DISCOVERY AND MECHANISM OF ACTION STUDY OF ANTI-VIRAL COMPOUNDS FOR DENGUE VIRUS

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SUMMARY

Dengue fever is a mosquito-borne disease that is prevalent in tropical and subtropical regions of the world. In some severe cases, this disease leads to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), which may lead to loss of life. The WHO estimates more than fifty million cases of dengue fever occurring every year, hence there is a need for drug-discovery and vaccine development for dengue fever. The aim of this thesis is to identify and characterize three antiviral compounds, NITD448, NITD770 and U18666A, as novel anti-dengue compounds.

In the first study, a rational approach was used to create a library of small molecules. These compounds were structurally predicted to bind to the dengue envelope protein. A medium throughput assay measuring cell-cell fusion activity was developed to screen this library and this screening led to the identification of a novel small molecule compound (NITD448) which was validated to block dengue fusion and infection.

In the second study, a small molecule mevalonate pyrophosphate decarboxylase (MVD) inhibitor (NITD770) was tested for anti-viral activity in DENV. It exhibited a good anti-viral activity with a therapeutic window of more than 100. Its anti-viral activity was also found to be specific against flaviviruses. However, subsequent studies confirmed that MVD was not the target of NITD770 and hence, there was a need to determine its mode of mechanism. During the studies to determine the mode of mechanism of NITD770, host lipid rafts (as suggested by chemogenomic profiling data) and cholesterol were confirmed not targeted by this compound. Gene sequencing of resistant viruses raised against the compound revealed that resistant mutations were within the NS5 RNA-dependent RNA polymerase (RdRp) coding region. When these mutations were introduced into wild type RdRp, an increased in

polymerase activity was observed but these mutations did not rescue the suppression effect of NITD770, implying that these were compensatory mutations.

In the final study, the importance of host cholesterol to dengue infection was investigated using an amphiphile, U18666A.When two main sources of cholesterol in the host cell, i.e., extracellular cholesterol intake and cholesterol biosynthesis, were inhibited by U18666A, dengue infection was suppressed. Subsequent studies further showed that when extracellular cholesterol transport into host cell was arrested by U18666A, it resulted in inefficient trafficking of dengue viruses. Immuno-flourescence studies revealed that these viruses were trapped in the host late endosomes, which were heavily loaded with the accumulated cholesterol, and unable to undergo fusion. This resulted in reduced infection. U18666A was also shown in this study to have a suppression effect on viral replication and further studies suggested that it could be caused by the reduction of host de-novo biosynthesis of cholesterol by this compound.

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LIST OF ABBREVIATIONS

βOG	*n*-octyl-b-D-glucosidase
C75	4-Methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid
CC_{50}	50% cytotoxic concentration
CFI	Cell-based Flavivirus Inhibition
CTL	Control
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Hemorrhagic Fever
DRM	Detergent Resistant Membrane
DSS	Dengue Shock Syndrome
E	Envelope
EC_{50}	Half maximal effective concentration
ER	Endoplasmic Reticulum
FASN	Fatty acid synthase
GC MS	Gas Chromatography Mass Spectrometry
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
ITC	Isothermal Titration Calorimetry
MBCD	Methyl-beta-cyclodextrin
MOI	Multiplicity of Infection
MVD	Mevalonate pyrophosphate decarboxylase
NGC	New Guinea C laboratory strain of dengue virus type 2

NS	Non Structural
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
PI	Propidium Iodide
RdRp	RNA dependent RNA polymerase
RFU	Relative Fluorescence Unit
SAR	Structure Activity Relation
TEM	Transmission Electron Microscopy
U18666A	3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one
WHO	World Health Organization
WNV	West Nile Virus

LIST OF PUBLICATIONS

First Author Publications:

Poh MK, Yip A, Zhang S, Priestle JP, Ma NL, Smit JM, Wilschut J, Shi PY, Wenk MR, Schul W (2009) A small molecule fusion inhibitor of dengue virus.. *Antiviral Res*. 84(3):260-6.

Poh MK, Shui GH, Shi PY, Wenk MR, Gu F (2011) U18666A, an intra-cellular cholesterol transport inhibitor, inhibits dengue virus entry and replication. Manuscript submitted to Antiviral Research Journal in May 2011.

Collaborative Publications:

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POSTER PRESENTATION

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Poh MK, Yip A, Zhang S, Priestle JP, Ma NL, Smit JM, Wilschut J, Shi PY, Wenk MR, Schul W. A screening program to look for dengue virus fusion inhibitors.

Gordon Research Conference 2009 (Virus & Cells)

IL Ciocco, Italy. 7-12th June 2009.

Poh MK, Shui GH, Shi PY, Wenk MR, Gu F. A study of the role of cholesterol in dengue infection.

12th Western Pacific Congress on Chemotherapy & Infectious Diseases

Shangri la, Singapore. 2-5th December 2010.

Poh MK, Shui GH, Shi PY, Wenk MR, Gu F. The role of cholesterol in dengue viral entry and replication.

1. INTRODUCTION

1.1. HISTORY OF DENGUE INFECTIONS

Dengue fever (DF) is a mosquito-borne viral disease that affects humans. The disease is caused by a virus known as dengue virus (DENV). DENV was first successfully isolated from human patients in Hawaii (DENV-1) and New Guinea (DENV-2) in 1944, and subsequently in the Philippines (DENV-3 & DENV-4). Dengue fever can be caused by four distinct but related serotypes of dengue virus (DENV-1 to 4). It made its deadly presence known to the medical field in the 1950s when a severe form of dengue fever, Dengue Hemorrhagic Fever (DHF), surfaced during epidemics in the Philippines and Thailand. This disease is also known as "break-bone" fever, perhaps owing to the symptoms observed in DF patients, which include intense headaches and body aches.

Dengue fever is classified as an emerging disease, with initially less than ten countries reporting to have DHF (prior to 1970), to more than hundred countries displaying cases of DHF. The World Health Organization (WHO) currently estimates more than fifty million cases of dengue fever every year. The escalating number of cases is a worrying issue as there is no cure to date. Tropical and sub-tropical climates are environments where dengue thrives and with the increasing global travelling, a spread in the disease is thought to be inevitable (Gubler 2002). Reports of epidemics in several countries are occurring more frequently in this century; often with more severity than ever displayed before. This is of particular concern to countries with limited resources in medical care, as patients require constant and careful monitoring.

1.2. BIOLOGY OF DENGUE VIRUS

1.2.1. Taxonomy of dengue virus

Dengue virus (DENV) belongs to the family of Flaviviridae that consists of three genera, flavivirus (e.g. dengue virus, West Nile virus, and yellow fever virus), hepacivirus (hepatitis C virus) and pestivirus (e.g. bovine viral diarrhea virus), as shown in figure 1. The detailed taxonomy and classification can be found at the International Committee on Taxonomy of Viruses website at http://www.ictvonline.org/virusTaxonomy.asp. This family of viruses is mostly arthropod-borne, with a complex transmission cycling between the vectors (mostly mosquitoes and ticks) and host vertebrates. There are two known transmission cycles for DENV: (i) human hosts and Aedes mosquitoes (mainly, Aedes Aegypti) and (ii) a sylvatic cycle involving non-human primates and Aedes mosquitoes (Gubler 1988; Gubler and Trent 1993).

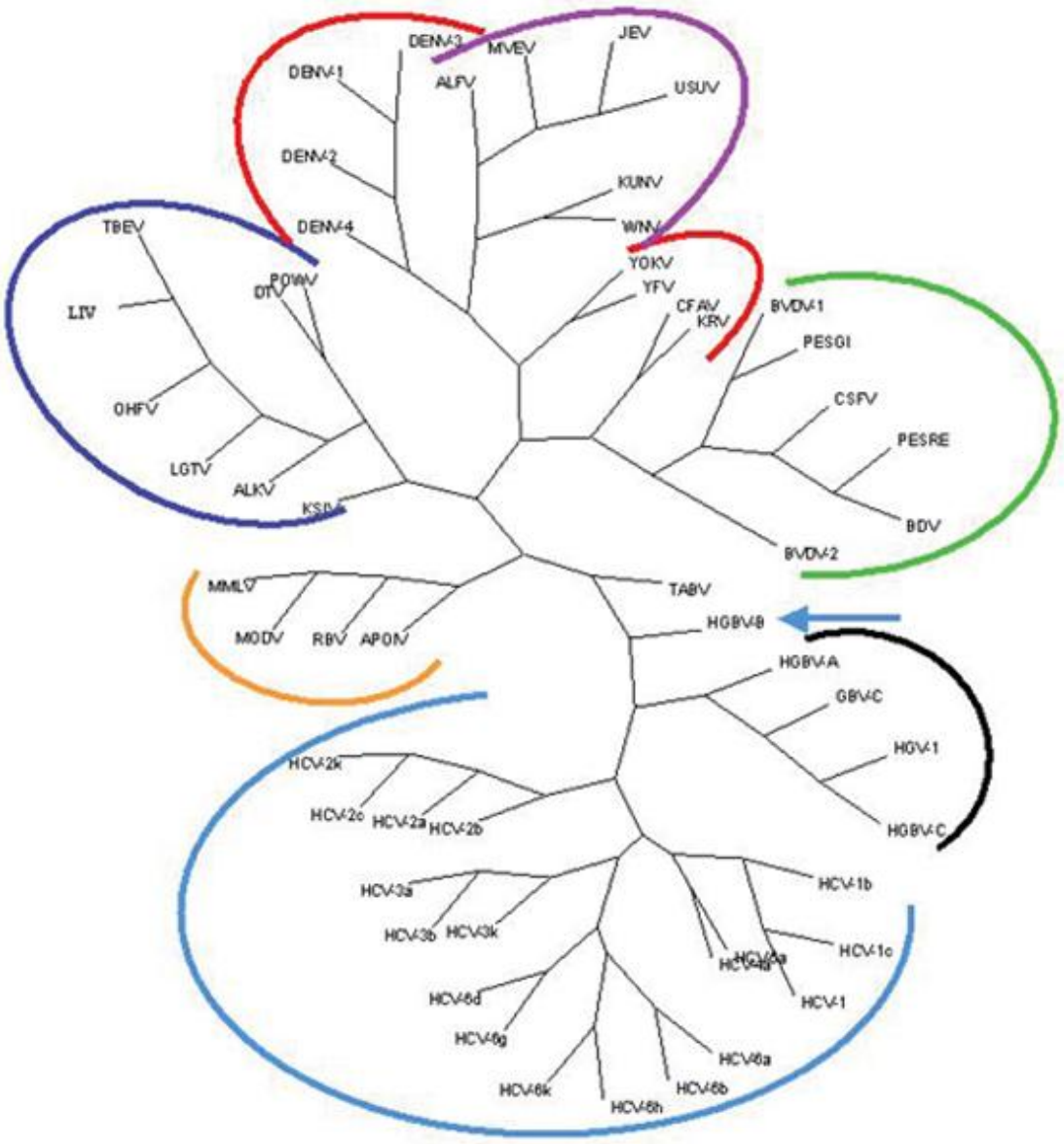


Figure 1-1: Whole genome phylogenetic tree of family Flaviviridae. This tree is reconstructed using maximum parsimony. Color coding for arcs is as follows: Red (Aedes borne Flaviviruses), Purple (Culex borne Flaviviruses), Blue (Tick borne Flaviviruses), Orange (No known vector Flaviviruses), Green (Pestiviruses), Cyan (Hepaciviruses) and Black (unassigned members of family Flaviviridae). Reprinted by permission from BioMed Central: [BMC Bioinformatics] (Kulkarni-Kale U, Bhosle SG, Manjari GS, Joshi M, Bansode S, and Kolaskar AS. 2006. Curation of viral genomes: challenges, applications and the way forward. BMC Bioinformatics 7 Suppl 5:S12.)

1.2.2. Structure and genetic organization of dengue virus

Previous ultra-structural studies showed that dengue virus is a 50 nm icosahedral entity with a surprisingly smooth surface. DENV particle is made up of a RNA core that is encapsidated by nucleo-capsid, which is wrapped by a lipid bilayer membrane, followed by an organized outer protein shell (Zhang et al. 2003).

The dengue genome (as shown in figure 2) is composed of a positive single stranded RNA of approximately 11 kb in size. It is organized into three structural viral proteins (capsid (C), pre-membrane (prM) and envelope (E)), and seven non-structural proteins essential for viral replication (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Lindenbach et al. 2007).

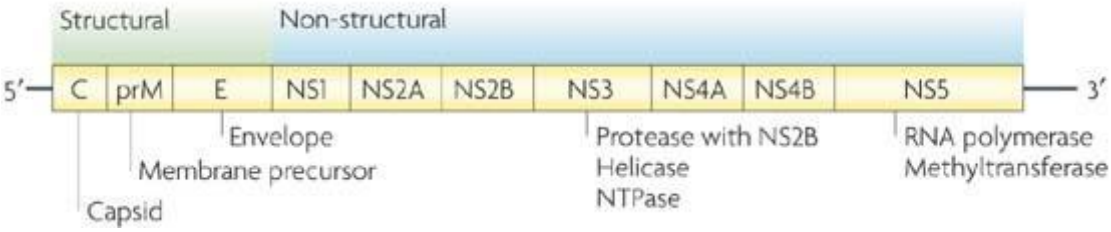


Figure 1-2: Dengue virus genome: It is a polyprotein composed of three structural proteins (highlighted in green) and seven non-structural proteins (highlighted in blue). Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] (Whitehead S, Blaney J, Durbin A, and Murphy B. 2007. Prospects for a dengue virus vaccine. Nat Rev Microbiol 5(7):518-528), copyright (2007).

1.2.3. Viral infection cycle

The infection cycle of DENV (as depicted in Figure 3) begins with the binding of DENV onto the host cell surface with a receptor, probably a low affinity but abundance receptor, such as DC-SIGN (Tassaneetrithep et al. 2003). The virus is then endocytosed into the cell via an unknown high affinity specific receptor (Lozach et al. 2005). When encountering a change in pH within the acidic environment of the late endosome, protonation of the histidine residues of the viral envelope occurs (Mueller et al, 2008). This triggers the E protein to undergo major conformational changes which leads to the fusion of the viral membrane with host endosomal membrane. The fusion event releases the nucleocapsid, containing the genetic material, into the cytoplasm. Positive strand viral RNA is translated into a single polyprotein that is further processed into structural and non structural viral proteins by viral and host cellular proteases. Viral replication is initiated on the intercellular membranes near to the host endoplasmic reticulum (ER). Newly synthesized viral proteins are assembled, in the ER, into immature non-infectious virions. These immature virions are subsequently transported to the trans-Golgi apparatus for processing; resulting in the release of the mature infectious virions to the extracellular environment via the host secretory pathway.

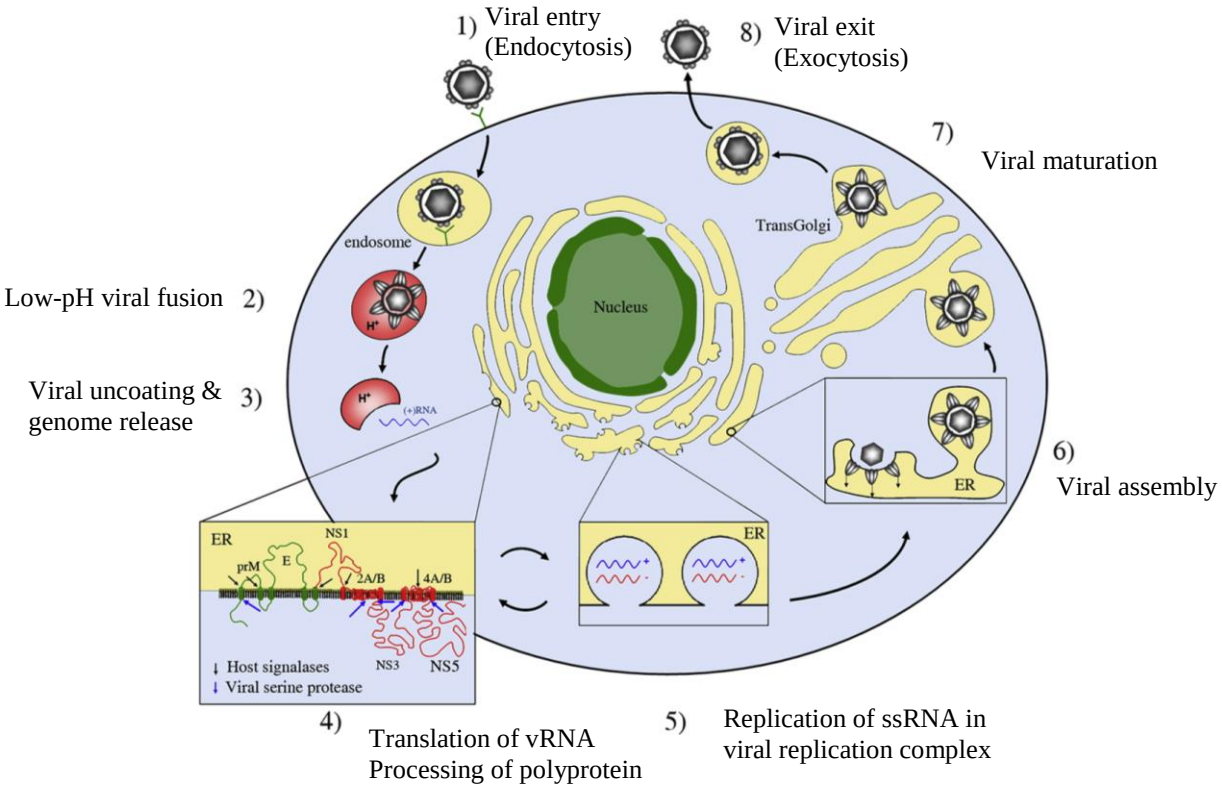


Figure 1-3: Infection cycle of dengue virus in host cell.
Reprinted from Host Cell. Cell Host & Microbe, 5(4), Fernandez-Garcia M-D, Mazzon M, Jacobs M, and Amara, A., Pathogenesis of Flavivirus Infections: Using and Abusing the Host Cell, p318-328, Copyright (2009), with permission from Elsevier.

1.2.4. Viral proteins

Viral RNA is packaged inside the DENV capsid to forms the RNA core, known as nucleocapsid. This RNA core protects the viral genome before its delivery into the host cell cytoplasm for initiation of viral replication. After viral fusion, the viral capsid localizes to both cytoplasm and nuclease of the host cell. The reason for nuclear localization is still poorly understood. There were studies done suggesting the possible roles of capsid in (i) virus-induced apoptosis, (ii) viral assembly (Khromykh and Westaway 1996), (iii) viral morphogenesis (Samsa et al. 2009) and (iv) acting as an antagonist of an excocyst protein (hSec3p), which is a repressor of viral replication (Bhuvanakantham et al. 2009).

The membrane protein of DENV is initially presented as a pre-membrane form (prM). It acts as a shield covering the fusion peptides of DENV envelope protein, preventing it from premature fusion with the cellular membrane during the synthesis of new viral particles. During the final step of viral assembly (known as maturation), it is cleaved by a host protease, furin, with the "pr" peptide remains associated with the virion in the environment of the TGN. This association keeps the virus in a non-infectious state inside the cell. Upon exiting the cell, "pr" peptide dissociates from the virus, making the virion infectious. This primes the mature virion for fusion upon entry into acidic compartments of host cells.

DENV is an enveloped virus which contains a lipid bilayer that has 180 copies of E protein and M protein embedded in it. The E proteins are arranged in an icosahedral scaffold of 90 dimers (Kuhn et al. 2002). The fusion protein of DENV E protein is classified as a class II fusion protein, similar to those belonging to alphaviruses and flaviviruses. The E protein has three domains, domain I being the central domain, flanked by a dimerization domain (domain II) on one end and

immunoglobulin-like domain (domain III) on the other end (Mukhopadhyay et al. 2005). The discovery of a hydrophobic pocket, occupied by a small detergent molecule, n-octyl-β-D-glucoside (βOG), near to the hinge region of the E protein, highlighted an attractive region for anti-viral targeting (details are to be further discussed in later section, see chapter 1.3.3). Other important roles of E proteins include receptor-mediated binding, neutralization and viral assembly (Chin et al. 2007; Crill and Roehrig 2001; Hiramatsu et al. 1996; Stiasny et al. 2006)

The non-structural protein, NS1, exists in both soluble and insoluble forms (Winkler et al. 1989). During the replication event of DENV, NS1 is anchored to the intracellular membrane of the endoplasmic reticulum, mainly as homodimers (Falgout and Markoff 1995; Mackenzie et al. 1996), and is implicated to participate in viral replication. It is also found to be associated with the cell surface via a GPI-anchor and is capable of triggering signal transduction (Jacobs et al. 2000). The soluble form of NS1 circulates in the extra-cellular compartment in the form of a hexamer (Flamand et al. 1999). Soluble NS1 levels were found to be elevated in the blood serum of dengue fever patients during the acute phase of the disease (Young et al. 2000). It is believed that these circulating soluble NS1 hexamers could contribute to the pathogenesis of the disease by reacting with the host complement system, causing activation and the onset of host immune responses, leading to a vascular leakage (Avirutnan et al. 2006).

NS2B-NS3 complex of DENV is an excellent example of efficient fusion of various functional proteins into one protein which allows sequential processes to take place in close association. NS3 has the viral protease at its N-terminal end that cleaves the viral polyprotein together with host proteases. At the C-terminal end of NS3 is the viral helicase with a RNA-stimulated nucleoside triphosphatase to provide the energy

to unwind viral RNA replication intermediates during amplification of viral RNA. NS2B is a co-factor of NS3, acting as a putative anchor for NS3 protease to the host membrane in order to allow efficient proteolytic activity. Other non-enzymatic functions of flaviviral NS3 have been suggested, such as substrate recognition (capped viral RNA) (Luo et al. 2008; Patkar and Kuhn 2008) and the recruitment of fatty acid synthase to the site of replication for fatty acid biosynthesis, which is necessary for viral replication (Heaton et al. 2010).

NS5 is another non-structural protein of DENV with multi-enzymatic properties. At the N-terminus of NS5 is the viral methyltransferase, which is involved in the methylation of the N7 and 2'-O positions of viral RNA cap. RNA capping stabilizes viral mRNA for efficient translation (Furuichi and Shatkin 2000; Wengler 1993). The RNA-dependent RNA polymerase (RdRp) domain is found in the C-terminal region of NS5 and is responsible for the amplification of viral RNA (Ackermann and Padmanabhan 2001; Tan et al. 1996; Yap et al. 2007). NS5 also has a non-enzymatic function in modulating the host responses by binding to STAT2, resulting in the suppression of interferon signalling involved in anti-viral response in host (Ashour et al. 2009; Mazzon et al. 2009).

NS2A, NS4A and NS4B are non-structural proteins that are generally believed to possess non-enzymatic functions. All have been shown to antagonize IFN signalling with NS4B having the most potent anti-IFN activity. The function of NS4A is still relatively elusive, although that it is known to be localized to the replication site, and is shown to induce the formation of membranous structures similar to those observed in infected cells (Miller et al. 2007). NS4B has been implicated in enhancing the overall helicase activity of NS3 by causing the dissociation of NS3 from single-stranded RNA (Umareddy et al. 2006).

1.3. PATHOGENESIS OF DENGUE INFECTION

1.3.1. The course of dengue infection

There is a usual incubation period of one week, upon bitten by an infected mosquito, before viremia is detected in the infected human. Commonly observed in dengue fever (DF) patient is the sudden onset of illness which has the following phases: febrile, critical and recovery (Dengue guidelines by WHO, 2009 report). In the febrile period, patient encounters an abrupt onset of high fever lasting more than a week and other symptoms such as red-spots in the skin, myalgia, retro-orbital pain, muscle and joints pains and headache. In the case of dengue fever, the acute febrile period usually comes and goes away within a week of illness, with patients recovering from the disease. However, in some unfortunate cases, Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS) may occur whereby patient's health deteriorates further and experiences serious complications, progressing into what is known as the critical period which lasts from 24 to 48 hours.

During the critical period, patient's platelet count starts to drop critically, together with a rapid rise in haematocrit, due to plasma leakage (Epidemiological News Bulletin by Tan Tock Seng Hospital, 2005). Other clinical symptoms observed in DHF patients include thrombocytopenia and hemorrhagic manifestation. DSS occurs when there is a critical volume of plasma lost through increased in capillary permeability. Fluid resuscitation is required to treat the resulting hypovolemic shock as patient may die within 12-36 hours if there is no immediate treatment of this complication (Martina et al. 2009). If the patient recovers from the critical phase, platelet count will gradually return to normal with a restoration of the general well-being of the patient's health.

1.3.2. Cross reactive T cells and deregulation of cytokine production

The cellular immune response consists of heterogeneous populations of antigen-specific T cells (CD4 & CD8) that effectively eradicate invading pathogens. Although these virus-specific T cells clear viruses, these T cells can also exacerbate tissue injury and induce pathogenesis of the diseases (Cannon et al. 1988; Klavinskis et al. 1989; Kurane and Ennis 1992). In the case of DF, the severity of the disease is believed to be attributed from the amplification of cytokine release caused by secondary infection occurring in the presence of memory T cells (Rothman and Ennis 1999).

DENV-specific CD4+ and CD8+ T lymphocytes are detected in patients with primary infection. Studies showed that beside the predominant response, in uncloned PBMC, to the serotype of DENV that the donors were exposed to, there was also presence of serotype cross-reactive responses detected (Dharakul et al. 1994; Gagnon et al. 1999). This is probably due to the high homology between the four serotypes of DENV. The kinetics of the T cell response in a secondary infection caused by dengue infection is also different from a primary infection. The dengue-specific memory T cells respond more rapidly during a secondary infection compared to naïve T cells, resulting in faster proliferation of dengue-specific T cells. This is also believed to be the same for DENV serotype cross-reactive memory T cells during secondary exposure to the virus (Beaumier et al. 2008; Mathew et al. 1998).

These virus-specific CD4+ and CD8+ T cells lyse infected cells and produce cytokines such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα). Several clinical studies have reported elevated levels of cytokines such as TFNα, IFNγ and IL-2 in patients with DHF compared to those having DF (Bethell et al.

1998; Green et al. 1999; Hober et al. 1993). The deregulation of the lymphokine production may further activate complement cascade and contribute to the overall pathogenesis, such as plasma leakage observed in DHF patients.

1.3.3. Antibody-dependent enhancement of dengue virus infection

DENV is shown to replicate in macrophages in-vivo and cause enhanced infection known as antibody dependent enhancement (ADE) (Halstead 1982; Halstead and O'Rourke 1977). Recovery from first time infection with DENV normally provides a life-long immunity to the particular serotype of DENV. However, sequential infection by another serotype often leads to the development of the more severe form of illness, DHF or DSS (Halstead 2002). This ADE phenomenon occurs during a secondary infection when the viruses enter via non-neutralizing IgG antibody complexes (generated during previous infection) through idiosyncractic Fcγ-receptors. Due to the inability of the non-neutralizing antibodies to activate phagocytosis, the virus-IgG immune complexes escape the host defense and gain a free ride into the host cells. Epidemiology studies reporting higher occurrence of DHF/DSS in patients who have previous infection with the virus validated this hypothesis (Halstead et al. 1970; Sangkawibha et al. 1984; Thein et al. 1997). This is further strengthened by the findings of increased risk of DHF experienced by infants born from dengue-immune mothers, probably due to the presence of pre-existing maternal dengue antibodies in these children (Kliks et al. 1988). However, there were also few reported cases of DHF/DSS patients who did not have pre-existing dengue antibodies and showed primary immune response, implying the possibility of other factors responsible for causing the severity of the disease (Scott et al. 1976).

1.3.4. Genotype and viral factors involvement in pathogenesis of DHF

All four serotypes of DENV can cause the severe form of the disease; with an implicated higher risk in patients infected with dengue serotype 2 virus (Burke et al. 1988; Sangkawibha et al. 1984) or dengue serotype 3 (Messer et al. 2003) . There were also studies describing the existence of "virulence" and "avirulent" genotypes that differ in causing the severity of the disease. This was based on the observation of DHF/DSS epidemic appearing in the western hemisphere after the introduction of a southeastern Asian DENV2 genotype in 1981 (Guzman et al. 1995; Rico-Hesse et al. 1997). Asian DENV2 genotype was also shown in in-vitro experiments to produce higher titers in macrophages and dendritic cells compared to the American DENV2 genotype (Cologna and Rico-Hesse 2003; Pryor et al. 2001). As a result, it is believed that these Asian genotypes are more "virulent" than those native genotypes found in Americas and the South Pacific.

Beside genotype difference in causing severity of the disease, the presence of more viruses or viral proteins in bloodstream of patient may also be responsible for causing DHF/DSS. Higher viremia load in patients' blood was found to correlate with the severity outcome of the disease (Endy et al. 2004; Libraty et al. 2002). Circulating viral protein in the bloodstream, sNS1, was also frequently found in elevated levels in patients with DHF (Alcon et al. 2002; Libraty et al. 2002; Young et al. 2000). NS1 is strongly immunogenic and there were studies done showing protection achieved against the disease by using anti-NS1 antibodies (Falgout et al. 1990; Henchal et al. 1988; Schlesinger et al. 1987). Other viral protein, NS2A, NS4A and NS4B are also implicated to play a role in causing the severity of the disease, possibly via

interference in the signaling of interferon during immune responses (Jones et al. 2005; Munoz-Jordan et al. 2005; Munoz-Jordan et al. 2003)

1.4. DRUG DISCOVERY OF DENGUE VIRUS

1.4.1. Vector control

With no cure for dengue fever, vector control is used as an emergency measure throughout many countries where outbreaks are reported. Chemical intervention, such as space spraying, is commonly used but it is not considered viable either as a long-term or effective strategy. This is probably due to its perceived negative impact on human health and environment (Curtis and Lines 2000). Furthermore, once spraying activity stops, the mosquito population returns. Success has been reported in cases where huge amounts of resources were expended, in terms of both labor and costs. These included the implementation of a vigilant surveillance system and the strict observance of vector control programs (Erlanger et al. 2008).

Biological intervention is another area of research aimed at controlling the transmission of dengue. Mosquitoes are genetically modified (GM) to carry destructive genetic traits to the mosquitoes and are subsequently released to mate with the wild mosquitoes. One key component to the success of this strategy is the ability of these GM mosquitoes to survive, mate and pass on the destructive genetic traits (Scott et al. 2002).

One such biological strategy is the use of the sterile insect technique (SIT) on mosquitoes. It was first used in the early 1950s on the New World screwworm Cochliomyia hominivorax, and since its success, scientists have attempted similar approaches to control other disease-carrying vectors (Benedict and Robinson 2003). Laboratory-grown mosquitoes are sterilized by either ionizing radiation (IR) or

chemo-sterilization, with the first sterile mosquitoes by IR released into the wild in 1959 by the United States Department of Agriculture in South Florida (Weidhaas et al. 1962).

Other biological interventions include genetically modifying the genome of these disease-carrying mosquitoes, using transposable elements or bacterial symbionts to introduce harmful genes into the mosquito population (Beerntsen et al. 2000; Ribeiro and Kidwell 1994). Another interesting approach is the shortening of the life span of mosquitoes by introducing life-shortening strains of the obligate intracellular bacterium *Wolbachia pipientis* into laboratory mosquitoes. These mosquitoes are then released to mate with the wild pool (Cook and McGraw 2010; McMeniman and O'Neill 2010). Results from all these studies conclude that seasonal patterns of the targeted species distribution and competiveness of the wild type mosquitoes versus the released sterile populations (Helinski and Knols 2008) are crucial determinants in the successful outcome of this strategy. Realizing these factors, scientists are currently placing more effort on their mosquito population dynamics surveillance studies and on the comprehension of the ecology and biology of these arthropods.

1.4.2. Vaccine development

To understand the daunting task faced in developing a vaccine for dengue fever, we need to understand that this disease can be caused by any of the four serotypes of the virus. Gaining immunity against one serotype does not confer immunity to the other three. Another factor to consider is the antibody-dependent enhancement mechanism implicated with this disease when administering a monovalent vaccine to a patient who has previous infection with a different DENV serotype. Hence, the logical strategy adopted by many is to develop a tetravalent

vaccine that protects against all four serotypes (Halstead 1988). Furthermore, there is no available animal model to date that can mimic the same pathological scenarios in the DHF/DSS in human infection. Thus, this makes the research on the pathogenesis of this disease challenging.

To achieve global vaccination against dengue fever has been a priority of the WHO (Brandt 1990). Two pharmaceutical companies, Sanofi Pasteur and GlaxoSmithKline (GSK), are currently leading the field of dengue vaccine development to come up with the first live attenuated tetravalent vaccine. The Sanofi Pasteur vaccine candidate, ChimeriVax, is composed of recombinant live attenuated vaccines of all four serotype envelope genes based on a yellow fever vaccine 17D vector backbone (Deauvieau et al. 2007; Lang 2009). GSK, in collaboration with Walter Reed Army Institute of Research, developed a dengue vaccine based on a different strategy: their vaccine candidate is a cocktail of individual monovalent vaccine against each of the four serotypes of dengue virus (Edelman et al. 2003; Innis and Eckels 2003; Sun et al. 2003). Other approaches include the usage of reverse genetic techniques to introduce attenuating deletion mutations into the 3'-untranslated region (UTR) of cDNA clones of DENV (Men et al. 1996; Whitehead et al. 2003), thereby developing recombinant sub-unit vaccines, based on the viral E proteins as antigens (Guzman et al. 2003; Robert Putnak et al. 2005). DNA vaccine, which expresses viral structural proteins to elicit immune responses that produce neutralizing antibodies against DENV, has also been attempted (Blair et al. 2006; De Paula et al. 2008; Raviprakash et al. 2003).

1.4.3. Targeting the E protein to inhibit viral fusion

DENV is known to enter host cells via clathrin-mediated endocytosis (Acosta et al. 2009; Gollins and Porterfield 1985; Krishnan et al. 2007; Mosso et al. 2008; Schaar et al. 2008). The virus requires an acidic pH environment in the late maturing endosomes (Schaar et al. 2008; Zaitseva et al. 2010) for viral fusion with host membrane, in order to release its genetic material for successful establishment in the host. The glycoprotein envelope (E) protein mediates this viral fusion. The viral fusion protein of the E protein is maintained initially in the virion in a metastable state. This fusion protein contains a conserved, mostly hydrophobic and glycine-rich segment, known as the fusion peptide (FP), which is inserted into the target membrane during the fusion event. The energy released from the maneuvering of the E protein to its most stable form drives the merging of the viral membrane with the host membrane (Weissenhorn et al. 1999). Viral fusion proteins are classified into two classes: class I and class II, defined by their structural properties (Kielian and Rey 2006; Lescar et al. 2001).

Class I fusion protein

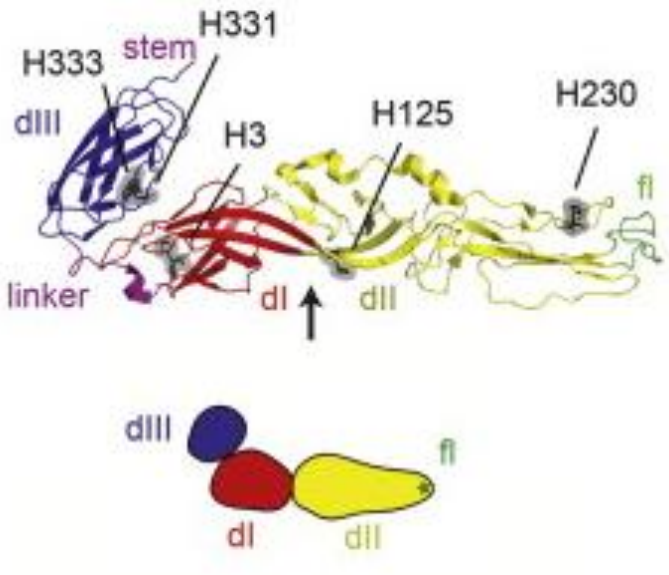
The class I fusion proteins exhibit structural analogies to the cellular SNARE fusion proteins (Skehel and Wiley 1998; Söllner 2004). They are found in a diverse family of viruses such as retroviruses, coronaviruses, paramyxoviruses, and filoviruses. Class I fusion protein is composed of mostly alpha-helical structures and is characterized by having the fusion peptide, in the precursor form, at the C-terminal to the cleavage point. The fusion protein is then brought closer to the N terminus upon maturation. For example, influenza virus hemagglutinin fusion protein, at neutral pH, is presented in the virion as fusion incompetent precursors (HA0). Upon protein cleavage, this generates two subunits, HA1 and HA2, with HA1 mediating the

receptor attachment and the other subunit HA2 mediating the fusion event (Skehel and Wiley 2000). Protein cleavage also primes the fusion protein into a metastable fusogenic state, exposing the fusion peptide (FP) on the N terminus of the protein subunit HA2. Within the acidic environment of the endosomal compartment, the HA2 undergoes radical conformation change (Huang et al. 1981), resulting in the dissociation of HA1 and refolding of the HA2 protein (Godley et al. 1992). The free energy released by this refolding and rearrangement of the HA structure at low pH is believed to drive the final lipid bilayer fusion between the virus membrane and the host membrane.

Class II fusion protein

Class II fusion protein is different from class I fusion protein. It is composed of mainly beta sheets and has an internal fusion peptide rather than N-proximal peptide which does not require cleavage for maturation. Class II fusion proteins include those found in alphaviruses and flaviviruses whose structural similarities are shown in figure 1-4. The E protein of DENV exists in dimeric structure at neutral pH with the highly conserved fusion peptide buried in a hydrophobic pocket at the tip of DII between DI and DIII, shielded from interaction with cellular membranes (Rey et al. 1995; Zhang et al. 2004). Upon encountering a drop in pH within the acidic compartment, selectively, highly conserved histidine residues in the E protein are protonated. This event triggers major rearrangement of this protein, with the homodimers dissociating to form intermediate monomers (Fritz et al. 2008; Kampmann et al. 2006; Stiasny et al. 2007). The resulting exposed fusion protein at the C-terminal of the E protein, primes it for contact with the host endosomal membrane. A flexible region between domain I (DI) and II (DII) of the E protein, also known as the “hinge”, acts like a spring to bring the fusion peptide in close proximity

(A) Alphavirus (SFV)



(B) Flavivirus (TBE)

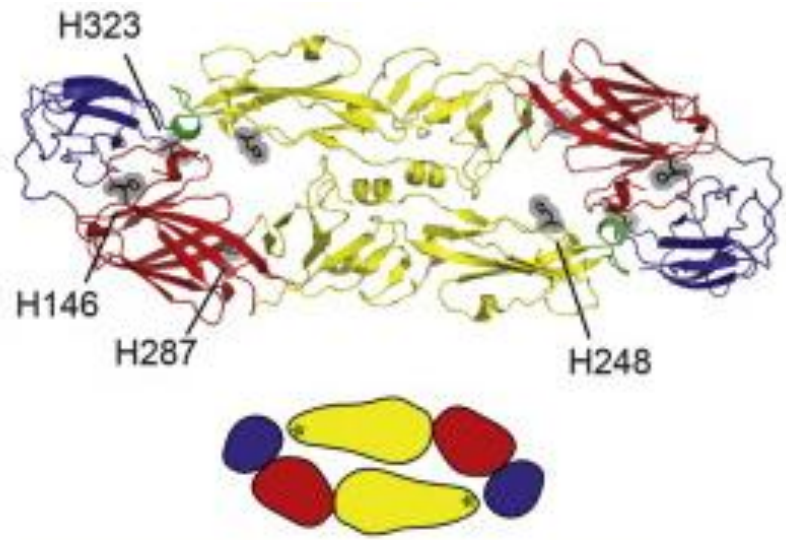


Figure 1-4: Overall architecture of class II fusion protein of (A) Semliki forest virus (SFV) which is an alphavirus and (B) Tick-borne encephalitis (TBE) virus, which is a flavivirus that carries a class II fusion protein which is hidden within DI and DIII of the E dimer. The various domains of the E protein are highlighted in red (DI), purple (DII), yellow (DIII) and green (fusion protein). Histidine residues involved in low-pH triggered conformation of E protein are also highlighted in the pre-fusion structures.
Reprinted from Trends Microbiology, 17(11), Sanchez-San Martin C, Liu CY, and Kielian M., Dealing with low pH: entry and exit of alphaviruses and flaviviruses., pg 514-521., Copyright (2009), with permission from Elsevier.

(insertion) with the host membrane during viral fusion (Modis et al. 2004; Zhang et al. 2004). There are four loosely packed DI-DII interface peptides, H1-H4, that contribute to the overall flexibility within this region during the low pH catalyzed conformations in the E protein (Hurrelbrink and McMinn 2001). The fusion ends with the transformation of the E monomers into homotrimers, which then pull back the anchor of the E protein with the fusion peptide-inserted endosome membrane to complete the fusion (Allison et al. 1995; Stiasny et al. 2004).

Small molecule fusion inhibitors

Cryo EM fitting of mature and immature DENV virions into their respective density revealed that the domains of E protein undergo major re-organization during the maturation event (Zhang et al. 2003). Crystallography of the trimeric post-fusion soluble ectodomain of E protein (sE) also showed large-scale orientation of the three E domains relative to each other; such as the overall differences of 35^0 in the angular orientation between domain I and II in the postfusion structure (Harrison et al. 2004). A serendipitous discovery of a hydrophobic pocket near the hinge region of E protein occupied by a small detergent molecule led to an interest for designing fusion inhibitors targeting this pocket of E protein (Figure 1-5; Modis et al. 2003). This pocket is proposed to be "fit-induced" as other structures of E proteins from DENV and TBE do not show the existence of such a pocket; suggesting molecule that binds to the pocket will result in the steric hindrance to the hinge movement, hence hampering fusion in the process. There were several studies reported that aimed to identify fusion inhibitor of DENV by using in-silico screening of small molecules predicted to bind to βOG pocket, and these resulted in the identification of several novel anti-viral compounds. A summary of these findings is found in table 1-1.

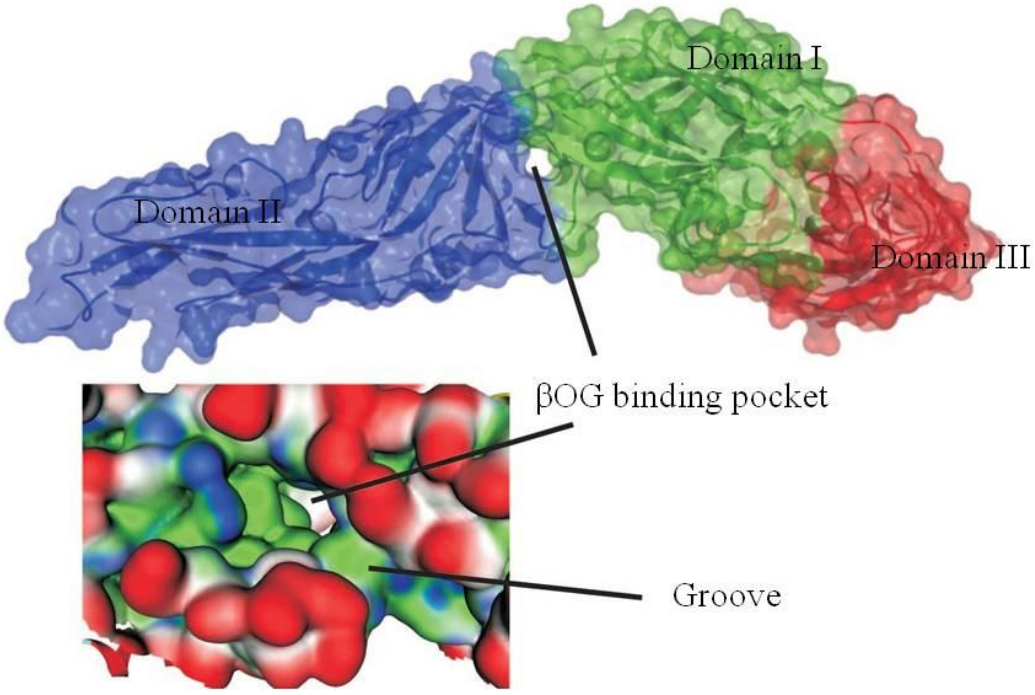


Figure 1-5: A pictorial representation of the DENV envelope monomer highlighting the BOG site. The enlarged viewed of the groove region with solvent-accessible surface with 1.4 Å radius solvent probe (red) and buried surface (green for hydrophobic, blue for hydrophilic).
Reprinted with permission from Zhou Z, Khaliq M, Suk JE, Patkar C, Li L, Kuhn RJ, and Post CB. 2008. Antiviral compounds discovered by virtual screening of small-molecule libraries against dengue virus E protein. ACS Chem Biol 3(12):765-775, Copyright (2008). American Chemical Society.

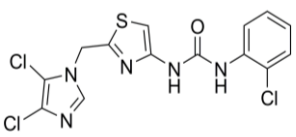
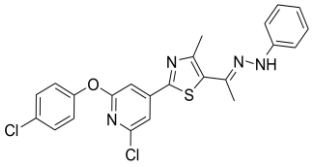
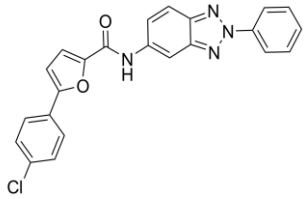
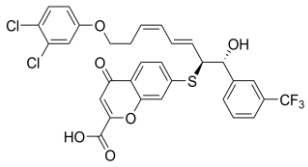
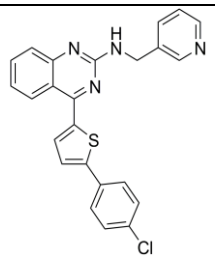
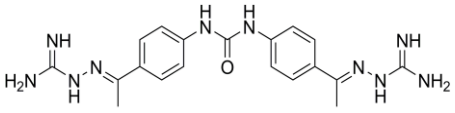
Compound	Assays tested	References
 CLogP= 3.578 MW = 402.69  CLogP= 6.367 MW = 469.40	Immunodetection Plaque reduction Cell-cell fusion	Kampmann et al. 2009. In silico screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with anti-viral activity against multiple flaviviruses. Antiviral Res. 84(3):234-41.
 LogP= 6.638 W = 414.86	Plaque reduction	Yennamalli et al., 2009. Identification of novel target sites and an inhibitor of the dengue virus E protein J Comput Aided Mol Des. 23(6):333-41.
 CLogP= 7.950 MW = 659.49	Plaque reduction Cell-cell fusion Liposome-based fusion	Poh et al., 2009. A small molecule fusion inhibitor of dengue virus. Antiviral Res. 84(3):260-6.
 CLogP= 6.650 MW =428.95	Immunodetection Virus-cell binding Plaque reduction	Wang et al., 2009. A small molecule dengue entry inhibitor. Antimicrob Agents Chemother.53(5):1823-31
 CLogP= 0.008 MW = 408.47	Cell-based virus growth Cell-based replication NMR binding	Zhou et al., 2008. Antiviral compounds discovered by virtual screening of small-molecule libraries against dengue virus E protein. ACS Chem Biol. 3(12):765-75

Table 1-1: Summary of small molecule fusion inhibitors of DENV

Peptide fusion inhibitors

Apart from small molecule inhibitors, peptide inhibitors are also investigated for their ability to inhibit viral fusion. Results from these investigations led to the conclusion: a ligand that can bind to the intermediate conformations of the fusion E protein (prior to the fusion of lipid bilayer with the host membrane) can retard or block the fusion event (Harrison 2008). Hence, synthetic peptides, reflecting the various regions of the DENV E proteins, were made to test for fusion inhibition. These peptides are usually first screened for their affinity to the E protein. Strong binders are subsequently tested for their ability to block fusion and viral infection. Several reports have shown peptides targeting domain III and the membrane-proximal regions inhibit dengue fusion and result in anti-viral activity (Hrobowski et al. 2005; Liao and Kielian 2005; Schmidt et al. 2010). Other regions of the E protein were also shown to be targetable by peptide inhibitors targeting the hinge region and the inter-domain region between domain I and II of E protein (Costin et al. 2010).

Antibodies that block fusion

The dengue E protein is involved in various stages of the viral infection such as receptor attachment, entry and membrane fusion and thus, this protein undergoes dynamic re-arrangements to form distinct functional conformations. The central domain (DI) of E protein is connected to DIII and DII by flexible linkers that allow the E protein to form such distinct conformations required during the various stages of viral infection. Antibodies that target the fusion loop of DENV E protein have been identified but they exhibit modest efficacy both in-vitro and in-vivo (Crill and Chang, 2004; Gocalvez et al. 2004; Nelson et al. 2008; Throsby et al. 2006). It was puzzling to scientists how this fusion-loop antibodies are able to recognize the mature form of the virus as DENV is known to have the fusion loop buried within the DI/DIII

interface of the antiparallel dimer (Kuhn et al. 2002; Nelson et al. 2008; Stiasny et al. 2006). This question is subsequently answered with the observation reported by Lok and colleagues whom showed that temperature can affect the affinity of a fusion-loop antibody (1A1D-2 Fab) to the virion, with enhanced affinity at 37 ^{0}C compared to 4 ^{0}C (Lok et al. 2008). They demonstrated that the conformation adopted by DENV E protein at 4 ^{0}C has limited access for the Fab to bind compared to the conformation adopted at physiological temperature. The immunoglobulin-like DIII undergoes significant displacement during the fusion transition. It has been demonstrated that both viral fusion and infection are inhibited by exogenous DIII fragments that bound to fusion intermediate following E trimerization (Chin et al. 2007; Chu et al. 2005; Liao and Kielian 2005). Hence antibodies that bind to DIII could potentially trap fusion intermediates preceding viral fusion, making them promising anti-dengue tool.

1.4.4. Targeting viral enzymes involved in viral replication

1.4.4.1. NS2B-NS3 protease

NS2B-NS3 protease (NS2B-NS3pro) is an attractive target as it is a crucial enzyme required to cleave viral precursor polyprotein during the initial establishment of viral genome replication in host cell. The viral protease is a trypsin-like serine protease possessing a catalytic triad (His51, Asp75 & Ser135) (Bazan and Fletterick 1989; Gorbalenya et al. 1989). The dengue protease requires the ~40 residues hydrophilic domain from NS2B to cleaves the newly translated polyprotein at the junctions NS2A-NS2B, NS2B-NS3, NS3-NS4A, NS4B-NS5 and also internal sites within C, NS2A, NS3 and NS4A (Falgout et al. 1991; Lobigs 1993; Nestorowicz et al. 1994; Stocks and Lobigs 1998; Teo and Wright 1997; Yusof et al. 2000; Zhang et al. 1992).

Structural-based studies have greatly aid in the development of inhibitors for dengue proteases. Brinkworth and colleagues did some pioneering work on deciphering the structural properties of dengue protease using sequence homology modeling between the DENV NS3 with HCV NS3 protease as comparison model. The X-ray crystal structure of NS3 protease in complex with the mung-bean Bowman-Birk inhibitor, although not a representative of the active form of the enzyme, provided the initial structural basis for studying enzyme-inhibitor interaction (Krishna Murthy et al. 1999). Recent development in X-ray crystallography with the active form of NS3 with its co-factor NS2B showed large differences in the substrate binding region between the NS3 structure and NS3-NS2B structure. This further highlights the importance of role of NS2B in the viral protease activity (D'Arcy et al. 2006; Erbel et al. 2006). One major challenge is to find suitable inhibitors that can bind with high affinity to the active site of protease, given that the active site is flat (Erbel et al. 2006) and negatively charged (Noble et al. 2010).

The substrate specificity of the viral protease is also investigated. It was found that NS3 protease has specific binding sites for amino acid chains of the substrate(s) and the S1-S4 region of the substrate pocket is highly conserved across the four serotypes, implying the possibility of developing a pan-dengue protease inhibitor (Li et al. 2005) There were several studies done on peptidic inhibitors that disrupt the viral protease activity (Chanprapaph et al. 2005; Ekonomiuk et al. 2009; Ganesh et al. 2005; Yin et al. 2006). This approach is based on using peptidomimetics similar to the viral protease catalytic substrate. One such substrate reported was the tetrapetide benzoyl-norleucine (P4)-lysine (P3)-arginine (P2)-arginine (P1)-ACMC (Bz-Nle-Lys-Arg-Arg-ACMC) (Li et al. 2005) which forms the basis for subsequent studies involving

HTS and SAR-based screening for more potent peptide inhibitors (Niyomrattanakit et al. 2006; Yin et al. 2006).

Beside compounds that target the viral protease activity, compounds that disrupt the interaction of NS3 with the NS2B co-factor could be of interest in anti-dengue drug design. There are two proposed sites ("site 1" and "site 2") for NS2B binding sites, making them possible targets for allosteric inhibition (Chappell et al. 2008). Two teams have reported their HTS screening of small molecules protease inhibitors that led to the identification of such non-competitive compounds (Johnston et al. 2007; Yang et al. 2010).

1.4.4.2. NS3 helicase

The NS3 NTPase/helicase domain is responsible for the initiation of viral RNA synthesis by driving the energy required for this event and unwinding the secondary structure of the 3'-UTR region (Takegami et al. 1995). It has a DExH-subfamily helicase; helicases within this family have the primary sequence of a conserved motif called motif 2 or Walker B (Walker et al. 1982). Compared to the well studied HCV NS3 helicase, DENV NS3 helicase unwinding mechanism is still not well characterized such as the polarity of unwinding, loading strand requirements and specificity for duplex substrate (Eoff and Raney 2005; Lindqvist et al. 2008). Crystallization of NS3 helicase (Xu et al. 2006) has provided initial information about this enzyme and this led to the development of helicase inhibitors for DENV (Sampath et al. 2006). The high-degree of similarity observed among the catalytic regions in various flaviviral helicases also indicates the possibility of developing a broad spectrum anti-viral effective against all flaviviruses (Bollati et al. 2009). Since NS3 helicases interact with both nucleotide and nucleic acids, targeting these two

interactions could lead to inhibition of this viral enzyme. As reviewed by Sampath and colleagues, there are three strategies to design an inhibitor for helicase: (1) inhibition of the nucleotide binding or ATPase activity of NS3hel, (2) blocking RNA substrate binding or processing and (3) blocking the translocation of NS3hel (Sampath and Padmanabhan 2009). These inhibitors could either be competitive by direct competing with the substrates for binding to NS3, or bind to other sites of the protein to cause an allosteric effect on the enzyme. The task of finding a potent inhibitor against dengue NS3 NTPase/helicase is faced with difficulties such as toxicity of ATPase inhibitors towards the host and the lack of good physiologically relevant in-vitro assays that are suitable for high throughput screening (Lescar et al. 2008).

X-ray crystallography studies done on NS3hel bound with nucleotide revealed several structural features that were overall not appealing for designing nucleotide competitive inhibitors for NS3hel (Davis and Teague 1999; Luo et al. 2008; Wu et al. 2005). Several studies also concluded that inhibition of the ATPase activity of NS3hel was not sufficient to inhibit its helicase activity. Borowski and colleagues found that a broad range of structurally unrelated compounds were able to inhibit/enhance the ATPase activity of WNV NTPase/helicase without affecting the helicase activity and conversely, other compounds that stimulated the helicase activity without affecting the ATPase activity (Borowski et al. 2001). Two mutational studies investigated on dengue helicase also showed the existence of strong residual helicase activities that were ATPase independent (Sampath et al. 2006; Xu et al. 2005). This led to the debate as to whether the screening of inhibitors based on helicase assay is a wiser choice over NTPase assay or RNA binding assay. Co-crystal structures of NS4hel with RNA substrate reveal that the size of its nucleic acid substrate binding site is big

enough to accommodate small molecule inhibitors (Luo et al. 2008). Although it was demonstrated in HCV helicase with the identification of a small molecule helicase inhibitor QU663, the substrate binding site shows low druggability because of the few hydrophobic residues present in this region for compound binding (Maga et al. 2005)

Disrupting the conformational changes of NS3hel required for its function can provide an alternative strategy to targeting substrate binding sites. Luo and colleagues recently reported the functional requirement of the inter-domain flexibility between DENV protease and helicase (Luo et al. 2010). This flexibility is conferred by a linker region (residues ^{169}ERIGEPDYEVD^{129}) that is required for both the enzymatic activity and RNA replication. Hence, it may be feasible to design inhibitors that bind to the region of NS3 which would prevent the subsequent conformational changes required for the enzyme active form.

1.4.4.3. NS5 methyltransferase

The capping of viral RNA, in which a guanosine monophosphate (GMP) moiety is covalently attached to the 5' end of the RNA transcript, stabilizes the mRNA and regulates its translation initiation (Furuichi and Shatkin 2000). This capping process is done by four enzymatic reactions (see figure 1-6). The dengue NS5 methyltransferase is involved in the last two modifications: the N-7 methylation of the cap (guanine) by a guanine-N7-methyltransferase (N7 Mtase) and the 2'-OH methylation of the first transcribed nucleotide by nucleoside-2'-O-methyltransferase (2'-O-MTase). This RNA capping process is important for mRNA stability, protein synthesis and also virus replication, making NS5 methyltransferase attractive target for anti-dengue drug design.

The understanding of how NS5 MTase recognizes and binds RNA capped structures required for viral replication is useful when designing MTase inhibitors. Crystal structures of dengue MTase domain in complex with its co-substrate, *S*-adenosyl-L-methionine (AdoMet), or its capped substrate analogues and also the product of the reaction, S-adenosyl-L-hohomcysteine (AdoHcy), are useful data for designing inhibitors against MTase (Benarroch et al. 2004; Egloff et al. 2002; Egloff et al. 2007; Geiss et al. 2009). Structural studies reveal both high affinity binding site (HBS) and secondary putative low affinity binding site (LBS) located close to the AdoMet binding site for the capped RNA (Dong et al. 2008; Mastrangelo et al. 2007); making theses sites potential drug targets. Targeting MTase has been shown to be successful in other viral systems, using GTP analogues and AdoMet/AdoHcy analogues (Li et al. 2007; Pugh et al. 1978; Woyciniuk et al. 1995). Sinefungin, a well-known anti-fungal AdoMet analogue, was shown to inhibit methylation and viral replication in another flavivirus, West Nile virus (Dong et al. 2008), making MTase a valid target for anti-dengue drug discovery.

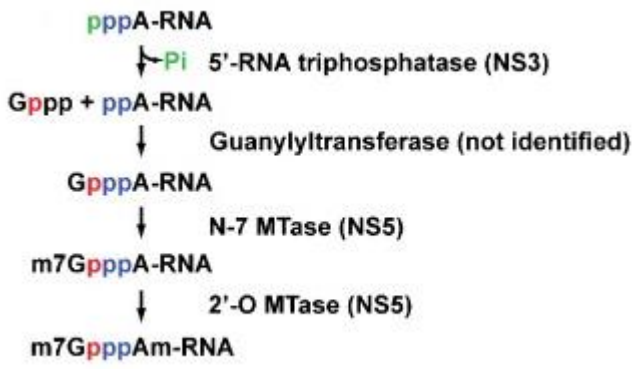


Figure 1-6: Dengue RNA 5′ cap formation. Four enzymatic modifications are required for cap formation that are carried out by NS3, a yet unidentified enzyme, and NS5 methytransferase as depicted in the diagram.
Reprinted from Antivral Research, 80(1), Dong H, Zhang B, and Shi PY, Flavivirus methyltransferase: a novel antiviral target, pg 1-10, Copyright (2008), with permission from Elsevier.

The knowledge of DENV RNA cap methylation is mostly derived from WNV MTases that exhibits a sequential event with the N-7 methylation event preceding the 2'O-methylation (Zhou et al. 2007). However, with the recent finding suggesting that DENV N-7 methylation, rather than 2'-O methylation, is essential for DENV replication, inhibitors that target N-7 methlyation may be a better choice for DENV drug discovery (Dong et al. 2010)

1.4.4.4. NS5 polymerase

The NS5 RNA-dependent RNA polymerase (RdRp) is a vital enzyme during viral replication as it is the core machinery for making new strands of viral RNA. This important function makes RdRp a target of particular interest in anti-viral drug design. Inhibitors that are currently developed for the polymerase are either nucleoside inhibitors or non-nucleoside inhibitors. High throughput screening of NS5 RdRp inhibitors has been greatly aided by the availability of cell-based sub-genomic RNA replicons containing only the non-structural proteins involved in the viral replication (Ng et al. 2007). Such tool not only allows the monitoring of viral replication in-vivo but also addresses other questions not possible in in-vitro polymerase assay, such as cell permeability and host toxicity.

In flavivirus drug research, many of the nucleoside inhibitors are presented as pro-drugs, requiring host kinases to phosphorylate and modify the compounds into active forms for inhibition of the viral enzyme (Carroll and Olsen 2006; De Clercq and Neyts 2009). These nucleoside inhibitors target the active site of the viral polymerase, functioning as competitors with natural NTP substrates or/and as 'chain terminators' (De Francesco and Carfi 2007). Many of the nucleoside inhibitors are discovered through the rational search of substrate analogues. An adenosine analogue

(7-deaza-2'C-acetylene-adenosine) is one such nucleoside reported to inhibit dengue NS5 polymerase through termination of the viral RNA synthesis. The compound exhibits potency in both cell lines and animal models (Yin et al. 2009). Non-nucleoside inhibitors are compounds that are bound to allosteric sites of the polymerase, affecting the conformational changes of the dynamic enzyme during replication, rendering the enzyme inactive. There are two potential allosteric sites proposed for dengue polymerase (Malet et al. 2008) that are targeted for the design of small molecule inhibitors. A non-nucleoside inhibitor for DENV polymerase (NITD002), reported by Niyomrattanakit and colleagues, was suggested to affect the polymerase activity by blocking the RNA template entry (Niyomrattanakit et al. 2010). However, like the fate of many non-nucleoside inhibitors, NITD002 failed to exhibit anti-viral activity in cell-based assay, possibly due to cell permeability issue. Studies done on DENV RdRp found that the de-novo RNA synthesis of the negative strand is enhanced by a large stem-loop structure (SLA) located near the promoter region (Filomatori et al. 2006that binds and interferes with the interaction of this SLA region with the RdRp domains may possibly block the conformation of active viral polymerase, thus making this region a potential drug target.

In summary, a structure activity relationship (SAR)-based approach is widely employed when looking for inhibitors of these various dengue viral enzymes. Despite having the knowledge of the crystal structures of these NS proteins, we have yet to find a potent anti-viral compound. This is often due to the challenges faced, such as the lack of relevant functional assays and the enigmatic complexity of dynamic conformational changes of these viral enzymes.

1.4.5. Host lipids as targets for anti-viral compounds

The revelation that many cellular host factors are important for replication fitness of the virus leads to the possibility of targeting host factors for anti-viral activity (Krishnan et al. 2008; Sessions et al. 2009; Tai et al. 2009). Targeting host factors is appealing as it removes the selective pressure of resistant mutations on the virus, which arise from drugs targeting viral proteins. Examples of host targets are host glucosidases that are involved in folding and glycosylation of viral proteins (Wu et al. 2002) and C-type lectin domain family 5, member A (CLEC5A), a lectin receptor, which is shown to involve in DHF/DSS pathogenesis (Chen et al. 2008) and host lipid metabolism. In the context of the work done in this thesis, I will focus on how host cell lipids contribute to viral infection and targeting them for anti-viral therapy.

1.4.5.1. Host cholesterol metabolism

Lipids have been shown to be important for many viruses and are required in various stages of the viral life cycle. Many plus-strand RNA viruses have been shown to induce membranous structures in host cells, which are believed to be sites of the viral replication machinery (Knoops et al. 2008; Ng and Hong 1989; Schaff et al. 1992; Welsch et al. 2009). There are implications of viral regulation on the host cholesterol homeostasis in order to supply lipids for the formation of these membranous structures (Deng et al. 2010). Cholesterol metabolism has been shown to be altered by many viruses, including WNV (Mackenzie et al. 2007), HCV (Fujino et al. 2010) and DENV (Rothwell et al. 2009). Robinzon and colleagues reported in their study of measles virus that genes in host cholesterol biosynthesis were up-regulated

during acute infection of neuronal cell lines (Robinzon et al. 2009). This led to the speculation that the measles virus regulates the host cholesterol synthesis to establish persistent infection in the host.

A popular class of cholesterol-lowering drugs, statins, has been shown to exhibit anti-viral activity against several viruses such as HIV (Amet et al. 2008), Poliovirus (Liu et al. 2006) and cytomegalovirus (Potena et al. 2004). For flaviviruses, statins were also shown to affect DENV (Rothwell et al. 2009) and WNV (Mackenzie et al. 2007). Statins, initially developed for treating atherosclerosis, act on inhibiting an important cholesterol synthesis enzyme, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase). There is a recent interest in using statins for HCV and HIV treatment, due to the frequent observations of lipid abnormalities associated with chronically infected patients suffering from these diseases. In one study, it was found that statins when used in combination with current HCV drugs resulted in a delay in resistance against these viral-targeted drugs (Delang et al. 2009).

However, there are controversial results arising from clinical studies with the use of statins: in one study, statin was found to improve the efficacy of HCV drug (Milazzo et al. 2010; Sezaki et al. 2009), whereas in another study, there was a noticeable increase in viral loads in patients treated with flustatin (Milazzo et al. 2009). These conflicting results could be due to the pleiotropic actions of statins in HCV infection (Lonardo et al. 2007; Milazzo et al. 2009). This discrepancy in the effect of statins in patients highlights a need for further clinical studies, in order to get a clearer understanding of the mode of mechanism of statins in fighting viral infections. This would help to address the validity of host cholesterol metabolism as an anti-viral target for drug discovery.

1.4.5.2. Host fatty acids metabolism

Fatty acids play an important role in dengue replication (Heaton et al. 2010). One study demonstrated that DENV NS3 protein was involved in the distribution of fatty acid synthase (a key enzyme in the synthesis of fatty acid) to the site of viral replication. In-vitro experiment showed that NS3 increases fatty acid synthesis. The favouring towards fatty acid synthesis in viral infection is also observed in HCV (Su et al. 2002; Yang et al. 2008) and HIV (Rasheed et al. 2008), hinting the possible regulation of fatty acids metabolism by these viruses to establish themselves in host cells.

Fatty acids, like cholesterol, have been suggested to be involved in maintaining structure and fluidity of the membranes associated with the viral replication machinery. It has been shown that saturated fatty acids, which enhance membrane fluidity, stimulate HCV replication, whereas the unsaturated form, for example, polyunsaturated fatty acid (PUFA), which decreases fluidity, inhibits HCV replication (Kapadia and Chisari 2005).

Fatty acids are also important for the post-translational modification of proteins. These fatty-acylated proteins are synthesized by acyltransferases that utilize fatty acid–CoA substrates to yield *N*-myristoylated proteins (Farazi et al. 2001) or *S*-palmitoylated proteins (Linder and Deschenes 2007). Myristoylation of structural proteins of viruses is required for targeting these modified viral proteins to membranes that are important for processes such as cell entry and viral assembly and budding (Ansardi et al. 1992; Hearps and Jans 2007; Perez et al. 2004). Palmitoylation of viral proteins is equally important during viral infection. It has been

shown to be important for protein-protein interaction in HCV replication machinery (Yu et al. 2006) and also for coronavirus entry and assembly (Boscarino et al. 2008; Shulla and Gallagher 2009).

In summary, host fatty acid metabolism is regulated by viruses during primary infection, and this could facilitate the formation of specialised membranes required for the replication machinery. It is also required for targeting viral proteins to designated sites of the host cells for viral infection. Hence, suppressing fatty acid synthesis to suppress viral infection could be an avenue for anti-viral strategy. Targeting fatty acid synthase has been shown to reduce viral infection in-vitro, so perhaps an approach of designing novel FASN-targeted small molecule inhibitors may lead to a potent anti-viral compound.

1.4.5.3. Host ceramides

Ceramide, a molecule composed of a sphingosine and a fatty acid, is produced by hydrolysis of sphingomyelin by sphingomyelinases, or via de-novo synthesis catalysed by serine-palmitoyl-CoA transferase and ceramide synthase (Marchesini and Hannun 2004). Cellular ceramide levels were found to be elevated within twenty-four hours of infection with human cytomegalovirus (HCMV), concurrent with the stimulation of de-novo sphingolipid metabolism (Machesky et al. 2008). Sindbis virus, rhinovirus and adenovirus were also found to induce host ceramide synthesis, and were affected when ceramide production was inhibited (Grassme et al. 2005; Kanj et al. 2006; Ng and Griffin 2006). Gassert and colleagues showed that measles virus counteracts host immune response by activating host sphingomyelinases and ceramide accumulation in T cell membranes (Gassert et al. 2009). This resulted in an aberration in the overall actin cytoskeleton remodelling in T-cells, which was required for functional activation of host immune responses against the invading pathogens.

Another similar mechanism is observed in the impairment of host's innate response by lymphocytic choriomengitis virus (LCMV) via manipulation of host ceramide levels. This was observed in mouse model having acid sphingomelinase (ASMase) deficiency. The suppressed cytotoxic activity of cytotoxic T lymphocytes (CTL) was reported in these ASMase-/-mice and resulted in a delayed clearance of LCM viruses from the organs of these mice (Herz et al. 2009). Results showed that ASMase was required for efficient fusion of the host secretory lysosomes with the plasma membrane of CTL, required for virus clearance. It was therefore proposed that LCM virus impaired the fusiogenic of these two by disrupting ceramide levels in the membrane via ASMase suppression (see figure 1-7).

In HIV infection, ceramide plays a role in the entry step of infection. Galatosylceramide (GalCer) was discovered to act as a co-factor in HIV envelope binding to CD4 cells (Hammache et al. 1998). Studies showed that stimulating ceramide accumulation in host membranes resulted in inhibition of HIV, underlying the importance of ceramide levels in host cell membranes. This was consequently looked into as a possible target for HIV drugs (Nguyen and Taub 2004). Several analogs of GalCer were shown to inhibit HIV infection (Mahfoud et al. 2002; Villard et al. 2002). For HCV, ceramide accumulation at the plasma membrane by SMase treatment triggers the internalization of CD81, a major receptor of HCV, resulting in viral inhibition (Voisset et al. 2008). In one study, the use of ceramide analogue, N-(3-Hydroxy-1-hydroxymethyl-3-phenylpropyl) dodecanamide (HPA-12), which inhibits ceramide trafficking from the endoplasmic reticulum to the Golgi apparatus, caused a suppression in HCV production (Aizaki et al. 2008). A separate study from the isolation of a secondary fungal metabolite led to identification of a novel HCV inhibitor, NA255. The mechanism of action of NA255 was elucidated to be targeting the de-novo synthesis of sphingolipids (Sakamoto et al. 2005). This compound was also found to inhibit the association of HCV non-structural proteins with lipid rafts.

The role of ceramides in flaviviruses is still underexplored. Recently, JEV production was shown to be enhanced with SMase treatment of cells, and that regulation of host ceramide level is important for JEV viral entry and egress (Tani et al. 2010). As reviewed by Han and Jiang, perhaps with the recent advancement in mass spectrometry, extensive sphingolipids profiling (known as sphingolipidomics) could allow us to gain more insights into the relationship between host sphingolipid metabolism and viral infection (Han and Jiang 2009).

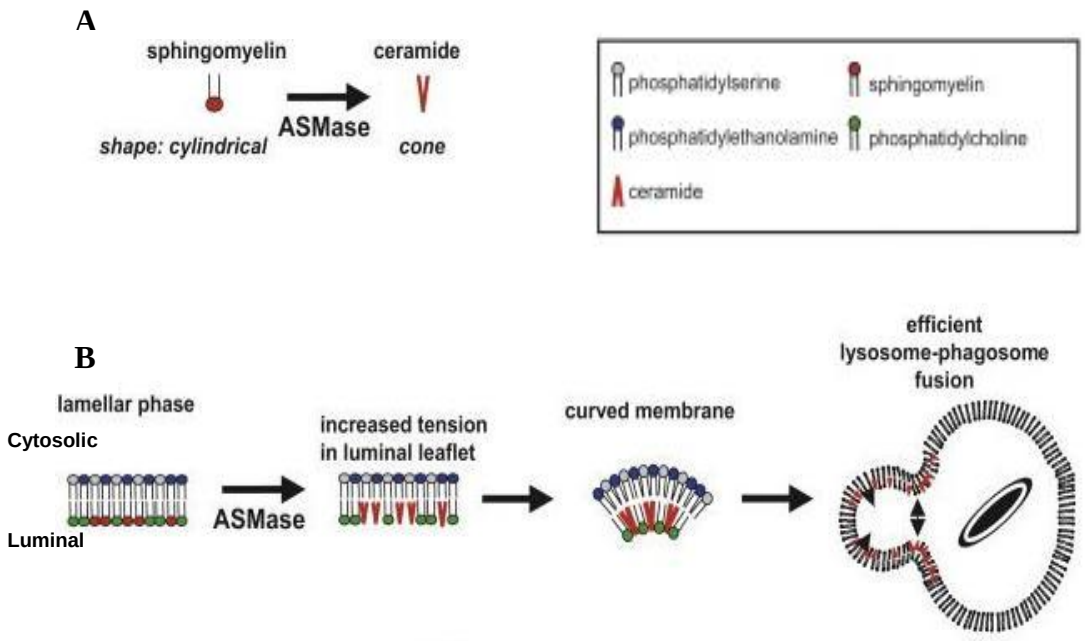


Figure 1-7: A cartoon depicting the mechanistic effects of ceramides in mediating cell bending and fusion. (A) Induction of ceramides via hydrolysis of sphingomyelin by acid sphingomyelinase (ASMase). (B) In biomembranes, lipids are asymmetrically distributed with sphingomyelin largely confined to the luminal and extracellular leaflets. Generation of cone-shaped ceramide within the luminal leaflet of vesicular membranes results in regions of bending thereby promoting full fusion of, e.g., lysosomes with CTL.
Reprinted from Immunobiology, 213(3-4), Utermohlen O, Herz J, Schramm M, and Kronke M., Fusogenicity of membranes: the impact of acid sphingomyelinase on innate immune responses, pg 307-314, Copyright (2008), with permission from Elsevier.

1.5. SCOPE AND OUTLINE OF THIS THESIS

The main objective of this thesis is to find novel compounds that inhibit dengue infection. The possibility of targeting a viral factor (viral fusion) or host factor (host cholesterol metabolism) using small molecule compounds will be investigated.

(1) Dengue virus fusion inhibitors

Viral fusion is a crucial event for the release of viral genomic material into host cytoplasm. This leads to the establishment of viral replication. The envelope (E) protein of dengue virus mediates the fusion event with host membrane. In Chapter 3, a rational approach is used to identify small molecules that can inhibit viral fusion. First, a cell-cell fusion assay will be developed to enable the screening of compounds in a 96-well plate format. Compounds identified from the cell-cell fusion assay will be validated and characterized for their anti-viral activity.

(2) A study of mechanism of action of NITD770, a compound discovered to inhibit host cholesterol biosynthesis

Work done in Chapter 4 is an extension of a study previously reported by Rothwell and colleagues (2009). They found that modulation of host cholesterol metabolism can influence dengue infection and that targeting the host mevalonate pyrophosphate decarboxylase (MVD) resulted in the suppression of viral replication. NITD770, a small molecule inhibitor of MVD, was found to inhibit dengue infection. Its mode of action and anti-viral activity will be validated in this study.

(3) U18666A, a cholesterol transport inhibitor: The identification and mechanism of action study of a new type of anti-dengue compound

In Chapter 5, the anti-viral effect of U18666A will be investigated. It is an amphiphile known to arrest cholesterol transport and inhibit cholesterol biosynthesis in cells. This study will investigate how the compound affects viral entry and viral replication, which aims to gain insight into the dependency of dengue infection on host cholesterol.

2. METHODS AND MATERIALS

2.1. CELL-BASED VIRAL FUSION ASSAY

The primary assay was adapted from a method previously described by Randolph and Stollar (1990). Mosquito cell line, C6/36, of a cell density of 1.5 x 10^5 cells per well were seeded together with dengue virus strain TSV01 DENV at a multiplicity of infection (MOI) of 0.1 in 96-well plates (Nunc, Denmark). Three days post-infection, each compound was added to the infected cells and incubated for one hour at 28°C. The medium was acidified to induce fusion by adding 5 μL of 0.5 M 2-(N-morpholino) ethanesulfonic acid (MES) (pH 5.0), and incubated for 1 hour at 37°C. The fused cells were then stained with propidium iodide (0.025 mg/mL) for 30 min at 28°C before proceeding to fluorescence reading with a Tecan Safire II plate reader using an excitation wavelength of 537 nm and an emission wavelength of 617 nm, with a bottom fluorescence measurement, and 3 readings per well. The quality of the assay can be determined by the dimensionless Z' value based on positive and negative control samples. The calculation, $Z'=1-(3xSD_{blank}+3xSD_{positive})/ (|Mean_{blank}-Mean_{positive}|)$, takes into account the signal dynamic range and the data variation associated with the signal measurement (Zhang et al. 1999). Only assays with a Z' value above 0.5, indicating a good dynamic range and a low variation, were accepted.

2.2. CELL-BASED FLAVIVIRUS IMMUNODETECTION (CFI) ASSAY

This is an in-house based assay developed in NITD. A549 cells were seeded one day before with a cell density of 20,000 cells per well in a 96-well plate. The cells were then cultured in 2%FBS in Hams F12 with 1% Penicillin/Streptomycin. Next day, the cells were infected with 1.09 μL (5500,000 PFU/mL stock) of NGC in the presence of 0.5 μL compound (100X) in 48.41 μL cell culture medium. A set of two-

fold serial dilutions was performed for each compound tested. After 48h incubation at 37^0C, viral antigen production was quantified by immuno-detecting using the anti-E antibody, 4G2) and secondary goat anti-mouse antibody conjugated with horse radish peroxidise. The dose-response curve was determined using nonlinear regression analysis by Prizm.

2.3. CELL CULTURES

All mammalian cell lines used in this thesis were cultured at 37^0C with 5% CO_2 except for insect cell line, C6/36 that was cultured at 37^0C without the requirement of 5% CO_2. Cell culture media and antibiotics, Penicillin and Streptomycin used in 1:100 dilution, were purchased from Gibco, Invitrogen, U.S.A. Fetal bovine serum (FBS) from Hyclone, Thermo Scientific, U.S.A. was supplied as 10% v/v during cell maintenance.

2.4. CYTOTOXICITY DETERMINATION

BHK21 cells were seeded with a density of 2 x 10^4 cells/well into a 96 well plate (NUNC, U.S.A) one day before treating them with increasing concentration of compound for 3 days. The CellTiter® Glo assay kit (Promega Corporation, Madison, WI, USA) was then used to determine the viability of the cells by quantifying the cellular ATP levels via a luciferase-based reporter system.

2.5. DRUG SYNERGY STUDY USING MACSYNERGY II

Drug interaction was performed on Huh7 DENV replicon cells using MacSynergy II platform, which look at synergy of two drugs based on the Bliss independence model (Prichard and Shipman 1990). The combination matrix was set

up with a 6 x 6 dose matrices with 5 serially diluted concentrations (33.3 μM, 11.1 μM, 3.7 μM, 1.2 μM and 0.4 μM) of each compound. A three dimensional plot was done which displayed synergy as peak above the calculated additive plane and depression below this plane as antagonism. A series of eight replicates were calculated to be statistically significant at 95% confidence level. The strength of synergy between the two compounds were determined by looking at the volume under the curve whereby synergy volume of <25 $\mu M^{2\%}$ is additive or non-synergy, 25-50 $\mu M^{2\%}$ is minor but significant synergy, 50-100 $\mu M^{2\%}$ is moderate synergy and >100 $\mu M^{2\%}$ is strong synergy.

2.6. EXPRESSION AND PURIFICATION OF DENV NS5 PROTEIN AND MUTANTS

Competent BL21rIL Escherichia coli (E.coli) cells were transformed with NS5 expression vectors and grown in LB plates containing 25 μg/mL of Kanamycin at 37^{o}C overnight. Next day, the colonies were scrapped and inoculated into 600mL fresh LB media (25 μg/mL of Kanamycin) and incubated at 37^{o}C with shaking (220 rpm) until an OD_{595} of 0.6 to 0.8. Induction of protein expression was done by addition of 0.4 mM of isopropyl-b-D-thiogalactopyranoside (IPTG) and placed in a pre-cooled 16^{0}C incubator with shaking set at 220 rpm for overnight. Cells were then harvested by centrifugation at 6000 rpm for 10 minutes at 40^{o}C and sonicated (34 % amplitude, 10 sec on, 10 sec rest, 5-7 min using Digital Sonifier® homogenizer, Branson, USA) in NTA-A buffer (20 mM Tris, 500 mM NaCl, 2 mM βMe, 5% Glycerol) with protease inhibitor (complete, mini, EDTA-free, Roche, U.S.A.) and 0.01% CHAPS. Resulting cellular debris were removed by centrifugation at 20500 rpm for 1 hr at 4^{o}C. Supernatant was then passed through 0.22 μm filters before

loading into a HisTRAP HP, 5 mL column (GE Healthcare, U.K) pre-equilibrated with cold NTA-A using AKTA FPLC Chromatographic System (GE Healthcare, U.K). The HIS-tagged NS5 protein was eluted from column using Buffer NTA-B (20 mM Tris-HCl at pH7.4, 500 mM NaCl, 2 mM βME, 5% Glycerol and 500 mM imidazole). Fractions as well as peak fractions were analyzed by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel stained with InstantBlue (Expedeon, UK) before pooling, desalting and concentrating to a volume of 0.5 mL using Amicon Ultra tube (Millipore, Billerica, USA) with a molecular weight cut-off of 50 kDa. The protein was then further purified by gel filtration using Superdex 200 10/300 GL column (GE Healthcare, U.K) that was pre-equilibrated with 20 mM Tris, 550 mM NaCl, 2 mM βMe, 5% Glycerol. Collected fractions were once again analyzed by SDS-PAGE and stained to identify fractions enriched with NS5. These enriched fractions were subsequently pooled together to be concentrated to approximately 8 mg/mL before snap freezing in liquid nitrogen and stored at -80^{0}C.

2.7. HPLC/APCI/MS ANALYSIS OF CHOLESTEROL AND ZYMOSTEROL

Control and treated cells were washed with PBS and harvested. Lipid was extracted from cells in chloroform: methanol (1:2) and the organic fraction was dried to lipid film. The dried lipid fraction was re-constituted in chloroform: methanol (1:1) for mass spectrometry analysis. Sterols were analyzed using an Agilent HPLC 1100 system (Agilent) coupled with an Applied Biosystems 3200 QTrap mass spectrometer (Applied Biosystems, Foster City, CA). In brief, separation of sterols was carried out using an Agilent Zorbax Eclipse XDB-C18 column (i.d. 4.6 × 150 mm) (HPLC conditions were as follows: (1) chloroform: methanol 1:1 (v/v) as the mobile phase at

a flow rate of 0.5 ml/min; (2) column temperature: 30°C; (3) injection volume: 10 ml. The LC-MS instrument was operated in the positive atmospheric pressure chemical ionization (APCI) mode with a vaporizer temperature of 500ºC and corona current of 3 µA. Multiple reaction monitoring (MRM) transitions for endogenous sterols as well as cholesterol-26,26,26,27,27,27-d6 (CDN Isotopes Inc., Quebec, Canada) and zymosterol-2,2,3,4,4-d5 (Avanti Polar Lipids, AL, USA) were used for sterol quantification (Huang et al. 2006).

2.8. INDIRECT IMMUNO-FLUORESCENCE MICROSCOPY

2.8.1. Immuno-fluorescence microscopy for DENV envelope in C6/36 cells

C6/36 cells were infected with viruses with a multiplicity of infection (MOI) of 1 for two days at 28°C. The infected cells were fixed with 2% paraformaldehyde in PBS for 15 min and washed with PBS. The fixed cells were then incubated with 1% FBS in PBS for 1 hr, and labeled with monoclonal antibody 4G2 (1:50 dilution from in-house antibody from hybridomas) against the E-protein overnight. This was followed by PBS washings before labeling with the secondary antibody, FITC-labeled goat anti-mouse IgG (1:1000 dilution; catalogue number #F0257 from Sigma, U.S.A.). The cells were counterstained with DAPI to visualize the nuclei. The labeled cells were observed through a laser-scanning microscope (Leica FW 4000) with a 63x oil-immersion objective.

2.8.2. Immuno-fluorescence microscopy for viral trafficking and co-labeling of DENV envelope protein with endosomes

BHK21 cells were infected with dengue virus strain NGC (with a multiplicity of infection (MOI) of 50) for various time points at 37°C to look at the trafficking of viruses. The infected cells were then fixed with 4% paraformaldehyde in PBS for 20 min, washed with PBS, permeablized with 0.05% saponin, 15 FBS, 10 mM Hepes, 10 mM Glycine in PBS, pH 7.5 for 30 min, and labeled with monoclonal antibody 4G2 (1:50) against the E-protein and either with anti-early endosomal antigen 1(1:50 dilution; rabbit polyclonal; catalogue number #2411 from Cell Signaling technology ™, U.S.A) or anti-Lamp1 (1:100 dilution; rabbit polyclonal; catalogue number #ab24170 from Abcam, U.K. U.S.A) overnight for co-labeling. This was followed by PBS washings before labeling with the secondary antibody, conjugated with Alexa Fluor 488 and 594 dyes (1:800 dilution; Catalogue number: #A10680 & #A11012 from Molecular probes, Invitrogen, U.S.A) . The cells were counterstained with DAPI to visualize the nuclei. The labeled cells were observed through a laser-scanning microscope (Leica FW 4000).

2.8.3. Cholesterol staining using FILIPIN III

For cholesterol staining, the cells were fixed with 4% PFA and washed several times with PBS before staining for 1 hour in dark with 70 μg/mL Filipin III (Cayman chemicals, U.S.A). The labeled cells were observed through a laser-scanning microscope (Leica FW 4000).

2.9. IN- VITRO FLUORESCENCE POLYMERASE ASSAY

The polymerase activity was determined using BBT-ATP (synthesized by Jena Bioscience GmbH, Germany), as a substrate for the polymerase reaction (Niyomrattanakit et al. 2011). This adenosine nucleotide was modified by attaching the BBT fluorophore group to the γ-phosphate of adenosine triphopshate. During the polymerization, adenosine monophosphate was incorporated into the RNA chain, and BBT_{ppi} was released as a by-product of the reaction. BBT_{ppi} , was then cleaved by calf intestinal alkaline phosphatase (CIP) to produce inorganic phosphate (Pi) and the alcohol, 2´-[2-benzothiazoyl]-6´-hydroxybenzothiazole (BBT), which gave fluorescence. The experimental procedure is described below.

The RNA template, 3'UTR-U_{30} (5'-bio-U_{30}-AACAGGUUCUAGAACCUGUU-3' purchased from Dharmacon, USA) was re-suspended to 200 μM in a buffer consisting of 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl in 0.1% diethyl pyrocarbonate (DEPC) water. The solution was then incubated at 60^0C for 5 min and placed at room temperature to allow the formation of the intra-molecular hairpin.

To measure the polymerase activity, a master-mix of 20 nM of NS5 protein and 50 nM of the RNA template were added into the RdRp assay buffer (50 mM Tris-HCl (pH 7.0), 1 mM $MnCl_2$ and 0.01% Triton) and left at room temperature for 30 min of incubation. Aliquots of 10 μL of the master-mix were transferred into wells of a 96 well assay plate (black plates from Corning Costar, USA). This was followed by 10 μL of 2 μM BBT-ATP in the same RdRp assay buffer. We then did a time-course study of 120 min. At every 10 min interval, a volume of 20 μL of stop buffer (200 mM NaCl, 25 mM $MgCl_2$, 1.5 M DEA) containing 25 nM calf intestinal alkaline (CIP), was added to terminate the reaction. The released BBT was monitored (after 1

hour incubation at room temperature) at ex/em 422/566 nm using Tecan SaffireII (Tecan, USA).

2.10. ISOLATION OF LIPID RAFTS

This protocol was modified from a published protocol (Lee et al, 2008). BHK21 cells were washed two times with ice cold PBS and scraped into falcon tubes. The pelleted cells were homogenized in low salt buffer (LSB) containing 10 mM Tris-HCL, pH 7.5, 25 mM KCl and 5 mM $MgCl_2$ with 1% triton on ice. Nuclei and unbroken cells were removed by centrifugation at 1000 g for 8 min. The post nuclei supernatant was then overlaid on a sucrose gradient composed of 10%-55%-75% and subjected to 16h centrifugation at 38000 rpm using a SW41 rotor (BECKMAN, U.S.A.) at 4^0C. The fractions were isolated in 1mL aliquots and cleaned up using a 10 kDa filter (Millipore, U.S.A.). The cleaned fractions were then loaded to a SDS-PAGE and viral proteins, NS3 and NS4B (GeneTex, Inc. #GTX103349), were immuno-blotted for lipid raft associations.

2.11. LIPID EXTRACTION

Cells or other types of samples were re-suspended in 100 μL of buffer (PBS, or other buffer depending on application). A volume of 0.6 mL of chloroform: methanol (1:2) was added and mixed by vortexing thoroughly for one minute. This suspension was then incubated on ice and subjected to vortexing for every 5 min of incubation on ice (performed twice). A further incubation period of 15 min on ice was done before addition of 300 μL of chloroform and 200 μL of water and mixed by vortex. The samples were centrifuged at ~8000 g force for 2 min at 4^0C. The organic phase (bottom layer) was collected and dried and stored at -80^0C. When setting up

the lipids for analysis, lipid pellet was re-dissolved in 100 µL of chloroform: methanol (1:1).

2.12. LIPOSOME-BASED VIRAL FUSION ASSAY

The assay was modified from a previously described experiment (White and Helenius 1980). Liposomes were prepared from a phospholipid (Avanti Polar Lipids, Alabaster, Ala.) mixture with a 1:1:1:1.5 molar ratio of phosphatidylcholine (PC; from egg yolk, Avanti), phosphatidylethanolamine (PE; prepared by transphosphatidylation from egg PC, Avanti), sphingomyelin (Sph; from bovine brain) and cholesterol. The amount of lipids used was 1 µmol PC, 1 µmol PE, 1 µmol Sph, 1.5 µmol Chol. The lipid mixture was then dried under a stream of nitrogen gas and reconstituted in HNE buffer (5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4) containing 10 mg/mL of trypsin (from bovine pancreas, Sigma, U.S.A). The lipid mixture was extruded through two pieces of polycarbonate membrane (0.2 µm hole size, Whatman, Clifton, NJ, USA) with a Mini-Extruder (Avanti, Polar Lipids, Inc., Alabaster, AL). Liposomes were stored at 4°C and used within one week. Fusion reactions were performed in 0.1 mL final volume containing 100,000 particles of purified viruses and 1 mM of trypsin-containing liposome. The samples were adjusted to the indicated pH values by adding a small pre-titrated volume of 0.1 M MES and 0.2 M acetic acid, and incubated for 2 min at 37°C for fusion to occur. The reaction was then neutralized to pH 8.0 using a pre-titrated volume of 0.1 M NaOH, and incubated at 37°C for an hour to allow trypsin digestion. The integrity of viral capsid was determined by SDS-PAGE of the reaction mixture followed by immuno-blotting using an anti-capsid antibody.

2.13. PLAQUE ASSAY FOR VIRAL TITER DETERMINATION

BHK21 cells (ATCC, U.S.A.) were seeded with a cell density of $2x10^5$ per well in a 24 wells plate (Nunc, Denmark) one day prior to infection. Undiluted and 10-fold dilutions of viral supernatant were prepared in RPMI-1640 medium containing 2% FBS and antibiotics (Pen Strep), and were loaded (0.2 mL) onto each well of BHK21 cells. After incubating the cells for 1 hour at 37°C (with 5% CO_2), the virus inoculums were replaced with 0.5 mL of 0.8% methylcellulose Aquacide (Calbiochem, USA) containing 2% FBS in RPMI-1640. After 4 days of incubation at 37°C with 5% CO_2, the overlay medium were fixed with 3.7% formalin in a formalin tank for 30 minutes, washed with tap water, and stained with 1% of crystal violet (200 μL). The plates were then dried in 50^0C oven and the plaques were visually counted .

2.14. PURIFICATION OF DENGUE VIRUS

The experimental procedures were performed as previously described (Kuhn et al. 2002). Briefly, the virus was centrifuged overnight (15 h) using a Beckman type 19 rotor at 30,000 x *g*. The virus pellets were then allowed to soak in HNE buffer (5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4) for 4 h on ice before thorough re-suspension by pipetting. Two different types of medium were used to set the discontinuous gradients in this thesis that are described below.

2.14.1. Dengue virus purification using potassium tartrate.

Overlay the re-suspended viral harvest to a SW41 tartrate gradient of 1.5 mL 35% (w/v) potassium tartrate in HNE-buffer, followed by 1.5 mL fractions of 30%, 25%, 20%, 15% and 1 mL fraction of 10% potassium tartrate in HNE (Fig.2A).

Subject the gradient to centrifugation for 2 hours at 4^0C in a Beckman SW41 rotor at a setting of 125,000 x g. The fractions were harvested in 1 mL aliquots.

2.14.2. Dengue virus purification using Optiprep.

The re-suspended viruses were overlaid to a SW41 Optiprep gradient of 1.5 mL 55% (w/v) Optiprep in HNE-buffer, followed by 1 mL fractions of 45%, 40%, 35%, 30%, 25%, 20% and 15% Optiprep in HNE (Fig. 2-1). Subsequently, the gradient was subjected to centrifugation for 2 hours at 4^0C in Beckman SW41 rotor at a setting of 125,000 x g. The fractions were harvested in 1 mL aliqouts.

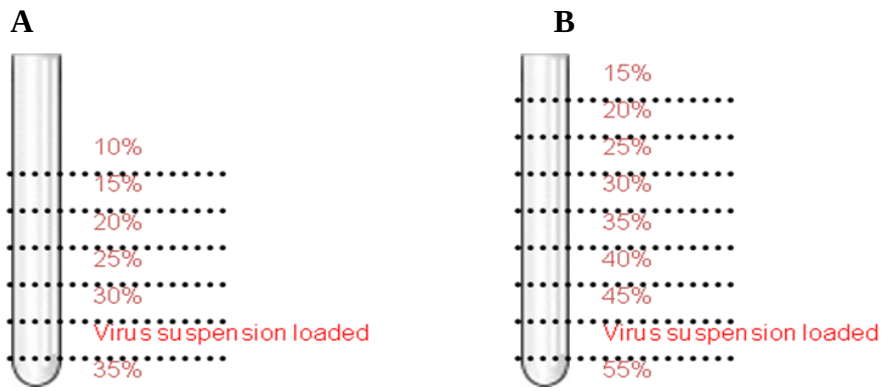


Figure 2-1: A cartoon representation of the gradient set up for (A) Potassium-tartrate medium and (B) Optiprep medium.

2.15. QUANTITATIVE REAL-TIME RT-PCR

Total RNA was extracted from viral supernatant using the RNeasy kit from Qiagen (USA). Reverse transcription was performed using the SuperScript™ III Reverse Transcriptase kit (Invitrogen, USA) with random hexamer primers (1.5 μg) (Roche, Switzerland). The reaction was carried out at 25°C for 10 min, 50°C for 60 min and 75°C for 15 min. For real-time PCR, a 20 μL reaction mix was set up using 4 μL of cDNA, 10 μL of iQ™ SYBR®Green Supermix (BioRad, USA) and 10 nM of primers. The sequences of the primers used were: Forward 5′-ACA AGT CGA ACA ACC TGG TCC AT-3′ and Reverse 5′-GCC GCA CCA TTG GTC TTC TC-3′ (Laue et al. 1999). PCR reactions were then cycled at 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec in the iCycler Thermal Cycler from the iQ5 Real-Time PCR Detection System (BioRad). A standard curve was established using a dilution series of NGC viral RNA extracted from a known titer as determined by plaque assay.

2.16. RAISING AND SEQUENCING RESISTANT VIRUSES

NITD770-resistant DENV-2 was generated by passaging the DENV-2 on A549 cells in the presence of NITD770. For each round of passaging, cells (2×10^5 per well) in 24-well plates were infected with 100 μL of DENV-2 with an MOI of 0.1 in the presence of 10 μM or 20 μM of NITD770. Passage 1 (P1) to passage 10 were performed. For each passage, viral supernatants were harvested at 48 hours post infection, viral titers were then quantified by plaque assays. The resistant pool in P10 was further passaged for three passages (P13) in 10 μM NITD770 before using it to check the establishment of resistance to the compound. Viral RNAs from passage 13 were extracted from the culture supernatant using a QiAamp viral RNA minikit (Qiagen, Valencia, CA). The RNAs were subjected to amplification using SuperScript III one-step RT-PCR kits (Invitrogen). The PCR products were gel purified and sequenced using the Sanger method provided by commercial company.

Fragment	Primer I.D	Primers Sequence	Nucleotide position
1	d2s23	5'- AGTWGTTAGTCTACGTGGAC	1 -20
	d2a18	5'- CCACTGCCACATTTCAGTTC	2455-2474
2	d2s5	5'- GGTGACACAGCCTGGGATTT	2182-2201
	d3a14	5'- ACTGTGATCATTAARTTGTGGGA	4334-4356
3	d2s9	5'- GCATTTTRGCCAGTTCTCTCCTA	4175-4197
	d2a10	5'- TACGCCCTTCCRCCTGCTTCA	6477-6497
4	d2s13	5'- GCAGACAGAAGGTGGTGTTTT	6193-6213
	d2a6	5'- CATGGTAWGCCCAYGTTTTGT	8468-8488
5	d2s16	5'- CAGGAAGTGGATAGAACCTTAGCA	7669-7692
	d2a23	5'- AGAACCTGTTGATTCAACAG	10704-10723

Table 2-1: Primers used for amplification of dengue viral genome.

Total RNA was extracted from viral supernatant using the RNeasy kit from Qiagen (USA). A combined one-step reverse transcription and PCR amplification was performed using the SuperScript™ III Reverse Transcriptase/ Platinum ® *Taq* Mix kit (Invitrogen, USA) with random hexamer primers (1.5 μg) (Roche, Switzerland). The cDNA reaction was carried out at 55°C for 30 min, 94°C for 2 min followed by PCR amplification at 40 cycles of 94^0C for 15 sec, 55^0C for 30 sec for fragment 1-4 and 42^0C, 68^0C for 2:30 min with a final extension cycle of 68^0C for 5 min using 1 μg of template RNA and 10 μM of primers.

2.17. REPLICON ASSAY FOR VIRAL REPLICATION STUDY

In-house stably transfected dengue replicons (Ng et al. 2007) were used in this thesis. Subgenomic genotype 1b HCV replicon cell line used was from Ralf Bartenschlager/ReBLikon GmbH. Non-cytopathic Venezuelan equine encephalitis virus (VEEV) replicon was a kind gift from Nancy L. Davis, University of North Carolina. . Please see table 2-2 below for information on the cell seeding density and culturing medium used for the respective replicon.

Cell Line	Cell seeding density	Cell culture medium
Dengue replicon in Huh7	20,000	2% FBS, DMEM
Dengue replicon in A549	10,000	10% FBS, Hams' F12
Dengue replicon in BHK21	10,000	2% FBS, RPMI
HCV replicon in Huh7	10,000	10% FBS, DMEM
VEEV replicon in BHK21	10,000	5% FBS, DMEM, High glucose

Table 2-2: Seeding density and cell culture conditions for replicon cell lines

The next day, the seeded cells were placed in fresh medium containing 2% FBS (instead of 10% FBS) and no selection compounds (e.g. puromycin, G418.) The compounds to be tested were then added in 2-fold serial dilution in the range of 0.195-100uM. The cells were incubated with the compounds for 48 hours before detection using EnduRen™ (Promega) added at 1:1000 dilution and incubated for 1hour before the luminescence was read. Using Prism v.4 (Graphpad software, San Diego, CA) software, the relative fluorescent unit (RFU) or luminescence reading was plotted against the log transformation of the concentration of the compound and a sigmoidal curve fit with variable slope was done to obtain the effective concentration (EC_{50}) value.

2.18. TRANSMISSION ELECTRON MICROSCOPY

The cell culture medium was removed from the cells and rinsed once with lukewarm PBS gently and quickly before fixation in 2.5% glutaraldehdye for an hour at room temperature. This was followed by three times PBS wash and treatment with 1% osmium tetroxide for another hour at room temperature in the fume cabinet. The fixed cells were then washed three times in PBS for 5-10 min in each wash. The cells were finally scraped into eppendorf tubes and centrifuged at low speed to create a cell pellet for gelatin packing (6-10%) at 4^0C for 45 min to solidify. The gelatin-embedded cell pellet was then soaked in 2.5% glutraldehyde for overnight at 4^0C. Next day, the gelatin-embedded cell pellet were cut into 1-2 mm cubes and dehydrated successively in ethanol (25% to 50% to 75% to 95% to absolute ethanol) and followed by absolute acetone. After dehydration, the cubes were infiltrated with resin, Epon-Resin (Pelco, Clovis, CA, USA) and left in 60^0C oven for 24 hours to polymerize the resin. The resin-embedded cubes were then cut to obtain ultrathin (70-90 nm) sections. The ultrathin sections were then stained with 3% uranyl acetate for 10 min, rinsed with water and then stained with lead citrate for 10 min, followed by rinsing with water. The processed sections were viewed using a transmission electron microscope (model 1010, Jeol Ltd., Akishima, Japan).

3. DISCOVERY OF SMALL MOLECULE FUSION INHIBITOR OF DENGUE VIRUS

3.1. INTRODUCTION

Dengue virus (DENV) is an enveloped virus belonging to the *Flaviviridae* family that includes other significant disease-causing members such as yellow fever virus (YFV), West Nile virus (WNV), and tick-borne encephalitis virus (TBE) (Chambers and Monath 2003). DENV is transmitted to humans via the bite of an infected mosquito (Vasilakis and Weaver 2008; Wang et al. 2000). Reported dengue cases are on the increase, with an emerging trend of dengue fever affecting both the affluent segment of society and the poor in tropical countries (Mathers et al. 2007; Normile 2007). The four serotypes of DENV (serotype 1-4) can cause a range of symptoms, from a mild fever to severe hemorrhagic manifestations (Chambers and Monath 2003; Kuno 1995). There has been an on-going effort to develop a vaccine for dengue fever since the 1940s (Batson et al. 2003; Mahoney et al. 2011). Vaccine development for DENV is challenging due to the antibody-dependent enhancement concern (Halstead et al. 1970) and the need to create a tetra-valent vaccine for protection against the four serotypes (Halstead 1988; Whitehead et al. 2007). An alternative approach to vaccine development is to develop anti-viral compounds to treat dengue infection. The multiple steps in DENV infection cycle such as viral entry, viral membrane fusion, replication, virus particle assembly and maturation are all potential anti-viral targets.

DENV enters the cell mainly by receptor-mediated endocytosis (Acosta et al. 2008; Suksanpaisan et al. 2009; van der Schaar et al. 2007) followed by viral envelope (E) protein mediated membrane fusion. The low pH-dependent fusion event between the viral membrane and the host endosome membrane releases the viral genetic material into the cytoplasm (Mukhopadhyay et al. 2005). The DENV E-

protein is composed of three main domains, with the central domain (Domain I) flanked by the immunoglobulin-like C-terminal domain (Domain III) and the dimerization N-terminal domain (Domain II), that carries the fusion peptide (Modis et al. 2004). The DENV E-protein belongs to the class II category of viral fusion proteins, which consist mainly of beta-sheets, compared to class I fusion proteins, which contain a more alpha-helical content (Kielian 2006). Other distinct characteristics of class II fusion proteins are the possession of an internal fusion peptide and the requirement for low pH initiation of viral fusion (Kielian and Rey 2006).

Upon lowering of the pH, the E-protein undergoes major conformational changes, with the particular region between Domain II and Domain I, known as the "hinge" springing upwards to bring the fusion peptide closer to the host membrane for fusion to occur (Modis et al. 2003). This hinge region is believed to be important during the early event of fusion. In the crystallization study reported by Modis and colleagues, a small detergent molecule, *n*-octyl-β-D-glucoside (βOG), was found to occupy a hydrophobic pocket near the hinge region; mutations within this binding pocket resulted in alteration of pH threshold for fusion. This has been interpreted as an avenue to look for occupants in this potentially druggable pocket in order to inhibit dengue viral fusion.

In this chapter, a screening program (Fig.3-1) that aims at finding small molecules that can inhibit DENV E-protein mediated membrane fusion is reported. By combining in-silico virtual screening with a 96-well based functional assay, and a low throughput secondary fusion assay, a novel compound, NITD448, was identified to inhibit DENV membrane fusion and viral production.

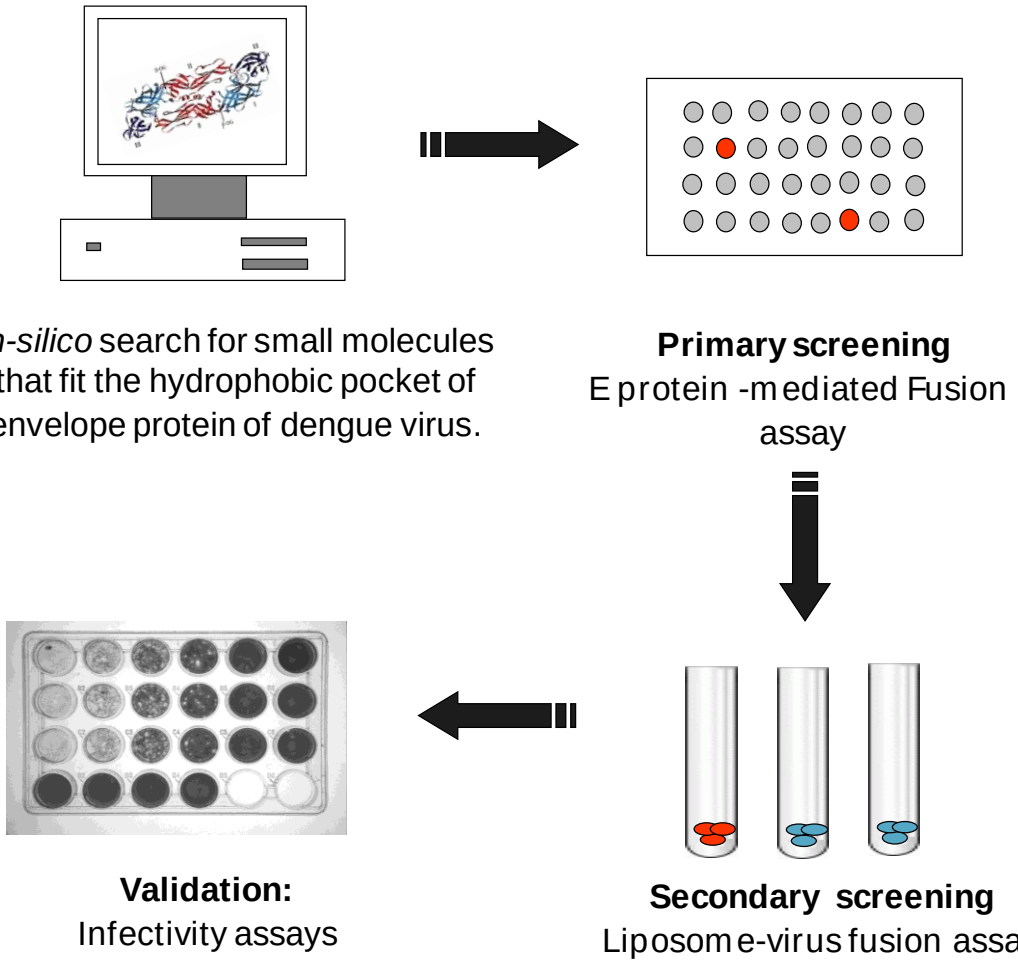


Figure 3-1: A diagram depicting the outline of the screening program to look for small molecules, which are able to inhibit dengue virus fusion.

3.2. RESULTS

3.2.1. In-silico virtual screening to build a focused library of dengue envelope protein binding compounds

Structure used as a target for in-silico docking of molecules onto the hydrophobic pocket of E protein

This study (done my Dr Ida Ma and Dr John Priestle from Novartis) used similar in-silico approach as reported previously by my colleagues in NITD (Wang et al. 2009) to define the βOG ligand (reported by Modis et al., 2003) as the ligand-binding site with potential interaction grids around this ligand generated. Each E-protein monomer forms its own βOG -binding pocket without direct contribution from the neighboring molecule. Although both chains were fully defined, the B chain was selected for the docking model as it possessed overall lower temperature factors, implying that its coordinates were more accurately determined. All solvent molecules were removed. There were three glycosylation sites on each chain. Since the glycosylation sites were far away (>25A) from the βOG binding site, they were also removed during virtual screening. The Protein Preparation module of Maestro (Schrödinger LLC, Portland, OR, USA) was used to prepare the protein for docking. The protein structure was also energy-minimized, with hydrogen atoms added, and their positions determined to maximize internal hydrogen bond formation.

Selection criteria for molecules predicted to bind to the hydrophobic ligand of E protein

From over 1 million compounds of the Novartis in house compound archive, 85,000 were removed as structural duplicates, possessing molecular weight >660, >2 undefined chiral centers or >12 freely rotatable bonds. The remaining compounds

were expanded for unknown chirality and alternative charge states using Pipeline Pilot (Accelrys Software Inc., San Diego, CA), generating 2.4 million structures to dock. These were docked using Glide SP (standard precision, Schrödinger LLC) on a Linux cluster. No constraints, e.g. a compulsory specific hydrogen-bond formation, were applied. Pipeline Pilot was used to sort the compounds by Glide_gscore and the top 3000 scoring compounds were kept. These were re-scored using consensus scoring (CScore) as implemented in Sybyl (Tripos, Inc., St. Louis, MI). The scoring results were normalized and combined. Low-scoring isomers (e.g. same molecules, different charge state) were removed and the remainder clustered with Pipeline Pilot using Functional Class FingerPrint, 6-bond radius fingerprints and a similarity distance threshold of 0.4 to remove some of the very similar compounds. The remaining 1367 top-scoring unique poses were then visually inspected until acceptable compounds were selected. The same procedure was carried out for docking the Novartis in-house natural product collection except that the chemical property limits for acceptable compounds was relaxed considerably, e.g. up to 15 rotatable bonds were accepted. In total, a library of 365 high-scoring, chemically reasonable small molecules was generated for the primary fusion assay.

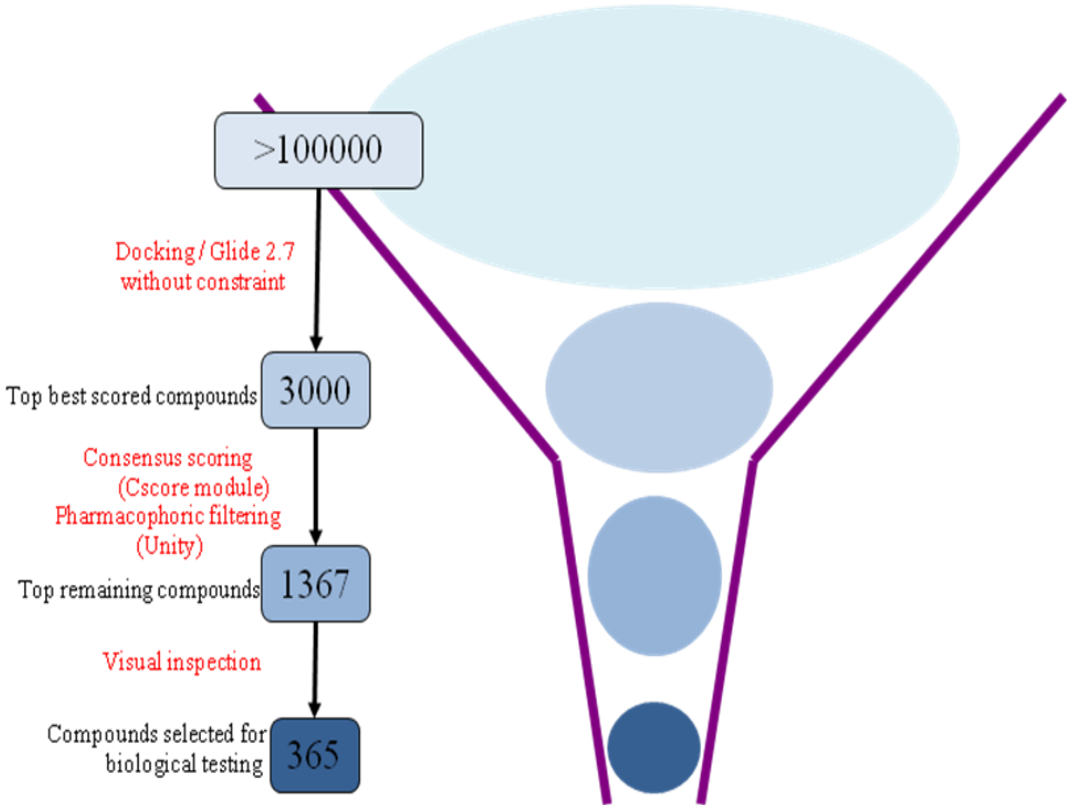


Figure 3-2: A schematic representation of the virtual screening process used to assemble a focused library of small molecules for the primary fusion assay. The βOG ligand, located in the region of DENV envelope protein important for fusion, was used to define the ligand-binding site.

3.2.2. Development of a medium throughput cell-based fusion assay to screen a focused compound library

Randolph and Stollar reported dengue infected C6/36 cells undergo cell-cell fusion (also known as syncytium) upon low-pH exposure (Randolph and Stollar 1990). This cell-cell fusion phenomenon is believed to be mediated by the viral E protein expressed on the infected cell surface. Immuno-fluorescence labeling of non-permeabilized dengue infected cells allows visualization of E-protein on the cell surface without labeling of E-protein present in the cell interior. As shown in figure 3-3A, E-protein is expressed on the cell surface of infected cells, including the interface between adjacent cellular membranes. Incubating at low pH of infected cells resulted in widespread cell-to-cell fusion (Fig. 3-3D). A cell-based fusion assay based on this cell-cell fusion property of the infected mosquito cells was developed to facilitate screening of Novartis compounds for fusion inhibition in a 96-well plate format

Propidium iodide (PI) staining was used to quantify the formation of cell-cell fusion. This method is based on the observations that viral protein-induced membrane fusion can damage membrane integrity (Bonnafous and Stegmann 2000; Frolov et al. 2003). PI dye cannot penetrate intact cellular membranes, resulting in very little staining of uninfected cells (Fig. 3-3B) or in infected cells maintaining a neutral pH (Fig. 3-2C). However, infected cells exposed to low pH showed bright PI labeling (Fig. 3-3D), allowing a fluorescent readout of cell-cell fusion. As a control, treatment with 4G2, a monoclonal antibody known to bind to the fusion loop of the E-protein (Crill and Chang 2004; Henchal et al. 1988) before exposure to low pH, inhibited cell fusion and PI staining (fig. 3-3E). In contrast, treatment of the infected cells with 9F12, a monoclonal antibody against Domain III of the E-protein, which is distal from

the fusion domain, did not interfere with fusion at low pH (Rajamanonmani et al. 2009).

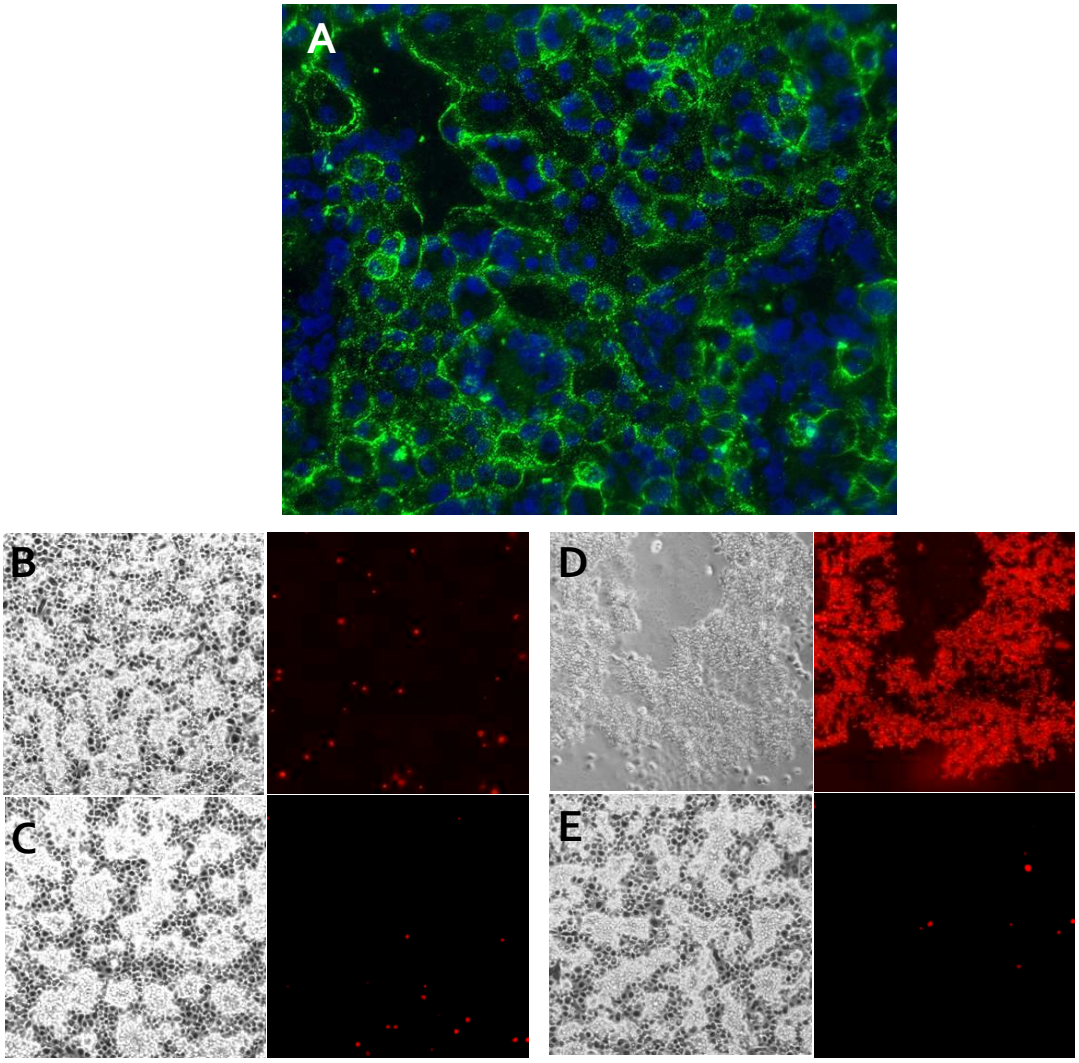


Figure 3-3: Low pH induced fusion of dengue infected C6/36 cells mediated by viral E-protein on the cell surface. (A) Cells infected with dengue virus display viral E-protein (green) on the cell surface shown by immunofluorescent labeling of fixed non-permeabilized cells. Nuclei were counterstained with DAPI (blue). (B) Uninfected cells did not fuse under acidic conditions, as shown in phase contrast (left panels), and did not exhibit appreciable propidium iodide (PI) staining (right panels). (C) Infected cells three days post-infection maintained at neutral pH also did not fuse and did not show PI staining. (D) Infected cells showed widespread fusion under acidic conditions resulting in PI staining of the syncytiated cell mass. (E) Infected cells three days post-infection that were pre-incubated for one hour with antibody 4G2 against the fusion loop of the E-protein, did not fuse and did not show syncytia formation nor PI staining after acidification.

Optimization experiments were performed (together with Mr Andy Yip from Novartis) to develop this cell-based fusion assay into a robust 96-well format. Cell seeding density was found to be an important factor in obtaining a reliable cell fusion signal, with an optimum between 1×10^4 to 2×10^4 cells per well (Fig. 3-4A). The assay signal was relatively insensitive to the Multiplicity of Infection (MOI) from 0.01 to 0.3, whereas a higher MOI of 0.5 reduced the assay signal (Fig. 3-4B). As expected, cell fusion was found to be pH-dependent, starting at an acidic pH value lower than 6.6 (Fig. 3-4C).

Using the above cell-based fusion assay, 365 small molecules identified from the in-silico docking were tested in triplicate at 25 μM. The assay had an a good Z' factor of 0.7. It was found that compound NITD448 (Fig. 3-5A) reproducibly inhibited the PI-mediated fluorescent staining in a dose-responsive manner, with a 50% inhibitory effect (IC_{50}) of 6.8 μM (Fig. 3-5B). These results suggest that NITD448 inhibits dengue virus membrane fusion.

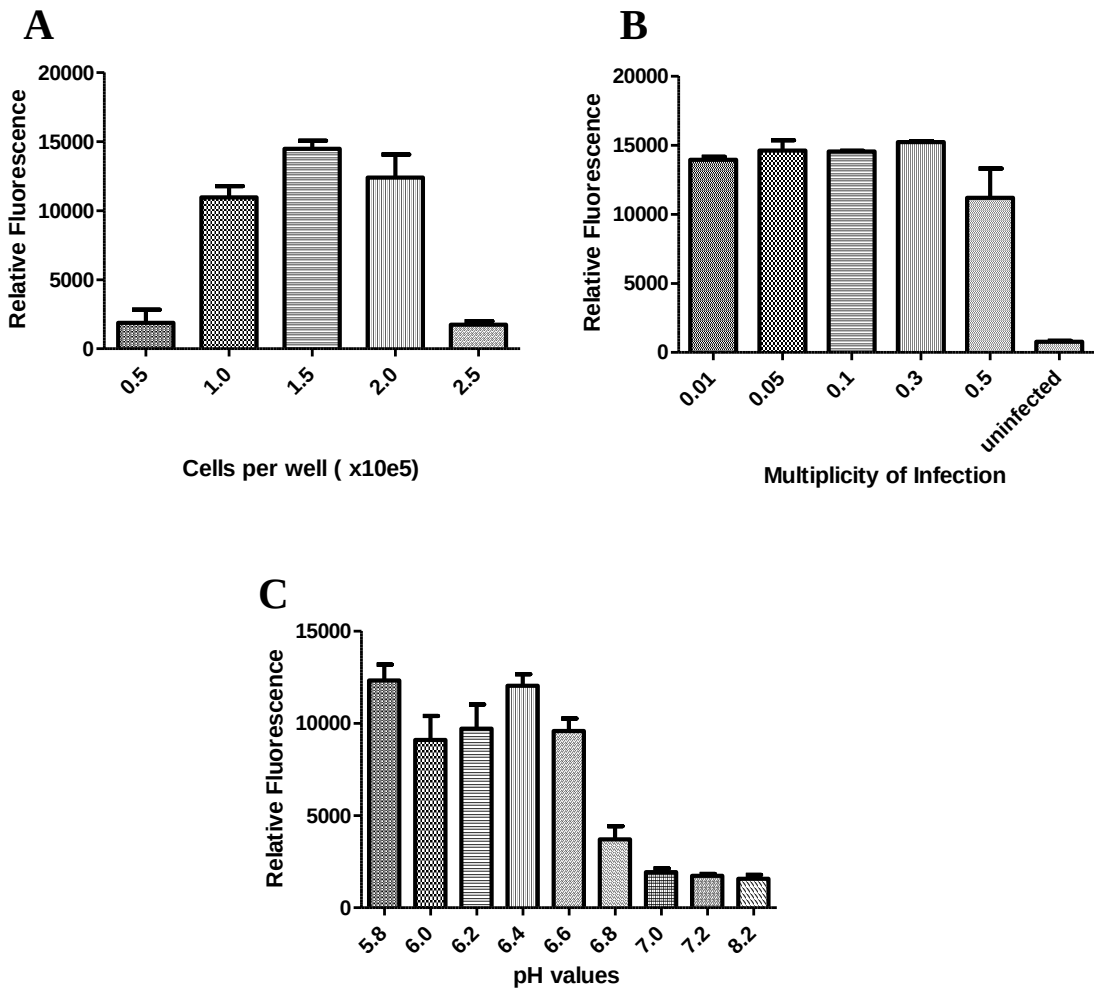
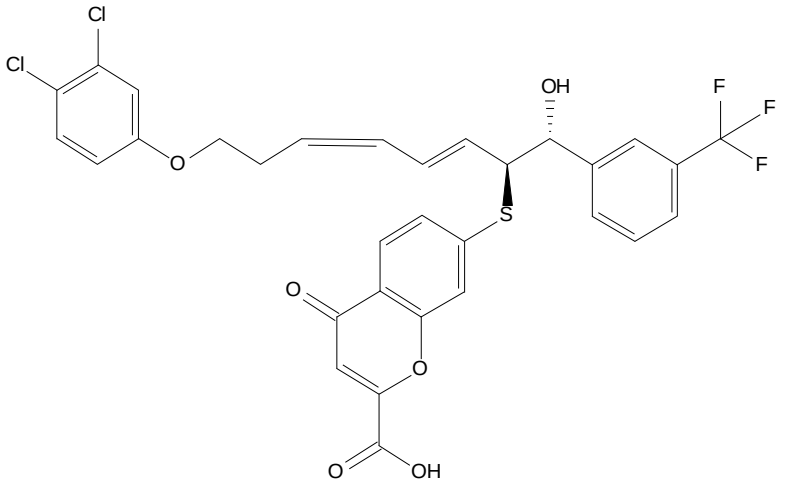


Figure 3-4: Characterization and optimization of the primary cell-cell fusion assay. (A) The wells of a 96-well plate were seeded with different cell densities and subsequently infected with dengue virus using an MOI of 0.1, followed by the acidification, staining and fluorescent readout on day 3 post-infection. Results shown are from four independent experiments, using two batches of cell stocks (P10 and P17). (B) Wells were seeded with a density of 1.5 x 10^5 cells per well and subsequently infected with different MOI, followed by the acidification, staining and readout on day 3 post-infection. Results are from two independent experiments. (C) Wells were seeded with a density of 1.5 x 10^5 cells per well and infected with dengue virus with an MOI of 0.1. On day 3, post-infection cells were exposed to media of different pH before staining and readout. Results are from two independent experiments. Error bars represent standard deviations.

A



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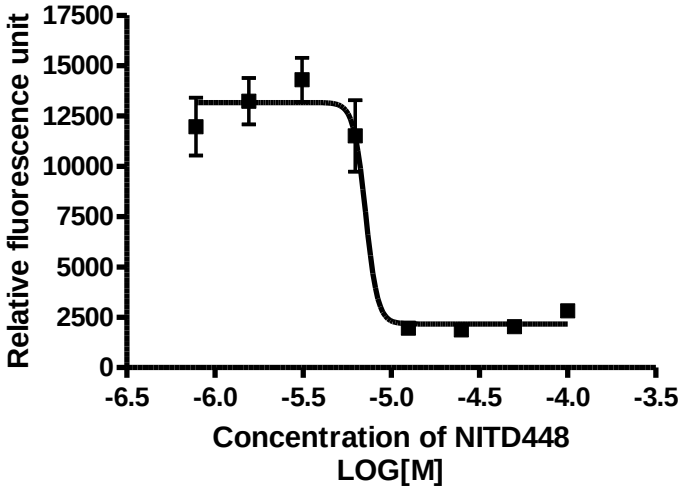


Figure 3-5: Compound structure and inhibition of fusion in primary assay. (A) The molecular structure of fusion inhibitor NITD448. (B) Compound NITD448 inhibited fusion in the cell-cell fusion assay with an IC_{50} of 6.8 μM. Error bars represent standard deviations from two replicates.

3.2.3. Compound NITD448 inhibits E protein-mediated membrane fusion in liposome-based fusion assay

Based on liposome fusion assays using isotope-labeled virus (White and Helenius 1980), a non-radioactive content-mixing fusion assay using immuno-detection of nucleocapsid protein was developed. Purified dengue virus mixed with liposomes containing the protease trypsin. Membrane fusion between these two particles allowed the trypsin to access the interior of the virions, resulting in digestion of the capsid (C) protein (Fig. 3-6).

Firstly, two different purification protocols were tested to find out which protocol would result in higher yield of viral particles for subsequent use in the liposome based fusion assay. It was noted that there were different viral banding profile obtained for the two protocols: the tartrate protocol resulted in a single visible viral band at 20% of tartrate, whereas three bands (20%, 25% & 30% optiprep) were visible for the optiprep protocol (Fig.3-7A). Further quantification of the viral titers of each of the gradient fractions resulted in the observation that the optiprep protocol had a higher recovery of infectious viruses compared to tartrate protocol (Fig.3-7B). SDS-PAGE analysis was performed to examine the purity of the viruses isolated from the fractions of optiprep gradient, as shown in figure 3-8, the envelope protein and possibly capsid protein were the main components in the purified fractions from the gradient. From this finding, optiprep protocol was used for purification of viruses used in subsequent liposome based fusion assay.

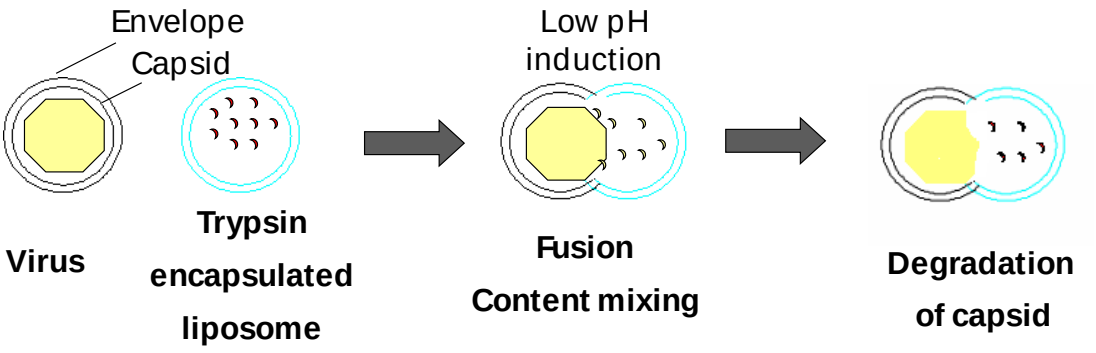
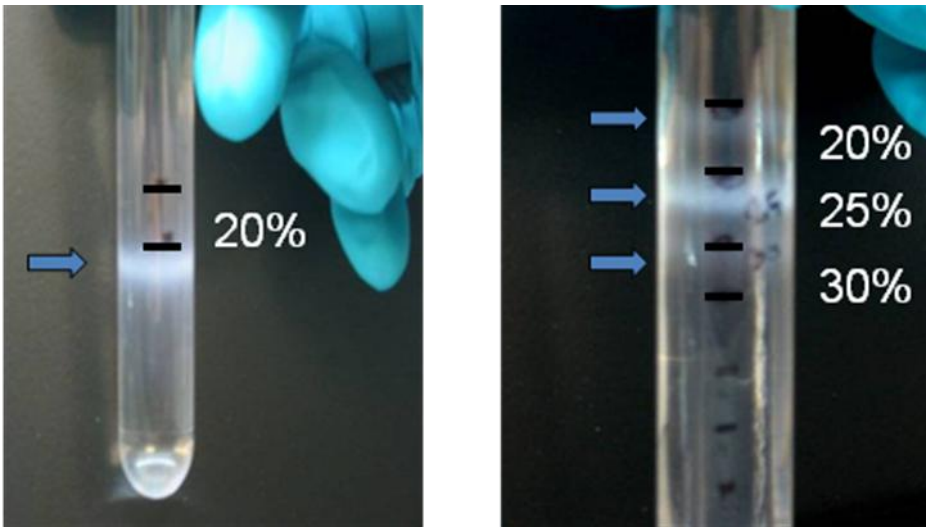


Figure 3-6: A cartoon representation of the liposome based viral fusion.

A

Tartrate **Optiprep**



B

Tartrate			Optiprep		
	pfu/mL	**% recovery**		**pfu/mL**	**% recovery**
Input	1.8 x 10^7		**Input**	5.9 x 10^7	
10%	2.0 x10^4	0.11	**15%**	7.0 x10^5	1.19
15%	1.5 x10^5	0.83	**20%**	1.0 x10^6	1.69
20%	7.0 x10^5	3.89	**25%**	1.3 x10^7	22.03
25%	8.0 x10^5	4.44	**30%**	1.3 x10^7	22.03
30%	1.5 x10^5	0.83	**35%**	8.0 x10^5	1.36
32%	1.0 x10^5	0.56	**45%**	7.0 x10^5	1.19
35%	2.5 x10^4	0.14	**50%**	4.5 x10^4	0.08
			55%	3.5 x10^3	0.01

Figure 3-7: Purification of DENV using two different mediums for density gradient. (A) Photographs taken after high-speed centrifugation resulting in concentration of viral bands around 20% for tartrate gradient and 20%, 25% and 30% for optiprep gradient. (B)Viral titer quantification using plaque assay to determine fractions of the gradient containing the most infectious viral viruses.

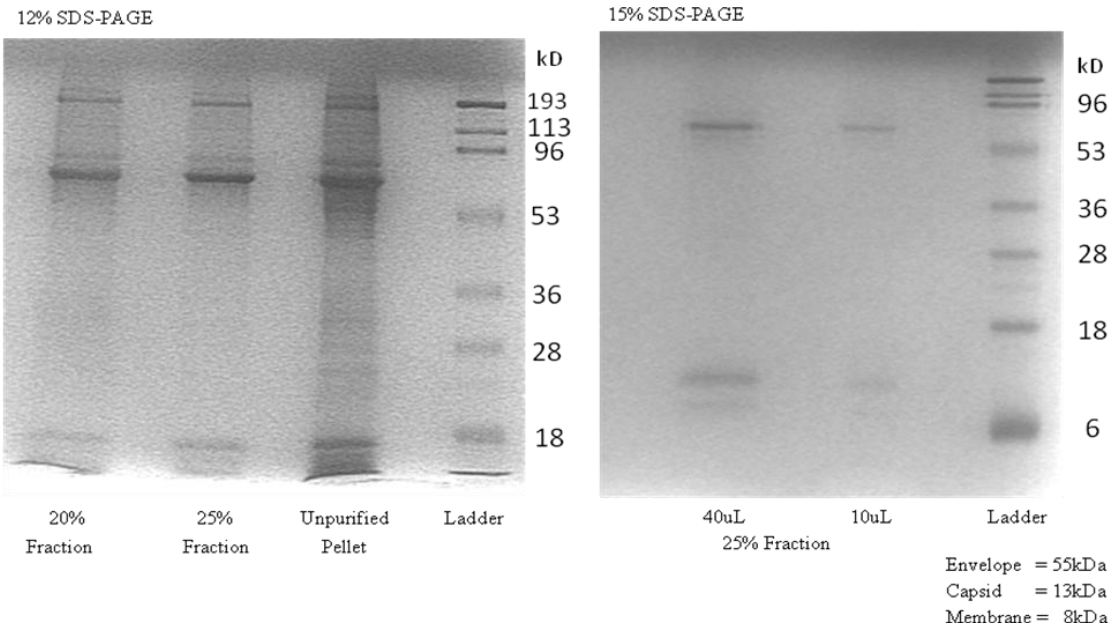


Figure 3-8: SDS-PAGE analysis to check for the purity of the purified viruses. Two resolution SDS-PAGE gels of 12% and 15% Bis-acrylamide were run. The viral proteins were visualized using coomassie blue protein staining reagent.

Western blot analysis showed intact C-protein when the mixture of virus and liposomes was kept at neutral pH. In contrast to mixture that was exposed to low pH, degradation of C-protein was observed, but only if trypsin was present in the liposomes (Fig. 3-9A). Addition of the 4G2 antibody against the E-protein was able to prevent degradation of C-protein in a dose-dependent way. The results indicate that the assay reliably monitors the pH-dependent E-protein membrane fusion.

Compound NITD448 was tested in this liposome based virus fusion assay and showed a clear and robust dose-dependent inhibition of membrane fusion (Fig. 3-9B). Taken together, the results clearly indicate that compound NITD448 can inhibit E-protein mediated membrane fusion.

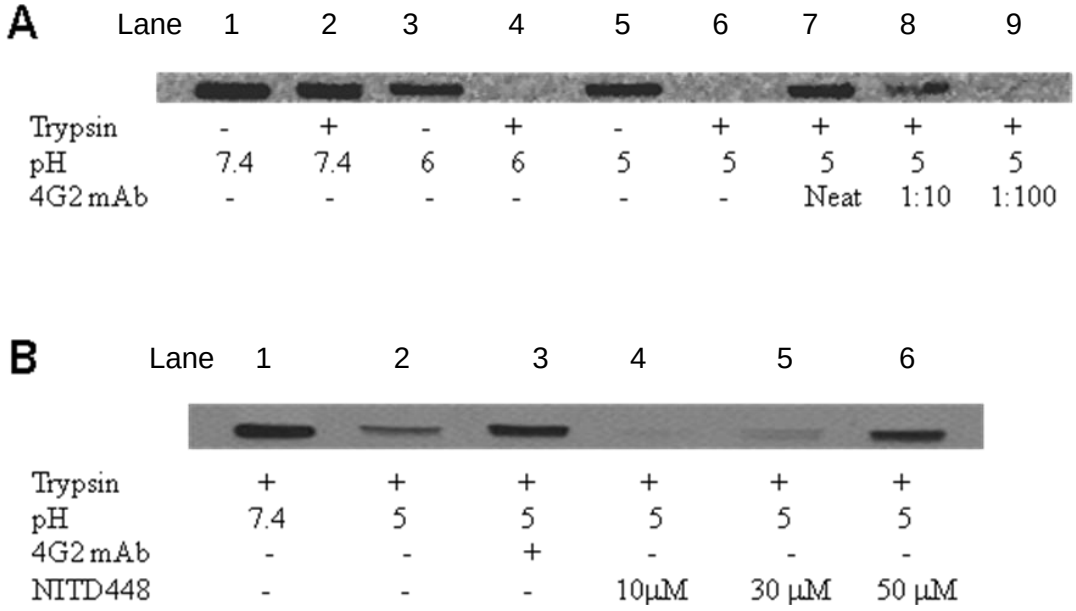


Figure 3-9: Inhibition of fusion in secondary assay. Mixtures of purified dengue virus and liposomes were exposed to various conditions and analyzed for the presence of intact viral capsid protein. (A) Capsid protein was not degraded if liposomes did not contain trypsin regardless of pH (lanes 1,3, 5), but if liposomes contained trypsin the viral capsid protein was degraded when the mixture was exposed to low pH causing fusion between liposomes and dengue virions (lanes 2, 4, 6). This degradation could be prevented in a dose dependent manner if the mixture was pre-incubated with antibody 4G2 against the fusion loop of the Envelope protein (lanes 7, 8, 9). (B) Similarly, compound NITD448 could inhibit fusion and trypsin degradation of capsid in a dose dependent manner.

3.2.4. Anti-viral activity of compound NITD448

To determine the anti-viral activity of compound NITD448, DEN2 NGC-infected cells were treated with various concentrations of the compound for three days, followed by determination of viruses produced in the supernatant. NITD448 was found to inhibit dengue infection with an EC_{50} value of 9.8 μM (Fig.3-10A). Its cytotoxicity was determined at a CC_{50} of 49 μM, resulting in a selectivity index of 5 (Fig. 3-10B).

Next, time-of-addition experiments were carried out to investigate the mechanism of viral inhibition. NITD448 was added to cells 1 hour before, during, or 1 hour post viral infection. As shown in figure 3-10C, the compound did not reduce virus production when present just before or after infection. NITD448 exhibited the strongest inhibition on viral production when added during the 1 h virus infection of the cells. The results once again indicate that the compound inhibits membrane fusion during viral entry into the cells.

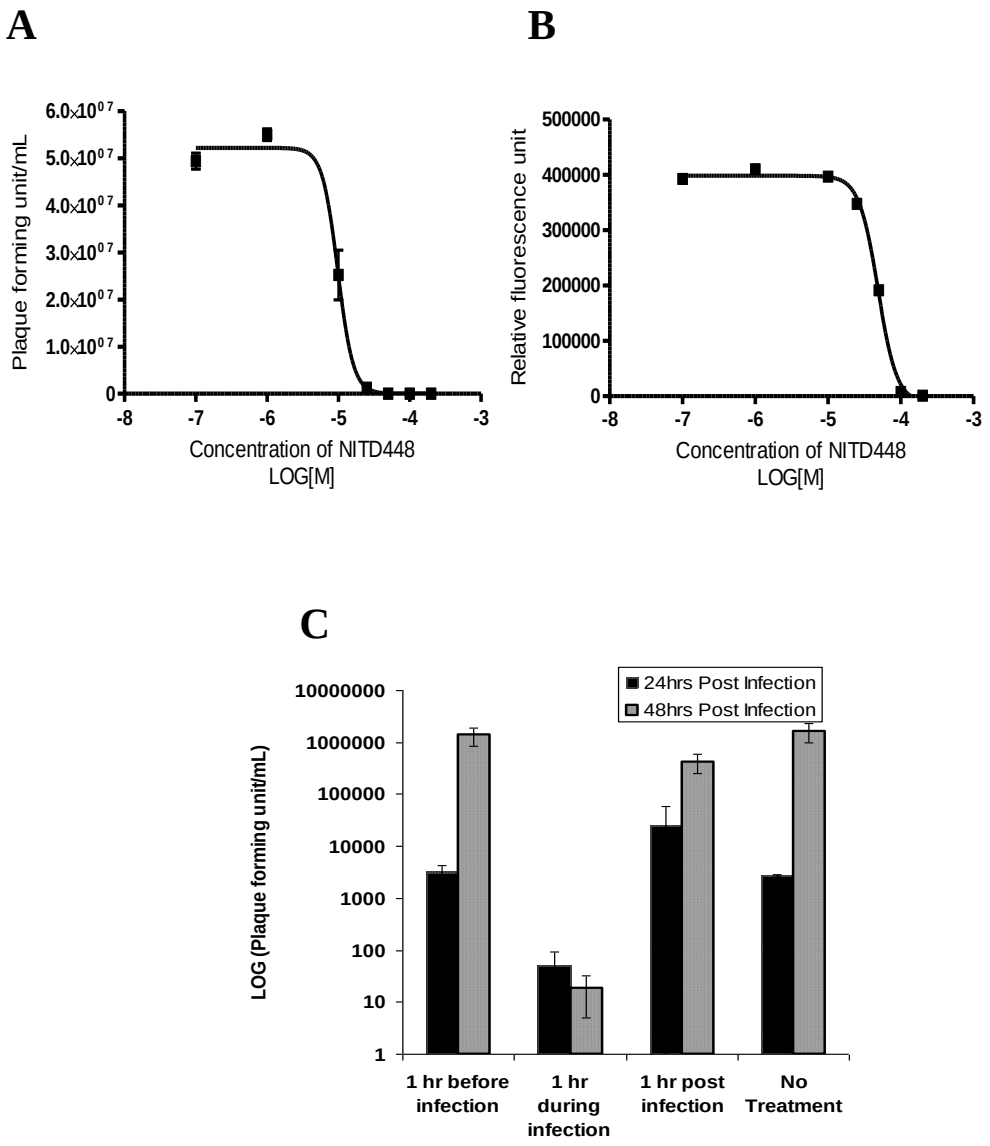


Figure 3-10: Antiviral activity of compound NITD448. (A) Cells were treated with a concentration range of compound NITD448 during infection with dengue virus for three days. Supernatant was subsequently harvested and analyzed by quantitative RT-PCR expressed in equivalents of plaque forming units (PFU) per mL. The compound showed a dose dependent inhibition of viral yield with an EC_{50} of 9.8 μM. (B) Cells were treated with a concentration range of compound NITD448 during infection with dengue virus for three days. Infected cells were then lysed and quantified using CellTiter® Glo assay kit for cell cyto-toxicity. The compound showed a CC_{50} of 48.7 μM. (C) Reduction of viral yield could be observed when NITD448 was present only during the one hour of infection, i.e. when virus and cells were incubated together, but not when cells were incubated only one hour before or only one hour after infection. Error bars represent standard deviations.

3.3. DISCUSSION

In the efforts towards developing an anti-viral treatment for dengue, a screening program was set up in NITD, aimed at identifying dengue E-protein mediated membrane fusion inhibitors. Previous studies only investigated the anti-viral activity of molecules, with the potential to bind to the E- protein, as predicted from in-silico docking (Wang et al. 2009; Yennamalli et al. 2009). The approach used in this study complements in-silico studies, with functional assays that monitor membrane fusion. A two-step screening approach was developed employing a 96-well primary cell-based fusion assay and a subsequent low-throughput secondary liposome-based fusion assay to validate viral fusion of primary hits. The primary assay was based on previously published (Randolph and Stollar 1990) cell-cell membrane fusion of dengue-infected cells, leading to syncytia formation, brought about by cell surface expressed E-protein. This study reports a convenient novel detection method for quantification of syncytia formation that allows monitoring of fusion activity in a 96-well plate.

A secondary assay was established based on a cell-free system containing purified dengue virus and liposomes, allowing detection of content mixing of these two membrane enveloped particles as a readout of the fusion event. By using antibody to detect capsid protein in Western blot to simplify the assay, there was no need for production and purification of radioactively labeled dengue virus.

Since the primary assay capacity did not allow high throughput screening, a focused library was made that was composed of compounds predicted to bind inside a putative binding pocket identified in the E-protein (Modis et al. 2003). Screening this focused library resulted in three primary hits, one of which was validated in the secondary assay, showing dose dependent inhibition of fusion.

The confirmed fusion inhibitor NITD448 proved to have anti-viral properties exhibiting an EC_{50} of 9.8 μM, comparable to the IC_{50} of 6.8 μM found when titrated into the cell-based fusion assay. This anti-viral effect works only if the compound is present during the viral entry phase of infection, consistent with the mechanism of action of a fusion inhibitor.

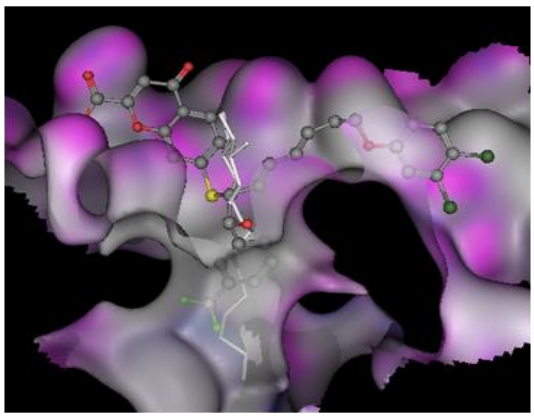
NITD448 is a novel structure, different from dengue entry inhibitors published previously (Li et al. 2008; Wang et al. 2009; Yennamalli et al. 2009). GLIDE docking suggests that the carboxylate on the chromenone ring of NITD448 interacts with Lys128 and Gln52 of the E-protein, with the trifluoro-phenyl motif of the molecule well buried into the hydrophobic βOG pocket (Fig. 3-11). This is similar to the chloro-phenyl-thiophene tail of the previously reported dengue entry inhibitors such as compound 6 reported by our colleague in NITD (Wang et al. 2009), an effective small molecule that shows good sub-micromolar EC_{50} in cell-based flavivirus inhibition assay and was shown by immuno-labeling to cause virus particles arrestment in the acidic endosomal compartments. Interestingly, compound 6, when tested in the cell-cell fusion assay and the liposome-based validation assay, failed to exhibit any inhibition in these assays (data not shown). This raised a question about the limitation of the fusion assay used in this screening program, such as the possibility of failing to identify a good anti-viral compound that was not showing activity in the cell-cell fusion assay.

In terms of its drug-like properties, based on the criteria stated by Lipinski's rule of five, it had a calculated octanol-water partition coefficient log P value (CLogP = 7.95) more than 5 and a molecular weight (659.49) of more than 500 Daltons. This might present a problem of oral bioavailability and hence, further chemical modification is necessary for this small molecule.

Chemists in NITD had also tried to examine the molecular properties of the other primary hits to look for similarity between the molecular properties of the two hits and that of NITD448 but found none. Furthermore, a family of compounds (from the Novartis compound library) that was structurally similar to NITD448 was tested on the primary fusion assay. They all were unable to block fusion in the liposome based fusion assay and none showed better EC_{50} in cell-cell fusion assay (data not shown). Hence, these compounds were not considered for drug discovery. The original NITD448 was not available in sufficient amount in Novartis compound archive. It is therefore not possible to perform further in-vivo validation of this compound (such as pharmacokinetic studies or mice studies) to further confirm its in-vivo efficacy.

Conceding the above fact that NITD448 was not selected as a candidate for drug discovery, it is the first reported small molecule with confirmed fusion inhibition activity against dengue. It may be the starting point for further development of more potent compounds with higher selectivity index. NITD448 can also be used as a tool in future screening campaigns for fusion inhibitors. In addition, it may be of value in studying the multi-step class II membrane fusion process.

A



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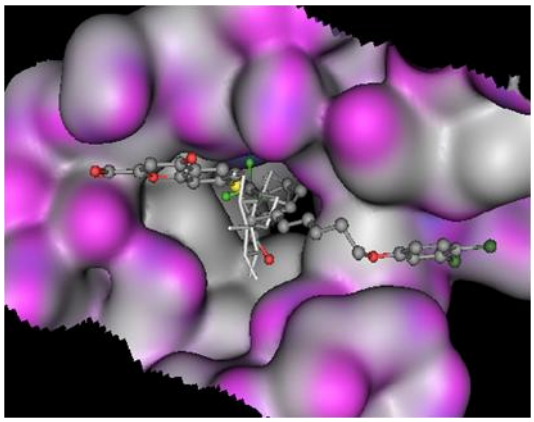

Figure 3-11: Putative binding mode of NITD448. The proposed binding modes by GLIDE for NITD448 (ball-and-stick) in DEN2 E-protein (PDB# 1OKE): (A) side view (B) top view. The protein surface is coded grey and purple for hydrophobic and hydrogen bonding, respectively. The βOG molecule in 1OKE is depicted as white sticks.

4. A STUDY OF THE MODE OF ACTION OF NITD770, A SMALL MOLECULE INHIBITOR OF DENGUE VIRUS

4.1. INTRODUCTION

Targeting host processes essential for viral infection is an alternative approach to look for anti-viral compounds. This study is a continuation of a previous effort in targeting host cholesterol metabolism pathway host for anti-dengue drug discovery (Rothwell et al. 2009). Host cholesterol pathway genes were initially examined via a candidate gene-by-gene approach and by using siRNA to knock down the various biosynthetic genes in host cholesterol pathway. Pharmacological inhibitors of the various enzymes in the pathway were also tested for an effect on dengue replication. Inhibition of mevalonate pyrophosphate decarboxylase (MVD), an intermediate enzyme of the sterol biosynthesis, results in suppressed dengue replication (figure 4-1). These efforts led to the discovery of a novel host target, MVD. A small molecule from the Novartis archive compound library, NITD770, a mevalonate inhibitor, was tested in dengue replicon system and was found to exhibit anti-viral activity.

In this study, the anti-viral profile of NITD770 was investigated and found to be effective at sub-micromolar concentration with a good selective index. The anti-viral efficacy of the compound was also investigated in other flaviviruses and non-flaviruses. Validation experiment done on NITD770 confirmed that it was not a MVD inhibitor. This led to a series of cross-disciplinary approaches to determine the mechanism of action of this compound.

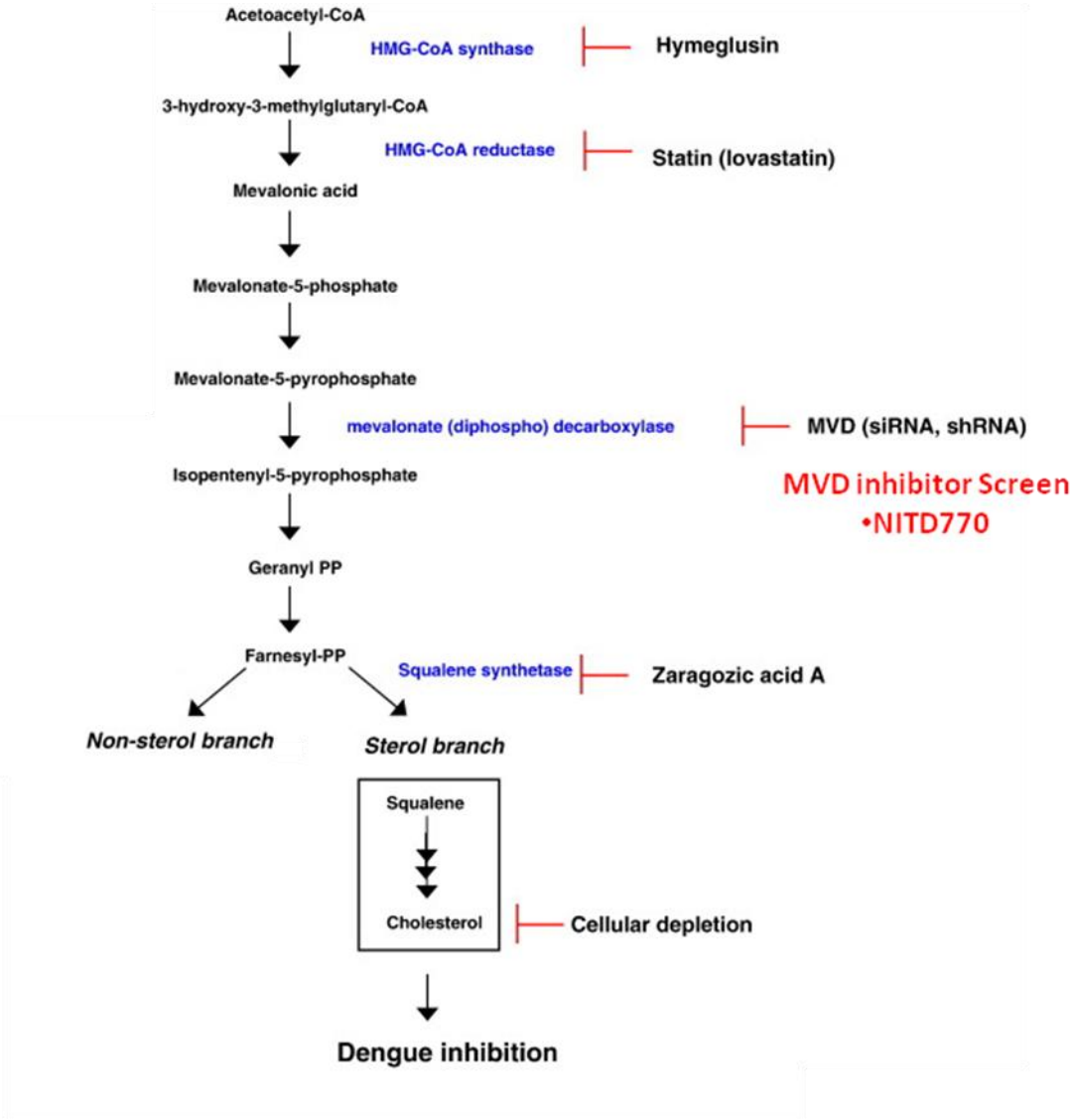

Figure 4-1: The disruption of host cholesterol biosynthesis using various inhibitors that target the intermediate biosynthesis enzymes resulted in dengue inhibition (figure modified from Rothwell and colleagues, 2009). Anti-dengue compound NITD770 was identified from a screen of compounds that targets the enzyme, mevalonate (diphospho) decarboxylase.

4.2. RESULTS

4.2.1. NITD770 shows specific anti-viral activity across several flaviviruses

NITD770 was found to inhibit dengue activity in cell-based flavivirus inhibition (CFI) assay, replicon assay and plaque assay (see Table 4-1). It showed a general half maximal effective concentration (EC_{50}) within 0.5-1.5μM. This compound was also effective against other members of the flaviviridae family, having a EC_{50} of 3.5 μM in HCV replicon cells and 0.6 μM in yellow fever virus CFI assay (done by Miss Haoying Xu). When tested against non-flaviviruses, the compound lost its potency, as observed with alphaviruses (highlighted in blue), Venezuelan equine encephalitis virus (VEEV) (done by Dr Paola Florez de Sessions) and Chikungunya virus (work done by Mr Liu Wei).

Assay	EC_{50} μM	CC_{50} μM
Dengue (NGC strain) A549 CFI	0.58	50-100
Dengue (NGC strain) A549 Plaque	0.6	50-100
Dengue (NGC strain) BHK21 Plaque	0.5	50-100
Dengue (NGC strain) A549 replicon	0.6	16.6
Dengue (NGC strain) Huh7 replicon	1.4	67
Yellow Fever (17D vaccine strain) A549 CFI	0.6	>100
HCV Huh7 replicon	3.5	>100
VEEV BHK21 replicon	>20	>100
Chikungunya (Ross strain) BHK21 Plaque	>10	>100

Table 4-1: Anti-viral activity profile of NITD770 in several assays. The potency of the compound is determined by the calculation of the concentration at 50% inhibition, IC50 value. Yellow fever A549 CFI assay was done by Miss Haoying Xu, VEEV BHK21 replicon assay was done by Dr Paola Florez de Sessions and chikungunya BHK21 plaque was done by Mr Wei Lu.

4.2.2. The lack of inhibition of NITD770 in MVD enzymatic assay and host cholesterol biosynthesis pathway

NITD770 was reported to be a MVD inhibitor in the 1990s. At that time, a crude lysate of HepG2 cells (enriched with MVD) was used to measure the conversion of radioactive C_{14} labeled mevalonate di-phosphate to cholesterol. In order to validate once more the compound's inhibition on MVD, an affinity-purified recombinant MVD was used in the enzymatic assay carried out by Dr Christian Noble in NITD. As shown in figure 4-2, 100 μM of NITD770 did not affect the MVD enzymatic activity.

Further investigations were carried out to examine whether NITD770 affects the host cholesterol metabolism. This was done by measuring the total cholesterol levels and zymosterol levels in host cells. Zymosterol is an intermediate product along the sterol biosynthesis pathway. An increase in zymosterol levels indicates an increase in the host sterol biosynthesis. Cells were treated with NITD770 for 48 hours followed by lipid extraction using organic solvent. Next, Dr Guanghou Shui from LipidProfiles in NUS performed the quantification of lipids using gas-chromatography mass spectrometry (GC-MS). As shown in figure 4-3, neither total cholesterol levels (p-values for BHK21=0.40 and A549=0.24) nor zymosterol levels (p-values for BHK21=0.18 and A549=0.39), were significantly altered by the compound.

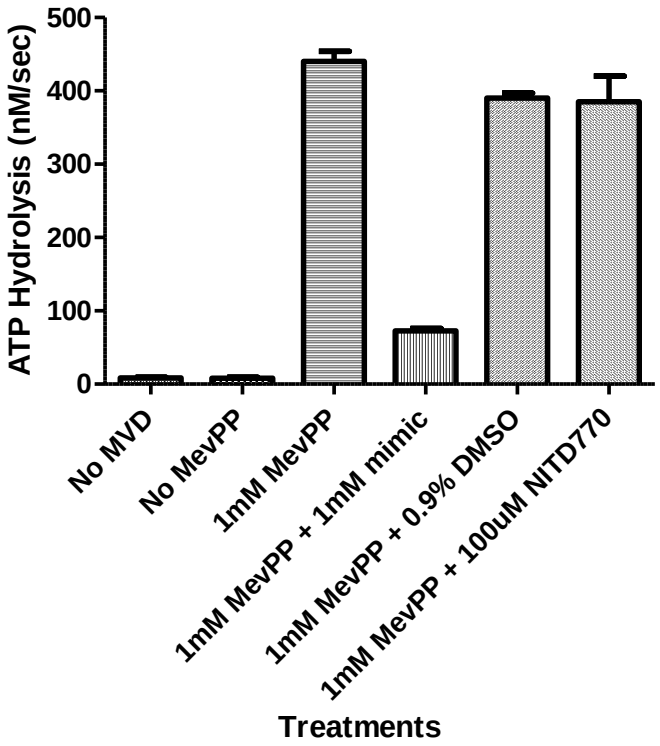

Figure 4-2: Mevalonate diphospho decarboxylase (MVD) enzymatic assay. This was done by measuring indirectly the hydrolysis of ATP by MVD. The substrate for MVD is MevPP and mimic used as a positive control of inhibiting the enzyme MVD. This experiment was done by Dr Christian Noble from NITD.

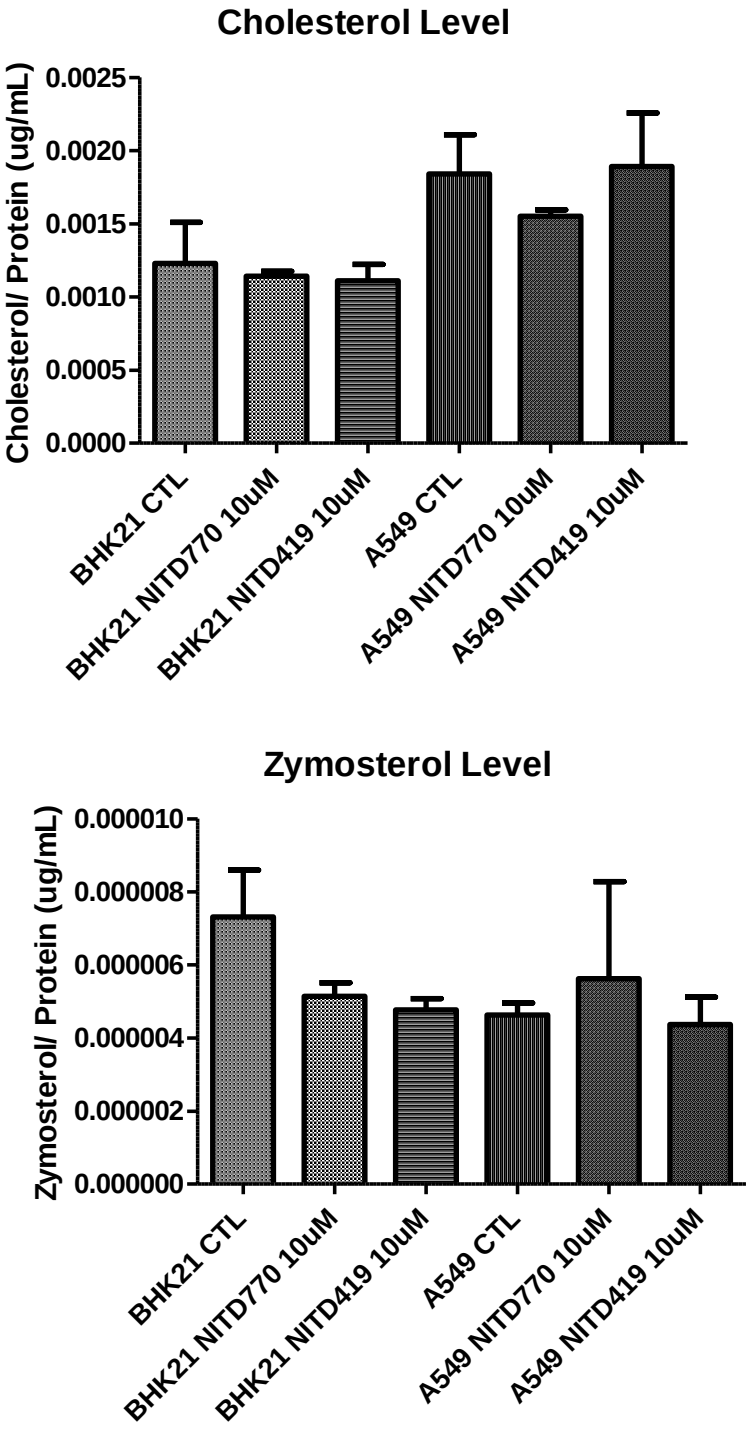

Figure 4-3: Determination of total cholesterol and zymosterol level in cells using GC-MS. The values were normalized with total protein concentration. The cells used for quantification were control (CTL), treated with NITD770 and treated with the inactive analogue, NITD419. T-test (unpaired unequal variance) was performed to compare the significant differences using p value of <0.05).

4.2.3. Validating host lipids as potential host target of NITD770

We enlisted help from our collaborators (Novartis, NIBR) to look for likely host targets using HIP-HOP in yeasts. This is a genome-wide approach done in yeasts to look for potential drug target or mechanism of action by looking at the cellular response to perturbations caused by the drug of interest (Hillenmeyer et al. 2010). In the haploid insufficiency profiling (HIP) assay, a library of approximately 6,000 haploid yeast mutants (each strain having one copy of the gene deleted) is assessed for their growth ability in a culture medium with the presence of the drug. The deletion of one gene copy sensitizes the yeast mutant to the drug effect and hence, mutant that does not grow well in the presence of the drug indicates that particular gene as a possible target. Each mutant has a "tag" containing hybridization signal that can be identified in an array-style format using high-density oligonucleotide array (Giaever et al. 2002). Homozygous profiling (HOP) is an alternative approach used to identify gene required for growth in the presence of the drug. For HOP, the library of yeast mutants consists of strains having both copies of a particular nonessential gene deleted (homozygous deletion mutants). The identification of the slow grower highlights the possible pathway affected by the drug of interest.

The preliminary HOP data had a relatively clean profile, with only a few sensitive and significant strains (due to confidentiality issue, data is not shown here). These hits are similar to a few compounds previously screened in other HOP assays done by our collaborators (these yeast profiling experiments were not related to our HOP profiling), that were found to be affecting host lipid rafts. Others have previously reported the compounds that inhibit the same genes (co-inhibition) may

have similar chemical structure and mechanism of action (Giaever et al. 2004), suggesting that NITD770 may target the lipid raft pathway in the host.

The integrity of lipid rafts in NITD770-treated cells was examined to see whether the compound targeted lipid rafts, as suggested by the yeast HOP studies. Lipid rafts were isolated from detergent resistant membrane (DRM) fractions, using detergent extraction and sucrose gradient density centrifugation. Two lipid rafts markers, anti-flotilin and anti-caveolin, were used to identify the DRM. As shown in figure 4-4A, there was no observed effect on the lipid raft integrity (fractions 2 to 4) isolated from cells treated with NITD770 compared to the control untreated cells. In the next experiment, immuno-fluorescence microscopy, using cholera toxin sub-unit A to label lipid rafts, was performed to examine the lipid raft integrity. The compound methyl-beta-cyclo-dextrin (MBCD) was used as a positive control, which is known to disrupt lipid rafts by extracting cholesterol from the cell plasma membrane. As shown in figure 4-4B, MBCD treatment destroyed the cholera toxin staining for lipid rafts on the cell plasma membrane. NITD770 has no effect on the lipid raft integrity because the treated cell plasma membrane labeled by cholera toxin showed similar labeling pattern as those in the control untreated cells.

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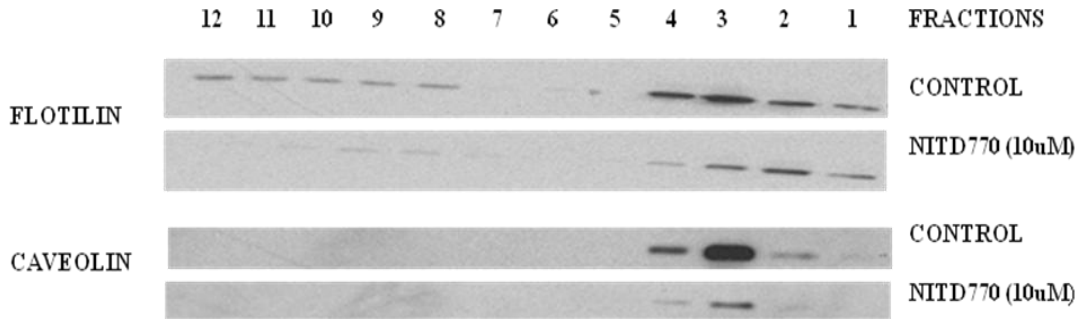

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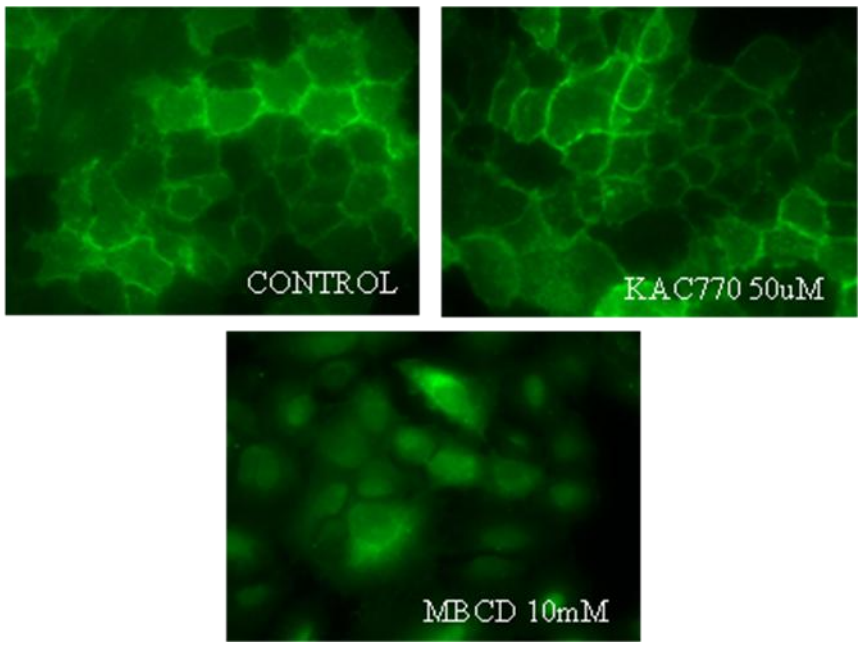

Figure 4-4: Looking at the integrity of lipid rafts in cells upon treatment with NITD770. (A) Lipid rafts were isolated using detergent (1% triton) on ice. Flotilin and caveolin were used as markers for fractions enriched with lipid rafts. (B) Lipid rafts were stained using cholera toxin sub-unit A conjugated to Alexa-448 (green).

4.2.4. Raising resistant mutant viruses against NITD770

Drug-resistant isolates are obtained by continuously passaging the viruses in the presence of anti-viral compound. Following several rounds of infection cycle, some mutant viruses may become increasingly resistant to the compound. The genome for these resistant viruses is then sequenced to identify mutations that may contribute to the resistance displayed. My colleagues, Dr Gu FENG and Mr Cedric NG, used this technique to find out the likely viral targets of NITD770. They started the experiment with two concentrations of NITD770, 10 μM and 20 μM, and harvested resistant viruses at 48 hours intervals. As shown in figure 4-5A, there was an increase in resistance to 10 μM NITD770 in passages 8, 9 and 10. From this, they took passage 10 10uM resistant viruses to validate the resistance achieved in this pool of mutants. Data in figure 4-5B shows that mutants were resistant to both 10 μM and 20 μM treatments of NITD770.

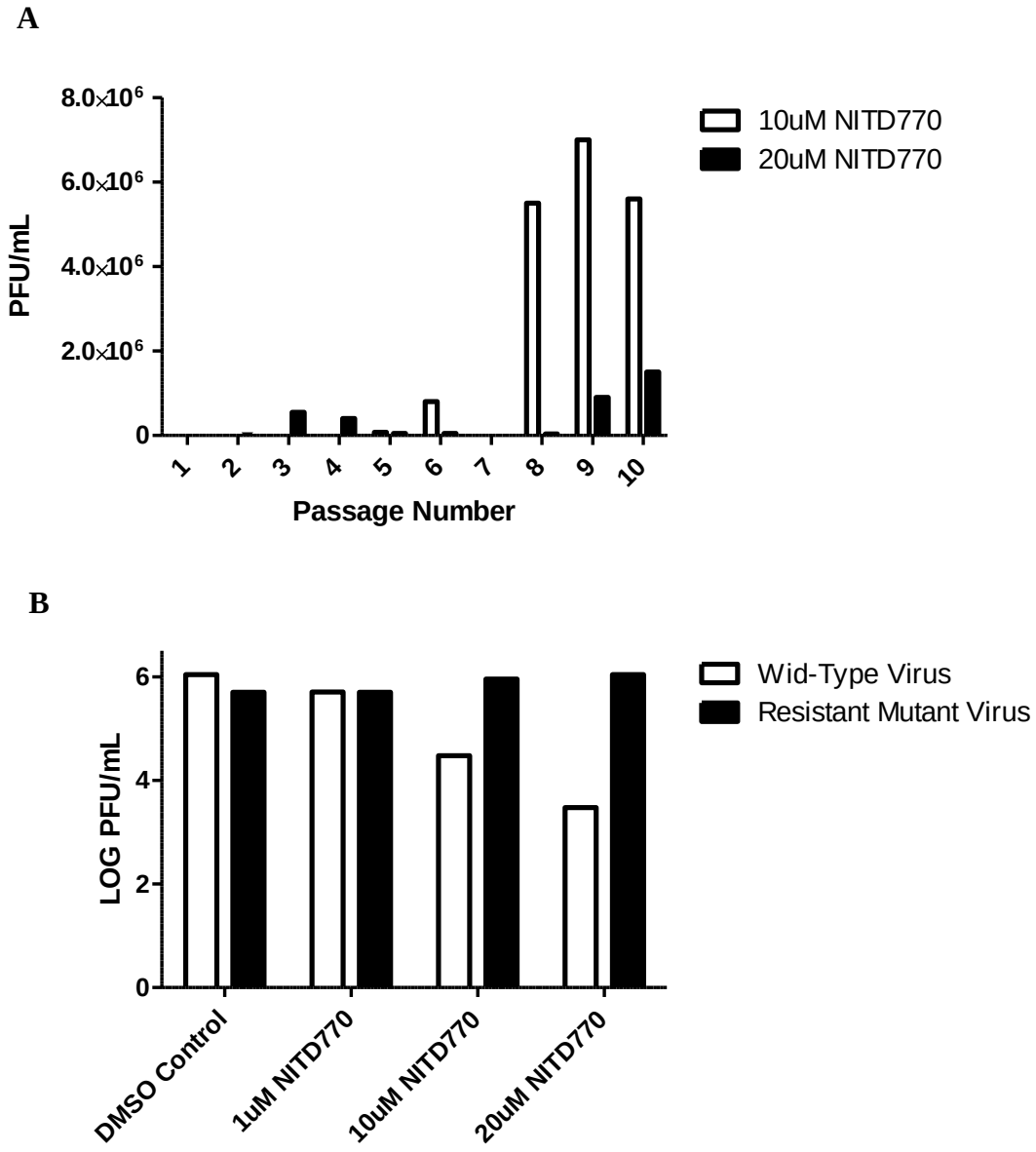

Figure 4-5: Raising resistant viruses against NITD770. (A) BHK21 cells were used to infect with dengue viruses, in presence of either 10 μM or 20 μM of compound. The newly produced viruses were harvested 48 hours post infection and used to re-infect new uninfected BHK21 cells in succession up to ten passages. The viral titer of each passage was determined using plaque assay and expressed as plaques per mL of virus harvest (PFU/mL). (B) The resistant viruses from 10 μM P10 batch were further passaged for three passages (P13) in 10 μM NITD. P13 resistant viruses were used to determine the resistance against up to 20 μM of NITD770 against the wild type viruses. This set of experiments was performed once by Mr Cedric NG and Dr Gu Feng from NITD.

4.2.5. Isolation and sequencing of individual isolate of viruses resistant to NITD770 found conserved mutations within the NS5 polymerase, near to the surface of the RNA entry tunnel

The genomes of six isolates were sequenced and the mutations are presented in figure 4-6. Five mutations were found in these resistant viruses, one in NS4B and four located within NS5. Of these mutations, only three are conserved across all four serotypes of DENV and are located within the RdRp of NS5.

Like WNV, DENV RdRp adopts a right-hand structure with three subdomains: fingers, palm and thumb (Malet et al. 2008; Yap et al. 2007). The three resistant mutations of NITD770-resistant virus are located in the palm domain of RdRp. The mutation at nucleotide 535A-V is located near to the helix α13, and mutations 607G-A and 615E-G are located within helix α16 of the palm domain. As seen in figure 4-7, all three mutations are clustered around the palm domain, near to the surface of the RNA template tunnel.

	NGC (NITD) wt			NGC (770-Mut)		Region
nt position	**nt seq**	**a.a seq**	**a.a position**	**nt seq**	**a.a seq**	
7361	CGTGA**C**TCAAG	LCV**T**QVL	179in NS4B	CGTGA**T**TCAAG	LCV**I**QVL	NS4B (in the middle of transmembrane 4)
9176	CACCG**C**AGGAT	DDT**A**GWD	535 in NS5	CACCG**T**AGGAT	DDT**V**GWD	NS5 (RdRp) conserved
9392	CCTATG**G**ACTC	GTY**G**LNT	607 in NS5	CCTATG**C**ACTC	GTY**A**LNT	NS5 (RdRp) conserved
9416	TATGG**A**AGCC	TNM**E**AQL	615 in NS5	TATGG**G**AGCC	TNM**G**AQL	NS5 (RdRp) conserved
9225	AGAAA**T**GGTA	NEE**M**VT	551 in NS5	AGAAA**A**GGTA	NEE**K**VT	NS5 (RdRp) not conserved

Table 4-2: Sequencing results of the genome of the NITD770 resistant viruses. In total, six isolates from the 20 μM resistant dosage pool were sequenced. The mutated nucleotide and amino acid residues are highlighted in both wild type (RED) and resistant virus (GREEN). Some mutations were conserved in all four dengue serotypes 1-4.

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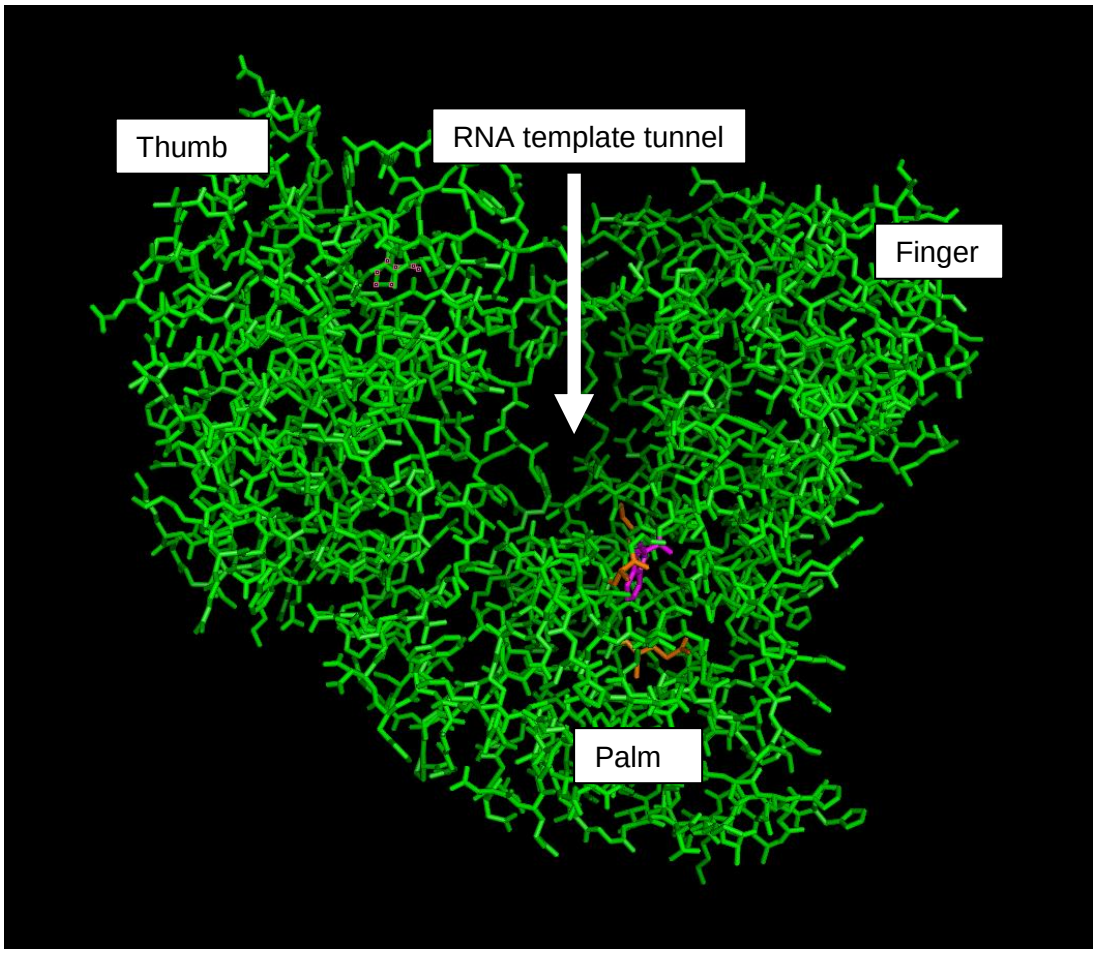

Figure 4-6: Location of the conserved mutations in the NITD770-resistant viruses. A schematic representation of the structure of the DENV-2 NS5 polymerase domain with the three subdomains (Finger, Thumb & Palm) highlighted. The three conserved resistant mutations are colored in orange and the tryptophan residue near to these mutations is colored in pink.

4.2.6. Studying the effect of NITD770 and its resistant mutations on the polymerase activity of NS5 protein

Observations made from the resistant mutations all point to the palm domain of the NS5 RdRp region that is highly conserved across many structurally known polymerases. Hence, it would be interesting to find out if NITD770 interferes with the viral polymerase function.

An in-vitro RdRp assay was carried out to examine the effect of the resistant mutations on the viral polymerase activity. This was done by introducing the resistant mutations into NS5 protein (single mutants, double mutants and triple mutants) and measuring the polymerase activity of each NS5 mutant proteins, in comparison to wild type NS5 protein. Only the triple mutant protein exhibited a noticeable increase in the polymerase activity (slope of best-fit line = 249.2) compared to wild type protein (slope of best-fit line = 144.1) (Figure 4-8A).

Next, the effect of NITD770 on the viral polymerase activity was studied to see if the engineered mutant NS5 can rescue the suppression of polymerase activity by the compound. Increasing concentration of NITD770 did not significantly inhibit the RdRp reaction (Figure 4-8B), having a calculated IC_{50} of 42 μM. When the triple mutant protein was tested against NITD770, the mutant protein did not relieve the dose-response inhibitory effect of the compound on the viral polymerase activity (Figure 4.8B; an IC_{50} value of 49 μM).

Based on the data from these two studies, it was concluded that these resistant mutations resulted from an indirect consequence of creating a faster polymerase, in order to overcome the compound anti-viral activity on the viruses.

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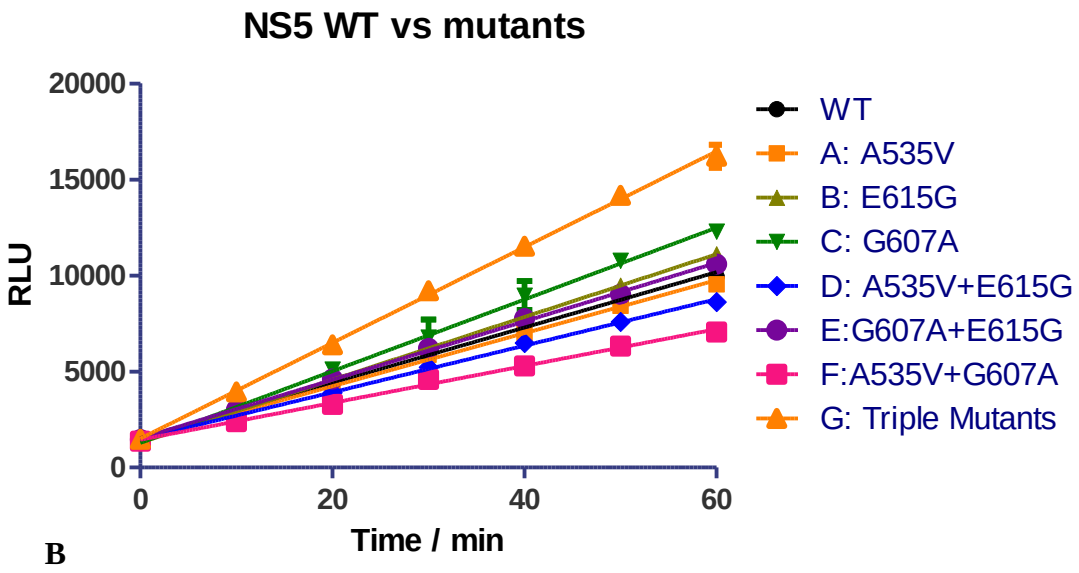

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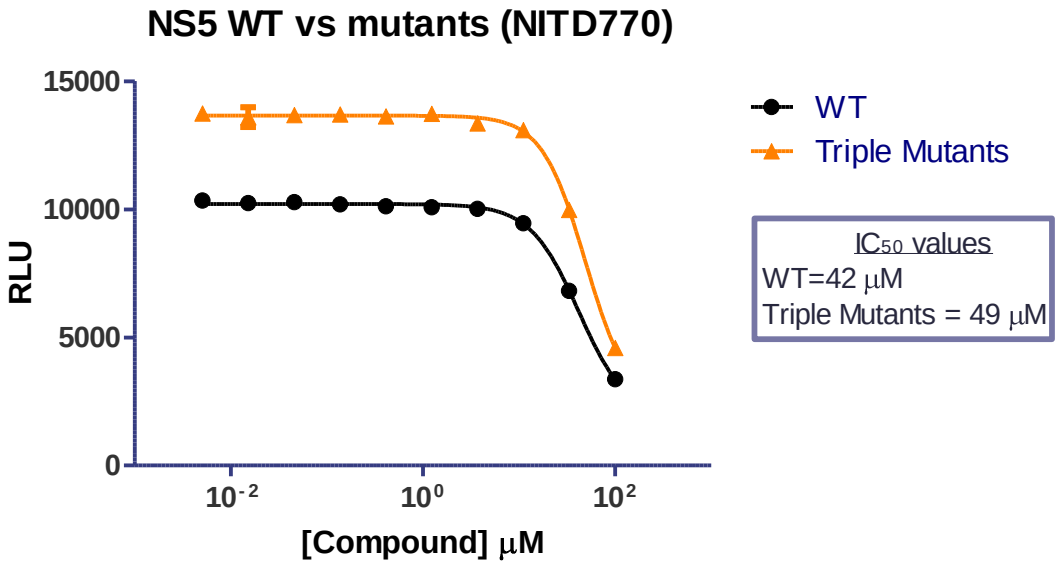

Figure 4-7: Determination of polymerase activity of NS5 and its resistant mutants. An in-house in-vitro RNA-dependent RNA polymerase (RdRp) assay was set up to measure polymerase activity, by measuring the amount of fluorescent-RNA substrate incorporated into the polymerase. (A) A profile of the polymerase activity of the various resistant NS5 proteins in the absence of inhibitor. (B) NITD770 was added in an increasing concentration to wild type (WT), double mutant (A535V+G607A) and triple mutant NS5 individually.

4.3. DISCUSSION

NITD770's anti-dengue mechanism of action was investigated in this study. Even though it was reported as an MVD inhibitor in the archive compound library, during the validation experiment, NITD770 was confirmed to be not an inhibitor of MVD. Discrepancies between the results from the initial cell-free MVD assay and the validation experiment could be due to the presence of unknown factors in the crude cell lysate used in the initial MVD enzymatic experiment, compared to the purified recombinant MVD used in this study. Although MVD was not a target of NITD770, given the acceptable anti-viral profile and potency of this compound, it was kept in the drug discovery program.

From anti-viral profiling experiments, NITD770 exhibited a good anti-viral activity with an EC_{50} value of low micro molar range and a wide selective index (CC_{50}/EC_{50}) of more than 100. It was also shown to be specific to viruses of flaviviridae family compared to alphaviruses.

As the mechanism of action of NITD770 was unknown, a chemo-genetic profiling of this compound was done in yeast to determine other host targets. HIP-HOP data showed an effect of NITD770 on the yeast lipid raft pathway, implying host lipid raft to be a likely target. However, based on data obtained from the lipid-raft detergent extraction experiment and the immuno-staining of lipid-rafts by cholera-toxin, it was concluded that lipid rafts in host cells were unaffected by NITD770. The inconclusive outcome from the HIP-HOP experiment could be due to the limitations of this assay such as the target of NITD770 is not being represented in the yeast mutant pool used. It is an important issue to consider when using this approach, as some human genes are not represented in the yeast genome. Another possible explanation is the differences in permeability of the compound through the yeast cell

compared to the mammalian cell wall. Another limitation of using HIP-HOP is the restriction of identifying only protein targets due to their reliance on gene effect observations, so if indeed NITD770 targets something other than a protein, HIP-HOP would not be able to allow the identification of this target.

The effect of NITD770 on host cholesterol was investigated. It was speculated that the crude cell lysate, enriched with MVD, used for the preliminary MVD assay might contain other sterol biosynthesis enzymes that could be affected by NITD770. However, data showed that host total cholesterol levels and the cholesterol biosynthesis were unaffected, as shown by unaltered zymosterol levels, an intermediate product along the cholesterol biosynthesis pathway.

To identify the possible viral target of NITD770, resistant viruses were raised in the presence of this compound. By mapping the causal mutations raised from the NITD770-resistant viruses, three conserved mutations were identified within the NS5 polymerase domain and this led to the investigation whether the viral polymerase is the target of this compound. Data showed that the triple mutations in NS5 resulted in an increase (approximately 1.7 times) in the polymerase activity compared to wild type protein. However, the IC_{50} values of NITD770 in the RdRp assay were high and similar in both the wild type (42 μM) and the triple mutant protein (49 μM). These high inhibitory concentrations suggested that NS5 polymerase activity is not a target of this compound and that the three conserved mutations within the NS5 are indirect resistant mutations, often referred to as compensating mutations. An example of such phenomenon is observed in the case of neuraminidase-resistant influenza viruses, which exhibit weaker binding hemagglutinins that enhance viral binding to host cells, rather than alter the effect on neuraminidase itself (Gubareva et al. 1998; McKimm-Breschkin 2000). In this study, it was concluded that the mutations in the resistant

virus resulted in a viral polymerase that is more active in order to overcome the anti-viral effect caused by the compound.

The possible interaction of NITD770 with NS5 was also studied by examining the effect of the compound on the intrinsic fluorescence property of NS5. It was observed that NITD770 quenches the NS5 protein's fluorescence (Annex-2). However, both the spectroscopy properties of NITD770 and the abundance of tryptophan residues in NS5 (ninety tryptophan residues) prevented a straightforward interpretation as to whether the interaction is specific. Our colleague, Dr Christian Noble, had recently attempted to co-crystallize the compound with NS5 without any success. Further studies needed to be performed, to this end, a multi-disciplinary effort, such as studying the kinetics of the association/disassociation of NITD770 with NS5 using isothermal calorimetry (ITC) or using surface plasma resonance (SPR) technology, is required to determine the specificity of this compound's interaction with NS5.

This study provided a more detailed characterization of the anti-viral profile of NITD770 in dengue infection. The inhibition of viral replication by this compound is shown not to be via its action on the RdRp activity and was concluded that the resulting resistant mutations were compensatory mutations. Colleagues (Wouter Schul and Andy Yip) in NITD tested NITD770 in mice model and found a 42% percent in reduction of viremia in mice after 3 days of treatment with 100mg/Kg twice daily oral dosing (data shown in Annex 3). Although the compound did reduce the viremia in this study, the effect was small in relation to the high dosage and long exposure used during the study. This further highlighted the need to determine the target of NITD770 to prevent any side effect that may arise from this compound. There are on-

going efforts to improve the potency of this compound and identification of the target of NITD770.

5. U18666A, A CHOLESTEROL TRANSPORT INHIBITOR AND ITS EFFECTS ON DENGUE VIRAL ENTRY AND REPLICATION

5.1. INTRODUCTION

In drug research for anti-dengue therapy, compounds were designed to target viral proteins such as the viral envelope (during the entry/fusion step) (Costin et al. 2010; Poh et al. 2009; Schmidt et al. 2010; Wang et al. 2009) and viral polymerase, NS5 (during the viral replication) (Latour et al. 2010; Niyomrattanakit et al. 2010; Yin et al. 2009). Alternative targets include host targets that have the advantage of reduced drug resistance faced by viral protein targeted drugs. For example, targeting host processes such as glucosidase (Chang et al. 2009; Liang et al. 2006), cholesterol modulation (Lee et al. 2008; Mackenzie et al. 2007; Rothwell et al. 2009) and host factors leading to the pathogenesis of DHF (Pastorino et al. 2010; Subramanya et al. 2009).

Cholesterol is essential to various cellular structures and processes. It is required predominantly for the formation of cellular membranes (Cannon et al. 2006) and for the production of other important components, such as bile acid and steroid hormone synthesis. In a cell, the level of cholesterol is constantly regulated at three levels: during the intake of extracellular cholesterol in the form of light density lipoprotein (LDL) via endocytosis; during de-novo biosynthesis of cholesterol (Brown and Goldstein 1983) and during the efflux of cholesterol by ABCA1, a transporter found associated in the cell periphery (Higgins 1992). Viruses have been shown to affect cholesterol levels during infection (Syed et al. 2009) and when cholesterol is reduced in host cells, viral production is affected (Desplanques et al. ; Medigeshi et al. 2008). Enzymes involved in the intermediate steps of biosynthesis, such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and mevalonate (diphospho) decarboxylase (MVD) were shown to be important in viral replication

(Sidorkiewicz et al. 2009). Statins, a widely known class of inhibitors that have been used to lower cholesterol levels in the treatment of cardiovascular diseases. Inhibiting HMG-CoA reductase was also found to be effective against several viruses such as HIV, influenza virus and HCV (Bader et al. 2008; Kruger et al. 2006; Oh and Hegele 2007). These findings highlight the possibility that modulating host cholesterol may inhibit dengue infection.

In this study, a cholesterol transport inhibitor drug, U18666A (3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one) (Liscum and Faust 1989) was used as a tool to allow us to study the role of cholesterol during DENV infection. Time-point-of-addition experiment done in cells infected with DENV showed that U18666A reduces viral production at multiple steps of the infection. Subsequent studies showed that this compound inhibits viral trafficking and replication. The combined treatment of C75, a fatty acid synthase inhibitor and U18666A was also shown to have an additive anti-viral effect, suggesting the role of cholesterol and fatty acid in dengue replication.

5.2. RESULTS

5.2.1. Anti-viral activity of U18666A, a cholesterol transport inhibitor and its effect during viral infection

The compound, U18666A affects cholesterol levels in cells mainly via two mechanisms, the transportation of cholesterol in the endocytosis of host cells and the inhibition of oxidosqualene cyclase, an intermediate enzyme in the sterol biosynthesis. We observed that cholesterol is ubiquitously distributed in host cells (Fig. 5-1A; Control: FILIPIN III). When these cells were treated with U18666A for 16 hours, it resulted in an accumulation of cholesterol in the late endosome (Fig. 5-1A; U18666A: Co-labeled). This treatment resulted in significant reduction in viral production with more than 1 log of viral production observed at 16h pre-treatment and after entry (Fig. 5-1B; VIRAL PRODUCTION). The effect of this cholesterol transport inhibitor was even more pronounced when presented throughout the infection, as shown with a 3-Log reduction in viral production. Although the 1 hour entry did not cause as much viral reduction compared to 16 hour pre-treatment, it could be due to insufficient perturbation of host cholesterol homeostasis to see a pronounced effect on viral infection. The cell viability experiment was done in parallel with the anti-viral experiment and showed that U18666A's anti-viral effect was not the cause of host toxicity (Fig. 5-1B; VIABILITY).

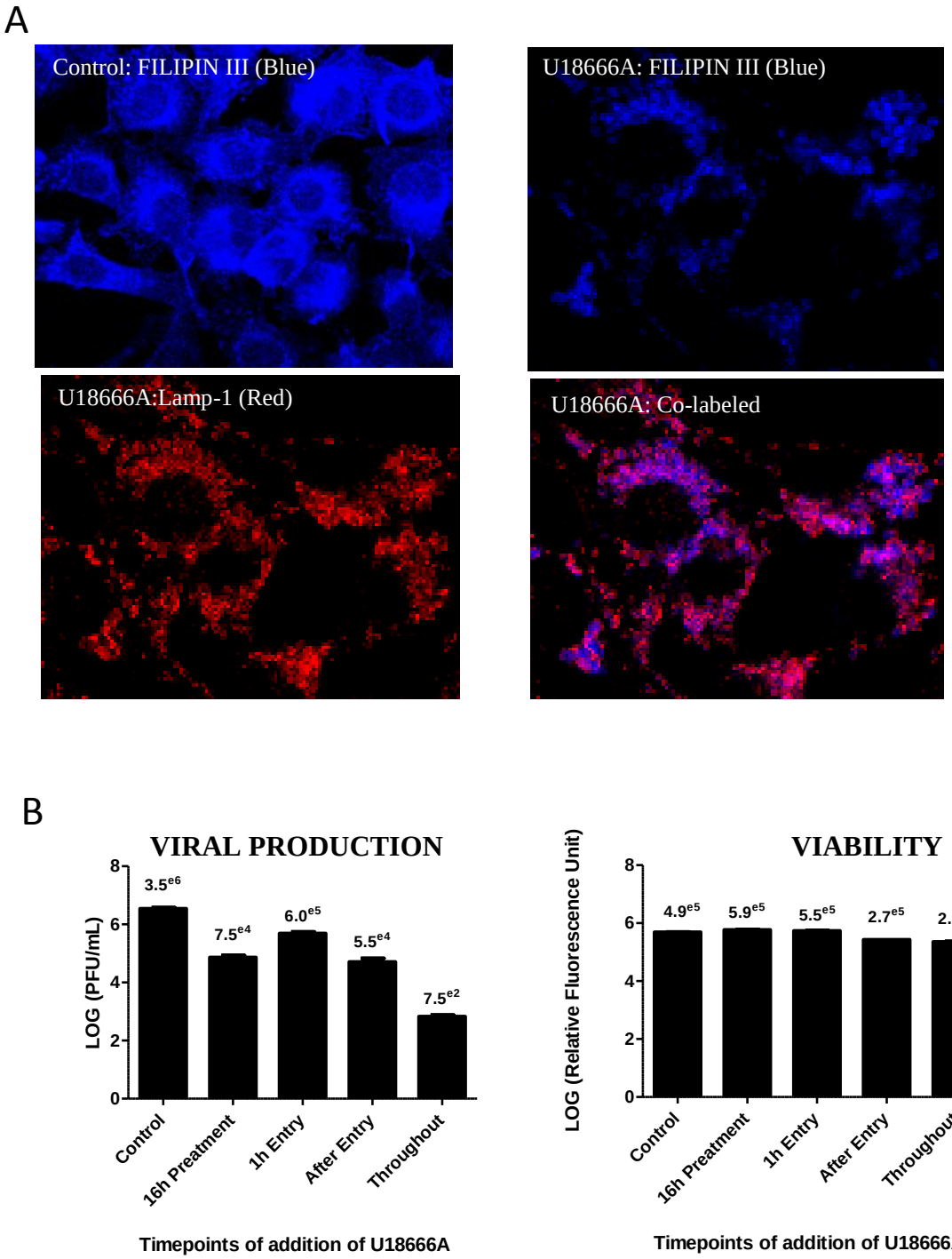

Figure 5-1: Antiviral effect of U18666A on dengue viruses. (A) BHK21 cells were treated either with (i) 0.9% DMSO (control) or (ii) 6 μM of U18666A for 16 hours before fixation and labeled with either FILIPIN III (blue) or Lamp-1 (red). A co-labeling was performed for U18666A treated cells. (B) Time-point-of-additions: BHK21 was treated with U18666A at 3 μg/mL at various time-points during the course of infection by dengue viruses at MOI of 1. Viral supernatants were harvested 48 hours post infection and plaque assay was used to determine the viral titer. Cell viability was determined using CellTiter® Glo assay kit

5.2.2. The importance of cholesterol in viral trafficking in cells

To study the early event of infection, immuno-fluorescence microscopy was used to examine viral binding on host cells and trafficking event of the endocytosed viruses. For virus binding experiments, viruses with a MOI of 50 were added onto chilled BHK21 cells and incubated on ice for an hour. These virus bound cells were then fixed for immuno-labeling with anti-E antibody to visualize the viruses. In cold condition, viruses are able to bind to the cell surface but are unable to undergo endocytosis (Helenius et al. 1980). This experiment showed that viruses were still able to bind as efficiently on to host cells in the presence of U18666A (Fig. 5-2).

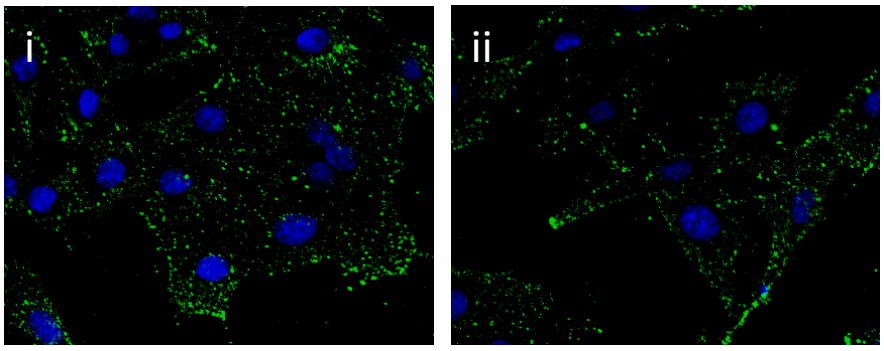

Figure 5-2: Characterization of the effect of U18666A on the viral binding. (A) For cell binding study: Dengue viruses were added to BHK21 treated either with (i) 0.9% DMSO as control or (ii)16h pre-treatment with U18666A (3 μg/mL). This was incubated on ice for one hour to allow the viruses to bind onto the cell surface. The cells were then washed and fixed, followed by immuno-labeling with anti-Env antibody (4G2) (Green) to visualize the bound viruses. DAPI (Blue) was used as a counter-stain for cell nuclei.

In order to study the post-attachment event, infected cells were fixed at different timepoints over a time course of 24 hours post infection. Fixed cells were subsequently immuno-labeled with anti-E antibody to monitor the endocytosed viral particles. One hour post infection, viruses were observed in the cytoplasm of the host cells (Fig 5-3, Control 1h). Fusion of the viral particles was believed to have taken place before the time-point of 4 hours post infection as the endocytosed viruses could no longer be detected (Fig.5-3, Control 4h). Newly synthesized viral proteins were detected at the time-point of 12 hours post infection, with the labeling of E protein localized in the peri-nuclear regions of the host cells (Fig. 5-3, Control 12h). However, in cells where the cholesterol transport was arrested by U18666A, many endocytosed viral particles could still be labeled with the anti-E antibody (Fig 5-3, U18666A 4h). There was also less labeling of the newly synthesized viral protein at the time-point of 12h post infection (Fig. 5-3, U18666A 12h). Further investigation showed that these trapped endocytosed viruses were not found in the early endosomes (immuno-labeled with anti-EEA1 antibody; Fig. 5-4i), but found mainly in the late endosomes (immuno-labeled with anti-Lamp-1 antibody; Fig. 5-4ii). This resulted in a reduced level of infection as evidenced in the 12h and 24h time points, with reduced labeling of the synthesized envelope proteins (Fig.5-3, U18666A 12h and 24h).

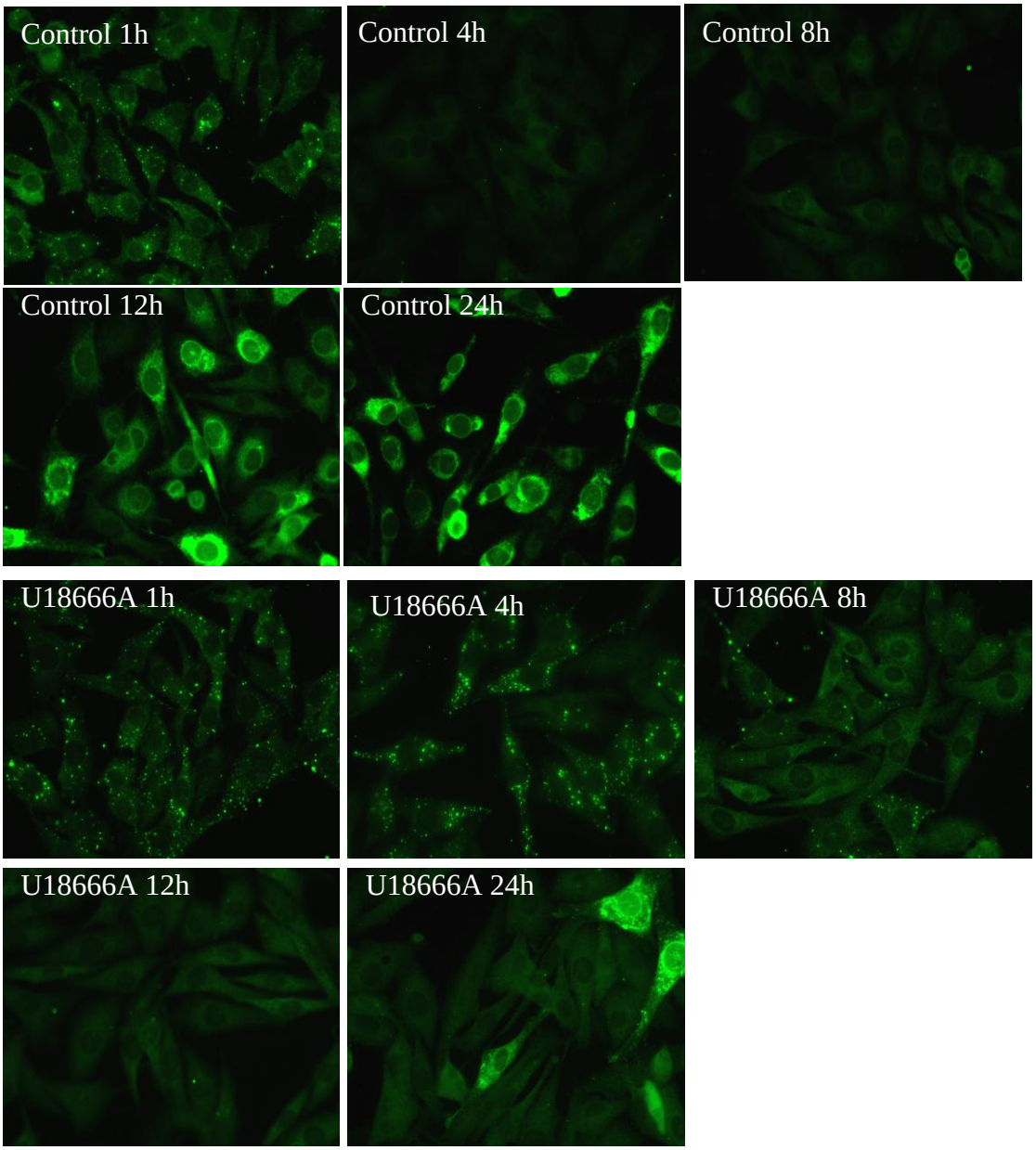

Figure 5-3: The effects of U18666A on viral trafficking. The control and U18666A-treated BHK21 cells were infected with dengue viruses (MOI of 50) and at various time-points of infections (1h, 4h, 8h, 12h and 24h), followed by fixing of these cells and immuno-labeling with anti-Env antibody (4G2) (Green).

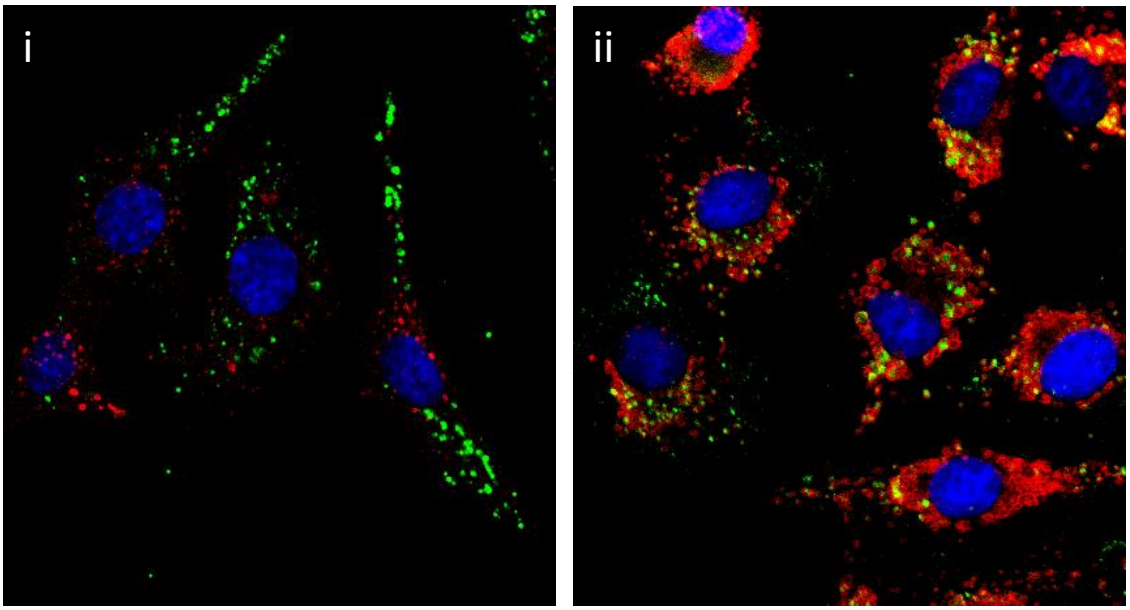

Figure 5-4: Association of trapped viruses in Lamp-1 labeled compartments. BHK21 cells were infected (moi of 50) and treated with U18666A (6 μM) for 4h and then fixed and immuno-labeled with anti-Env antibody (4G2) (Green) and with either (i) anti-EEA1 antibody (Red) or (ii) anti-Lamp-1 antibody (Red). DAPI (Blue) was used as a counterstain for cell nuclei.

5.2.3. The importance of cholesterol in the replication of dengue viruses

Two in-house stablly transfected replicon cell lines (DENV replicon in A549 cell line and DENV replicon in Huh7 cell line), carrying a reporter gene fused to the non-structural genes of DENV, were used to study the effect of U18666A on viral replication (Ng et al., 2007). This assay focused on the replication event, isolating this event from viral entry, trafficking and post replication. A range of concentrations of U18666A (0 to 100 μM) were added to the replicon cell line for 48 hours. The treated replicon cell line was then assayed for the luciferase activity of the replicons. U18666A showed an inhibitory effect on the replicon activity, with a half maximal effective concentration (EC_{50}) of 6.2 μM in dengue replicon A549 cell line (Fig. 5-5A) and 2.9 μM in dengue replicon Huh7 cell line (Fig. 5-5B).

5.2.4. Modulation of host cholesterol and zymosterol levels by U18666A.

The cholesterol level in host cells was measured using gas phase chromatography mass spectrometry (GC-MS). Quantification of lipids using GC-MS was performed by Dr Guanghou Shui. The total cholesterol was found to be slightly increased in infected cells (MOI of 1) treated with U18666A (48 hours treatment) (Fig. 5-6A). Zymosterol, an intermediate product of the sterol biosynthesis pathway, was measured using GC-MS, to monitor the de-novo biosynthesis of cholesterol in host cells. The data showed that zymosterol level was significantly reduced (p-value=1.15E-06) in infected cells treated with U18666A (Fig.5-6B), indicating a suppression in host cholesterol biosynthesis.

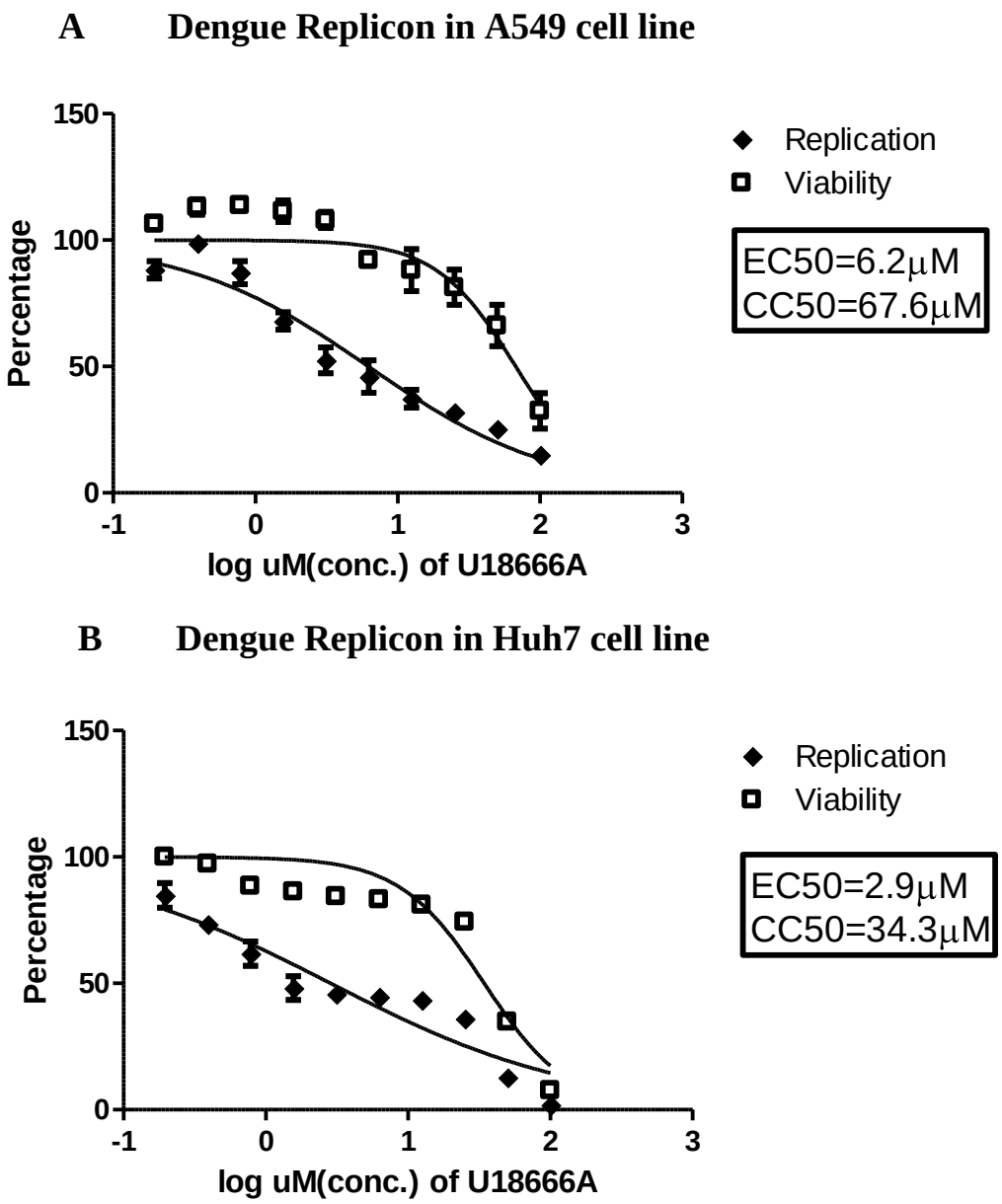

Figure 5-5: Inhibition of viral replication by U18666A. A stably transfected dengue replicon in (A) A549 (B) Huh7 cell lines were used. These cells were treated with a concentration range of U18666A for 48h. The activity of replicons was measured via luciferase activity using Enduren™ live cell substrate. The replicon cells were then lysed and quantified using CellTiter® Glo assay kit for cell cyto-toxicity. Both the replication and viability activity is expressed as a percentage with the untreated control cells set as 100%.

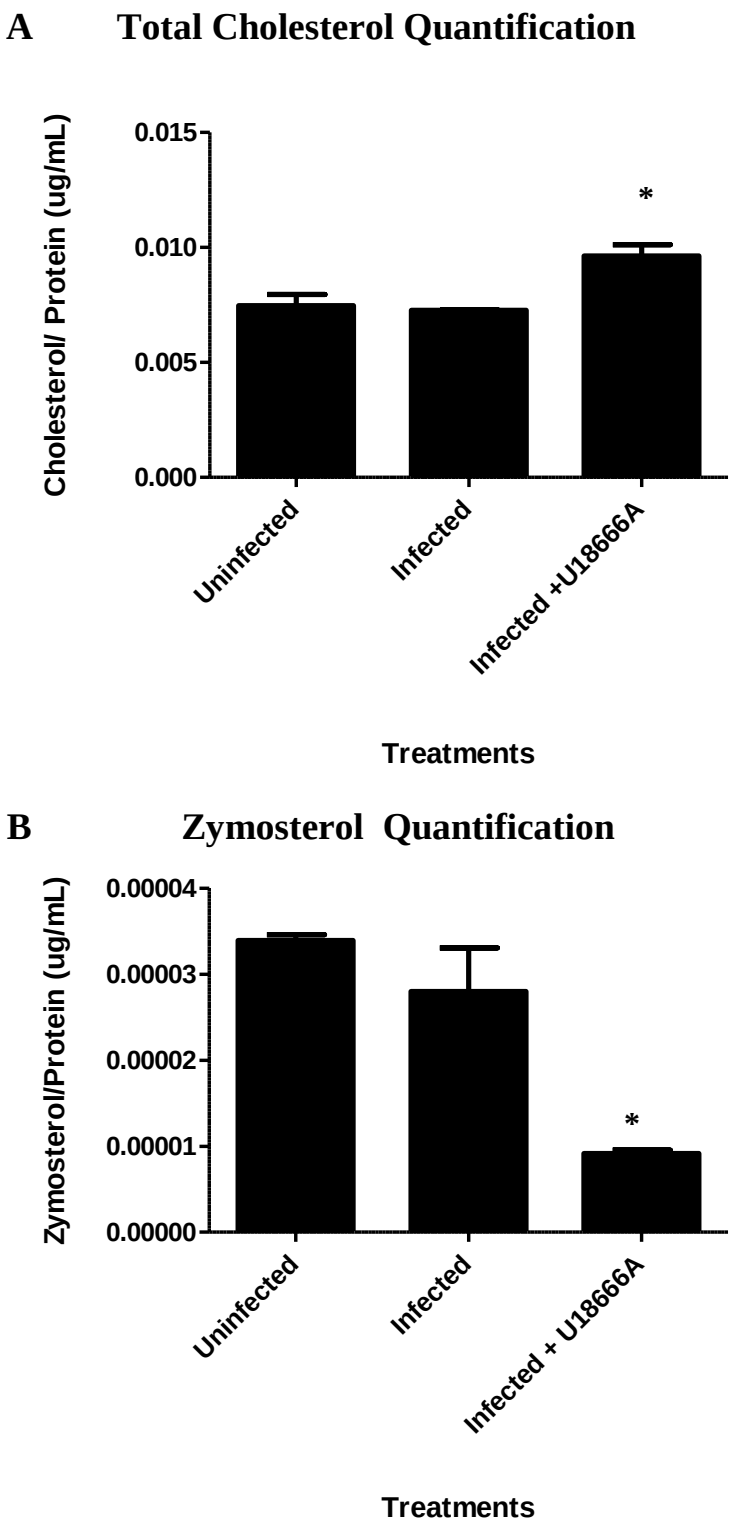

Figure 5-6: Quantification of cholesterol and zymosterol level. BHK21 cells were treated with either 0.9% DMSO as control or 6μM U18666A, then infected with dengue viruses (moi of 1) for 48h before lipids were extracted and quantified using GC-MS. Both (A) cholesterol and (B) zymosterol levels were normalised with total cell protein and expressed as a ratio of cholesterol / protein (μg/mL). T-test (unpaired unequal variances) was performed to compare significant differences using p values of < 0.05. The sign * denotes significant differences.

5.2.5. U18666A has no effect on the association of viral proteins with lipid rafts and the formation of viral induced membranous structure

The effect of suppressed cholesterol biosynthesis (induced by U18666A as observed in figure 5-6B) on the viral replication machinery was investigated using both biochemical and ultra-structural approaches.

DENV's non structural proteins had been shown by Lee and colleagues to be associated with lipid rafts isolated from infected cells (Lee et al. 2008). Their lipid raft isolation protocol was used in this study with infected cells treated with U18666A to find out if reduced cholesterol transport and biosynthesis caused by U18666A would disrupt the association of the viral proteins with lipid rafts. The isolation of detergent resistant membrane (DRM) fraction, enriched for lipid rafts, was performed by DRM extraction with 1% Triton on ice, followed by using a sucrose-density gradient ultracentrifuge protocol (see Chapter 2, methods and materials). There was no noted difference, in the association of NS3 or NS4B with lipid rafts in the caveolin-rich fractions, between the control and U18666A- treated cells (Fig. 5-7).

In the ultra-structural studies, transmission electron microscopy (TEM) was used to observe dengue viral replication. The formation of viral induced membranous structures in both dengue replicon BHK21 cell line (Fig. 5-8A) and live virus infections (Fig.5-8B) were examined. It was observed that viral induced membranous structures could be found in U18666A treated cells, similar to the control infected cells.

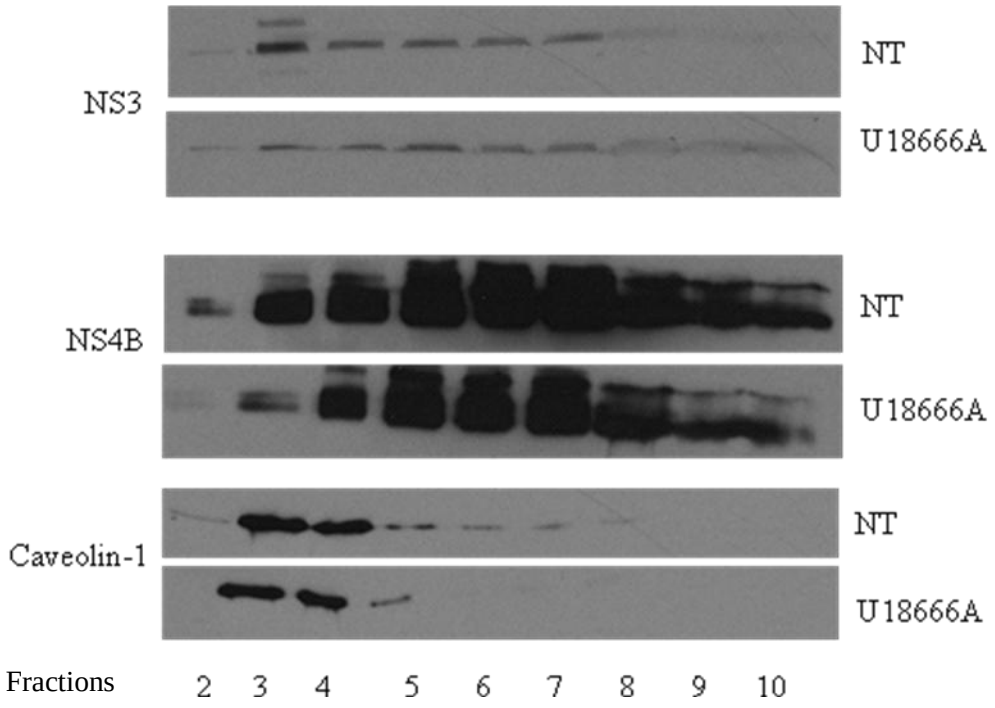

Figure 5-7: Association of viral proteins with lipid rafts. BHK21 cells were treated with either 0.9% DMSO as control or 6μM U18666A and infected with dengue viruses (MOI of 10) for 24h and then harvested for lipid raft extraction in 1% triton on ice. The detergent resistant fractions were separated from the soluble fractions by sucrose density gradient. Fractions 2 to 4 represented the lighter density fractions collected from the top of the tube and fraction 5 to 10 represented fractions collected from the bottom half of the tubes. Non-structural proteins, NS3 and NS4B, were immuno-blotted for and caveolin-1 was used as a lipid raft maker (as shown to be enriched in fractions 3 and 4).

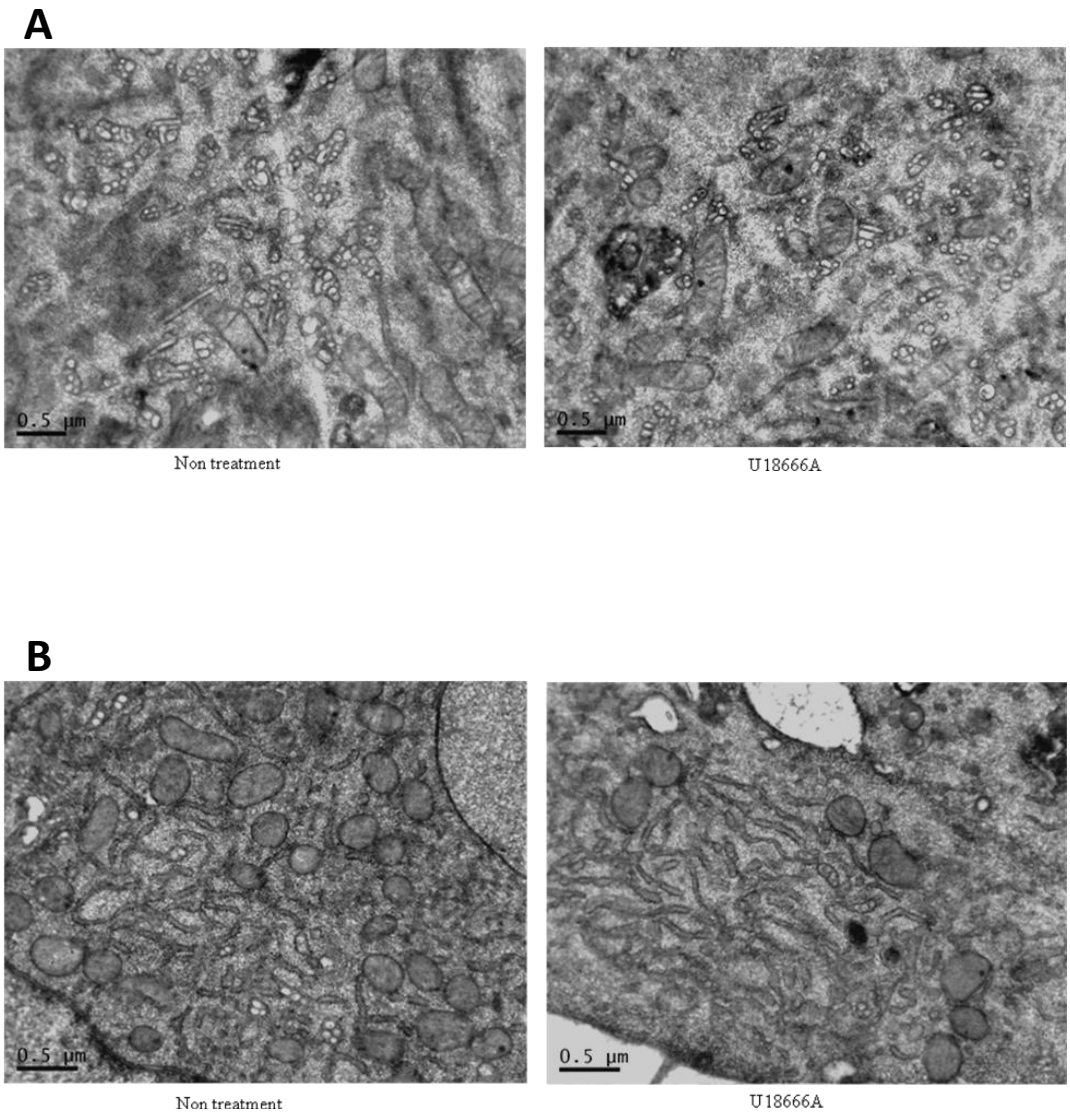

Figure 5-8: Ultra-structural study of viral induced membranous structures. (A)Stably transfected dengue replicons in BHK21 cells were treated with either 0.9% DMSO, control or 6 µm U18666A for 24h before fixation for transmission electron microscopy (TEM). (B) For live virus infection, BHK21 cells (control) or U18666A treated cells, were infected with a MOI of 10 for 24 hours before fixation for TEM sample processing.

5.2.6. Effect of various intermediate sterol inhibitors on dengue replication

A set of sterol inhibitors were screened for anti-viral activity in both dengue replicon and HCV replicon cell lines (Table. 5-1). This experiment was based on a recent study (Owens et al. 2010) indicating that targeting the intermediate steps of the sterol pathway using commercially available sterol inhibitors can affect HCV replication. In the case of dengue replication, only U18666A exhibits anti-viral activity within the non-toxic concentration range, while other sterol inhibitors were either not effective or exhibited very narrow selective index (with CC_{50}/EC_{50} values less than 4). Contrary to dengue replication, HCV replication was affected by most of the inhibitors save SR-12813 and R0 48-8071. It was also noted that U18666A had a lower EC_{50} value of 0.2 μM in HCV replicon compared to 2.8 μM for dengue replicon.

	DENV Replicon			HCV Replicon		
	EC_{50}	**CC_{50}**	**CC_{50}/EC_{50}**	**EC_{50}**	**CC_{50}**	**CC_{50}/EC_{50}**
Sterol inhibitors						
U18666A	2.8	34	12.1	<0.2	33.9	>30
SR 12813	20.2	29.5	0.7	24.1	34.1	0.7
Simvastatin	32.9	73.5	2.2	12.4	82.3	6.6
Squalestatin	No inhibition			Increased replication		
Clomiphene	5.8	13.9	2.4	2.4	22.4	9.3
R0 48-8071	4.1	9.5	2.3	7.1	14.9	2.1
Triparanol	3.7	11.1	3	3.7	14.6	3.9
AY-9944	7.8	15.9	2	3.6	20.4	6.7

Table 5-1: Effect of sterol inhibitors on DENV and HCV replicon cell lines. A class of sterol inhibitors targeting various intermediate pathways of sterol biosynthesis was tested in both dengue replicon-Huh7 cell line and HCV replicons-Huh7 cell line. The efficiency concentration (μM) to bring down 50% replication (EC_{50}) and cyto-toxicity concentration (μM) for 50% killing (CC_{50}) were tabulated in the table. The selective index is calculated and represented as CC_{50}/EC_{50} ratio.

5.2.7. C75, a fatty acid synthase inhibitor, has an additive anti-viral effect when used in combination with U18666A

Recent reports on the importance of lipid droplets (Samsa et al. 2009) and fatty acids (Heaton et al. 2010) in dengue infection both showed anti-viral activity of C75, a fatty acid synthase inhibitor. Figure 5-9 showed that C75 has anti-viral activity in dengue replicon-Huh7 cell line with an EC_{50} of 2.3 μM and a CC_{50} value greater than 100 μM.

These results prompted further investigation to perform a drug synergy experiment with C75 and U18666A and find out whether C75 can lead to an enhanced anti-viral activity with U18666A in the replicon cell line system. An ordered combination of both compounds, of varying concentrations, was set up in dengue replicon cells based on MacSynergy II format that relies on a mathematical model using the Bliss Independence theory (Prichard and Shipman 1990). This experiment allows the assessment of whether the anti-viral effect for the combination of C75 and U18666A is synergistic, additive or antagonistic. A series of eight replicates were calculated to be statistically significant at 95% confidence level. The strength of synergy between the C75 and U18666A were determined by looking at the volume under the curve whereby synergy volume of <25 μM^2 unit % is additive or non-synergy, 25-50 μM^2 unit % is minor but significant synergy, 50-100 μM^2 unit % is moderate synergy and >100 μM^2 unit % is strong synergy. A synergy volume value of 12 μM^2 unit % was calculated at 95% confidence interval for the two compounds (Fig. 5-10A), indicating that these two compounds acted in an overall additive manner. The three dimensional synergy plot, illustrated in figure 5-10B, showing low

peaks above the horizontal plane also represented the overall additive effect of the two compounds.

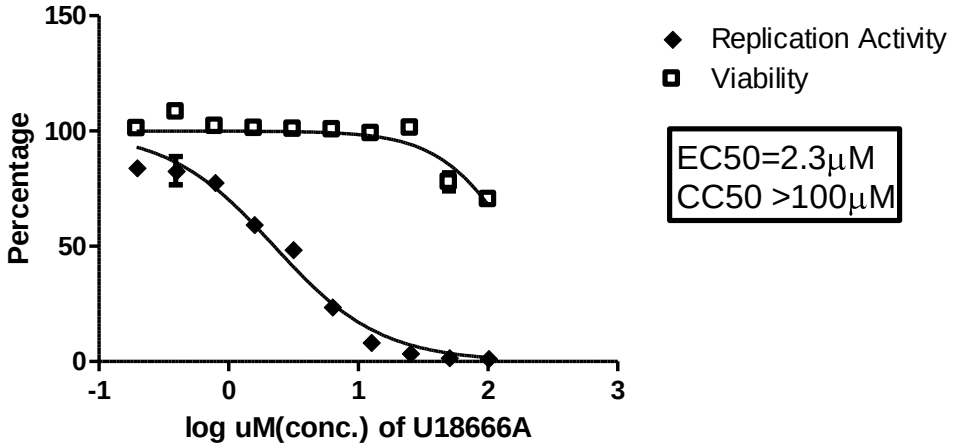

Figure 5-9: Inhibition of viral replication by C75, a fatty acid synthase inhibitor. Stably transfected dengue replicon in Huh7 cell lines were used. These cells were treated with a concentration range of C75 for 48h. The activity of replicons was measured via luciferase activity using Enduren™ live cell substrate. The replicon cells were then lysed and quantified using CellTiter® Glo assay kit for cell cytotoxicity. Both replication activity and viability were expressed in percentage with the control untreated cells defined as 100%.

A

SYNERGY PLOT
(95%)

C75 uM							
25	0	0	0	-1	0	-1	
8.33	0	4	1	1	0	0	
2.78	0	0	0	0	0	0	
0.93	0	3	1	1	1	0	
0.31	0	0	0	0	0	0	
0	0	0	0	0	0	0	
	0	0.41	1.23	3.7	11.11	33.33	U18666A uM

B

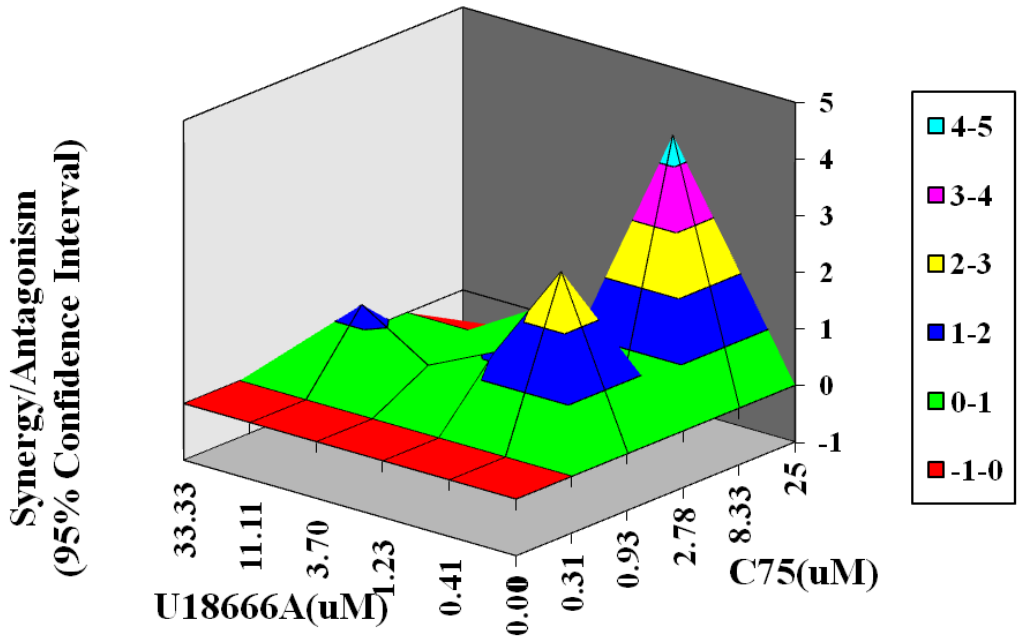

Figure 5-10: A detailed calculation of combined dose effect of U18666A and C75 in inhibition of dengue replication using MacSynergy II. A five point dilution of U18666A was tested in matrix combination format with C75 to obtain the synergy calculation. (A) A table showing the synergy calculation and the total synergy value calculated was 12. (B) A three dimensional synergy plot with 95% confidence interval was used to illustrate non-synergy between the two drugs as shown with few low peaks (less than 5) above the plane.

5.3. DISCUSSION

Previous studies on the role of cholesterol in flaviviruses predominantly used statins to inhibit sterol synthesis in host cells and methyl-beta-cyclo-dextrin (MBCD) to extract cholesterol from cell plasma membrane. U18666A was used in this study because it has been shown to act on host cholesterol via two main pathways: (i) by blocking the transport of endocytosed cholesterol in the form LDL, resulting in an accumulation of cholesterol in the late endosome and (ii) by suppressing the intermediate steps of the de-novo sterol biosynthesis via oxidosqualene cyclase (Sexton et al. 1983). This study aimed to find out whether disrupting these two processes in cholesterol homeostasis would have an impact on DENV infection.

Plaque assay data indicated that the early event of infection is affected by U18666A. The virus binding experiment indicated that cholesterol does not play a role in virus binding onto the host cell, which is in consistent with earlier studies by others (Reyes-Del Valle et al. 2005) using MBCD. These studies showed that viruses were still able to bind to cells from which plasma membrane cholesterol had been extracted by the cholesterol compound.

This study showed that dengue virus infection depends on host cholesterol in a post-attachment manner, i.e. during viral trafficking in host. The arrestment of the endocytosed viruses in the late endosomes of treated cells (4 hours into infection) resulted in a severe reduction of newly synthesized viral proteins at the later time-points of 12 hours and 24 hours post infection. This might be due to the high level of accumulated cholesterol in the late endosomes caused by U186666A, which created a perturbance in the cholesterol level in these trafficking vesicles, for efficient viral fusion to occur. Hence, cholesterol homeostasis is important in the trafficking of DENV for successful infection.

Furthermore, in this study, it was demonstrated that DENV replication is reliant on its host de-novo cholesterol biosynthesis. An anti-viral effect in infected cells was observed when level of zymosterol (an intermediate product of synthesized cholesterol) in host cells was suppressed by U18666A.

Earlier work done by others on the characterization of cholesterol levels in ER membranes in U18666A-treated cells showed a 40% reduction in cholesterol in these treated cells (Lange et al. 1999). Hence, one question asked in this study is whether reduction in the cholesterol levels in E.R, caused by U18666A, would affect the viral replication machinery, believed to be located on membranous structure derived from E.R. However, it was observed that U18666A treatment did not disrupt the association of viral proteins, NS3 and NS4B, with lipid rafts isolated from infected cells. Ultra-structural study using TEM also showed that the viral induced membranes were still formed under the effect of U18666A in the DENV infected cells in 24h post infection.

An attempt to find a more potent cholesterol biosynthesis suppressing compound than U18666A was also tried. A list of various commercially available sterol inhibitors was tested in the dengue replicon cell line. The result from this study argues against any potential use of these inhibitors for anti-dengue therapy due to the toxicity observed. This prompted further investigation to find out whether other lipids played a role in the replication event. Fatty acids have recently been shown to be important to dengue replication (Heaton et al. 2010). A fatty acid synthase inhibitor, C75, was tested on dengue replicon assay and was found to be effective against dengue replication. C75 gave an overall additive suppression on dengue replication when used in combination with U18666A. This implies that both cholesterol and fatty acids are important for successful dengue infection.

6. SUMMARIZING CONCLUSION AND FUTURE OUTLOOK

6.1. TARGETING VIRAL FUSION VIA THE βOG POCKET NEAR TO THE HINGE REGION OF DENV E PROTEIN

Targeting viral entry via inhibition of membrane fusion is attractive for antiviral strategy because it is inhibitory at the early step of infection. With the elucidation of the dengue envelope (E) protein structure (both pre-and post-fusion structures), there is information available for a structure activity relationship (SAR) approach to design small molecules inhibitors. In Chapter 3, a rational approach using in-silico selection of predicted E-binders was used to look for dengue virus fusion inhibitor. Faced with a lack of suitable fusion assay that would allow the monitoring of fusion-inhibition in a high throughput manner, an assay based on cell-cell fusion in mosquito cell line was set up. This assay was used for compound screening in a 96 wells format. This assay is relatively easy to set up once the parameters (such as cell seeding density, viral titer for infection, and pH value for fusion) are optimized. It does not require the need of molecular cloning of reporter genes, unlike reported cell-cell fusion assay for HCV which is based on reporter system (Kobayashi et al. 2006). A similar fusion assay based on cell-cell fusion was also adopted by Kampmann and colleagues to validate the fusion inhibitors they had identified from the in-silico screening of E binders (Kampmann et al. 2006). They used a mixture of anti-prM and anti-E antibodies to visualize infected mosquito cells and then count for cell-cell fusion event by looking for fluorescently tagged mutli-nucleated cells.

To date, there are several published studies (see in table 1.1 page 23) using βOG binding pocket as the defined ligand for an in-silico search for small molecules that bind and block dengue viral fusion. These reported fusion inhibitors are structurally distinct. As mentioned in chapter 3, the trifluoro-phenyl motif of

NITD448 (predicted to be buried into the hydrophobic βOG pocket) shares some similarity to the chloro-phenyl-thiophene tail of compound 6 reported by our colleague in NITD (Wang et al. 2009). Two teams identified inhibitors that shared the common feature of a central thiazole ring in their most active compounds (Kampmann et al. 2009; Li et al. 2008). Another team reported fusion compounds which are tetracycline derivatives (Yang et al. 2007). However, none of the reported inhibitors, including NITD449, made it to the clinical studies. This raised a question on the validity of using βOG-binding pocket as a target for rational drug design.

The molecular properties of how antiviral compounds enter and bind onto a hydrophobic pocket are unknown and experimentally difficult to be determined. A compound's ability to enter and bind can be attributed to the compound's structural flexibility and also the compound's interaction with the residues found within the pocket of the protein. Often in drug discovery, a co-crystallization of the compound with the target can provide hints to the binding interaction of the compound. Such structural information would greatly aid in the validation and future design of better generation of fusion inhibitors. This is hampered by the current protocol for the expression and purification of DENV E protein that is unable to yield large amount of E protein required for structural studies. Only one team managed to show the binding of the inhibitor they identified (PO2) with the DENV E protein using preliminary NMR spectrometry studies. Much effort and improvement in physical interaction studies are required in order for SAR improvements in these reported fusion inhibitors.

The discovery of βOG binding pocket is only a starting point for small molecular fusion inhibitor research. With the increasing knowledge learnt about the dynamics of E protein during the different stages of viral fusion, it would be

worthwhile to explore these transition stages as alternative target site beside the βOG pocket. Such alternative sites as proposed by Yennamalli and colleagues could be a cavity that is either present in the dimer but not in the trimer (blocking trimerization) or cavity that is present in the dimer interface (blocking transition to post-fusion state) (Yennamalli et al. 2009).

6.2. NITD770, AN ANTI-VIRAL SMALL MOLECULE WITH UNKNOWN MECHANISM OF ACTION

Determining how a compound acts on the targeted disease is an important factor in the development of clinical drugs. Understanding the way in which a compound interacts with its target can help chemists design a second-generation of compounds with improved potency. Structural studies, such as X-ray crystallography, are often used to study compound binding site. This facilitates a rational improvement on the molecular properties of the compound, which can result in a higher affinity to the target and thereby potentially resulting in increased potency of the compound. The other positive highlight of identifying the mechanism of action of the compound is to eliminate possible biological side effects. Many clinical candidates did not progress to the market due to the unexpected side effects exhibited by the compound. Hence, knowing which biological pathway(s) is/are being affected by the compound, one can make a Go or No-go decision in further drug development. This decision is also based on the predicted toxicity to the patient at an earlier phase of the drug discovery progress. These factors potentially save time and money on failed outcomes of clinical candidates.

In Chapter 4, a small molecule mevalonate pyrophosphate decarboxylase (MVD) inhibitor (NITD770) was found to have a good potency with a selective index

of EC_{50}/CC_{50} of more than 100 and specificity towards the members of the flaviviridae family. Target validation of this compound revealed that it was not targeting MVD. Various approaches involving cross-disciplinary studies were done without any success to explain the anti-dengue activity exhibited by this compound. There is an on-going effort to decipher the mode of action for NITD770. Although it was observed in this study to have no effect on the lipid raft or cholesterol metabolism, a more thorough lipid profiling, looking into other species of lipids in treated cells, may provide insight and perhaps answer the question as to whether host lipid is a target of this compound. In the quest to find NITD770's target, it would be useful if a fluorescent probe could be tagged on to NITD770 to visualize, by microscopy, the entry and localization of this compound into host cell in order to aid prediction of site of action. An affinity pull-down of host cell materials would also be an alternative approach to look for interacting host targets. Both techniques require some form of chemical manipulation to attach a linker to the small molecule, and this was found to compromise NITD770's bioactivity. A new technique used for target identification, known as Drug Affinity Responsive Target Stability (DARTS), which does not require any modification to the drug to be investigated (Lomenick et al. 2009) could potentially be used. This technique is based on the conferred stabilization of targeted proteins by the compound upon binding. Stability of the bound proteins can be caused by either induced conformation stability or masking of the protease recognition sites present in the proteins. Hence, the compound bound proteins, among the pool of protein mixtures in a cell lysate, become resistant to protease treatment, which can then be identified using mass spectrometry. This could be applicable for future work in finding the target of NITD770.

6.3. THE DEPENDENCE OF HOST CHOLESTEROL BY DENGUE VIRUS AND TARGETING HOST LIPID METABOLISM AS AN ANTI-VIRAL STRATEGY

Caution is needed when dealing with host factors as a target class for anti-dengue therapy because potential harmful effects to human may occur. However, there is still a therapeutic window to work with if the drug exhibits greater potency against the viral target than it does against the host homologue. There have been an increasing number of findings pointing towards the importance of perturbation of host lipid metabolism (as discussed in the introductory chapter of this thesis). The work done in Chapter 5 allows us to gain insight into the dependence of DENV on host cholesterol homeostasis.

Targeting lipid in viral entry

This study described that the level of cholesterol in the late endosome of host endocytosis pathway is important for efficient trafficking of DENV for successful infection. This could be due to a reduction in the fluidity of the host endosome membrane (caused by the U18666A induced accumulation of cholesterol, a saturating lipid) resulting in hampering of fusion with the viruses. It is generally believed that different viruses fuse with the host trafficking vesicles based on the pH threshold value of its E protein (pH threshold value was determined using liposome based fusion assay) for triggering viral fusion (White and Helenius 1980). In the field of DENV research, scientists are unable to explain the observation of viral fusion occurring in late endosome even though early endosome has an acidic content sufficient to trigger viral fusion in-vitro. This question could perhaps be answered by the recent study done by Zaitseva's team proposing that dengue virus engineers it own lipid bilayer to contain late endosome enriched lipid, bis(monoacylglycero)phosphate,

for its timely fusion at this compartment (Zaitseva et al. 2010). This further strengthens the point that targeting host lipid important for viral fusion can result in an anti-viral activity. Targeting host lipid can also provide an alternative or complement strategy to targeting the viral E protein for designing a fusion inhibitor of dengue virus.

Targeting lipid in viral replication

Host membrane lipids and proteins could serve as scaffolds for the assembly of viral replication complex. They can also mediate the targeting of the viral replication proteins to the site for replication. Based on immuno-labeling studies and ultra-structural studies, we now know that viral replication takes place in close association with the membranous structure near to the proximity of the host endoplasmic reticulum. Several (+) RNA viruses have been observed to induce membranous proliferation (Bienz et al. 1992; Egger et al. 2002; Salonen et al. 2005). Membrane-bound viral cytoplasmic replication is not restricted to RNA viruses, DNA virus such as poxvirus, also replicates in the cytoplasm (Schramm and Locker 2005). Hence, there is a consensus that these viruses depend on the host lipids for establishment of viral replication in host. Several plus-strand viruses remodel their host membranes to create structures, known as spherules that may serve to concentrate the viral replication proteins for efficient replication. These induced membranous structures may even act as a shelter from the harmful cellular proteases and nucleases, during the synthesis of new viral genetic material. Mackenzie and colleagues did some pioneering work in flavivirus, West Nile virus (WNV), where they observed the redistribution of the host cholesterol in the plasma membrane to the site of viral replication during infection (Mackenzie et al. 2007). Of recent year, dengue viral protein NS3 was shown to recruit fatty acid to the site of replication by

targeting fatty acid synthase (FASN) to the viral factory and possibly also responsible for stimulating the biosynthesis of this lipid needed for efficient viral replication (Heaton et al. 2010).

Limiting the de-novo synthesis of host cholesterol level is also explored in anti-viral therapy. This approach is encouraged by the fact that there are commonly used cholesterol-lowering drugs (statins) taken by patients for treating other diseases, emphasizing the possibility of developing a cholesterol anti-dengue drug that is safe to humans. In chapter 5, the study showed that dengue viral replication is severely suppressed when the de-novo synthesis of a sterol intermediate was inhibited (shown by reduced zymosterol levels) by U18666A. Subsequent investigation was done to determine whether other intermediate steps in the sterol biosynthesis can be targeted using pharmacological intervention in the dengue replicon screening assay. The results showed that, besides U18666A, the remaining compounds were either ineffective or had narrow selective index to avoid host toxicity. Interestingly, in parallel screening using HCV replicons, several sterol inhibitors were found to be suppressing HCV replicons at lower concentration within the non-toxicity concentration. This data implies that HCV is more dependent on host cholesterol biosynthesis compared to DENV. HCV has been the focus of intense drug discovery for the last two decades. Many findings previously found in HCV are currently being tested in anti-dengue research. One needs to note that the disease caused by HCV is different from DENV, with HCV causing a chronic infection whereas DENV causes an acute infection. This may explains the difference observed in the potency of the sterol inhibitors between these two viruses. Hence, it would be interesting for future studies to compare the efficacy of these sterol inhibitors in other viruses which cause

either chronic infection or acute infection. This may help to gain insights into the mechanism of viral establishment in relation to host cholesterol modulation.

Targeting host fatty acid metabolism remains an unexplored territory in anti-dengue therapy. As mentioned earlier in this section, with the recent finding of regulation of fatty acid by the dengue viral protein, NS3 (Heaton et al. 2010), this metabolic pathway could be investigated in more detail as a host target for an anti-dengue drug. The studies reported in chapter 5 place emphasis on the fact that viral infection can be targeted via regulation of biosynthesis of host cholesterol and fatty acid.

6.4. TARGETING VIRAL AND HOST FACTORS IN ANTI-DENGUE DRUG DISCOVERY

In summary, the work reported in this thesis explored the possibility of targeting two processes, the viral fusion event (targeting E protein) and the host cholesterol metabolism, in the search for compounds that inhibit dengue infection.

Using a rational approach to look for an entry-inhibitor drug has the advantage of specificity to viral target. It also accelerates the hit-to-lead progression in drug discovery pipeline, by reducing the chance of biological side effects and aiding SAR improvement for secondary compounds. In the case of designing a cholesterol-targeted drug in anti-dengue treatment, it is important that the drug has a well defined mechanism of action with specificity towards the targeted protein or process, due to the possible cross toxicity to host. In my studies, there were similar encounters with narrow therapeutic range for most of the cholesterol compounds targeting the various intermediate steps in host cholesterol biosynthesis. In order to overcome the host toxicity, more studies are necessary to fine-tune the balance between anti-viral activity and host toxicity. Cholesterol is an essential component of many cellular processes in host cell and hence, this may be the reason of the limited therapeutic range observed when targeting the host cholesterol metabolism in dengue infection. Perhaps, it would be more successful for an anti-dengue compound if the lipid target is required for a unique function found in the pathogen, compared to the ubiquitous cholesterol. Identification of such lipid will require studies involving detail lipid profiling of the dengue virus particles or infected host cells.

The paradigm of anti-viral drugs has been to achieve a high anti-viral effect and low host toxicity. Targeting a viral protein reduces the chance of cross-inhibition of host protein but it is often only effective against one type of virus and encounters

the problem of the emergence of resistant viruses. It is an arduous journey to develop a drug from bench to patients. Hence, having a drug that has broad-spectrum activity seems a worthwhile effort to be pursued by scientists. With the increasing reports on studies using genome scale analysis on the host-pathogen interactions, it seems promising that these studies could lead to the identification of unique host processes as potential targets for inhibiting viral infection in future.

Another issue to consider when developing anti-dengue drug is the pathogenesis of this disease, with the likelihood to progress from the milder case of DF to the more severe case of DHF/DSS where the host immune responses are perturbed. In the latter case, anti-dengue drugs that modulate the host response would be a more effective approach to treat these patients.

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ANNEXES

ANNEX 1: CLONING OF DENGUE 4 NS5 MUTANT PROTEINS

Total RNA was extracted from tissue culture media supernatant containing D4MY01-22713 virus using the QIAamp Viral RNA kit (Qiagen, USA) following the manufacturer's instructions. The purified RNA was then reverse-transcribed with Superscript III (Invitrogen, USA) using the primer 5′-CAATGGTCTCTTTGGTGT TTG-3′. After reverse transcription, the primers 5′-CATATGGCTAGCGGAACTGG GACCACAGGA-3′ and 5′-GCGGCCGCTTACAGAACTCCTTCACTCTC3′ were used to amplify NS5. The amplified PCR band was subsequently cloned into a zero blunt TOPO PCR cloning vector. After verification of the DNA sequence, the TOPO vector was cut with *NheI* and *NotI* restriction enzymes to release the DENV-4 NS5 fragment and cloned into pET28a cut with the same enzymes. The ligation reaction was transformed into Top10 cells (Invitrogen, USA).

The single mutants were made using appropriate primers together with the wild-type DNA template. The double mutants were created with appropriate primers and single mutant as template and the triple mutant were created using appropriate primers and double mutant as template as listed in the tables below.

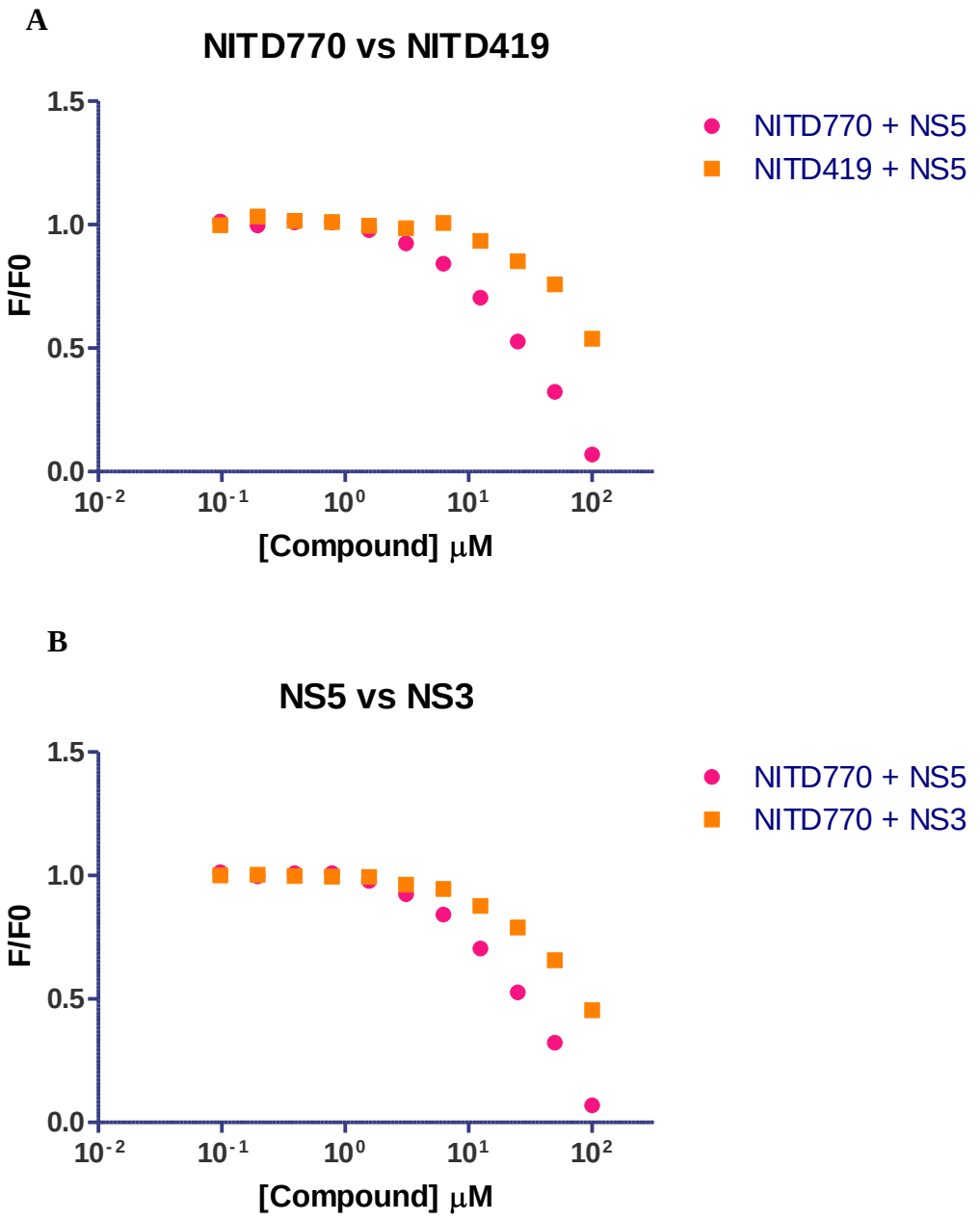
Primers	**Nucleotide sequence**
8171S	5'-gacgacacagtaggctgggacacgagaatc -3'
8172A	5'-gtcccagcctactgtgtcgtcagcatatat -3'
8173S	5'-gtgggaacatacgctttaaacacattcactaac -3'
8174A	5'-tgtgtttaaagcgtatgttcccacctgtccact -3'
8175S	5'-actaacatgggagttcaactcatccgccaa -3'
8176A	5'-gagttgaactcccatgttagtgaatgtgtt -3'

Mutants	PCR TEMPLATE	Primers
pET28a D4 NS5FL A535V	pET28a D4NS5FL	8171S and 8172A
pET28a D4 NS5FL G607A	pET28a D4NS5FL	8173S and 8174A
pET28a D4 NS5FL E615G	pET28a D4NS5FL	8175S and 8176A
pET28a D4 NS5FL AG535/607VA	pET28a D4 NS5FL A535V	8173S and 8174A
pET28a D4 NS5FL AE535/615VG	pET28a D4 NS5FL A535V	8175S and 8176A
pET28a D4 NS5FL GE607/615AG	pET28a D4 NS5FL G607A	8175S and 8176A
pET28a D4 NS5FL AGE535/607/615VAE	pET28a D4 NS5FL AG535/607VA	8175S and 8176A

Annex 1: Primers Sequence

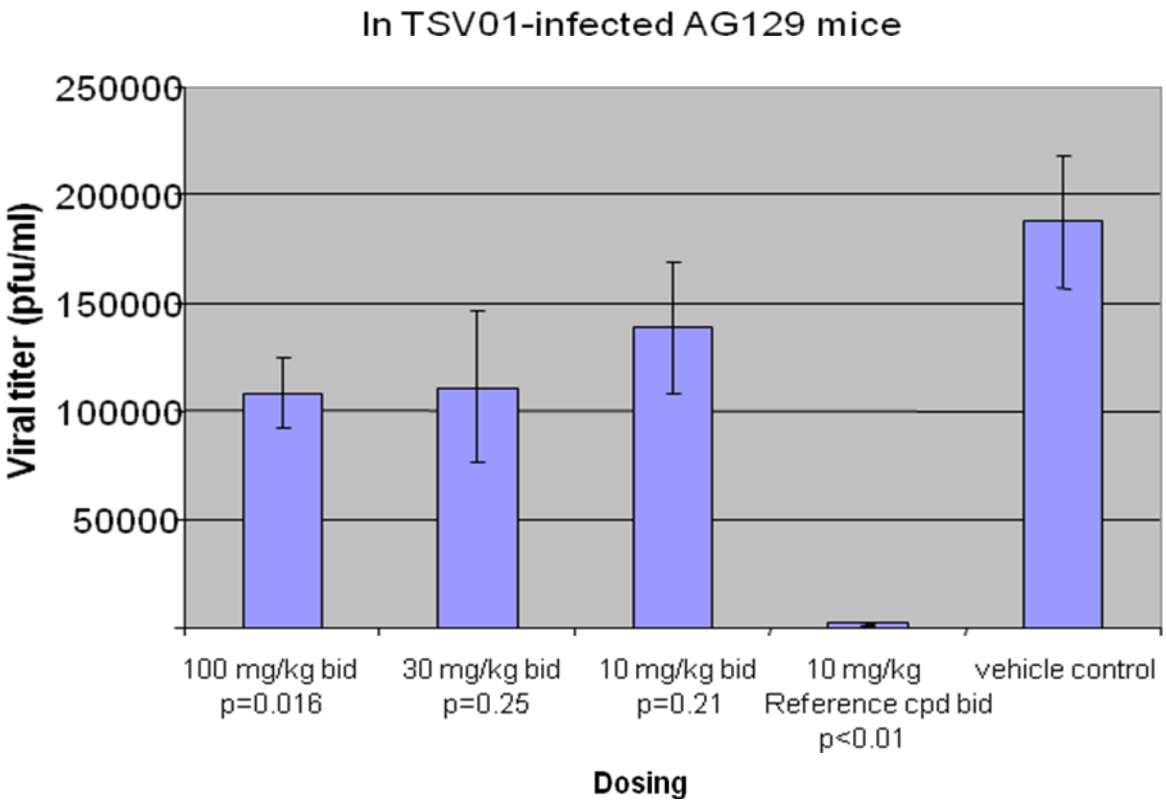
The PCR reaction was carried in 50 μL of reaction volume consisting of 50 ng of template (pET28a D4 NS5 FL or mutants), 200 μM of dNTP mix, 1 μM of primers and 1 μL of pfu polymerase (Stratagene, USA). This PCR reaction was performed at 94^0C for 2 minutes, followed by 16 cycles of 94 ^{0}C for 30 seconds, 55^0C for 30 seconds and 68^0C for 16 minutes. A final extension step was carried out for 10 minutes at 72^0C. DpnI restriction enzyme (1 μL) was used to digest away the input DNA template at 37 °C for 1 hour. After the digestion, the resulting PCR product was transformed into top10 bacteria cells. Colonies were picked and the presence of mutation was verified with direct sequencing.

ANNEX 2: QUENCHING OF INTRINSIC FLUORESCENCE OF NS5 BY NITD770



Annex 2. Quenching of NS5 intrinsic fluorescence by NITD770. Specific fluorescence emission was measured at a wavelength of 350nm. Normalized fluorescence (F/F_0 where F_0 is the fluorescence without compound) was then plotted against increasing concentrations of compound. Two sets of experiments were set up to test the specificity of NITD770 effect on NS5. (A) NITD770 versus NITD419 (an inactive analogue of NITD770). (B) Effect of NITD770 on NS5 versus NS3.

ANNEX 3: IN-VIVO EFFICACY OF NITD770 IN MOUSE VIREMIA MODEL



Annex 3. An evaluation of NITD770 efficacy in a dengue viremia model in mice AG129 mice (with knockout interferon α/β receptor and interferon γ receptor) purchased from B&K Universal were injected intraperitoneally with 0.4 mL of RPMI-1640 medium containing 5000,000 PFU/mL of DENV-2 (TSV01). The infected mice were then dosed with either NTD770 or vehicle (100% corn oil) by s.c injection. Various concentration of NITD770 was tested in TSV01-infected AG129 mice for three days followed by the determination of the viremia load in these mice using plaque assay.

ANNEX 4: CHIKUNGUNIA VIRUS CPE ASSAY

BHK21 cells were seeded, one day before, at a cell density of 20,000 cells/well (RPMI-1640 containing 2% FBS) in a 96 well plate. On the following day, the cells were infected with 200μL of 10,000 PFU/mL chikungunya viruses. NITD770 was then added and incubated for 2 days treatment before viral titer determination using plaque assay.