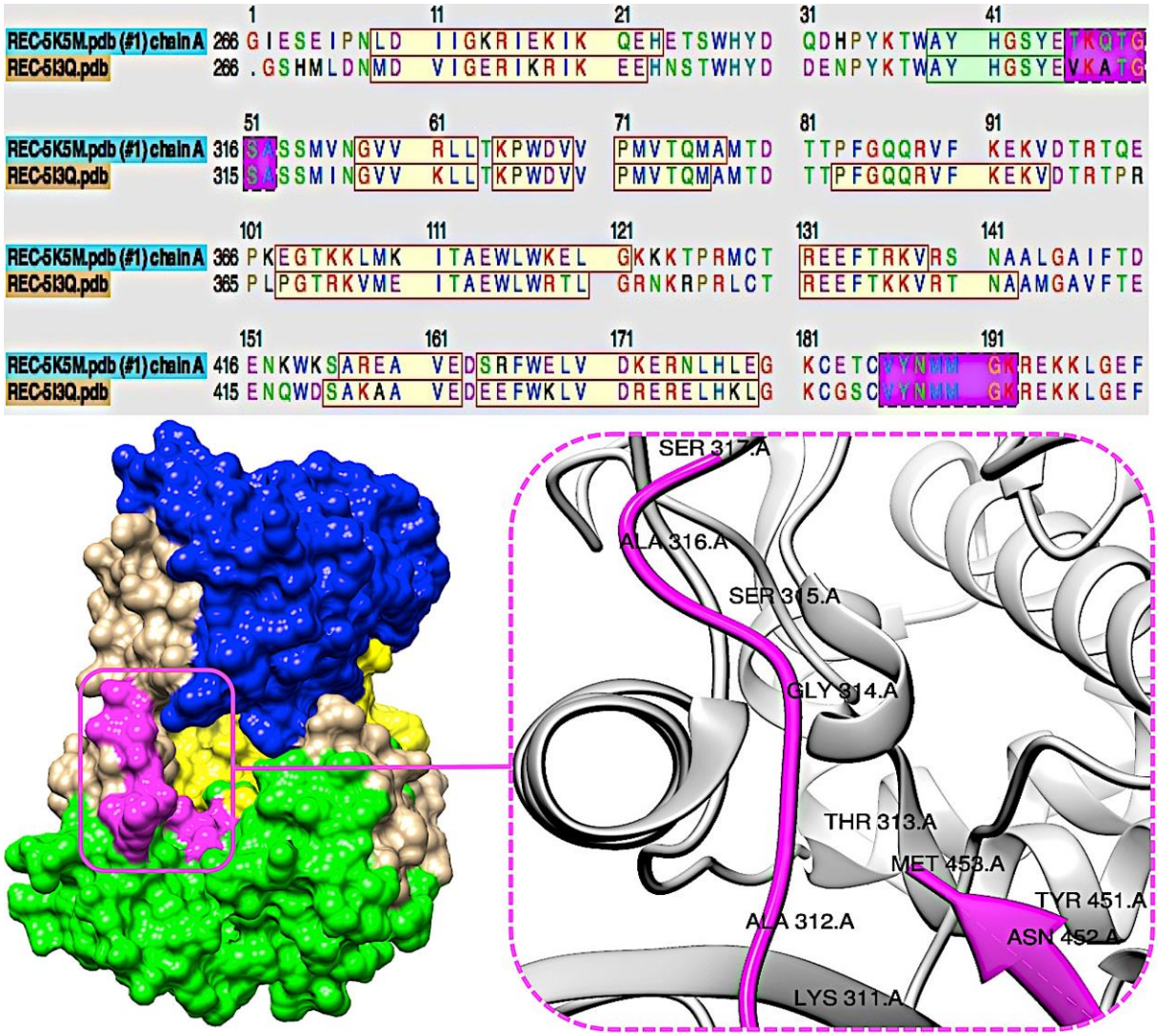


Figure 5.4: The active site region and sequence analysis of DENV.

The RdRp active site is characterized by a conserved region comprising of a glycine-aspartate core section located in the palm domain (Jablonski, Luo & Morrow, 1991; Routhier & Bruenn, 1998; Wu, Liu & Gong, 2015). The active site of DENV is made up of hydrophobic residues 311-317 and 451-457 (Source et al., 2013; Klema et al., 2016), which are located above the palm domain (Ago et al., 1999; Lesburg et al., 1999) (Figure

4). One of the unique characteristics of the active site is that it is located at the intersections of two tunnels. The finger and thumb domain form the first tunnel, which is responsible for coordinating the single-stranded RNA, while the second tunnel coordinates the nascent double-stranded RNA (Yap et al., 2007).

Mutations on the active site have contributed to challenges in finding inhibitors for DENV (Mateo, Nagamine & Kirkegaard, 2015), thus analysis of the active site will enable researchers to find broad spectrum inhibitors against both serotypes of DENV.

Seven catalytic motifs, A to G, have been identified for DENV RdRp. These motifs contribute to the sequence and structural conservation of the RdRp active site. Motifs A and C contain two aspartic acid residues that are universally conserved amongst *Flaviviruses*. Motif B has a highly conserved RdRp-specific serine-glycine sequence, which is replaced by threonine in drug-resistant strains (Perera & Kuhn, 2008; Klema et al., 2016; Yap et al., 2007). The glycine adjacent to motif B provides the backbone flexibility needed for conformational switches around the adjacent serine. The sequence is also vital for allowing large-scale conformational changes of the motif B loop. Motif D does not have conserved residues, however, it contains a lysine residue that has been shown to contribute in catalysis. Motif E and G do not contain conserved residues, but contribute to the composition of the active site of the RdRp (Yap et al., 2007; Lim et al., 2016; Zhao et al., 2012).

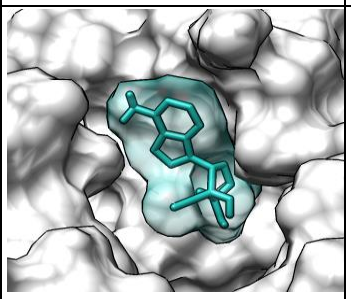
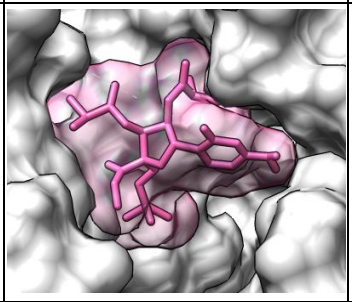
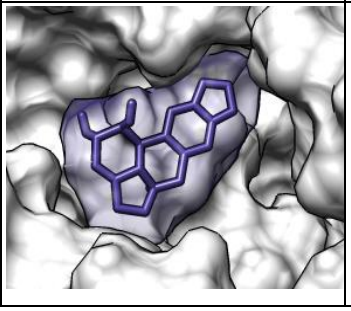
Conquering Targeted Therapy with Popular Drugs

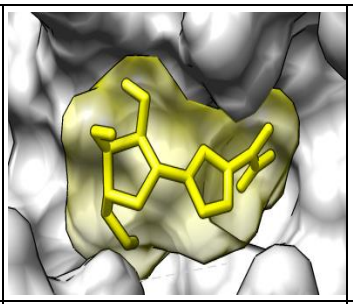
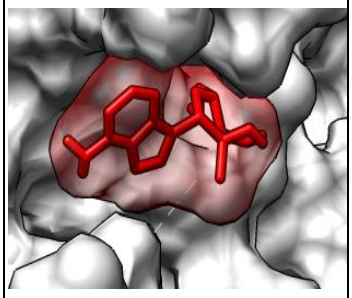
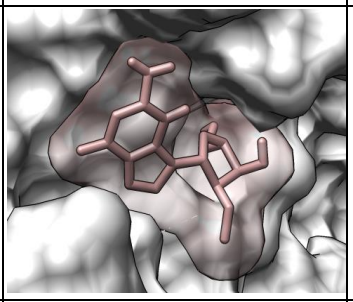
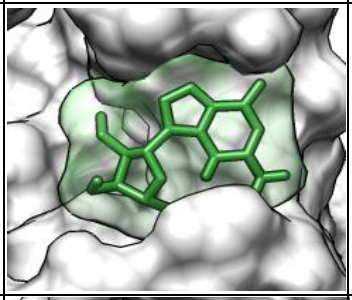
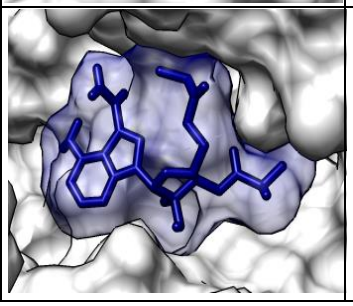
Studies have identified multiple general *flavivirus* RdRp inhibitors, however, there are currently no FDA approved drugs that are specific to all serotypes of the RdRp region of DENV. The development of an antiviral therapy for DENV is further complicated by the fact that protection against one serotype leads to increased vulnerability against the other serotypes (Heinz & Stiasny, 2012). This study therefore seeks to fill the gap between the increase in DENV case reports and absence of antivirals. Over the years, inhibitors that have shown potential antiviral activity come with multiple challenges including elevated toxicity levels. Scientists are therefore still battling to find an inhibitor that is potent, efficacious and non-toxic for the treatment of DENV (Galiano et al., 2016; García, Padilla & Castaño, 2017; Ramharack & Soliman, 2017).

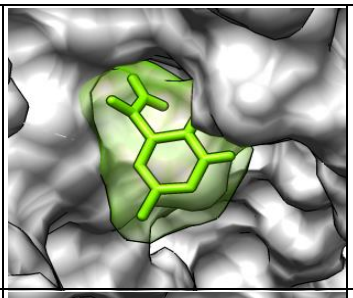
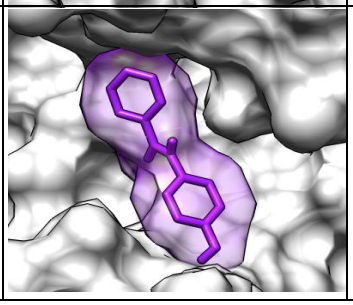
In this study, various potent inhibitors of the RdRp region of DENV were assessed. Based on a study by García *et al*., 2017, experimental compounds that demonstrated compelling inhibition of DENV were chosen and docked into the active site of DENV RdRp. The ten best docked poses were reported in Table 1. The RdRp residues interacting with the docked compounds were identified, thus adding to the requirements needed when designing a possible inhibitor of DENV. Of these residues, TYR 451 was found in nine out of ten of the complexes, indicating its importance as an interacting residue for both serotypes. Various studies have proved NITD008 to be a potent *flavivirus* inhibitor (Yin et al., 2009; Shan et al., 2013; Deng et al., 2014, 2016). The binding affinities, however, showed that NITD-203 had the best docking score (-6.4 kcal/mol). The NITD-203 compound is an adenosine analog that has been shown to have potent competitive inhibition of adenosine triphosphate (ATP) at the active site of RdRp. It is, however, important to note that one of

the most common adverse effect of nucleoside compounds such as NITD-203 is mitochondrial toxicity (Garcia et al., 2017). This dismisses the compound's progression to FDA approval. Nonetheless, NITD-203 may still be utilized in the development of DENV antiviral therapy.

Table 5.1: Popular *flavivirus* inhibitors bound to DENV RdRp (Adapted from (García, Padilla & Castaño, 2017)).

Compound	**Mechanism of Action**	**Binding Energy (kcal/mol)**	**Pose**	**Amino acid residues**
NITD008	Inhibitor of all 4 DENV serotypes (Lim et al., 2013; Caillet-Saguy et al., 2014a; Lo et al., 2016).	-5.5		PHE 127 ARG 210 LYS 130 VAL 131 ASN 134 ALA 135
Balapiravir	A cytidine nucleoside that is a domain RdRp inhibitor (Lim et al., 2013).	-6.1		TYR451 ASN452 VAL577 ARG594 VAL576
Lycorine	A potent *flavivirus* inhibitor in cell culture through suppression of viral RNA replication (Zou et al., 2009; Lim et al., 2013).	-5.8		TRP 474 TRP 477 TYR 451 SER 470 VAL 579 LYS 578

Ribavirin	Significant *in vitro* activity against *flaviviruses* (Gilbert et al., 1985; Feld et al., 2017).	-5.0		TYR 451 ASN 452 GLN 580 VAL 579 VAL 310 ILE 473
7-deaza-2'methyladenosine	Potent ZIKV inhibitor that reduces viremia (Zmurko et al., 2016)	-5.5		ILE 283 GLU 286 ILE 473 MET 453 MET 589 TYR 451
3'Dgtp	GTP analogue that inhibits DENV-2 RdRp but has a low IC50 value (Malet et al., 2008)	-5.0		MET 589 VAL 579 LYS 578 ASN 452 TYR 451 VAL 310
2'O-metil GTP	Showed low IC in vitro DENV-2 (Malet et al., 2008).	-5.3		THR 343 THR 313 TYR 451 TRP 477 GLY 349 ASN 452
NITD 203	Inhibitor of all 4 DENV serotypes (Caillet-Saguy et al., 2014b).	-6.4		VAL 358 GLY 599 TYR 451 ILE 591 GLY 601 SER 600

Favipiravir	A pyrazine-substitute compound that has shown to inhibit *flavivirus* mortality in rodents (Furuta et al., 2009).	-4.0		ASN 452 TYR 451 VAL 579 LYS 578
Ivermectin	Pretreatment of ivermectin with N-(-4-hydroxyphenyl) inhibits the nuclear localization of NS5 (Tay et al., 2013).	-5.3		ASN 452 LYS 578 TYR 451 VAL 579 VAL 310 MET 453

Based on the “prodrug” characteristics of NITD-203, it was chosen as a model to identify specific pharmacophoric elements that are required when designing an efficient inhibitor of all five serotypes. Pharmacophore modeling is a pivotal tool exploited in rational drug design, providing crucial insights into the nature of the interactions between a drug target and ligand. It involves the concept of "privileged structures", which are molecular frameworks capable of providing useful ligands for more than one type of protein. Pharmacophore models are vital in drug design as they act as templates for screening compounds that have similar structural and chemical features. These ligands could then be used as lead compounds against various diseases (Wolber & Langer, 2005; Gao et al., 2010; Qing et al., 2014).

In this study, we have therefore utilized this pharmacophoric approach to design a model based on NITD-203 that may be used as a stepping stone toward efficient DENV inhibitors. The Ligandscout (Wolber & Langer, 2005) software was used to demonstrate the vital pharmacophoric elements required when designing a DENV RdRp inhibitor. These chemical features were based on active site residue interactions with functional groups of NITD-203 (Figure 5).

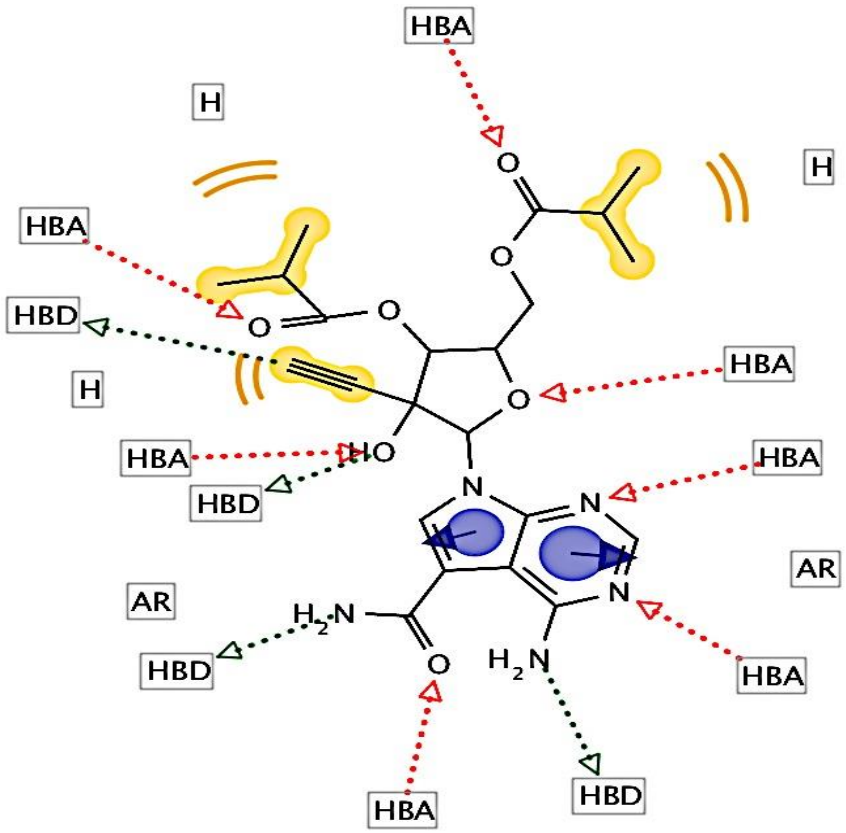


Figure 5.5: Significant pharmacophoric elements of NITD-203 for the design of target-specific inhibitors of DENV RdRp (HBA/D- Hydrogen bond acceptor/donor, H- Hydrophobic, AR- Aromatic).

Based on the pharmacophore model identified in Figure 5, chemical features such as hydrogen bond donors/acceptors as well as aromatic rings are crucial elements that are required in constructing an efficacious DENV inhibitor. We believe that this study will be a cornerstone in paving the road toward the design of target-specific inhibitors against DENV RdRp.

Conclusions

Dengue is an established *flavivirus* that is causing distress in the lives of many. The development of an antiviral against DENV is further complicated by its manifestation into various serotypes. This augments the need for innovative research methods in DENV drug design. The bioinformatics techniques discussed in this paper will aid in the identification of potential RdRp inhibitors, thus mitigating the effects of DENV in the lives of compromised individuals, as well as prevent the transmission of DENV on a global scale.

Conflict of interest

Authors declare no conflicts of interest.

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CHAPTER 6

6.1 Conclusion

The present study sought to investigate a notorious flavivirus that has been in circulation for over 30 years. Over the last few decades, DENV has re-emerged in various serotypes and is causing mayhem in the lives of many [1,2]. Dengue is dreaded for the severe fever it causes in its advanced stage. Dengue has the reputation of what is known as Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), a leading cause of death in children in some Asian countries [3]. The vertical transmission of Dengue is also a cause of concern as it has teratogenic effects [4]. Its rapid spread has made it arduous to restrain and eradicate.

Attempts have been made to design vaccines and small drug molecules as potential inhibitors against DENV. The virus however is resilient, and exists in 5 serotypes with numerous strains under them, thwarting the efforts of researchers to curb its spread. This prompted us to design a study that would address the above challenges by use of CADD tools, which elaborated on the design of target-specific inhibitors of DENV from an atomistic perspective.

In this study, there were two aims, which were to design an "All-in-one" pharmacophoric architecture for the discovery of potential broad spectrum anti-*flavivirus* drugs, and to provide a comprehensive cheminformatics based review for the discovery of DENV serotypes 2 and 3 inhibitors.

A renowned *flaviviral* inhibitor (NITD008) was used to analyze the structural binding landscape of five *flavivirus* enzymes. The NITD008-*flavivirus* systems were subjected to 100ns MD simulations and the trajectories evaluated using free energy decomposition analysis (NITD008-HCV complex as a reference). Binding affinities were ranked and the results demonstrated similar binding interactions to the NITD008-HCV positive control, with the NITD008-ZIKV system portraying the greatest similarity. Furthermore, the study prompted us to map out a set of structural criteria that could be crucial for further optimization of novel *flavivirus* inhibitors. Chemical features that met the desired criteria were then assembled to form a pharmacophore model of the ligand that could pave the road toward the design of one inhibitor to treat multiple *flaviviruses*. This pharmacophore approach may assist medicinal chemists in the design of small molecules that may overcome cross-resistance and increase patient adherence.

The development of an antiviral against DENV is further complicated by its manifestation into five serotypes. This augmented the need for innovative research methods in DENV drug design. The cheminformatics and bioinformatics discussed herein resulted in a comprehensive and comparative review of the two most

prominent serotypes of DENV. The analysis of popular *flavivirus* inhibitors laid the foundation to identify a molecular framework that will aid in the identification of potential DENV inhibitors. We believe that these findings will aid in mitigating the effects of the DENV in the lives of the compromised individuals, as well as prevent the transmission of DENV from patients to healthy individuals.

Overall, this study has provided valuable insights into the design and development of DENV inhibitors through molecular modeling and CADD.

6.2 Future Perspectives

The potential inhibitors of the study have presented promising protein-ligand interactions and binding energies and therefore maybe utilized as the lead compounds. Prospective biological experimentation is however mandatory for the testing of these compounds in order to verify these *in silico* studies.

The ramifications of DENV infection have led to a multitude of potential small molecule inhibitors and vaccines that are currently in clinical trials. The virus nonetheless has other challenging defense mechanisms that may render most drugs and vaccines ineffective. The co-existence of 5 serotypes has regressed efforts made by researchers as DENV is ever mutating.

With regard to this investigative study, a purely computational perspective was used. To further the DENV drug design research toward targeted therapy, the design of an experiment is warranted to analyze the efficacy of the pharmacophores mentioned above. Screened inhibitors based on the pharmacophores designed can be procured and tested through rational drug design to find lead compounds that are effective against DENV. As far we know, this is the first study that has mapped out a path for small molecules that are target-specific to the DENV RdRp.

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