PROBING THE FLAVIVIRUS LIFE CYCLE: REPURPOSING AMODIAQUINE AS AN
INHIBITOR OF FLAVIVIRUS INFECTIVITY AND FUNCTIONAL ANALYSIS OF
FLAVIVIRAL NS5 IN 5’CAPPING

A Dissertation
submitted to the Faculty of the
Graduate School of Arts and Sciences
of Georgetown University
in partial fulfillment of the requirements for the
degree of
Doctor of Philosophy
in Microbiology and Immunology

By

Siwaporn Boonyasuppayakorn, M.D.

Washington, DC
December 3, 2013
PROBING THE FLAVIVIRUS LIFE CYCLE: REPURPOSING AMODIAQUINE AS AN INHIBITOR OF FLAVIVIRUS INFECTIVITY AND FUNCTIONAL ANALYSIS OF FLAVIVIRAL NS5 IN 5’CAPPING

Siwaporn Boonyasuppayakorn, M.D.

Thesis Advisor: Radhakrishnan Padmanabhan, Ph.D.

ABSTRACT

Dengue virus serotypes 1-4 (DENV1-4) are transmitted by mosquitoes and are the most frequent cause of arboviral infections in the world. Neither vaccine nor antiviral drug is currently available. In this study, we discovered amodiaquine (AQ), one of 4-aminoquinoline drugs, inhibited DENV2 infectivity with an EC$_{90}$ of 2.69 ± 0.47 μM and DENV2 RNA replication with an EC$_{50}$ of 7.41 ± 1.09 μM in the replicon expressing cells. Cytotoxic concentration on BHK-21 cells was 52.09 ± 4.25 μM. The replication inhibition was confirmed by an infectivity assay measured by plaque assay of the extracellular virions, and by qRT-PCR of the intracellular and extracellular viral RNA levels. AQ was stable for at least 96 h and had minor inhibitory effect on entry, translation, and post-replication stages in the viral life cycle. Flaviviral enzymes including protease, methyltransferase, and RNA dependent RNA polymerase do not seem to be targets of AQ. Both $p$-hydroxyanilino and diethylaminomethyl moieties are important for AQ to inhibit DENV2 replication and infectivity. With a proven efficacy in vivo, AQ will become a new anti-flaviviral candidate for clinical trials.

Flaviviral genome contains a $^7$MeGpppA$_2$OMe cap structure mimicking that of eukaryotic mRNA so as to evade the host immune system and initiate translation. Synthesis of this cap requires the enzymatic functions of NS3, NS5, and additional unknown factors. Full-length flaviviral NS5 (NS5$_{FL}$) displayed higher methyltransferase activity than the N-terminal domain alone.
Heterologous flaviviral NS5\textsubscript{FL} proteins were equally active as methyltransferases. A complete understanding of guanylyltransferase activity remains to be explored as NS5 alone is insufficient to add the GMP cap to the 5′-diphosphorylated RNA substrate. An additional cofactor may be required in the transfer of GMP to diphosphorylated RNA, the second step of 5′-capping. Both NS3 and NS5 possess GTP hydrolysis activities resulting in GDP and GMP products, respectively. NS3 is more active than NS5 in GTP hydrolysis such that GDP becomes the predominant product. NS5 acquires higher efficiency in GTP hydrolysis to GMP in the presence of 5′terminal RNA\textsubscript{nt1-200}. These findings will guide further investigations towards a complete understanding of the flaviviral capping process.
ACKNOWLEDGEMENT

First of all, I would like to thank my advisor, Dr. Radhakrishnan Padmanabhan, for giving me this opportunity to work in his lab. I have learnt a lot during these five years. He allows me to explore the scientific world through basic and translational projects. He always inspires students with his insight, knowledge, and experience in the field. He never runs out of new ideas and always surprises us with his extraordinary problem-solving skills. And most of all, he is a living example of success is gained through perseverance.

Next, I would like to thank my Committee Chair, Dr. William Fonzi, for his continuous supports throughout the Ph.D. study. He is kind, supportive, and thoughtful, without compromising the precision in his academic profession. He also challenges students with high expectations. Indeed, he is a role model of a good teacher. Moreover, I would like to thank my committee members; Dr. John Casey, Dr. Brent Korba, and Dr. David Yang for valuable inputs. Insights from their specialties have polished this thesis to perfection. Thanks to Dr. Kuppuswami Nagarajan for suggesting the antimalarial drugs to the replicon assays for the first time.

In addition, I would like to express my gratitude to Faculty of Medicine, Chulalongkorn University for sponsoring my Ph.D. education. I would like to thank Dr. Parvapan Bhattarakosol for introducing me to the Virology world, and for never giving up on me. Thanks to Dr. Pokrath Hansasuta, Dr. Ekasit Kowitdamrong, and all staffs from the Virology unit for helpful instruction, tips, and methods in Virology. Thanks to Dr. Kamjorn Tatiyakavee for his help during the process of my scholarship application. Thanks to Dr. Taweesak
Tirawatanapong, and Dr. Nattiya Hirankarn for introducing me to Dr. Padmanabhan and Georgetown University.

In addition, I appreciate a good collaboration from my colleagues; Dr. Tadahisa Teramoto, Dr. Priya Srinivasan, Dr. Huiguo Lai, Dr. Mark Manzano, Rupa Guha, Shira Saperstein, Rohit Mukherjee, Jessica Connor, and Stephen Lim. Special thanks to my friends; Dr. Peera Hemarajata, Dr. Yuwares Malila, Dr. Prasanth Viswanathan, Dr. Satjana Patanasak, Shruti Jayakumar, Brittany Griffin, Chris Obara, Mounavya Agileti, and Minnie An. Without them, my life in a foreign country would have been difficult and lonely. And I am deeply thankful for Dr. Sarapee Hirankarn for her supporting hands in my first rough winter.

Finally, I would like to thank Dr. Saran Salakij for his positive thinking and mental support. Most importantly, thanks to my family, Sanguan and Kunwadee Boonyasuppayakorn, Lim Jung, and The Siripatrachais for their holistic supports. I am grateful for their love, care, inspiration, and encouragement throughout my Ph.D. life.

Many thanks,
Siwaporn Boonyasuppayakorn
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<tbody>
<tr>
<td>AST</td>
<td>aspartate aminotransferase (liver function test)</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase (liver function test)</td>
</tr>
<tr>
<td>AQ</td>
<td>Amodiaquine</td>
</tr>
<tr>
<td>AQD</td>
<td>Amodiaquine derivative</td>
</tr>
<tr>
<td>BHK-21</td>
<td>Baby Hamster Kidney cell line</td>
</tr>
<tr>
<td>BHK-21/DENV2</td>
<td>subgenomic DENV2 replicon stably expressed in BHK-21 cell</td>
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<td>C</td>
<td>capsid protein</td>
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<td>CC&lt;sub&gt;50&lt;/sub&gt;</td>
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<td>cluster of differentiation</td>
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<td>CDC</td>
<td>Center of disease control</td>
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<td>CobY</td>
<td>GTP:adenosylcobinamide-phosphate guanylyltransferase</td>
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<td>CQ</td>
<td>Chloroquine</td>
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<td>DENV</td>
<td>dengue virus</td>
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<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DMSO</td>
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xvi
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<td>multiplicity of infection</td>
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PART I
REPURPOSING AMODIAQUINE AS AN INHIBITOR OF FLAVIVIRUS INFECTIVITY
CHAPTER 1
INTRODUCTION

1.1. General knowledge on Dengue diseases

1.1.1. Epidemiology

Dengue virus (DENV) infection is widespread in more than 100 countries worldwide affecting over 2.5 billion people (Gubler, 2002, WHO, 2012). The incidence of dengue disease has increased 30-fold in the past 50 years by geographical expansion to new countries, and from urban to rural settings (Gubler and Clark, 1995, Gubler, 2002) (reviewed by (Wilder-Smith et al., 2010)). Estimated case reports are 50-100 million per year, with 500,000 hospitalizations, and 12,000 deaths (Rodhain, 1996, Rigau-Perez et al., 1998, WHO, 2012). However, recent estimates (Bhatt et al., 2013), (Mitka, 2013) indicate there were 390 million dengue infections and 96 million clinically manifested cases annually. The number of cases varies from year to year, but usually peaks every 3-5 years. Factors contributing to this triennial trend are still to be explored (reviewed by (Wilder-Smith et al., 2010)).

Dengue is transmitted by Aedes mosquitoes, primarily Aedes aegypti (Halstead, 2008), and secondarily by Aedes albopictus. In tropical and subtropical areas, Aedes aegypti is a major contributing factor in spreading dengue virus for reasons as follows; 1) this mosquito prefers to breed in clean stagnant water near household areas; 2) it potentially takes a blood meal from multiple human hosts; and 3) the mosquito is capable of completing its life cycle in shorter period (reviewed by (Wilder-Smith et al., 2010) and (Juliano et al., 2002)). Global warming is predicted to facilitate the spread of the mosquito vector (Halstead, 2008) (reviewed by (Wilder-Smith et al., 2010)). Aedes albopictus, on the other hand, is responsible for the virus spread in
cold climates (Juliano et al., 2002). The mosquito was found northernmost in Chicago, IL (CDC, 2012).

In America, the disease incidence increased from 16.4/100,000 in the 1980s to 35.9/100,000 in 1990s, and to 71.5/100,000 in 2000-7 (San Martin et al., 2010). Most DENV infections in the United States are travel-associated (Wilder-Smith and Schwartz, 2005). There were sporadic outbreaks in Hawaii (2001) (Effler et al., 2005), and South Texas (2005) (Ramos et al., 2008). The disease is endemic in Puerto Rico with the latest outbreak in 2010 (Prince et al., 2011, Anez et al., 2012). Declared by the CDC, Dengue is an emerging infectious disease and since 2009, all dengue fever (DF) and dengue hemorrhagic fever (DHF) cases in the US must be reported to the CDC (CDC, 2012, Tomashek, 2012).

1.1.2. Classification

The World Health Organization has revised the classification criteria for diagnosis and management guideline of dengue diseases. The terminology dengue fever (DF) and grade 1-4 dengue hemorrhagic fever (DHF) were replaced with dengue (± warning signs) and severe dengue (WHO, 2009), respectively. The new criteria were designed to support a diversing clinical spectrum due to geographic expansion and increased incidence in older age groups (adolescence to young adult) (Srikiatkhachorn et al., 2011, Hadinegoro, 2012). Moreover, the new criteria are more sensitive and specific in detecting severe dengue in retrospective studies (Narvaez et al., 2011). However, in a prospective study, a significant number of false positive cases were recruited because the new criteria were not specific (Srikiatkhachorn et al., 2011).
Dengue virus (DENV) is classified as a member of the genus Flavivirus within a family Flaviviridae (Lindenbach et al., 2007). Other flaviviruses with clinical importance include Yellow fever virus (YFV), West Nile virus (WNV), and Japanese encephalitis virus (JEV). Flavivirus has a positive single stranded RNA genome with a methylated nucleotide cap at the 5’ terminus, but lacking a poly A tail at the 3’ terminus. Its enveloped virion is spherical to pleomorphic in shape, and 40-60 nm in diameter (Lindenbach et al., 2007). Most members are primarily arthropod-borne. There are 4 serologically distinct types of dengue (DENV 1-4) sharing about 65% homology at the amino acid level (Westaway, 1997).

1.1.3. Molecular biology and life cycle

An infectious virion contains a single-stranded positive-sense RNA genome of approximately 11 kilobases in length associated with the capsid protein (C), and covered with host derived lipid membrane containing precursor membrane proteins (prM), mature proteins (M), and envelope proteins (E) (Mukhopadhyay et al., 2005). The genome is decorated with a dimethylated cap called a type I cap ($\text{7MeGpppA}^{2’\text{OMe}}$) at the 5’terminus. A single open reading frame encoding all viral proteins is flanked with highly structured 5’ and 3’ untranslated regions (UTR) (Lindenbach et al., 2007). The viral polyprotein is synthesized by host ribosomes (cap-dependent translation), and processed by viral protease (NS3) and host proteases (signal peptidase, furin) into 3 structural proteins, C, prM, and E, and 7 nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.

DENV enters the cell via receptor-mediated endocytosis. To date, several host receptors have been reported including heparin sulphate, chondroitin sulphate, mannose binding lectin, and
Dendritic Cell - Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) or cluster of differentiation 209 (CD209) (reviewed by (Sampath and Padmanabhan, 2009)). DENV infects a wide range of cell lines from human, mosquito, monkey, and mice implying that the virus is capable of utilizing several receptor types for its binding (reviewed by (Rodenhuis-Zybert et al., 2010)). After internalization, the clathrin-coated viral endosome is acidified. This leads to a conformational change of envelope (E) proteins, which subsequently induces the fusion of endosomal membrane and viral envelope, and release of viral nucleocapsid into the cytoplasm (reviewed by (Rodenhuis-Zybert et al., 2010)).

The genomic RNA is a template of both translation and transcription. Firstly, cap dependent translation by host machinery gives rise to a single polyprotein containing all viral proteins (C, prM, E, NS1-5). NS3 and NS5 are multifunctional enzymes responsible for the viral replication (Morozova et al., 1991) (Kapoor et al., 1995). The viral replication occurs in ER-derived highly-structured membranous compartments (Welsch et al., 2009) by the replicative complex consisting of NS3, NS5, and unidentified host proteins (reviewed by (Nagy and Pogany, 2012)). A negative strand transcription starts when NS5 binds to the 5’ UTR of circularized RNA genome and then to the 3’UTR (Alvarez et al., 2008). The negative strands are transcribed into the nascent genomic RNA in a semi-conservative manner resulting in a 10:1 ratio of positive: negative RNA in the cytoplasm (Ackermann and Padmanabhan, 2001, Nomaguchi et al., 2003). Theoretically, the genomic RNA is capped and methylated co-transcriptionally.

The nascent RNA genome and capsid proteins are assembled to form a nucleocapsid. The prM and E proteins form a heterodimer. The nucleocapsid and the heterodimers are transported into the ER lumen where the immature virion is assembled. This process is not
completely understood. The nascent virion contains 180 copies of prM/E heterodimer on its surface (reviewed by (Rodenhuis-Zybert et al., 2010)). Immature virions mature during transport through the trans-golgi network (TGN), wherein the acidified environment (pH 5.8-6.0) triggers cleavage of prM, and M/E heterodimer dissociation. The prM protein is cleaved by the cellular endoprotease furin resulting in membrane associated M and secretory pr peptide. The E protein reorganizes and forms a homodimer.

1.1.4. Molecular Pathogenesis

The virus life cycle requires both mammalian and arthropod hosts. From current knowledge, dengue virus is still confined to the Aedes mosquito vector and human host whereas its neighbors, WNV, SLEV, YFV were reported with alternative vectors (Billoir et al., 2000). A DENV infection of the human host starts with an infected mosquito taking a blood meal. The virus from the saliva infects nearby immature dendritic cells (DC) via the DC-SIGN receptor. The infected DC matures while migrating to regional lymph nodes. At the lymph nodes, monocyte-macrophage lineage cells are highly susceptible to DENV, thereby amplifying the infection. Primary viremia is characterized by dissemination of the virus throughout the lymphatic and vascular system, establishing the infection in liver, lung, and spleen. Following this event, acute phase responses (prodromic symptoms) vary from asymptomatic, to acute febrile illness, to high fever with severe myalgia. Note that the more clinically severe the prodrome, the more likely severe hemorrhage will occur. Details will be discussed in subheading 1.5.
Severe manifestations occur in a secondary heterotypic DENV infection because the host immunity is driven towards the memorized primary infection. Robust, but incompetent, humoral and cell-mediated immunities play a role in this pathologic event. From several prospective cohort studies, a secondary infection is an epidemiological risk factor for severe dengue, whereas tertiary and quaternary heterotypic infections are mostly subclinical (Gibbons et al., 2007). The highest incidence of primary infection is found in 6-8 month old infants whose mother’s protective immunity is tapering off. Moreover, severe manifestation relies on host susceptibility (age, gender, ethnic group, and preexisting comorbidity) and viral factors (strain virulence).

1.1.4.1. Humoral immunity

Antibody dependent enhancement (ADE) was the first hypothesis put forth to explain the hemorrhagic disease (Halstead and O'Rourke, 1977). Overproduction of non-neutralizing immunoglobulin (Ig)G during a secondary heterotypic infection facilitates the viral entry mediated by Fc receptor. Mononuclear myeloid leukocytes become more permissive in such an environment, thus making them a major target of dengue replication (Green and Rothman, 2006, Kurane, 2007, Halstead et al., 2010, Whitehorn and Simmons, 2011, Srikiatkhachorn et al., 2012). Moreover, the level of antibody enhancement increases by several factors including the level of highly immunogenic target (prM) (Nelson et al., 2008), the number of antibodies per virion (Pierson et al., 2007), etc. Monoclonal antibody to prM was the most highly cross-reactive, non-neutralizing antibody (Dejnirattisai et al., 2010). Infants at a specific age range (6-8 months) from dengue immune mother are at risk for severe dengue since the antibody level is optimized to generate ADE (Kliks et al., 1988). Although the role of antibody enhancing the
disease severity has been extensively studied for decades, still more work remains to be done towards a complete understanding of the mechanisms.

Levels of complement components are reduced in patients with severe dengue. Excessive complement activation at endothelial cell surface was suggested. This effect alters the vascular permeability, causing plasma leakage, and finally leading to hypovolemic shock (Avirutnan et al., 2006). NS1 was recently discovered to degrade C4 to C4b, thus affecting the mannose binding lectin (MBL) activation pathway (Avirutnan et al., 2010, Shresta, 2012). Moreover, the plasma levels of NS1 and terminal complement components (C5b-9) were correlated with the disease severity (Avirutnan et al., 2006).

1.1.4.2. Cell mediated immunity

Original antigenic sin manifests not only in the antibody profile, but also in memory T-cell mediated responses (Mongkolsapaya et al., 2003). Triggered by viral peptides presented on the infected cell surface, memory T cells proliferate and produce pro-inflammatory cytokines that indirectly affect vascular endothelial cells (Mongkolsapaya et al., 2003). Correlations between the magnitude of T cell responses to DENV NS3 and severe disease is also documented (Duangchinda et al., 2010). Levels of pro-inflammatory cytokines such as IFN-γ, TNF-α, and CD107a are significantly increased in dengue hemorrhagic fever compared to dengue fever (Duangchinda et al., 2010). Besides IFN-γ and TNF-α, alteration of IL-6, IL-10 and nitric oxide (NO) levels is involved in dengue pathogenesis (Khare and Chaturvedi, 1997, Juffrie et al., 2001, Perez et al., 2004). Moreover, the relative level of cytokine can shift from a mild Th1 response to the more aggressive Th2 response resulting in severe dengue (Chaturvedi et al., 2000). However,
the actual roles of these cytokines and their interactions towards endothelial pathophysiology leading to plasma leakage are still to be elucidated.

TNF-α is one of the pro-inflammatory cytokines in acute phase reaction. It is one of the key triads observed in severe thrombocytopenic mice with hemorrhage besides high viral titer, and macrophage infiltration (Chen et al., 2007a). In the same study, high tissue level of TNF-α was also shown to correlate with endothelial cell apoptosis. Pathologically high level of TNF-α is also observed in autoimmune diseases such as rheumatoid arthritis, ankylosing spondylitis, psoriasis, and refractory asthma. TNF signaling is antagonized by histamine, the local inflammatory mediator (Wang et al., 2003).

1.1.5. **Clinical Manifestation, Diagnosis, and Treatment**

DENV infection, like most viral infections, follows the iceberg concept. The “tip of the iceberg” (symptomatic dengue and severe dengue) is a small subset of the number of exposures and asymptomatic dengue infections (Halstead, 2007). Clinical severity could range from asymptomatic to life-threatening conditions. Classical dengue manifestation consists of 3 phases; febrile, critical, and convalescent occurring 3-5 days after the mosquito bite (reviewed by (WHO, 2009, Whitehorn and Simmons, 2011)). Viremia and increasing levels of pro-inflammatory cytokines are observed at the febrile state. The fever is usually accompanied by headache and myalgia, which are mostly resolved within 4-7 days. In severe dengue, high-grade fever with severe myalgia (breakbone fever) has been documented. Clinical manifestation and disease severity differ in young children and adults. Moreover, mortality rate is significantly higher in young children than older children or adults (Anders et al., 2011) (reviewed by (Whitehorn and
Simmons, 2011). Upon the critical phase, the temperature drops rapidly along with a decreasing platelet count and increasing hematocrit. The period usually lasts 24-48 hours and the presence of warning signs will indicate the case severity. Some patients skip this phase and proceed directly to convalescence. Others have non-severe, self-limited plasma leakage. In severe cases, vascular hypoperfusion from extensive plasma leakage, profound bleeding, or multiple organ failure can lead to hypovolemic shock. Patients have to be closely monitored for a prompt treatment throughout this state. Surviving the critical period, patients’ clinical status will be stabilized in the recovery period. The hematological profile returns to normal as the fluid returns to the system. Generalized macular rash can be observed.

According to the 2009 guideline (WHO, 2009), probable symptomatic dengue infection is diagnosed by the presence of fever with at least two signs and symptoms as follows; nausea, vomiting, rash, aches and pain, tourniquet test positive, and any of the warning signs (abdominal pain and tenderness, persistent vomiting, mucosal bleeding, clinical fluid accumulation, lethargy or restlessness, liver enlargement > 2 cm, increase hematocrit with rapid decrease in platelet count). Serological confirmation is not strictly required if the case occurs at the same time and location with other confirmed dengue cases.

Severe dengue includes any of the following conditions; 1) severe plasma leakage leading to shock or respiratory distress, 2) severe bleeding (evaluated by clinicians), or 3) severe organ impairment such as liver (AST, ALT > 1000), central nervous system (impaired consciousness), heart and other organs; whereas the 1997 WHO guideline has focused only on the plasma leakage.
Currently, there is no vaccine or antiviral drug. Supportive treatment with adequate fluid management is of importance. Oral rehydration solution (ORS) can be supplemented in non-severe cases. In severe cases, warning signs and body temperature have to be frequently assessed (2-4 hours) to predict the timing and severity of the critical stage. Fluid resuscitation has to be carefully adjusted to maintain the volume in cardiovascular system without causing fluid overload. The benefit from platelet supplement is still controversial, lacking a clear evidence based support (Lye et al., 2009, Thomas et al., 2009) (reviewed by (Whitehorn and Simmons, 2011)).

1.2. Current knowledge on flaviviral drug discovery

Despite extensive attempts for decades, there is still no vaccine or drug treatment for dengue diseases. Several constructs of tetravalent and trivalent vaccines have been on trial (Durbin and Whitehead, 2010). Gaining a safe and highly immunogenic tetravalent vaccine is still a challenging quest for vaccine research. For antiviral drug development, the objective is to find a small molecule capable of inhibiting all serotypes. Developing HTS assays to screen millions of compounds in libraries is equally important to finding lead compounds. So far, HTS assays developed in flaviviral drug discovery employ various approaches, ranging from in silico structure-based, to in vitro biochemical, to in vivo cell culture assay systems (reviewed by (Noble et al., 2010)).
1.2.1. **Viral target-based approach**

The ~11 kilobase flaviviral RNA genome codes for a single polyprotein that is processed to yield 10 mature viral proteins. Of the seven nonstructural (NS) proteins, NS2B in conjunction with NS3, as well as mature NS3 and NS5, possess enzymatic functions. Targeting a flaviviral enzyme in a high throughput format is of importance in drug discovery because these enzymes are crucial and conserved in all serotypes. The small-molecule inhibitors are expected to interfere with the viral life cycle. For example, an inhibitor targeting DENV E protein fusion function was discovered and its structure activity relationship (SAR) was studied (Poh et al., 2009, Wang et al., 2009).

1.2.1.1. Protease

DENV protease is extensively studied as a drug target. The biochemical assay in the HTS format is continuously being improved (Johnston et al., 2007, Mueller et al., 2007, Mueller et al., 2008, Tomlinson and Watowich, 2012, Nitsche and Klein, 2013). Located at the N-terminal domain of NS3, the serine protease catalytic triad requires NS2B as a cofactor for protease activity in viral polyprotein processing. The NS2B/NS3pro prefers substrates containing basic amino acids (R, K) prior to the cleavage site and a small chain amino acid at the C-terminus of the cleavage site (Chambers et al., 1990, Preugschat et al., 1990), (reviewed by (Noble et al., 2010)). Several potential inhibitors have been identified by HTS using a synthetic tetrapeptide substrate attached to a 7-amino-4-methylcoumarin fluorophore (AMC). The release of AMC was measured and analyzed for calculation of kinetic parameters of the protease activity. A Bovine pancreatic trypsin inhibitor (BPTI), also known as aprotinin, is used as a positive control. The Ki of aprotinin to DENV2 protease is 26 nM (Mueller et al. 2007). Flaviviral proteases in the assay

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are constructed from hydrophilic NS2B and N-terminal NS3 domain linked together with the C-terminal residues of NS2B, QR, or non-viral, AAAAA, residues. This recombinant form is expressed and purified in soluble and enzymatically active form, thus suitable for HTS. One drawback is that the optimal condition of the *in vitro* flaviviral protease assay is nonphysiologic. To achieve the optimum activity, it requires high pH, 9.5, and 20-30% glycerol. The high pH condition could protonate particular classes of small-molecules, and the high viscosity could cause high pipetting error in a small assay volume. Positive hits identified from this method are mostly charged molecules which are poorly transported across the cell membrane. Subsequently, their nanomolar range IC$_{50}$s in the *in vitro* assays do not usually correlate well with those EC$_{50}$ obtained from the cell-based assay systems.

1.2.1.2. Helicase

The C-terminal domain of NS3 (amino acid residues 171-618) contains at least 4 enzymatic activities; nucleotide triphosphatase (NTPase), RNA triphosphatase (RTPase), RNA helicase (Takegami et al., 1995, Grassmann et al., 1999, Matusan et al., 2001)(reviewed by (Bollati et al., 2010)), and ATP independent RNA annealing activity (Gebhard et al., 2012). A flaviviral helicase assay has recently been developed for HTS using the molecular beacon-based technology (Belon and Frick, 2008) (Byrd et al., 2013). In principle, the fluorophore and its quencher are attached to each strand of the complementary oligonucleotides. A fluorescent signal increases as the helicase unwinds the duplex. However, a DENV helicase usually generates a weak signal in this assay. Improving sensitivity, specificity, and cost-effectiveness is needed for the assay to be suitable for HTS.
1.2.1.3. Methyltransferase

The N-terminal domain of flaviviral NS5 contains two methyltransferase activities (N7 and 2’O) requiring S-adedosylmethionine (SAM) as the methyl donor. A mutagenesis study revealed that the methyltransferase activity is essential for viral replication (Kroschewski et al., 2008). Small-molecular compounds inhibiting flaviviral methyltransferase are considered attractive candidates. Assays suitable for HTS have been described; for example, a scintillation proximity assay (SPA) detects $^3$H-labeled methyl group transferred from SAM to the guanylated RNA (Lim et al., 2008, Barral et al., 2013). Inhibitors, S-adenosylhomocysteine (SAH) and its derivatives, were identified using this assay. Fluorescence-based methyltransferase assays have not been successfully developed.

1.2.1.4. RNA dependent RNA polymerase

Flaviviral RdRP is located in the C-terminal domain of NS5, the most highly conserved protein across the mosquito-borne flaviviruses. Undoubtedly, RdRP is highly potential for drug development since it is essential for viral replication and encoded by all flaviviruses. In vitro RdRP assays have been developed to screen potential RdRP inhibitors (You and Padmanabhan, 1999). A fluorescence-based alkaline phosphatase coupled assay (FAPA) was recently developed for HTS (Niyomrattanakit et al., 2011). Polymerase inhibitors can be classified into 2 major groups; nucleoside analogs, and non-nucleoside analogs.

Nucleoside analogs are structurally resembled nucleoside/nucleotide substrates, in which some analogs function as chain terminators. The nucleoside analogs usually undergo structural modification by host enzymes; therefore, their efficacies are usually evaluated by cell-based assays rather than in vitro enzymatic assays. Most nucleoside analogs are synthesized and tested
in a triphosphorylated form. Moreover, in the cell-based assay, the candidate compound has to exhibit preference for the viral polymerase over host DNA-dependent RNA polymerase, without any detectable mitochondrial toxicity.

Non-nucleoside analogs usually target allosteric cavities or pockets and function as non-competitive inhibitors. Two common pockets were identified from crystal structures of DENV3 and WNV RdRp (Malet et al., 2007). A possible drawback of this approach is that the non-conserved region is highly mutable regarding from the heterogeneity of RNA viruses. The HTS assays have recently been developed; hence more potent inhibitors are expected to be identified in the near future. An example of successfully developed drugs is the NNRTIs (Non-Nucleoside Reverse Transcriptase Inhibitors) targeting RNA-dependent DNA polymerase of HIV.

1.2.1.5. NS3-NS5 binding inhibitors

NS3 and NS5 were shown to interact with each other in vivo (Kapoor et al., 1995) and in vitro (Johansson et al., 2001). Recently, using a protein-protein interaction assay (AlphaScreen) Takahashi et al. (Takahashi et al., 2012) detected a specific interaction between NS3 and NS5 with a Z-factor of 0.71. A Z-factor is used to interpret an efficacy of a high-throughput screening assay, in which the factor scoring between 0.5 and 1 indicates an excellent assay. The binding assay is available in HTS format (384-well) and ready for screening of NS3/NS5 interaction inhibitors.
1.2.2. *Host target based approach*

1.2.2.1. Host factors in the viral life cycle

Flaviviruses, like most viruses, utilize host machinery in every step of its life cycle. Blocking the role of host factors in a critical step of the virus life cycle can be useful for development of antivirals. However, cytotoxicity of the compounds targeting a host factor should be carefully evaluated since the factor may be equally crucial for the host. Significant host factors that are important for the flavivirus life cycle include host cell-specific receptors for entry, host proteases (furin and signal peptidase) (Stadler et al., 1997, Elshuber et al., 2003), glucosidase (Courageot et al., 2000), kinases (Hirsch et al., 2005, Chu and Yang, 2007), and host factors involved in cholesterol biosynthesis, assembly and egress (Stiasny et al., 2003, Hirsch et al., 2005, Lee et al., 2008, Rothwell et al., 2009) (reviewed by (Noble et al., 2010, Pastorino et al., 2010)).

An HTS assay is available for furin protease and several furin inhibitors have been identified. Furin catalyzes prM→M cleavage on the surface of the virion’s host-derived membrane in the low pH environment of the Trans-Golgi Network, triggering E protein rearrangement and virion maturation (described in 1.3.). However, a risk-benefit analysis of furin inhibition in flaviviral infection has not been conducted.

1.2.2.2. Host factors involved in severe clinical manifestations

As described in 1.4., several factors including pathologic immunoglobulin G and proinflammatory cytokines are involved in severe dengue. Fc modified antibody was proven effective in severe dengue prophylaxis and therapy in an animal model (Balsitis et al., 2010); whereas monoclonal antibodies targeting TNF-α prevented vascular leakage (Shresta et al.,
These proinflammatory cytokines are potential targets for development of small molecular inhibitors.

1.2.3. Structure based approach

High-resolution atomic structures of several DENV proteins, especially those that are viral enzymes, have been solved over the past decade, providing a protein database for computational analysis of protein-ligand binding (Murthy et al., 1999, Murthy et al., 2000, Egloff et al., 2002, Benarroch et al., 2004, Xu et al., 2005, Erbel et al., 2006, Egloff et al., 2007, Yap et al., 2007, Luo et al., 2008) (reviewed by (Tomlinson et al., 2009)). The three dimensional structures of proteins are essentially determined using X-ray crystallography, and to a lesser extent using cryo-EM and NMR spectroscopy. Computational approaches using the crystal structure coordinates of a target are useful for virtual screening that can be performed in an HTS format against millions of compounds available in large chemical libraries such as ZINC and PubChem databases.

1.2.3.1. Virtual screening

Virtual screening systematically evaluates each compound (or pharmacophore) interaction with each cavity of a macromolecular target, so called docking program (Jones et al., 1997, Campbell et al., 2003, Kitchen et al., 2004, Cummings et al., 2005, Mohan et al., 2005). Virtual screening requires a 3-dimensional compound library, a 3-dimensional structure of a target macromolecule (usually a protein), a defined region (cavity or pocket) on the target surface to be examined, and a docking program. Until now, many docking programs have been developed and some of them are commercially available (Morris et al., 1996, Jones et al., 1997,
Protein-ligand binding affinity is achieved by calculation and scoring all intermolecular interactions between the molecules. In order to perform virtual HTS with thousands to millions of 3D compounds in the library, a supercomputing resource is required. Identification of lead compounds can be further validated with other experimental methods such as biochemical and cell-based assays. In current virtual screening, the target is considered as ‘rigid’ and the conformational changes arising from induced-fit mechanisms of ligand binding to the target are not being examined.

1.2.3.2. Compound database

A compound database is a virtual library of 3D structures of small molecules determined by X-ray crystallography. An example of a large, publicly available compound database with cross-references is PubChem from NIH. Small molecules that are ‘drug-like’ usually fall into Lipinski’s rule of 5 (molecular weight < 500 Da, log P < 5, H-bond donor < 5, and H-bond acceptor < 10) determining proper solubility and permeability (Lipinski et al., 2001). Results from HTSs lead to structure activity relationship (SAR) analysis, which subsequently reveals potential lead compounds.

A fragment database is a library of chemical functional groups or very low molecular weight compounds (< 300 Da) containing less than 5,000 compounds (Leach et al., 2006, Hesterkamp and Whittaker, 2008). A fragment is mainly used to determine a manner of interactions in the subset of target protein binding site. Data from the fragment-based approach is
utilized to generate potential compounds with strong binding moieties. For lead optimization, the fragments can be screened with X-ray crystallography, or NMR spectroscopy (Mooij et al., 2006, Antonysamy et al., 2008) (reviewed by (van de Waterbeemd and Gifford, 2003, Jhoti et al., 2007, Tomlinson et al., 2009, Noble et al., 2010)).

1.2.3.3. Drug target

Biophysical techniques to determine the protein-ligand structures are X-ray crystallography and NMR spectroscopy. X-ray crystallography provides atomic positions as a framework, whereas NMR reveals the possible conformations from molecular rotations. Ligands are either co-crystallized or soaked in pre-formed protein crystals before solving the structures. Recently, HTS crystallization was done with 100 nl of compound-protein mixture per drop and crystals were analyzed by synchrotron radiation (reviewed by (Noble et al., 2010)).

1.2.4. Cell based approach

Cell-based assays have a role in confirming the positive hits identified by other methods or identifying new hits from primary screens. Classical assays (e.g., plaque reduction assay) usually are labor-intensive and time consuming, and not applicable for HTS. Attempts to develop high throughput assays are facilitated by advances in molecular biology techniques to generate infectious cDNA clones and cDNAs of self-replicating reporters expressing replicon RNAs (Puig-Basagoiti et al., 2005, Green et al., 2008, Alcaraz-Estrada et al., 2010, Manzano et al., 2011, Alcaraz-Estrada et al., 2013). Cell-based approaches cover multiple steps of the viral life cycle and are therefore a valuable part of drug discovery efforts for antivirals. One of the disadvantages of the cell-based assay is that the target is unknown and it could be either cellular
or viral. Identifying the target of a small molecule compound could become a major effort. One of the advantages of using cell-based approaches for screening is that compounds identified as inhibitors of the virus life cycle have already demonstrated membrane permeability and stability during the incubation period. A number of cell-based assays have been developed for antiviral screens in high-throughput format.

1.2.4.1. Live virus assay

Dengue virus infection of mammalian cells causes a cytopathic effect (CPE) and compounds inhibiting CPE could be scored as potential hits. An assay was developed to detect inhibition of CPE using DENV infected Huh-7 cells (Green et al., 2008). In principle, an inhibitor would impair at least one step of the viral infection cycle, reduce virulence, and thereby prolong cell survival. Instead of counting plaques to measure CPE, ATP levels from the surviving cells were measured using a commercial kit (CellTiterGlo from Promega) in a high throughput format. Designed as a rapid counter screen, highly cytotoxic or false positive compounds will be detected and eliminated from the pipeline.

1.2.4.2. Artificial system mimicking viral infection

In West Nile virus drug discovery, a luciferase-encoding gene was genetically engineered into cDNA clones of a WNV subgenomic replicon. Puig-Basagoiti et al. (Puig-Basagoiti et al., 2005) designed three high throughput assays with a luciferase reporting system for screening of WNV inhibitors.

First, a subgenomic replicon (Khromykh and Westaway, 1997) (i.e. cDNA encoding nonstructural proteins NS1-5) fused in-frame with a luciferase gene, as well as a selectable marker gene (e.g. neomycin resistant gene), was constructed for screening of replication
inhibitors (Shi et al., 2002, Ng et al., 2007, Mosimann et al., 2010). The replicon was stably expressed in Vero cells in the presence of the neomycin analog G418. The stable replicon expressing cells were used to screen 96,958 compounds from the commercial libraries collected at NSRB; Harvard medical school (Puig-Basagoiti et al., 2009). Positive hits were predicted to interfere with the viral replication either through inhibiting viral factors or host factors.

A second assay used a virus-like particle (VLP) containing subgenomic replicon RNA encoding a reporter such as GFP or luciferase gene. In principle, VLPs are made by co-transfection of cells with a subgenomic replicon and a plasmid encoding all structural genes. The VLPs are collected by harvesting the supernatant. Subgenomic RNA is delivered into mammalian (or insect) cells by infection of cells with VLPs. The infection is validated by an indirect immunofluorescent assay (IFA) using an antibody against a nonstructural protein such as NS1. A positive signal reflects the replication of replicon RNA. The infection proceeds only one round starting with the virus entry through translation and viral RNA replication (Khromykh et al., 1998, Pierson et al., 2006). However, the assembly, release, or cell-to-cell spread will not happen. The VLP assay was validated against known positive inhibitors (Puig-Basagoiti et al., 2005, Mueller et al., 2008) and was demonstrated for HTS (Qing et al., 2010).

Third, an assay using an infectious clone (cDNA of the genome) fused with a luciferase encoding gene was used for screening multiple steps in the virus life cycle including entry, replication, and assembly. The genetically engineered virus containing cap independent translation of luciferase encoding gene required BSL-2 containment for DENV and BSL-3 containment for WNV.
1.3. Scope of the thesis

Dr. Padmanabhan’s laboratory has recently established DENV2, DENV4, and WNV replicons stably maintained in BHK-21, Vero, and Vero cells, respectively for HTS. A number of potential inhibitors have been identified and confirmed using this approach. The constructs in plasmid pRS424 are shown in Fig. 1.1 (Reichert, unpublished) (Alcaraz-Estrada et al., 2010, Alcaraz-Estrada et al., 2013). Briefly, structural genes were replaced with the Renilla luciferase reporter gene (Rluc), the antibiotic resistant gene (Neo'), and the encephalomyocarditis virus internal ribosome entry site (EMCV IRES) element. Rluc and Neo' are expressed by cap-dependent translation, whereas nonstructural proteins (NS1-5) are produced by cap-independent translation directed by the IRES element.

Positive hits were identified from in vitro protease HTS of 32,337 compounds (Mueller et al., 2008). Compounds containing a 8-hydroxyquinoline core were identified as protease inhibitors (Mueller et al., 2008, Lai et al., 2013). We sought to establish a structure activity relationship by analysis of several quinolone derivatives in my thesis project.

The rationale for analysis of other quinoline derivatives was that several known antimalarial drugs contain a quinoline scaffold in their core structures. Quinine, the first antimalarial drug, was discovered in 1908 from the bark extracts of a cinchona tree (reviewed by (O’Neill et al., 1998)). In 1920-40, the 8-aminoquinoline (pamaquine), 7-chloroquinoline (chloroquine), 6-methoxyquinoline (primaquine), and others were synthesized and tested as antimalarial drugs. Chloroquine had a good activity/toxicity profile so it was used as a major chemotherapeutic for malaria eradication campaigns in the 1960s. Structural modification of 7-chloroquinoline was extensively studied after chloroquine-resistant strains of Plasmodium
*falciparum* emerged. Chloroquine and its derivatives kill asexual stages of the malarial parasite in the erythrocyte by at least 3 mechanisms; increasing intracellular pH, inhibiting heme polymerization, and inhibiting DNA and RNA synthesis (reviewed by (O'Neill et al., 1998)). Besides antimalarial activity, chloroquine is an immunomodulatory agent used in treating rheumatoid arthritis, ankylosing spondylitis, psoriasis, etc. (Goldman et al., 2000, Weber and Levitz, 2000, Wozniacka et al., 2008) (reviewed by (Lee et al., 2011, Ben-Zvi et al., 2012); as well as an antiviral agent inhibiting HIV replication (Savarino et al., 2004).

Aims of this dissertation are as follows;

Aim 1. Screen quinoline antimalarial drugs and derivatives to find potential inhibitor(s) of flaviviral replication.

Aim 2. Establish inhibitory effects of AQ (the positive hit) against DENV2 replication and infectivity by various methods

Aim 3. Study mode of action and target identification by *in vitro* and cell based assays

Aim 4. Correlate structure-activity relationships from currently available compounds acquired from National Cancer Institute/Developmental Therapeutics program (NCI/DTP)
Fig. 1.1 Constructs of plasmid pRS424 containing flaviviral replicons.

Sources:

DENV2 from Reichert’s dissertation 2005.

DENV4 from (Alcaraz-Estrada et al., 2010)

WNV from (Alcaraz-Estrada et al., 2013)
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Compounds

Quinoline compounds used in the primary screening were obtained from National Cancer Institute/Developmental Therapeutics program (NCI/DTP) in 10 mg quantities (Table 3.1). Amodiaquine dihydrochloride dihydrate (AQ) was obtained from Sigma Aldrich (St. Louis, MO). Derivatives of 8-hydroxyquinolines were obtained from Dr. Kuppuswamy Nagarajan (Alkem Laboratories, Inc., Bangalore, India) (Table 3.2). Moreover, chloroquine derivatives were obtained from Prof. Christian Wolf (Georgetown University, Washington, DC) (Table 3.3). Dimethyl sulfoxide (BP231-100 ml) was purchased from Fisher Scientific (Pittsburgh, PA). All chemical structures were drawn using ChemBioDraw Ultra 11 (trial version).

2.1.2 Cells and Virus

The BHK-21 cell line (BHK-21/DENV2) stably expressing a subgenomic DENV2 replicon with a Renilla luciferase reporter (Rluc) was established by Dr. Erin Reichert (Georgetown University, Washington, DC) (Reichert, unpublished). Stable subgenomic DENV4 replicon expressing Vero cells (Vero/DENV4) and WNV replicon expressing Vero cells (Vero/WNV) with a Renilla luciferase reporter (Rluc) were established by Dr. Sofía L. Alcaraz-Estrada (Division de Medicina Genomica, Centro Medico Nacional-ISSSTE, Mexico, DF, Mexico) (Alcaraz-Estrada et al., 2010, Alcaraz-Estrada et al., 2013). Baby hamster kidney cells (BHK-21) of passage 53 and Vero cells of passage 93 were originally purchased from ATCC.
DENV2 New Guinea C strain was obtained from Walter Reed Army Institute of Research (WRAIR), propagated and stored in aliquots by Dr. Ratree Takhampunya (Mahidol University, Bangkok, Thailand).

2.1.3 Mammalian cell culture media and reagents

Dulbecco’s Modified Eagle Medium (DMEM), Minimal Essential Media (MEM), 10,000 U/ml penicillin and 10,000 μg/ml streptomycin (100 X solution of 100 I.U./ml penicillin and 100 μg/ml streptomycin), fetal bovine serum (FBS), and nonessential amino acids were purchased from Mediatech, Inc. (Manassas, VA). G-418 powder was purchased from Fisher Scientific (Pittsburgh, PA). Cell culture dishes and plates were purchased from Greiner Bio-One (Monroe, NC).

2.1.4 Replicon assay reagents and equipment

The 96-well μClear black microtiter plates were purchased from Greiner Bio-One. The Renilla luciferase assay system was purchased from Promega (Madison, WI). Luciferase activity was measured in Centro LB 960 luminometer from Berthold technologies (Oak Ridge, TN).

2.1.5 Cytotoxicity assay reagents and equipment

The 96-well μClear black microtiter plates were purchased from Greiner Bio-One (Monroe, NC). Cell Counting Kit-8, which uses the WST-8 tetrazolium salt for determination of cell viability, was purchased from Dojindo Molecular Technologies (Rockville, MD). The colorimetric signal was read in a Concert TRIAD plate reader from Dynex (Chantilly, VA).
CellTiter-Glo Luminescence cell viability kit was purchased from Promega (Madison, WI). Centro LB 960 luminometer was from Berthold technologies (Oak Ridge, TN).

2.1.6 qRT-PCR reagents and equipment

TRIzol Reagent was purchased from Invitrogen (Grand Island, NY). QIAamp Viral RNA minikit was purchased from QIAGen (Valencia, CA). Amicon-0.5 was purchased from Millipore (Billerica, MA). iScript cDNA Synthesis Kit iQ SYBR Green Supermix and Bio-Rad iQ5 Multicolor Real-Time PCR detection system were from Bio-Rad (Hercules, CA). Forward and reverse primers amplifying DENV2 NS1 gene fragment were 5’-CTCGACTCAAAACTCATGTCAG-3’ and 5’-GGCTTTCTCTATCTTCCATGTCG3’, respectively. Forward and reverse primers amplifying glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene fragment were 5’-AACTCCCTCAAGATTGTCAGC-3’ and 5’-TGAGTCCCTCCACAATGCC-3’, respectively. All primers were purchased from Integrated DNA Technologies (Coralville, IA).

2.1.7 Reagents for plaque assay

Formaldehyde (37% by weight), isoproponal (99.9%), and Crystal Violet (powder) were purchased from Fisher Scientific (Pittsburgh, PA).

2.1.8 In vitro protease assay reagents and equipment

The 96-well half area black plates were purchased from Greiner Bio-One (Monroe, NC). Fluorogenic tetrapeptide substrate, Bz-Nle-Lys-Arg-Arg-AMC, was purchased from Bachem.
(Torrance, CA). The fluorescence plate reader, SpectraMax Gemini EM, was from Molecular Devices (Sunnyvale, CA). The DENV2 NS2B-NS3 expression plasmid encoding the protease precursor (DENV2 NS2BH-(QR) NS3pro) containing the hydrophilic domain of NS2B cofactor (48 amino acids) and the protease domain of NS3 protein (185 amino acids) was constructed by Dr. Tadahisa Teramoto (Yon et al., 2005). Talon Metal Affinity resin was purchased from Clontech (Mountain View, CA).

2.1.9 In vitro MTase assay reagents and equipment

The DENV2 RNA_{nt-200} template forward and reverse primers (5’-TAATACGACTCACTATAGTTGTTAGTCTACGTGGAC-3’ and 5’-TTATTCATCAGAGATCT-3’, respectively) were purchased from Integrated DNA Technologies (Coralville, IA). The pSY2 template was constructed by Dr. Shihyou You (You and Padmanabhan, 1999). MEGAshortscript T7 RNA polymerase was purchased from Ambion/Life Technologies (Grand Island, NY). ScriptCap m7G capping system was purchased from CellScript (Madison, WI). The [$\alpha^{32}$P]GTP isotope was purchased from Perkin-Elmer (Waltham, MA). Micro Bio-Spin 30 columns were purchased from Bio-Rad (Hercules, CA). RNA clean and concentrators (TM-5, and TM-25) were purchased from Zymo research (Irvine, CA). Nuclease P1 and cellulose PEI plates were purchased from Sigma Aldrich (St Louis, MO).

The DENV2 NS5_{FL} expression plasmid containing a small ubiquitin-like modifier 1 (35 amino acids) at N-terminus was a gift from Dr. Craig Cameron (Penn State University, State college, PA). SUMO protease or Ubl specific protease 1 was purchased from LifeSensors (Malvern, PA). HiPrep 16/60 Sephacryl S-100 HR column chromatography was purchased from
GE Healthcare Biosciences (Piscataway, NJ) and AKTAPrime Plus FPLC system was from Amersham Biosciences (Sunnyvale, CA).

2.1.10. **In vitro RdRP assay reagents and equipment.**

The assay was performed by Mark Manzano. Briefly, the positive sense RNA substrate was transcribed *in vitro* from pSY2 mini genome (719 nucleotides) RNA template (You and Padmanabhan, 1999). Negative sense DNA template was generated by PCR amplification of the pSY2 template with 5’ – AGTTGTTAGTCTACGTGGACC - 3’ and 5’ – TAATACGACTCATAAGGGAACCTGTTGATTCAACACGACCATTCCATTTCCTG G - 3’ as forward and reverse primers respectively; then, the negative sense RNA was transcribed *in vitro*. The RdRp assay buffer was prepared as a 5 X stock (250 mM Tris HCl, pH 8, 250 mM NaCl, 25 mM MgCl₂) and stored at ambient temperature. The assays were performed using DENV2 NS5FL enzyme as described (subheading 2.2.14) (Ackermann and Padmanabhan, 2001, Nomaguchi et al., 2003). AQ stock solution (50 μM) was prepared in DMSO.

2.1.11 **Analytical software**

Statistical analysis and 2D graphs were done using GraphPad Prism v5 (La Jolla, CA). Image J (http://rsbweb.nih.gov/ij/) was used for quantification of radioactive signal from phosphoimager screen (Amersham Bioscience, Sunnyvale, CA). Before quantification, pictures derived from the screen were processed using ImageJ program with the following steps.

1) Transform to 32-bit format using this command; (Image>Type>32-bit).
2) Optimize the signal using these commands; (Process>Math>square, and Process>Math>divided by 21,427, respectively).

3) Adjust the brightness and contrast using this command; (Image>Adjust>Brightness/Contrast).

2.2. Methods

2.2.1. Replicon inhibition assays for HTS

All compounds were dissolved in DMSO to prepare 50 mM stock solutions and stored in amber glass vials at -20°C. Replicon expressing cell lines (BHK-21/DENV2, Vero/DENV4, and Vero/WNV) were maintained in DMEM supplemented with 10% FBS, 100 I.U/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml G418.

Cells (~10⁴ in 100 μl) were seeded into each well of a 96-well plate containing DMEM supplemented with 10% FBS. Cells were incubated for 6 h at 37 °C in a humidified incubator under 5% CO₂ before addition of compounds to 50 μM final concentration. DMSO (1%) alone was used as the no-inhibitor control (100% Rluc activity or 0% inhibition). All compounds were assayed in triplicate. Following compound addition, cells were incubated at 37 °C in a humidified incubator under 5% CO₂ for 24 hours prior to lysis and measurement of Rluc activities. Medium was removed and cells were washed with PBS. Cells were lysed using 20 μl/well of lysis buffer from the Renilla luciferase assay kit and rocking for at least 40 min. The substrate was added and the Rluc activities were measured using a luminometer. Data was reported as percent inhibition (% inhibition = 100 - % activity) relative to DMSO (0%
inhibition). Mycophenolic acid (MPA) was used as a positive control referring to 100% inhibition.

The quinoline compounds from NCI/DTP and from Dr. K. Nagarajan’s collection (Alkem Laboratories, Inc., Bangalore, India), were screened at 50 μM final concentration. The chloroquine derivatives from Dr. Christian Wolf were assayed at 25 μM final concentration. Cytotoxicity was measured in parallel with the Rluc activity measurement.

2.2.2. Replicon inhibition assays for EC$_{50}$ measurement

Selected compounds were further analyzed to determine the concentration of each compound causing 50% inhibition of replication (EC$_{50}$). Compounds were serially diluted to final concentrations of $10^{-4} - 10^{-9}$ molar in the initial trial. Cells were plated, incubated with a compound, and assayed for Rluc activity as described in the screening section. The % activity values at various concentrations of the compound were analyzed by nonlinear regression using GraphPad Prism 5. Each concentration was tested in triplicate and the results were confirmed by two independent experiments.

In addition, AQ at final concentrations of 0, 0.01, 0.1, 1, 2.5, 5, 7.5, 10, 20, 30, 40, 50, 60, 70, 80, or 100 μM in DMSO concentration of 1% were added to BHK-21/DENV2, Vero/DENV4, and Vero/WNV cells (~$10^4$ in 100 μl). Cells were incubated at 37 °C for 48 h in a humidified incubator under 5% CO$_2$. Cells were washed, lysed, and Rluc activities from the replicons were measured according to manufacturer’s protocol. Each concentration was tested in triplicate and the results were confirmed by two independent experiments.
2.2.3. Cytotoxicity assays

In parallel to replicon assays, cell viability was assessed in order to exclude false positives arising from cytotoxicity of the compounds. The concentration at which the cell viability was reduced by 50% (CC$_{50}$) was determined for each compound. CC$_{50}$ values of NCI/DTP and KN compounds were determined using the same concentrations used in determining EC$_{50}$ values. Naïve BHK-21 and Vero cells (~$10^4$ in 100 μl) were seeded into each well of a 96-well plate and incubated for 6 h before compound addition. Each compound was assayed in triplicate and cells were incubated for 24 h before measuring the ATP level using CellTiter-Glo® luminescent cell viability assay kit. Data were plotted and the CC$_{50}$ values were calculated by nonlinear regression analysis. Results were confirmed by two independent experiments.

For chloroquine derivatives, cytotoxicity was measured simultaneously rather than in parallel. Before Rluc readout in replicon inhibition screening, highly soluble tetrazolium salt (WST-8 in CCK-8 cell viability assay kit) was introduced into the experimental cultures. After 2 h incubation at 37 °C under 5% CO$_2$, absorbance was read at 485 nm. Cells were subsequently washed, lysed, and Rluc activities were measured. Data were plotted and the CC$_{50}$ values were calculated by nonlinear regression analysis. Each drug concentration was tested in triplicate and the results were confirmed by two independent experiments. Therapeutic index (TI) was calculated as the ratio of CC$_{50}$/EC$_{50}$. 
2.2.4. Inhibition of Rluc reporter activity by AQ

To exclude the possibility that AQ inhibits Rluc activity directly, BHK-21/DENV2 cells (~10^4 in 100 μl) were plated into each well of a 96-well μClear black microtiter plate and incubated for 24 h at 37 °C. AQ at the final concentrations of 0, 0.1, 1, 2.5, 5, 10, 25, or 100 μM in DMSO concentration of 1% were added during cell lysis. The substrate was added and the Rluc activities were measured according to manufacturer’s protocol.

2.2.5. qRT-PCR quantification of BHK-21/DENV2 replication inhibition by AQ

BHK-21/DENV2 cells (~2.5 x 10^5 in 2.5 ml) were seeded into a 6-well plate and incubated overnight at 37 °C under 5% CO₂ in a humidified chamber. AQ, dissolved in DMSO, was added to final concentrations of 0, 0.1, 0.5, 1, 2.5, 5, 10, or 25 μM and the cultures were incubated for an additional 48 h. Total RNAs were extracted from the cells by treatment with TRIzol reagent according to the manufacturer’s protocol. Sample RNA concentration was calculated based on the absorbance at 260 nm and adjusted to 1 μg/μl. Quantitative RT-PCR (qRT-PCR) was performed as previously described (Manzano et al., 2011). Briefly, the region of viral RNA encoding DENV2 NS1 and the housekeeping reference gene GAPDH were copied by reverse transcriptase and amplified by quantitative PCR. The ratio (r) of NS1 to GAPDH calculated from the threshold cycle (Ct) values with the formula: Ratio = \[ \frac{2^{-(viral\ Ct\ sample-viral\ Ct\ DMSO)}}{2^{-(house\ Ct\ sample-house\ Ct\ DMSO)}} \] (Pfaffl, 2001). Each sample was tested in triplicate and the results were confirmed by two independent experiments.
2.2.6. Inhibition of DENV2 infectivity

BH2K-21 cells were cultured in MEM, supplemented with 10% FBS, 100 I.U./ml penicillin and 100 μg/ml streptomycin in MEM supplemented with 2% FBS and 100 I.U./ml penicillin and 100 μg/ml streptomycin for 1 at 37 °C under 5% CO₂. BHK-21 cells were seeded into 12-well plate and incubated overnight at 37 °C under 5% CO₂ in a humidified chamber. Cells were infected with DENV2 (New Guinea C strain) at a multiplicity of infection (MOI) of 1 with gentle rocking every 15 min. After infection, cells were washed with PBS and incubated with 1.5 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 μg/ml streptomycin. AQ at the final concentrations of 0.1, 0.5, 1, 2.5, 5, or 10 μM in DMSO concentration of 1%, or DMSO alone, was added during infection and to the maintenance medium (MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 μg/ml streptomycin). Cells were then incubated for 72 h at 37 °C. Supernatants were collected and DENV2 infectivity was analyzed by plaque assay. Data were plotted and the EC₉₀ values were calculated by nonlinear regression analysis. Each AQ concentration was tested in duplicate and the results were confirmed by two independent experiments.

Moreover, in structure-activity relationship study, CQ and AQD8 at the at the final concentrations of 0.1, 0.5, 1, 2.5, 5, 10, 25, or 50 μM in DMSO concentration of 1% were added onto DENV2 infected BHK-21 cells (MOI of 1) during infection and post-infection. Cells were incubated and supernatants were collected for analysis as described. EC₉₀ values were calculated by nonlinear regression analysis. Data was plotted in comparison to that of AQ. Each drug concentration was tested in duplicate and the results were confirmed by two independent experiments.
In addition, we performed AQ inhibition to DENV2 infectivity using a different viral load. Briefly, BHK-21 cells were infected with DENV2 (New Guinea C strain) at a multiplicity of infection (MOI) of 0.01. AQ at the final concentrations of 0, 0.01, 0.1, 0.5, 0.75, 1, 2.5, 5, 7.5, 10, or 25 μM in DMSO concentration of 1% was added during infection and to the maintenance medium. Cells were then incubated at 37 °C and supernatants were collected at 48, 72, and 96 h post-infection. DENV2 infectivity was analyzed by plaque assay. Data were plotted and the EC$_{90}$ values were calculated by nonlinear regression analysis from supernatants collected at 96 h. Each AQ concentration was tested in duplicate and was confirmed by two independent experiments.

2.2.7. Confirmation of AQ inhibition on DENV2 replication and infectivity

BH K-21 cells (10$^5$ cells in 1 ml) were seeded into a 12-well plate and incubated overnight at 37 °C under 5% CO$_2$ in a humidified chamber. Cells were infected with DENV2 (New Guinea C strain) at a multiplicity of infection (MOI) of 1 in MEM supplemented with 2% FBS and 100 I.U./ml penicillin and 100 μg/ml streptomycin for 1 h at 37 °C under 5% CO$_2$ with gentle rocking every 15 min. After infection, cells were washed with PBS and incubated with 1.5 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 μg/ml streptomycin. AQ (5 μM in DMSO concentration of 1%), or DMSO alone (concentration of 1%), was added during infection and post-infection to the maintenance medium (MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 μg/ml streptomycin). Cells were then incubated for 72 h at 37 °C in a humidified chamber under 5% CO$_2$. Intracellular RNAs were extracted from the infected cells by treatment with TRIzol reagent according to manufacturer’s protocol. Total RNAs were quantified by spectroscopy (Nanodrop 1000, Thermo Fisher Scientific, Waltham, MA) and
adjusted to 1 µg/µl for reverse transcription using the iScript cDNA synthesis protocol, (Bio-Rad). The qRT-PCR was performed as previously described (subheading 2.2.5). The relative amount of DENV2 NS1 RNA from AQ (5 µM in DMSO concentration of 1%) treated cells was based upon normalization to that of untreated cells (DMSO concentration of 1%). Supernatants from the experiment were also collected and stored in aliquots (1 ml) at -70 °C. Supernatants were concentrated by Amicon-15 (Millipore). Viral RNAs were extracted using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA) and reverse transcribed using the iScript cDNA synthesis kit according to the manufacturer’s protocol. Extracellular viral RNA copies were measured by quantification of NS1 gene by qRT-PCR. Moreover, DENV2 infectivity in the presence and absence of AQ was analyzed by plaque assay. Results were reported as the percentage of infectivity normalized by the DMSO treated group. Each set of samples was assayed in duplicate and the results were confirmed by three independent experiments. Differences between the AQ treated group and the DMSO treated group derived from each of the three approaches were evaluated by paired t-test, two tailed.

2.2.8. Plaque assay

BHK-21 cells (~10^5 cells in 1 ml, or ~5 x 10^4 cells in 0.5 ml) were seeded into 12-well or 24 well plates, respectively. Cells were incubated at 37 °C under 5% CO₂ in a humidified chamber until reaching 90% confluency. Cells were infected with previously collected supernatants for 1 h at 37 °C under 5% CO₂, and rocking gently every 15 min. Control samples were with 100% infectivity derived from DENV2-infected cells treated with DMSO concentration of 1%; whereas, the samples with 0% infectivity derived from non-infected cells.
Cells were washed with PBS, and maintained with 1.5 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 µg/ml streptomycin, and 1% methylcellulose. The plates were incubated for 3-4 days at 37 °C under 5% CO₂. After plaques became visually apparent by microscopy, cells were fixed with 11.1% formaldehyde, 4.75% isopropanol, and stained with 1% crystal violet for 30 min. Plaques were counted and the values of plaque forming units (PFU) per ml were determined. Each sample was tested in duplicate and the results were confirmed by two independent experiments.

2.2.9. Time-course analysis of AQ inhibition of DENV2 infectivity

The protocol was adapted from Stahla-Beek et al. (Stahla-Beek et al., 2012). Briefly, BHK-21 cells were cultured in MEM, supplemented with 10% FBS, 100 I.U./ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified chamber under 5% CO₂. Cells were seeded at ~2.5 x 10⁵ cells in 2.5 ml into a 6-well plate and incubated overnight at 37 °C. Cells were infected with DENV2 at an MOI of 0.01 in MEM supplemented with 2% FBS and 100 I.U./ml penicillin and 100 µg/ml streptomycin for 1 hour at 37 °C under 5% CO₂ by gentle rocking every 15 min. After infection, cells were washed with PBS. Cells were incubated with 3 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 µg/ml streptomycin. AQ at a final concentrations of 0, 1, 5, 10, or 25 µM in DMSO (1% final concentration) were added during adsorption and post-infection. Supernatants were sampled at 4, 12, 24, 36, 48, 72, and 96 h post-infection and stored in aliquots at -70 °C for plaque assays. Each sample was tested in duplicate and the results were confirmed by two independent experiments.
2.2.10 Measurement of AQ inhibition of DENV2 infectivity by direct plaque assay

Instead of determining PFU in the supernatants from the drug-treated BHK-21 cells as described above, the virus titers were determined directly in the infected and drug-treated cell monolayers and controls as follows: BHK-21 cells (~10^5 cells in 1 ml) were seeded into a 12-well plate and incubated at 37 °C under 5% CO_2 until reaching 90% confluence. AQ at the final concentrations of 0, 1, 5, 10, or 25 µM in DMSO concentration of 1% were added to DENV2 (MOI of 0.01) during adsorption for 1 h at 37 °C with gentle rocking every 15 min. Cells were washed with PBS and maintained with overlay medium (MEM supplemented with 2% FBS, 100 I.U/ml penicillin and 100 µg/ml streptomycin, and 1% methylcellulose). AQ was not present in the overlay medium post-infection. Cells were incubated for 3-4 days at 37 °C under 5% CO_2 in a humidified chamber. Cells were fixed and stained as previously described. The assay was done in duplicate and the results were confirmed by two independent experiments.

2.2.11 Order of addition assay

The protocol was adapted from Schmidt et al. (Schmidt et al., 2012). BHK-21 cells (~10^5 cells in 1 ml) were seeded into a 12-well plate and incubated at 37 °C under 5% CO_2 in a humidified chamber overnight. AQ (5 µM in DMSO concentration of 1%) was added to DENV2 (MOI of 1) diluted in growth medium for 15 min at 37 °C before absorption (pre-incubation), during absorption (co-infection), and after absorption, wash, and medium replacement (post-infection). The adsorption was done by incubating BHK-21 monolayer with the virus for 1 h at 37 °C with gentle rocking every 15 min. Then, cells were washed with PBS and maintained in MEM supplemented with 2% FBS, 100 I.U/ml penicillin and 100 µg/ml streptomycin. The result
from samples with virus in the medium containing DMSO alone (concentration of 1%) was taken as 100% infectivity. Supernatants were collected at 24, 48, and 72 h post-infection for plaque assay. Each sample was tested in duplicate and the results were confirmed by two independent experiments.

2.2.12 Time of addition assays

The protocol was adapted from Wang et al. (Wang et al., 2011). We designed the experiment to study the viral replication. Briefly, BHK-21 cells (~10^5 cells in 1 ml) were seeded into a 12-well plate and incubated at 37 °C under 5% CO₂ in a humidified chamber overnight. Cells were infected with DENV2 (MOI of 1) as described above. AQ (5 µM in DMSO concentration of 1%) or DMSO alone (concentration of 1%) was added to DENV2 (MOI of 1) at 1, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, or 48 h post-infection. Supernatants were collected at 72 h post-infection for plaque assay. Supernatants and pellets were collected for analysis by qRT-PCR as previously described. Each sample was tested in duplicate and the results were confirmed by two independent experiments.

In addition, we performed a time of addition assay with modification to study the viral translation focusing on the first 12 h post-infection period. Briefly, AQ (5 µM in DMSO concentration of 1%) or DMSO alone (concentration of 1%) was added to DENV2 (MOI of 1) at 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, or 48 h post-infection. Supernatants were collected at 48 h post-infection for plaque assay.
2.2.13 In vitro protease assay

Assays were performed in triplicate in a 96-well half area black plate (Greiner Bio-One). The reaction mixture (100 μl) contained 200 mM Tris-HCl, pH 9.5, 30% glycerol, 0.1% CHAPS, 1% DMSO, 50 nM DENV2 NS2BH-(QR)-NS3pro enzyme (Yon et al., 2005), 10 μM fluorogenic tetrapeptide substrate, Bz-Nle-Lys-Arg-Arg-AMC, and 0, 7.81, 15.63, 31.25, 62.5, 125, 250, or 500 μM AQ in DMSO (concentration of 1%). The compound-enzyme mixture was pre-incubated for 15 min at room temperature before addition of the substrate. The reaction was continued at 37 °C for 30 min. The release of AMC from the substrate was recorded every 1.5 min at 380 nm excitation and 460 nm emission in a SpectraMax Gemini EM spectrofluorometer (Molecular Devices, Sunnyvale, CA). DMSO alone (concentration of 1%) was used as the no-inhibitor control (100% protease activity) and the bovine pancreatic trypsin inhibitor (BPTI, also known as aprotinin), which has a Ki of 26 nM against the DENV2 protease, was used at 5 μM in DMSO concentration of 1% as a positive control (0% protease activity). Data were plotted and the IC_{50} value was calculated by nonlinear regression analysis using a GraphPad Prism software v5.

2.2.14 In vitro MTase assay

RNA_{nt1-200} substrate for MTase was derived from the PCR fragment amplified from pSY2 plasmid template (You and Padmanabhan, 1999) using forward and reverse primers as described in Materials section. The forward primer included the T7 promoter. The RNA substrate was synthesized by in vitro transcription using T7 RNA polymerase (MEGAscript). Radiolabeled capped RNA was generated in a reaction containing 1 μCi [α-^{32}P]GTP, 1 μM
unlabeled GTP and vaccinia virus-encoded capping enzyme (m7ScriptCap from CellScript) following the manufacturer’s protocol. The unincorporated nucleotides were removed by chromatography on a P-30 column (Bio-Rad), and the RNA was recovered by RNA clean and concentrator kit (Zymo research). The MTase reaction contained 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 4 units RNase Inhibitor, 500 nM DENV2 NS5FL, 1 μg RNAnt1-200, and 0, 100, 200, 400, 600, 800, or 1000 μM AQ in water. The compound-enzyme mixture was pre-incubated for 15 min at room temperature before addition of the substrate, 400 nM S-adenosylmethionine (SAM). The reaction was incubated at 37 °C for 1 h. RNA was extracted by RNA clean and concentrator (Zymo research), and treated with nuclease P1. The reaction products were fractionated by thin layer chromatography (TLC) on cellulose PEI plate using 0.45 M ammonium sulfate as solvent. The plate was dried, exposed to a phosphoimaging screen. Radioactive signals were quantified by ImageJ software.

2.2.15 In vitro RdRP assay

The positive and negative sense subgenomic RNA templates were constructed and transcribed in vitro (You and Padmanabhan, 1999, Nomaguchi et al., 2003). The RdRp assay was performed using DENV2 NS5FL enzyme as described (Ackermann and Padmanabhan, 2001, Nomaguchi et al., 2003). Briefly, the reaction mixture contained 50 mM Tris-HCl, pH 8, 50 mM NaCl, 5 mM MgCl₂, 1 μg RNA template, 500 μM of ATP, CTP, and UTP, 10 μM unlabeled GTP and 10 μCi [α-32P]GTP, 500 nM DENV2 NS5FL, and AQ (50 μM in DMSO concentration of 1%), and incubated for 1 hour at 30 °C. The RNA product was extracted by acid
phenol/chloroform, and then was analyzed by denaturing PAGE. Radioactive signals from the newly synthesized RNAs were quantified by phosphoimaging.
CHAPTER 3
SCREENING QUINOLINE DERIVATIVES FOR INHIBITION OF FLAVIVIRUS REPLICATION USING REPLICON ASSAYS

3.1 Initial screening with quinoline compounds from NCI/DTP and Dr. Nagarajan’s library

Previous work in our lab by N. Mueller identified 8-hydroxyquinoline as an important core inhibiting WNV protease in vitro (Mueller et al., 2008). Compound B, one of the 8-hydroxyquinoline derivatives, was later identified as having an EC$_{50}$ value of 1.4 ± 0.4 μM (Mueller et al., 2008). In addition, E. Reichert and S. Alcaraz-Estrada developed DENV2, DENV4, and WNV replicons stably expressed in mammalian cell lines for drug discovery. Based on these developments, we investigated the potencies of quinoline derivatives as potential inhibitors of flavivirus replication using replicon based assays. The initial screening involved quinoline derivatives obtained from NCI/DTP (11 compounds) and from Dr. K. Nagarajan’s library (19 KN compounds) at 50 μM (final concentration) on BHK-21/DENV2 cells and Vero/WNV cells (Table 3.1-3.2). From these results, four NCI/DTP compounds (AQ and 3 derivatives with a dichloroethylamino side chain) and eleven KN compounds were selected for further analysis based on strong (≥ 80%) replication inhibition of DENV2 and WNV replicons. We were interested in AQ because it is an FDA-approved antimalarial drug. Because of the promising results with AQ in the preliminary assays, we searched for other AQ derivatives within the NCI/DTP collection and obtained all available AQ derivatives (8 compounds). Upon screening these compounds at 50 μM (final concentration) (Table 3.1), most derivatives, except AQD5 and AQD8, showed strong replication inhibition of DENV2 and WNV replicons. These compounds were also selected for further analysis (Table 3.4).
3.2 Therapeutic indices of selected compounds

Compounds with potent inhibition (≥ 80%) of replication in the screen were selected for further analysis. Briefly, replicon cells were seeded and incubated for 6 h before compound addition. The experiments were continued for 24 h before Rluc measurement and calculation of EC\textsubscript{50} values. We also analyzed cytotoxicity to naïve BHK-21 and Vero cells in parallel. The CC\textsubscript{50} values were evaluated by measuring the cellular ATP level with CellTiterGlo assay kit as described in the Methods section. Therapeutic indices (TI) calculated from the ratios of CC\textsubscript{50} and EC\textsubscript{50} are shown (Table 3.4).

Therapeutic indices (TIs) of NCI/DTP compounds including amodiaquine, quinacrine mustard, chloroquine mustard, and chloroquine ethyl phenyl mustard, were higher for the BHK-21/DENV2 replicon system compared to Vero/WNV. We hypothesized that Vero cells were more sensitive to drug toxicities than BHK-21 cells. Moreover, the AQ derivatives had therapeutic indices close to one, suggesting that their effect on the replicons was a consequence of their cytotoxicity. Due to the fact that AQ has been used in a regimen for malarial treatment since the 1990s, this drug has a long history of pharmacological and toxicological investigations. We were interested in identifying its antiviral efficacy, which would be an alternative application of the drug. For the mustard derivatives, our literature search revealed that compounds with a dichloroethylamino side chain could potentially be persistent and powerful blister agents (Keyes, 2004). For this reason, we decided not to continue studying these derivatives. Results from KN compounds (or 8-hydroxyquinoline derivatives) showed that the high level of replicon inhibition observed in screening were false positives due to their cytotoxicities.
3.3 Screening of chloroquine derivatives from Dr. Wolf

We obtained 19 chloroquine derivatives from Dr. Christian Wolf (GU Chemistry department) for initial screening using our replicon system at 25 μM to minimize the false positive effect due to potential cytotoxicity (Noble et al., 2010), (self observation). BHK-21/DENV2 and Vero/DENV4 cells were treated with the compounds and Rluc activities were measured after 24 h incubation. Each compound was analyzed in triplicate and two independent experiments were performed to confirm the results (Table 3.3). Cell viability in the presence of these compounds was estimated using a WST-8 tetrazolium salt from the CCK-8 kit prior to measurement of Rluc activities as described earlier (Table 3.5). All CQ derivatives displayed a high degree of replicon inhibition and a similar degree of cytotoxicity. We concluded that the strong inhibitory effect on virus replication was most likely due to cytotoxicity. Hence, these derivatives were not pursued further.

3.4 Discussion

We utilized a cell-based approach to screen quinoline compounds for their antiviral activities. The positive hits from this type of screen are expected to be more practical for drug development. The cell-based assay is more biologically relevant compared to in vitro enzymatic assays, as the latter approach ignores permeability issues. However, the cytotoxicity of compounds can be a major false positive issue. It is necessary to exclude this effect by running parallel cytotoxicity assays. Our finding showed that most quinoline compounds except the FDA-approved drugs were most likely cytotoxic, rather than true replicon inhibitors. The only
drug that showed promising anti-DENV2 replicon replication effects was AQ. We have confirmed the finding with various experiments and controls which will be discussed in Chapter 4. Moreover, mode-of-action studies were later performed in order to find a specific target for further SAR development. Details will be discussed in Chapter 5.
Table 3.1 List of quinoline derivatives from NCI/DTP

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<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>NSC number</th>
<th>Structure</th>
<th>MW</th>
<th>DENV2 (%)</th>
<th>WNV (%)</th>
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<td>amodiaquine (AQ)</td>
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<td><img src="structure1.png" alt="Structure" /></td>
<td>356</td>
<td>76.31 ± 1.60</td>
<td>96.30 ± 0.39</td>
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<td>apoquinine,</td>
<td>10581</td>
<td><img src="structure2.png" alt="Structure" /></td>
<td>427</td>
<td>-18.68 ± 11.81</td>
<td>33.67 ± 2.93</td>
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<tr>
<td>3</td>
<td>chloroquine (CQ)</td>
<td>14050</td>
<td><img src="structure3.png" alt="Structure" /></td>
<td>516</td>
<td>54.76 ± 4.45</td>
<td>18.54 ± 2.81</td>
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<td>chloroquine-ethylphenyl mustard</td>
<td>50982</td>
<td><img src="structure4.png" alt="Structure" /></td>
<td>459</td>
<td>99.77 ± 0.03</td>
<td>99.48 ± 0.05</td>
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<td>Compound</td>
<td>MW</td>
<td>Caco-2 Permeability (nm/s)</td>
<td>Papp ± SE (nm/s)</td>
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<td>5</td>
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<td>99.78 ± 0.03</td>
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<td>2.77 ± 0.25</td>
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<td>3.91 ± 0.34</td>
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Table 3.2 List of 8-hydroxyquinoline derivatives from Dr. Nagarajan (Alkem laboratories, Inc., Bangalore, India)

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Structure</th>
<th>MW</th>
<th>% replicon inhibition at 50 μM (Means ± SEM)</th>
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<td>1</td>
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<td>73.20 ± 6.40 N/A</td>
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</table>
Table 3.3 List of CQ derivatives from Dr. Wolf (Georgetown University, Washington, DC, USA)

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<tr>
<th>Number</th>
<th>Name</th>
<th>Structure</th>
<th>MW</th>
<th>DENV2 (%) ± SEM</th>
<th>DENV4 (%) ± SEM</th>
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<tbody>
<tr>
<td>1</td>
<td>CQ derivative 1</td>
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<td>86.47 ± 3.21</td>
<td>79.27 ± 6.30</td>
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<td>CQ derivative 4</td>
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<td>384.14</td>
<td>77.87 ± 4.43</td>
<td>96.17 ± 0.41</td>
<td></td>
</tr>
<tr>
<td>400.11</td>
<td>94.12 ± 0.93</td>
<td>96.70 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>445.10</td>
<td>99.88 ± 0.03</td>
<td>99.92 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>413.14</td>
<td>97.55 ± 0.37</td>
<td>98.19 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>463.16</td>
<td>99.08 ± 0.18</td>
<td>99.06 ± 0.36</td>
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</table>
Table 3.4 EC₅₀, CC₅₀, and TIs of selected compounds

<table>
<thead>
<tr>
<th>Drugs/compounds</th>
<th>DENV2 replicon</th>
<th>WNV replicon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (µM)</td>
<td>CC₅₀ (µM)</td>
</tr>
<tr>
<td>amodiaquine</td>
<td>10.81 ± 1.43</td>
<td>80.01 ± 6.27</td>
</tr>
<tr>
<td>quinacrine mustard</td>
<td>0.39 ± 0.10</td>
<td>2.40 ± 0.63</td>
</tr>
<tr>
<td>chloroquine mustard</td>
<td>2.90 ± 0.78</td>
<td>30.47 ± 9.77</td>
</tr>
<tr>
<td>chloroquine ethyl phenyl mustard</td>
<td>3.60 ± 0.83</td>
<td>74.13 ± 21.50</td>
</tr>
<tr>
<td>AQ derivative 1</td>
<td>21.09 ± 1.22</td>
<td>44.57 ± 2.98</td>
</tr>
<tr>
<td>AQ derivative 2</td>
<td>29.68 ± 2.26</td>
<td>35.97 ± 5.04</td>
</tr>
<tr>
<td>AQ derivative 3</td>
<td>4.76 ± 0.63</td>
<td>14.05 ± 2.57</td>
</tr>
<tr>
<td>AQ derivative 4</td>
<td>15.36 ± 1.31</td>
<td>16.88 ± 2.06</td>
</tr>
<tr>
<td>AQ derivative 5</td>
<td>88.71 ± 3.88</td>
<td>&gt;100</td>
</tr>
<tr>
<td>AQ derivative 6</td>
<td>12.49 ± 2.19</td>
<td>16.39 ± 0.46</td>
</tr>
<tr>
<td>AQ derivative 7</td>
<td>18.97 ± 1.73</td>
<td>17.73 ± 1.96</td>
</tr>
<tr>
<td>AQ derivative 8</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>8-hydroxyquinoline</td>
<td>1.38 ± 0.35</td>
<td>2.49 ± 0.82</td>
</tr>
<tr>
<td>5-chloro-8-hydroxyquinoline</td>
<td>16.61 ± 2.31</td>
<td>20.65 ± 4.20</td>
</tr>
<tr>
<td>KN01</td>
<td>5.55 ± 1.88</td>
<td>4.22 ± 1.31</td>
</tr>
<tr>
<td>KN05</td>
<td>19.78 ± 5.48</td>
<td>17.71 ± 2.23</td>
</tr>
<tr>
<td>KN06</td>
<td>5.95 ± 0.59</td>
<td>6.86 ± 1.05</td>
</tr>
<tr>
<td>KN08</td>
<td>23.75 ± 6.48</td>
<td>37.16 ± 5.92</td>
</tr>
<tr>
<td>KN09</td>
<td>10.41 ± 0.85</td>
<td>28.58 ± 2.97</td>
</tr>
<tr>
<td>KN10</td>
<td>6.14 ± 1.10</td>
<td>21.26 ± 5.79</td>
</tr>
<tr>
<td>KN13</td>
<td>12.02 ± 1.40</td>
<td>&gt;100</td>
</tr>
<tr>
<td>KN6815</td>
<td>3.80 ± 0.50</td>
<td>13.82 ± 1.67</td>
</tr>
<tr>
<td>KN6899</td>
<td>3.86 ± 0.44</td>
<td>5.69 ± 0.59</td>
</tr>
<tr>
<td>KN6905</td>
<td>5.61 ± 0.52</td>
<td>19.91 ± 2.83</td>
</tr>
</tbody>
</table>

Note: TI of AQ on Vero/DENV4 = 2.76 (EC₅₀ 10.18 ± 0.46 µM, CC₅₀ 28.13 ± 5.45 µM)
### Table 3.5 Percent replicon inhibition and percent cell death from CQ derivatives screening

<table>
<thead>
<tr>
<th>Compounds</th>
<th>DENV2 replicon (means ± SEM)</th>
<th>DENV4 replicon (means ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% inhibition</td>
<td>% cell death</td>
</tr>
<tr>
<td>CQ derivative 1</td>
<td>91.69 ± 4.63</td>
<td>88.58 ± 2.72</td>
</tr>
<tr>
<td>CQ derivative 2</td>
<td>97.47 ± 1.18</td>
<td>93.59 ± 0.71</td>
</tr>
<tr>
<td>CQ derivative 3</td>
<td>86.47 ± 5.56</td>
<td>81.58 ± 8.29</td>
</tr>
<tr>
<td>CQ derivative 4</td>
<td>81.59 ± 10.05</td>
<td>70.78 ± 11.73</td>
</tr>
<tr>
<td>CQ derivative 5</td>
<td>97.48 ± 1.11</td>
<td>92.25 ± 2.68</td>
</tr>
<tr>
<td>CQ derivative 6</td>
<td>78.76 ± 12.30</td>
<td>75.75 ± 12.16</td>
</tr>
<tr>
<td>CQ derivative 7</td>
<td>92.16 ± 3.94</td>
<td>86.93 ± 4.98</td>
</tr>
<tr>
<td>CQ derivative 8</td>
<td>94.64 ± 3.96</td>
<td>88.29 ± 5.87</td>
</tr>
<tr>
<td>CQ derivative 9</td>
<td>82.85 ± 4.98</td>
<td>73.25 ± 5.37</td>
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<tr>
<td>CQ derivative 10</td>
<td>90.68 ± 1.67</td>
<td>83.40 ± 2.99</td>
</tr>
<tr>
<td>CQ derivative 11</td>
<td>63.14 ± 8.95</td>
<td>67.31 ± 8.21</td>
</tr>
<tr>
<td>CQ derivative 12</td>
<td>71.04 ± 6.27</td>
<td>60.86 ± 5.13</td>
</tr>
<tr>
<td>CQ derivative 13</td>
<td>78.12 ± 4.83</td>
<td>64.49 ± 3.84</td>
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<tr>
<td>CQ derivative 14</td>
<td>87.29 ± 3.43</td>
<td>69.75 ± 5.41</td>
</tr>
<tr>
<td>CQ derivative 15</td>
<td>77.87 ± 7.67</td>
<td>80.77 ± 5.40</td>
</tr>
<tr>
<td>CQ derivative 16</td>
<td>94.12 ± 1.62</td>
<td>87.97 ± 2.37</td>
</tr>
<tr>
<td>CQ derivative 17</td>
<td>99.88 ± 0.05</td>
<td>95.45 ± 0.33</td>
</tr>
<tr>
<td>CQ derivative 18</td>
<td>97.55 ± 0.64</td>
<td>89.90 ± 1.72</td>
</tr>
<tr>
<td>CQ derivative 19</td>
<td>99.08 ± 0.31</td>
<td>92.25 ± 0.52</td>
</tr>
</tbody>
</table>

- Proportion of % cell death to % inhibition
CHAPTER 4
CHARACTERIZATION OF AQ INHIBITION OF DENV2 REPLICATION USING REPLICON EXPRESSING CELLS AND INFECTIVITY ASSAYS

4.1 Inhibition of DENV2, DENV4, WNV replicon replication measured by Rluc activity

From the screening in Chapter 3, AQ inhibited replicon replication of BHK-21/DENV2 with a TI of 7.4 (Table 3.4), but did not inhibit Vero/DENV4 and Vero/WNV replicon replication. TIs of Vero/DENV4 and Vero/WNV were 2.76 and 1.66, respectively. In a detailed study of AQ, we extended the incubation period to 48 h with AQ at the final concentrations of 0, 0.01, 0.1, 1, 2.5, 5, 7.5, 10, 20, 30, 40, 50, 60, 70, 80, or 100 µM, each with a final DMSO concentration of 1%. TI values of AQ to BHK-21/DENV2, Vero/DENV4 and Vero/WNV replicon expressing cells were 7.31, 1.13, and 4.98, respectively (Fig. 4.1-4.3). In these experiments, we directly measured cytotoxicity of the replicon cells using highly soluble tetrazolium salt (WST-8) before reading the Rluc. EC$_{50}$ and CC$_{50}$ values were reduced in this experimental setting; however, therapeutic indices remained similar to those of 24 h experiments. For BHK-21/DENV2, TIs were 7.40, and 7.31 in the 24, and 48 h experiments, respectively, suggesting that AQ was consistently effective in inhibiting DENV2 replicon replication (Table 3.4, Fig. 4.1). TIs of Vero/DENV4 from both experiments were low suggesting cytotoxicity of AQ on both naïve Vero cells and replicon Vero cells (Table 3.4, Fig. 4.2). As of Vero/WNV, the TI of the 24 h experiment was low because of cytotoxicity but the TI of 48 h experiment was higher at 4.98 with increasing CC$_{50}$ at 39.94 ± 17.67 µM (Table 3.4, Fig. 4.3). Both Vero/WNV TIs were not statistically different because the CC$_{50}$ of the 48 h experiment spanned a wide-range. We concluded that replicon Vero cells were also sensitive to AQ cytotoxicity, similar to naïve
Vero cells. To accurately measure AQ inhibition on DENV4 and WNV, infectivity assays with qRT-PCR and/or plaque assay quantification were recommended.

4.2 AQ did not inhibit Rluc reporter

To exclude the possibility that AQ was directly inhibiting the Rluc reporter, rather than inhibiting replication, BHK-21/DENV2 cells (~$10^4$ cells in 100 μl) were plated in the exact condition as previous experiments and incubated for 24 h at 37 °C. AQ was added during cell lysis. Substrate was added and Rluc activities were measured. No Rluc inhibition was observed at any AQ concentration (0, 0.1, 1, 2.5, 5, 10, 25, or 100 μM) (Fig. 4.4) indicating that the luciferase activity per se was not inhibited by the drug. Thus, AQ appears to cause a dose-dependent inhibition of replicon replication.

4.3 AQ did not inhibit BHK-21/DENV2 replication as quantified by qRT-PCR

In addition to measuring the inhibition of Rluc reporter expression, we analyzed the AQ-mediated inhibition of viral replication by quantifying DENV2 replicon RNA levels. BHK-21/DENV2 cells were treated with AQ for 48 h at the final concentrations of 0, 0.1, 0.5, 1, 2, 2.5, 5, 10, or 25 μM containing in the presence of 1% DMSO. Replicon RNAs were extracted and analyzed by qRT-PCR, comparing DENV2 NS1 and the control GAPDH genes. Each sample was tested in triplicate and results were confirmed by two independent experiments. Data represented means and standard errors derived from two independent experiments (Fig. 4.5). From this experiment, AQ had no measurable effect on the amount of replicon RNA. We concluded that AQ had little effect on continuously replicating subgenomic RNA. In time-of-
addition assays (subheading 5.3), AQ was not effectively inhibiting DENV2 infectivity when added at 18 h post-infection or later. From these results, AQ was apparently ineffective to inhibit DENV2 infection that was already established. On the other hand, we detected AQ inhibition in replicon expressing cells with RLuc measurements (Table 3.4, Fig. 4.1). Under the influence of AQ, we believed that RLuc expression reflected recent RNA activities, including translation and replication, better than the RNA level.

4.4 Inhibition of DENV2 infectivity by AQ as quantified by plaque assay

Next, we studied the infectivity of the viral progeny in the presence of AQ in order to validate the drug effect. To measure the efficacy of AQ inhibition of DENV2 infectivity, AQ at the final concentrations of 0, 0.1, 1, 2, 2.5, 3, 4, 5, 7.5, or 10 µM in DMSO concentration of 1% were added to DENV2 infected BHK-21 cells (MOI of 1). Culture supernatants were harvested at 72 h post-infection and DENV2 infectivity was analyzed by plaque assay (Fig. 4.6). Data were collected from two independent experiments in which each experiment was done in duplicate. EC$_{50}$ and EC$_{90}$ values were 1.08 ± 0.09 µM and 2.69 ± 0.40 µM, respectively (Fig. 4.9).

Moreover, another set of experiments in which AQ added at final concentrations of 0, 0.01, 0.1, 0.5, 1, 2.5, 5, 7.5, 10, or 25 µM to DENV2-infected BHK-21 cells (MOI of 0.01) were also performed. Supernatant were collected at 48, 72, and 96 h post infection (Fig. 4.7-4.8). EC$_{50}$ and EC$_{90}$ at 96 h post-infection were 1.43 ± 0.17 µM, and 2.68 ± 0.52 µM, respectively (Fig. 4.9). Apparently, There were ≥ 90% plaque reduction in the presence of AQ (5 µM) at both viral loads tested in this study. Results of AQ inhibition of DENV2 infectivity measured by plaque assay were consistent with that of the replicon assays.
4.5 Confirmation of inhibition of DENV2 replication and infectivity by AQ

In addition, we verified AQ inhibition to DENV2 replication using DENV2 infected BHK-21 cells and measured the intracellular viral RNA, extracellular viral RNA quantified by qRT-PCR, and viral infectivity quantified by plaque assay. We expected significant reductions of the viral RNAs, and the viral infectivity in AQ treated samples as the drug stopped the replication. As described in subheading 4.4, we selected the AQ concentration of 5 μM because it was sufficient to inhibit DENV2 infectivity by ≥ 90% with no cytotoxic effect on BHK-21 cells. DENV2 infected BHK-21 cells (MOI of 1) were incubated for 72 h in the presence and absence of AQ (5 μM). Intracellular RNAs were collected from cell lysates using TRIzol and analyzed DENV2 NS1 by qRT-PCR. Extracellular RNAs were extracted using QIAamp kit from culture supernatants (10 ml) concentrated using Amicon-15 and analyzed DENV2 NS1 by qRT-PCR. The culture supernatants were also used to analysis DENV2 infectivity using plaque assay. Each sample was tested in triplicate and results were confirmed by three independent experiments. Results showed significant difference (p < 0.001) between the treated and untreated groups (paired t test, two tailed) (Fig. 4.10). Therefore, AQ effectively inhibited DENV2 replication so that the levels of intracellular and extracellular RNAs, as well as DENV2 infectivity were all reduced. Noted that within AQ treated group, we did not observed any significant difference between the levels of intracellular RNA and extracellular RNA, (unpaired t-test, two tailed), as well as extracellular RNA and infectivity (unpaired t-test, two tailed) (Fig. 4.10).
4.6 Discussion

Our results taken together indicate that AQ inhibited DENV2 replication. First, using RLuc reporter assays at two different incubation periods, 24 and 48 h, we observed similar inhibition of viral replicon replication. Although EC$_{50}$ and CC$_{50}$ values were reduced at 48 h incubation period compared to 24 h, TI values remained similar at 7.31 to 7.40 (Table 3.4, Fig. 4.1). The effect of AQ was not due to inhibition of RLuc enzyme activity as the drug did not have any effect when added during cell lysis (Fig. 4.4). These results support the conclusion that the reduction of RLuc activity when AQ was added to the DENV2 replicon expressing cells was due to an inhibitory effect of AQ on DENV2 replicon replication. However, we did not observe a corresponding reduction of DENV2 replicon RNA detected by qRT-PCR (Fig. 4.5). AQ potently inhibited DENV2 infectivity measured by plaque assays of the extracellular virions, as well as by measuring the intracellular and extracellular viral RNA levels using qRT-PCR (Fig. 4.10). AQ inhibited DENV2 infectivity similarly in both viral loads (MOI of 1 and 0.01) (Fig. 4.9). In conclusion, our results show that AQ inhibits DENV2 replication and infectivity.

AQ was first synthesized in the 1980s by addition of a 4-hydroxyanilino group to chloroquine (CQ). AQ was proven effective against chloroquine-resistant strains of the parasite Plasmodium falciparum (reviewed by (O'Neill et al., 1998)). In the 1990s, the drug was withdrawn from use in long-term antimalarial prophylaxis due to its rare but serious side effects, agranulocytosis and hepatotoxicity (WHO 1990; reviewed by (Olliaro and Mussano, 2003)). Cochrane’s systematic review in 1996, however, supported the use of AQ for treatment of uncomplicated malaria. The impact of this review forced WHO in 1997 to modify its recommendation and AQ became an optional treatment of uncomplicated malaria (reviewed by...
The typical AQ regimen for antimalarial treatment is three days of 600-800 mg/day for adults. Moreover, an adverse drug reactions (ADR) study found no significant difference among the antimalarials CQ, AQ, and sulfadoxine/pyrimethamine (reviewed by (Olliaro and Mussano, 2003)). Pharmacogenetic studies have found genetic variations (genetic polymorphisms) in one of the cytochrome P450 genes, called CYP2C8, that could explain hepatotoxicity. Poor metabolizer (PM) variants had higher AQ intoxication from prolonged metabolism and delayed clearance (reviewed by (Kerb et al., 2009)). There are two PM phenotypes (CYP2C8*2, and CYP2C8*3) in African and Caucasian populations respectively with the frequency of 11-17%, and 15%, respectively. Dose-adjustment and increased safety precautions will be applied to the variants in order to prevent the intoxication. With proven anti-DENV efficacy, AQ could be a powerful candidate for treatment of DENV infected patients.

Next, we explored whether AQ inhibited another stage in the viral life cycle in order to thoroughly understand the activity of the drug. The rationale was that its close 4-aminoquinoline relatives (chloroquine (CQ) and ferroquine (FQ)) possessed strong inhibitory effects on vesicular membrane associated stages (maturation (Randolph et al., 1990) and fusion (Vausselin et al., 2013), respectively). Moreover, we explored three critical flaviviral enzymes (protease, MTase and RdRP) for being possible targets of the drug by in vitro assays.
Fig. 4.1 EC$_{50}$ of AQ on DENV2 replicon replication (% Rluc) and CC$_{50}$ of AQ on BHK-21/DENV2 cells (% cell viability). BHK-21/DENV2 cells (~10$^4$ in 100 μl) were seeded and incubated for 24 h before compound addition. AQ at the final concentrations of 0, 0.01, 0.1, 1, 2.5, 5, 7.5, 10, 20, 30, 40, 50, 60, 70, 80, or 100 μM, in DMSO concentration of 1% were added to the cells. Cells were incubated at 37 °C for 48 h. Cells were washed, lysed, and Rluc signal from the replicons were measured. Cytotoxicity was measured simultaneously with highly soluble tetrazolium salt. Each concentration was tested in triplicate and the results were confirmed by two independent experiments.

EC$_{50}$ = 7.41 ± 1.09 μM
CC$_{50}$ = 52.09 ± 4.25 μM
Fig. 4.2 EC$_{50}$ of AQ on DENV4 replicon replication (% Rluc) and CC$_{50}$ of AQ on Vero/DENV4 cells (% cell viability). Vero/DENV4 cells (~10$^4$ in 100 μl) were seeded and incubated for 24 h before compound addition. AQ at the final concentrations of 0, 0.01, 0.1, 1, 2.5, 5, 7.5, 10, 20, 30, 40, 50, 60, 70, 80, or 100 μM, in DMSO concentration of 1% were added to the cells. Cells were incubated at 37 °C for 48 h. Cells were washed, lysed, and Rluc signal from the replicons were measured. Cytotoxicity was measured simultaneously with highly soluble tetrazolium salt. Each concentration was tested in triplicate and the results were confirmed by two independent experiments.
Fig. 4.3 EC$_{50}$ of AQ on WNV replicon replication (% Rluc) and CC$_{50}$ of AQ on Vero/WNV cells (% cell viability). Vero/WNV cells (~10$^4$ in 100 µl) were seeded and incubated for 24 h before compound addition. AQ at the final concentrations of 0, 0.01, 0.1, 1, 2.5, 5, 7.5, 10, 20, 30, 40, 50, 60, 70, 80, or 100 µM, in DMSO concentration of 1% were added to the cells. Cells were incubated at 37 °C for 48 h. Cells were washed, lysed, and Rluc signal from the replicons were measured. Cytotoxicity was measured simultaneously with highly soluble tetrazolium salt. Each concentration was tested in triplicate and the results were confirmed by two independent experiments.
**Fig. 4.4** AQ effect on Rluc reporter. BHK-21/DENV2 cells (~10^4 in 100 μl) were seeded and incubated for 24 h at 37 °C. AQ at the final concentrations 0, 0.1, 1, 2.5, 5, 10, 25, or 100 μM in DMSO concentration of 1% were added during cell lysis. The substrate was added and the Rluc activities were measured.
Fig. 4.5 AQ effect of DENV2 RNA levels in BHK-21/DENV2 cells measured by qRT-PCR. BHK-21/DENV2 cells (~2.5 x 10^5 in 2.5 ml) were seeded and incubated overnight at 37°C. AQ at the final concentrations of 0, 0.1, 0.5, 1, 2.5, 5, 10, or 25 μM in DMSO concentration of 1% were added. Cells were incubated at 37 °C for 48 h. Total RNAs were extracted from the cells by treatment with TRIzol reagent. Sample RNA concentration was adjusted to 1 μg/μl. The region of viral RNA encoding DENV2 NS1 and the housekeeping reference gene (GAPDH) were quantified by qRT-PCR. Each sample was tested in triplicate and results were confirmed by two independent experiments.
Fig. 4.6 AQ inhibition of DENV2 infectivity in BHK-21 cells (MOI of 1). BHK-21 cells were seeded into a 12-well plate and incubated overnight at 37 °C. Cells were infected with DENV2 (New Guinea C strain) at a MOI of 1 with gentle rocking every 15 min. After infection, cells were washed with PBS and incubated with 1.5 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 µg/ml streptomycin. AQ at the final concentrations of 0.1, 0.5, 1, 2.5, 5, or 10 µM in DMSO concentration of 1%, or DMSO alone, was added during infection and post-infection. Cells were then incubated for 72 h at 37 °C. Supernatants were collected and DENV2 infectivity was analyzed by plaque assay.
Fig. 4.7 AQ inhibition of DENV2 infectivity in BHK-21 cells (MOI of 0.01). BHK-21 cells were seeded into a 12-well plate and incubated overnight at 37 °C. Cells were infected with DENV2 (New Guinea C strain) at a MOI of 0.01 with gentle rocking every 15 min. After infection, cells were washed with PBS and incubated with 1.5 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 µg/ml streptomycin. AQ at the final concentrations of 0.01, 0.1, 0.5, 0.75, 1, 2.5, 5, 7.5, 10, or 25 μM in DMSO concentration of 1%, or DMSO alone, was added during infection and post-infection. Cells were then incubated for 96 h at 37 °C. Supernatants were collected and DENV2 infectivity was analyzed by plaque assay.
**Fig. 4.8** Titer (pfu/ml) showing AQ inhibition of DENV2 infectivity in BHK-21 cells (MOI of 0.01) from supernatants collected at 48, 72, 96 h post-infection. BHK-21 cells were seeded into a 12-well plate and incubated overnight at 37 °C. Cells were infected with DENV2 (New Guinea C strain) at a MOI of 0.01 with gentle rocking every 15 min. After infection, cells were washed with PBS and incubated with 1.5 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 µg/ml streptomycin. AQ at the final concentrations of 0.01, 0.1, 0.5, 0.75, 1, 2.5, 5, 7.5, 10, or 25 µM in DMSO concentration of 1%, or DMSO alone, was added during infection and post-infection. Cells were then incubated for 96 h at 37 °C. Supernatants were collected at 48, 72, and 96 h post-infection. DENV2 infectivity was analyzed by plaque assay. Each AQ concentration was tested in duplicate and was confirmed by two independent experiments.
Fig. 4.9 EC\textsubscript{90} values of AQ of DENV2 infectivity in BHK-21 cells (MOI of 1 and 0.01). BHK-21 cells were seeded into a 12-well plate and incubated overnight at 37 °C. Cells were infected with DENV2 (New Guinea C strain) at a MOI of 1 or 0.01 with gentle rocking every 15 min. After infection, cells were washed with PBS and incubated with 1.5 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 µg/ml streptomycin. AQ at the final concentrations of 0.1, 0.5, 1, 2.5, 5, or 10 µM in DMSO was added to DENV2 infected cells (MOI of 1) and the final concentrations of 0.01, 0.1, 0.5, 0.75, 1, 2.5, 5, 7.5, 10, or 25 µM in DMSO was added to DENV2 infected cells (MOI of 0.01) during infection and post-infection. Cells were then incubated at 37 °C for 72 h, or 96 h in DENV2 infected cells (MOI of 1, or 0.01), respectively. Supernatants were collected and DENV2 infectivity was analyzed by plaque assay. Data were plotted and the EC\textsubscript{90} values were calculated by nonlinear regression analysis. Data represented duplicates of two independent experiments.
**Fig. 4.10** Confirmation of AQ inhibition of DENV2 replication and infectivity. BHK-21 cells (10^5 cells in 1 ml) were seeded into a 12-well plate and incubated overnight at 37 °C. Cells were infected with DENV2 (New Guinea C strain) at a multiplicity of infection (MOI) of 1 for 1 h at 37 °C under 5% CO₂ with gentle rocking every 15 min. After infection, cells were washed with PBS and incubated with 1.5 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 µg/ml streptomycin. AQ (5 µM) in DMSO concentration of 1%, or DMSO alone, was added during infection and post-infection. Cells were then incubated for 72 h at 37 °C. Intracellular RNAs were extracted from the infected cells by treatment with TRIzol reagent. Sample RNA concentration was adjusted to 1 µg/µl. The region of viral RNA encoding DENV2 NS1 and the housekeeping reference gene (GAPDH) were quantified by qRT-PCR. Extracellular viral RNAs were extracted from supernatants using the QIAamp viral RNA mini kit. The region of viral RNA encoding DENV2 NS1 was quantified by qRT-PCR. Infectious virions were analyzed from DENV2 infectivity measured by plaque assay. The relative amount from AQ (5 µM in DMSO concentration of 1%) treated cells was based upon normalization to that of untreated cells (DMSO concentration of 1%). Each sample was tested in duplicate and results were confirmed by three independent experiments. Difference between AQ treated and DMSO treated groups derived from each approach was evaluated by paired t-test, two tailed.
CHAPTER 5
MODE OF ACTION AND TARGET IDENTIFICATION STUDY OF AQ INHIBITION OF DENV2 INFECTION

5.1. Time-course analysis of DENV2 infectivity

In this chapter, we asked the question whether AQ also inhibited any other stages of the DENV2 life cycle. As an antimalarial drug, AQ had several mechanisms of action including interfering with vesicular acidification and inhibiting RNA polymerase (reviewed by O'Neill et al., 1998). Although the drug efficacy to overall DENV2 infectivity was not significantly superior to DENV2 replication alone, the knowledge will strengthen our understanding of the drug property and learn to maximize its potency. We started investigating the drug effect on DENV2 infectivity pattern in time-course analysis with four different concentrations (0, 1, 5, 10, 25 μM) of AQ.

In this assay, we infected BHK-21 cells with DENV2 (MOI of 0.01) and collected the supernatants at 4, 12, 24, 36, 48, 72, and 96 h post-infection. The drug was introduced to the DENV2 infected cells during infection and post-infection. In the DMSO control (0 μM AQ), titer of DENV2 infectivity gradually increased over time. The number of plaques (pfu/ml) was significantly reduced in the presence of AQ in dose dependent manner ($p < 0.05$, one way ANOVA) (Fig. 5.1-5.2). At 5 μM, plaque formation was reduced ≥ 90%, corresponding to previous results on AQ efficacy. In addition, AQ was effectively inhibited DENV2 infectivity for 96 h period and did not require drug supplement at 48 h, or 72 h post-infection.
5.2. Effect of AQ on viral entry and early stages of the viral life cycle

To analyze the effect of AQ on viral entry, we used a direct plaque assay. A BHK-21 monolayer was infected with DENV2 (MOI of 0.01) in the presence of AQ (0, 1, 5, 10, 25 μM). After washing with PBS, cells were maintained with overlay medium (MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 μg/ml streptomycin, and 1% methylcellulose) and in the absence of drug for 3-4 days. The plaques were fixed, stained and counted as described earlier. Data obtained from this experiment is referred to as direct plaques (Fig. 5.3, dotted bars). Indirect plaques (Fig. 5.3, clear bars) reflected the data obtained from the previous time-course analysis after 96 h. Means and error bars represented duplicate results from two independent experiments. Indirect plaques (Fig. 5.3, clear bars) showed significant inhibition of DENV2 infectivity by AQ at 5 μM compared with its DMSO control ($p < 0.01$, paired t-test, two-tailed). However, with direct plaques, (Fig. 5.3, dotted bars), DENV2 infectivity by AQ at 5 μM was not significantly different from its DMSO control. These results indicate that the 5 μM does not inhibit DENV2 adsorption. However, the inhibition of DENV2 infectivity were increased to 69.23 ± 1.57 % and 82.70 ± 2.48 % by increasing the AQ concentrations to 10 and 25 μM, respectively, during adsorption (Fig. 5.3, dotted bars). We concluded that the inhibition of DENV2 entry by AQ was possible when the drug was provided in high dose (10 or 25 μM). Since much higher concentrations of AQ were required to detect an effect on adsorption, AQ seems to have its greatest effect post-infection.

In addition to direct plaque assay, we analyzed the levels of DENV2 infectivity impairments with order of addition assay. The assay was designed to characterize compounds with neutralization property (Schmidt et al., 2012). AQ was added to DENV2 infected BHK-21
cells in various orders mentioned as pre-incubation, co-infection and post-infection. Pre-incubation was the addition of AQ (5 μM) to DENV2 (MOI of 1) and incubated for 15 min at 37 °C. The mixture was later used to infect BHK-21 cells. Co-infection was the addition of AQ (5 μM) to DENV2 (MOI of 1) during BHK-21 cells infection (adsorption). Post-infection was the addition of AQ (5 μM) to BHK-21 cells after DENV2 infection was established. DENV2 infection was done by incubation of the virus at 37 °C under 5% CO₂ for 1 h with gentle rocking every 15 min. Supernatants were collected at 24, 48, and 72 h post-infection for analysis by plaque assay (Fig. 5.4). Results indicated that the inhibitions of DENV2 infectivity by AQ were maximized (≥ 90% plaque titer reduction from its DMSO control) when the drug was administered post-infection for 48 and 72 h PI (Fig. 5.4, striped bars). From these results, the major pathway for inhibition by AQ is obviously post-entry and fusion events. Late stages including viral translation, replication, and assembly of the virus life cycle were involved. So far, we have identified viral replication as the critical step affected by the drug.

5.3. Effect of AQ on late stages of the viral life cycle

Next, to pinpoint the post-infection stage of inhibition more precisely, we added AQ at 5 μM to DENV2 infected BHK-21 cells (MOI of 1) at 1, 3, 6, 9, 12, 15, 18, 21, 24, 30, and 36 h post-infection. Supernatants were collected at 72 h post-infection and were analyzed for time-dependent plaque reduction. The inhibition (≥ 90% plaque titer reduction) was found even when the drug was added as late as 15 h post-infection (Fig. 5.5, left upper panel). At 21 h post-infection and later time points, the drug lost its inhibitory effect to the viral infectivity. These results support our conclusion that AQ inhibits a late stage in the DENV2 life cycle and that viral
replication is one of the drug targets. It is unlikely that AQ is a translation inhibitor because a true translation inhibitor would lose its inhibitory effect within 12 h post-infection (Wang et al., 2011). For more understanding, we also quantified viral RNAs by qRT-PCR from supernatants (Fig. 5.5, right upper panel) and pellets (Fig. 5.5, middle panel) to measure extracellular and intracellular viral RNA copy numbers, respectively. Extracellular viral RNA copy numbers in AQ treated samples were at the limit of detection, but we still noticed the inhibition by AQ at early time-points (Fig. 5.5, right upper panel). The inhibition of intracellular viral RNA copy numbers by AQ (5 μM) was ≥ 90% at 1-12 h PI (Fig. 5.5, middle panel), and the inhibitory effect declined when AQ was introduced at later time-points. DENV2 replication starts after 12 h post-infection as suggested by rapidly increase of intracellular RNA levels in BHK-21 cells quantified by qRT-PCR (Shrivastava et al., 2011). We concluded that AQ effectively inhibited viral replication in the infected cells when introduced prior to 15 h post-infection. To validate the intracellular viral RNA quantification, GAPDH was analyzed and its Ct value was reported side by side to the Ct value of DENV2 NS1 (Fig. 5.5, lower panels).

In addition, we modified a time of addition assay focusing on the first 12 h post-infection to study the viral translation. Results indicated an effective inhibition of AQ when the drug was introduced at 1, 2, 3, 4, 5, 6, 8, 10, or 12 h post-infection (Fig. 5.6). The true translation inhibitor completely lost its efficacy by 12 h post-infection (Wang et al., 2011). Therefore, AQ was unlikely a translation inhibitor.
5.4. \textit{In vitro} assays of viral replication enzymes

The above findings supported the notion that AQ targets a factor functioning in viral replication. The factor could be of either viral or host origin. The flaviviral genome codes for seven nonstructural proteins (reviewed in Molecular biology and life cycle section under subheading 1.1.3). Two of these proteins (NS3 and NS5) are multifunctional proteins responsible for most enzymatic activities in the life cycle. Because established \textit{in vitro} assays are available in our laboratory for DENV2 protease, methyltransferase (MTase) and RNA dependent RNA polymerase (RdRP), we investigated whether any of these viral factors could be a target of AQ in inhibition of viral replication.

We first chose to test the effect of AQ on DENV2 NS3 protease using the \textit{in vitro} protease assay developed for high throughput screening (Yusof et al., 2000, Mueller et al., 2008, Lai et al., 2013). DENV2 protease was pre-incubated for 15 min with AQ at the final concentrations of 0, 7.81, 15.63, 31.25, 62.50, 125, 250, and 500 μM in DMSO concentration of 1%. The assay was done in triplicate. The IC$_{50}$ value of AQ for DENV2 protease inhibition was \(\geq 250 \text{ μM} \) (Fig. 5.6), indicating that protease was not the likely target of AQ.

Next, we investigated whether the flaviviral methyltransferase from the N-terminal domain of NS5 could be the target of AQ. It has been reported in the literature that AQ is a potent inhibitor of histamine-N-methyltransferase (E.C. 2.1.1.8) with a Ki of 18.6 nM (Horton et al., 2005). Two molecules of AQ in complex with histamine-N-methyltransferase at the SAM binding pocket, and the nearby outer pocket were solved using crystallography (Horton et al., 2005). Another crystal structure of AQ in complex with phosphoethanolamine methyltransferase from \textit{Plasmodium falciparum} at SAM binding pocket was also reported (Lee et al., 2012).
Flaviviral methyltransferase was responsible for N7 and 2’O methylations (Ray et al., 2006, Dong et al., 2008a). In this study, we used NS5FL for the assay because its catalytic efficiency is higher than that of the MTase domain alone (Fig. 9.6). DENV2 MTase was pre-incubated for 15 min with AQ at final concentrations of 0, 100, 200, 400, 600, 800, and 1000 μM in water. The results showed no inhibition of DENV2 MTase with AQ concentrations up to 1 mM (Fig. 5.7, upper panel). The values represent the mean and standard error of triplicate results from the experiment (Fig. 5.7, lower panel). We concluded that DENV2 methyltransferase was not the target of AQ drug.

Since multiple lines of evidence were consistent with inhibition of replication by AQ, we examined whether RNA synthesis by the flaviviral RdRP might be the target of AQ. Both positive (+) and negative (-) strand RNAs were used as templates in an in vitro RdRP assay in the presence of 50 μM AQ for 60 min. Inhibition of RdRP activity by AQ was only 10.58 ± 0.94 % for positive strand synthesis and -17.12 ± 4.37 % for the negative strand synthesis (Fig. 5.8, upper panel), implying that flaviviral RdRP is not a likely target of AQ. The values represent the mean and standard error of triplicate results from the experiment (Fig. 5.8, lower panel).

5.5. Discussion

In this section, we studied the mode of inhibition of DENV2 replication and infectivity by AQ and investigated potential targets of AQ using in vitro enzyme assays. Time-course analysis suggested that the drug was stable and persistently inhibited DENV2 infectivity up to 96 h post infection. We also learned that viral entry and internalization was partially inhibited by the drug but the major effect of drug occurred at a late stage of the viral life cycle. AQ was
ineffective to inhibit replication that is already established as seen in the time of addition assay. The drug was less effective when added later than 15 h post-infection measured by intracellular RNAs, and 18 h post-infection as measured by viral infectivity. It was unlikely that post replication processes are affected since AQ treatment had a similar quantitative effect on the amount of intracellular and extracellular RNAs (Fig. 4.10).

Viral replication requires both viral and host factors (reviewed by (Sampath and Padmanabhan, 2009, Bollati et al., 2010, Nagy and Pogany, 2012). Our findings excluded some enzymatic activities of NS3 and NS5 as targets of AQ. It is possible that AQ targets a nonstructural protein without an enzymatic function. Isolation and characterization of AQ resistant mutants and mapping these mutations by sequence analysis might reveal the identity of the viral targets, assuming viral proteins are the target. Although most DENV2 infected BHK-21 cells die within 12 days under 5 μM AQ selective pressure (unpublished results), a longer incubation period, up to 30 days, might reveal an escape mutant. An in vitro RdRP system (Ackermann and Padmanabhan, 2001, Nomaguchi et al., 2003) could provide an insight into whether the initiation factors of viral replication are targets of AQ. Although the flaviviral replication complex is not fully understood, some host components have been identified and characterized (reviewed by (Nagy and Pogany, 2012)). Searching for potential AQ targets could start with an examination of identified host factors necessary to provide optimal conditions for initiation of viral replication.
Fig. 5.1 Time-course analysis of AQ inhibition of DENV2 infectivity. BHK-21 cells were seeded into a 6-well plate and incubated overnight at 37 °C. Cells were infected with DENV2 (New Guinea C strain) at a multiplicity of infection (MOI) of 0.01 with gentle rocking every 15 min. After infection, cells were washed with PBS and incubated with 3 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin and 100 µg/ml streptomycin. AQ at the final concentrations of 0, 1, 5, 10, or 25 µM in DMSO concentration of 1%, or DMSO alone, was added during infection and post-infection. Cells were then incubated for 96 h at 37 °C. Supernatants were sampled at 4, 12, 24, 36, 48, 72, and 96 h post-infection DENV2 infectivity was analyzed by plaque assay (upper panel). The lower panel showed duplicate results of plaque formation from supernatant collected at 96 h PI.
**Fig. 5.2** Measurement of AQ inhibition of DENV2 infectivity by direct plaque assay. BHK-21 cells (~10^5 cells in 1 ml) were seeded into a 12-well plate and incubated at 37 °C until reaching 90% confluence. AQ at final concentrations of 0, 1, 5, 10, or 25 µM in DMSO concentration of 1% were added to DENV2 (MOI of 0.01) during adsorption for 1 h at 37°C with gentle rocking every 15 min. Cells were washed with PBS and maintained with overlay medium (MEM supplemented with 2% FBS, 100 I.U/ml penicillin and 100 µg/ml streptomycin, and 1% methylcellulose). AQ was not present in the overlay medium post-infection. Plaque formation (pfu/ml) after 3-4 days incubation was normalized using AQ at 0 µM as 100%. Indirect plaque assay was performed by addition of AQ at 0, 1, 5, 10, 25 µM in DMSO concentration of 1% during DENV2 infection (MOI of 0.01), and post-infection for 96 h. Supernatants were collected at 96 h PI for DENV2 infectivity analysis. Titer (pfu/ml) was normalized to using AQ at 0 µM as 100%. Data represented means and SEM of two independent experiments.
**Fig. 5.3 Order of addition assay.** BHK-21 cells (~$10^5$ cells in 1 ml) were seeded into a 12-well plate and incubated overnight at 37 °C. AQ (5 µM in DMSO concentration of 1%) and DENV2 (MOI of 1) were assayed by 1) pre-incubation of drug and virus at 37 °C for 15 min before adsorption for 1 h, 2) co-infection of drug and virus for 1 h, or 3) post-infection of drug after viral adsorption and PBS wash. Supernatants were collected at 24, 48, and 72 h post-infection for analysis by plaque assay. DMSO (concentration of 1%) was used as a mock treatment. Error bars indicated the standard error of the means of experiments done in duplicate. The assay was performed in duplicate and confirmed by two independent experiments.
Fig. 5.4 Time of addition assay following the viral replication. BHK-21 cells (~10^5 cells in 1 ml) were seeded into a 12-well plate and incubated overnight at 37 °C. AQ (5 µM in DMSO concentration of 1%) was added to DENV2 infected BHK-21 cells (MOI of 1) at 1, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, or 48 h post-infection. Supernatants were collected at 72 h post-infection for analysis of the viral infectivity by plaque assay (left upper panel) and the extracellular viral RNA by qRT-PCR (right upper panel). Pellets were also collected for analysis of intracellular RNAs by qRT-PCR (middle panel). Ct values of intracellular RNAs of DENV2 NS1 (left lower panel) and GAPDH (right lower panel) were shown for validation. DMSO (concentration of 1%) was used as a mock treatment. Error bars indicated the standard error of the means of experiments done in duplicate. Results were confirmed by two independent experiments.
Fig. 5.5 Time of addition assay following the viral translation. BHK-21 cells (~10^5 cells in 1 ml) were seeded into a 12-well plate and incubated overnight at 37 °C. AQ (5 µM in DMSO concentration of 1%) or DMSO alone (concentration of 1%) was added to DENV2 (MOI of 1) at 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, or 48 h post-infection. Supernatants were collected at 48 h post-infection for analysis by plaque assay. Error bars indicated the standard error of the means of experiments done in duplicate.
Fig. 5.6 AQ inhibition of DENV2 protease in vitro. The reaction mixture (100 μl) contained 200 mM Tris-HCl, pH 9.5, 30% glycerol, 0.1% CHAPS, 1% DMSO, 50 nM DENV2 NS2BH-(QR)-NS3pro enzyme (Yon et al., 2005), 10 μM fluorogenic tetrapeptide substrate, Bz-Nle-Lys-Arg-Arg-AMC, and 0, 7.81, 15.63, 31.25, 62.5, 125, 250, or 500 μM AQ in DMSO concentration of 1% at final. The compound-enzyme mixture was pre-incubated for 15 min at room temperature before addition of the substrate. The reaction was continued at 37 °C for 30 min. DMSO alone (concentration of 1%) was used as the no-inhibitor control (100% protease activity) and the bovine pancreatic trypsin inhibitor (BPTI, also known as aprotinin), which has a Ki of 26 nM against the DENV2 protease, was used at 5 μM in DMSO concentration of 1% as a positive control (0% protease activity). Data were plotted and the IC₅₀ value was calculated by nonlinear regression analysis.
Fig. 5.7 AQ inhibition of DENV2 MTase in vitro. The radiolabeled cap of RNA_{nt1-200} substrate (1 
μg) was methylated in the reaction containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM 
MgCl₂, 1 mM dithiothreitol, 4 units RNase Inhibitor, 500 nM DENV2 NS5_{FL}, and 0, 100, 200, 
400, 600, 800, or 1000 μM AQ in water. The compound-enzyme mixture was pre-incubated for 
15 min at room temperature before addition of the substrate, 400 nM S-adenosylmethionine 
(SAM). The reaction was incubated at 37 °C for 1 h. RNA was extracted and treated with 
nuclease P1. The products were fractionated by thin layer chromatography (TLC) on cellulose 
PEI plate using 0.45 M ammonium sulfate as solvent (upper panel). Radioactive guanine cap 
analogs were quantified by phosphoimaging. (lower panel).

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<th>[AQ] (μM)</th>
<th>7MeGpppA₂-O-Me</th>
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<tr>
<td>100</td>
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<td>24.79 ± 0.14</td>
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<tr>
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<td>1000</td>
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Fig. 5.8 AQ inhibition of DENV2 RdRP in vitro. The positive and negative sense subgenomic RNAs served as templates of the DENV2 RdRP reactions. The reaction contained 50 mM Tris-HCl, pH 8, 50 mM NaCl, 5 mM MgCl$_2$, 1 μg RNA template, 500 μM of ATP, CTP, and UTP, 10 μM unlabeled GTP and 10 μCi [$\alpha$-$^{32}$P]GTP, 500 nM DENV2 NS5$_{FL}$, and AQ (50 μM in DMSO concentration of 1%), and incubated for 1 hour at 30 °C. The RNA product was extracted by acid phenol/chloroform, and then was analyzed by denaturing PAGE (upper panel). Radioactive signals from the newly synthesized RNAs were quantified by phosphoimaging (lower panel).
CHAPTER 6
STRUCTURE-ACTIVITY RELATIONSHIP STUDY OF AQ AND DERIVATIVES USING A DENV2 INFECTIVITY ASSAY

6.1. Diethylaminomethyl group

From the analysis of AQ and its derivatives using BHK-21/DENV2 cells (subheading 3.2), compounds lacking a diethylaminomethyl group (AQD5 and AQD8) failed to inhibit replicon replication. The EC\textsubscript{50} values were ~100 μM (Table 3.4), at least 10 fold higher than that of AQ. We chose AQD8 for further study using the infectivity assay. AQD8 at various concentrations was added to DENV2 infected BHK-21 cells (MOI of 1). After 72 h incubation, supernatants were collected for plaque assay. Results showed an EC\textsubscript{90} of AQD8 at 31.20 ± 5.15 μM (Fig. 6.1), which is > 20 fold higher than that of AQ (EC\textsubscript{90} of 2.69 ± 0.47 μM). Thus, AQD8 failed not only to inhibit replicon replication, but also failed to suppress the viral infectivity. We concluded that the diethylaminomethyl group was a key chemical structure AQ contributing to inhibition of DENV2 replication and infectivity.

6.2. p-hydroxyanilino group

Recently, Farias et al. (Farias et al., 2013) demonstrated CQ as an inhibitor of DENV2 infectivity in Vero cells. We also found that CQ inhibited DENV2 infectivity (MOI of 1) in BHK-21 cells (EC\textsubscript{90} = 5.04 ± 0.72 μM) (Fig. 6.1), but not DENV2 replicon replication (Fig. 3.1). CQ at 100 μM increased prM/M composition of gradient-purified DENV2 extracellular virions (Randolph et al., 1990), suggesting that the drug inhibits virion maturation. Based on our
findings, we hypothesized that AQ and CQ inhibited DENV2 infectivity by different mechanisms.

6.3. Discussion

Due to limited availability of AQ derivatives, we could not thoroughly study structure-activity relationship of AQ. However, we obtained an important piece of information from the analysis of derivatives AQD5 and AQD8. We concluded that the presence of a diethylaminomethyl group was crucial for AQ to be active. Also, CQ was widely studied as an antiviral agent against many viruses including DENV2. CQ inhibited DENV2 infectivity by interfering with prM to M cleavage of the virion maturation. Moreover, ferroquine (FQ), a CQ derivative structurally similar to AQ, inhibited HCV infection at an early step by interfering with fusion (Vausselin et al., 2013) and had minor inhibitory effects on viral replication and assembly. From the currently available data, we conclude that each quinoline compound inhibits flaviviral infection with a unique mechanism of action.

Structurally, AQ contains intramolecular H-bonding (Warhurst et al., 2003) between the hydroxyl group of 4-aniline ring and N of diethylaminomethyl group. This bond generates an angle between the quinoline and the aniline rings (reviewed by (O'Neill et al., 1998)). This special character is absent in CQ since CQ does not possess an electron donor (hydroxyl group) for H-bonding. Since CQ was not a potent inhibitor of replicon replication, we hypothesized that the angular structure might be involved in contributing to replication inhibition. Future work on lead optimization should focus on modification of AQ taking into consideration this intramolecular H-bonding.
Fig. 6.1 Inhibition of DENV2 infectivity in BHK-21 cells (MOI of 1) by AQ, CQ, and AQD8. BHK-21 cells were seeded into a 12-well plate and incubated overnight at 37 °C. Cells were infected with DENV2 (New Guinea C strain) at a MOI of 1 with gentle rocking every 15 min. AQ at the final concentrations of 0.1, 0.5, 1, 2.5, 5, or 10 μM in DMSO concentration of 1%, or CQ or AQD8 at the final concentrations of 0.1, 0.5, 1, 2.5, 5, 10, 25, or 50 μM in DMSO concentration of 1% were added onto DENV2 infected BHK-21 cells (MOI of 1) during infection and post-infection. Cells were incubated at 37 °C for 72 h. Supernatants were collected and DENV2 infectivity were analyzed by plaque assay. Data were plotted and the EC$_{90}$ values were calculated by nonlinear regression analysis. Each drug concentration was tested in duplicate and results were confirmed by two independent experiments.
CHAPTER 7
CONCLUSION AND FUTURE DIRECTION

In the past decade, drug repositioning was one of the major areas of activity in academic drug discovery (Oprea et al., 2011). The field requires a multidisciplinary collaboration from basic, translational, to clinical research, and often times, new mode of actions of the approved drugs have been discovered. Examples of drugs or classes of drugs are raltegravir, cyclobenzaprine, benzbromarone, mometasone furoate, astemizole, R-naproxen, ketorolac, tolfenamic acid, phenothiazines, methylergonovine maleate and beta-adrenergic receptor drugs. Based on this idea and our previous data on 8-OHQ inhibiting flaviviruses (Mueller et al., 2008, Lai et al., 2013), we looked into related quinolone compounds for possible antiviral properties. Quinoline derivatives have been widely studied as antimalarial drugs since the 1900s ((O’Neill et al., 1998). However, several FDA-approved drugs were discontinued due to emergence of drug resistance. Drug resistant pathogens are a serious problem for long-term treatment regimens. However, since DENV causes acute infections, emergence of drug resistant strains of DENV may not be a serious concern.

AQ is an FDA-approved drug and is currently prescribed to treat uncomplicated malaria (600-800 mg/day for 3 days). As discussed in Chapter 4, extensive pharmacogenetic and toxicity studies have given clinically useful information for dose-adjustment and increased safety precautions for poor metabolizer patients.

A series of experiments were done to validate AQ efficacy against DENV2 replicon replication. AQ effectively inhibited DENV2 replicon replication measured by RLuc expression. In two different experimental settings, we showed that AQ inhibited DENV2 replicon replication
with a consistent therapeutic index of 7.3 and 7.4. The inhibition was not due to the drug interfering with the Rluc reporter activity. However, there was no reduction in replicon RNA levels between AQ-treated and untreated DENV2/BHK-21 cells measured by qRT-PCR. On the other hand, AQ effectively inhibited DENV2 infectivity with an EC\textsubscript{90} of 2.69 ± 0.40 μM as determined by plaque assays. We also detected corresponding significant reductions of intracellular RNA, extracellular RNA, and infectious virions in DENV2 infected BHK-21 cells. Our results suggested that AQ is a potentially good antiviral compound for treatment of DENV infection.

We studied the mode of action of AQ in order to understand the drug’s effect at each stage of the virus life cycle. Time-course analysis indicated that the drug was stable and effective until at least 96 h post-infection. Early stages including viral attachment, internalization, and fusion were slightly affected by the drug. However, the viral replication step was strongly affected by the drug. Inhibition of viral replication was maximized when AQ was added to DENV2 infected BHK-21 cells within 15 h post-infection. Data from the \textit{in vitro} enzyme assays indicated that AQ did not target viral protease, methyltransferase, or RNA-dependent RNA polymerase. It is tempting to speculate that AQ’s target is likely to be a component of viral replicase complex involved in the assembly of the complex, replication initiation, or viral replication. Deciphering the exact mechanism of action of the drug would be useful in lead optimization.

Structurally similar quinolines, chloroquine (CQ) and ferroquine (FQ), were also shown to inhibit infectivity of DENV2 (Farias et al., 2013), and HCV (Vausselin et al., 2013), respectively. Although these drugs acted at different stages, their mechanisms of action were
strongly associated with acidification of endosomes. However, we did not find any correlation between AQ and vesicular acidification in our study. Our structure-activity relationship study revealed the importance of diethylaminomethyl group of AQ for its antiviral activity.

As a follow-up to our in vitro studies, we will be studying the efficacy of AQ in vivo using the AG129 mouse model of DENV pathogenesis (Johnson and Roehrig, 1999) (Williams et al., 2009) (Calvert et al., 2006, Kyle et al., 2008, Perry et al., 2011, Tan et al., 2011). AG129 mouse has IFN-α/β and IFN-γ receptor deficiencies and is used for testing the efficacy of not only antivirals (Schul et al., 2007) (Schul et al., 2013) (Lim et al., 2013) (Chen et al., 2010) but also candidate vaccines for DENV (Huang et al., 2003) (Johnson and Roehrig, 1999) (Suzuki et al., 2009, Brewoo et al., 2012). Available data on AQ pharmacology and toxicology will be useful in designing proper dosing regimen in mice. According to the Registry of Toxic Effects of Chemical Substances (RTECS, 1985-1986), lethal doses (LD50) in mice were 225 mg/kg (intraperitoneal) and 550 mg/kg (oral) (Wayne A. Temple, 1993). We will first study the toxicity of AQ on AG129 mice and test the efficacy for protection of mice challenge with DENV.
PART II
FUNCTIONAL ANALYSIS OF FLAVIVIRAL NS5
IN 5’CAPPING
CHAPTER 1
INTRODUCTION II

1.1. Flaviviral RNA capping

The mosquito-borne Flaviviruses are classified as positive strand RNA viruses as their genomes serve as mRNAs in the infected host and are translated by the host translational machinery. The 5’-end of flavivirus RNA has a conserved dinucleotide AG and a type I cap structure ($^{7}$MeGpppA$_{2'OMe}$) in which the guanine base at the 7-position and the 2’OH of the ribose moiety of 5’-A are methylated (Wengler et al., 1978) like most eukaryotic mRNAs (reviewed by Decroly et al., 2012). Mimicking the host mRNA, the viral capped RNA can utilize the host’s cap-dependent translational machinery. The 5’-cap also protects the genome from 5’exonuclease-mediated degradation and/or evasion of innate immunity (RIG-I/MDA5 pathway) that targets uncapped ssRNA (Xagorari and Chlichlia, 2008). The 5’- capping of flavivirus RNA consists of 4 sequential steps: removal of the γ-phosphate of triphosphorylated RNA generating diphosphorylated RNA (pp-A—RNA), addition of guanine monophosphate from GTP to the diphosphorylated RNA to form GpppA—RNA, and the two methylation reactions catalyzed by NS5 MTase domain (N7 MTase and 2’O MTase) in the presence of SAM to form $^{7}$MeGpppA$_{2'OMe}$—RNA, $^{7}$MeGpppA—RNA, and GpppA$_{2'OMe}$—RNA (Egloff et al., 2002, Ray et al., 2006, Dong et al., 2007, Egloff et al., 2007, Zhou et al., 2007, Liu et al., 2010, Dong et al., 2012) (Fig. 7.1).

The N terminal domain (265 amino acids) of the nonstructural protein 5 (NS5) is sufficient for both N7 and 2’O methylations (reviewed by Bollati et al., 2010). Methyllations occur at N7 followed by 2’OH in both WNV (Zhou et al., 2007) and DENV2 (Chung et al.,...
An RNA substrate of at least 74 nucleotides in length is required for N7 MTase activity (Dong et al., 2007), whereas short RNA oligonucleotides \((\mathrm{7MeGpppAC}(5)\) and \(\mathrm{GpppAC}(5)\)) are sufficient to serve as substrates for the 2’O MTase activity (Selisko et al., 2010). Mutational analysis (Ray et al., 2006, Zhou et al., 2007) revealed distinct mechanisms involved in the two catalytic steps; D146 is critical for N7 methylation whereas the KDKE motif (K61-D146-K181-E217 in DENV2 and K61-D146-K182-E218 in WNV) is important for 2’O methylation. A substrate repositioning model has been proposed (Dong et al., 2008b) for sequential N7 and 2’O methylation reactions.

The C-terminal domain of flaviviral NS3 has an RNA triphosphatase activity that hydrolyzes the g-P of the triphosphorylated RNA (Yon et al., 2005) and this activity is enhanced by interaction with NS5. Recently, the N-terminal region of NS5 was also reported to have a guanylyltransferase activity (GTase) which transfers GMP from the hydrolysis of GTP to the diphosphorylated RNA produced by NS3 (Issur et al., 2009a). The GTase activity was reported to be enhanced by interaction with NS3. NS3 and NS5 proteins are two crucial components of the replication and 5’-capping complexes involved in the flaviviral life cycle (Kapoor et al., 1995). Their interaction domains have been mapped (Johansson et al., 2001).

1.2. Scope of the thesis II

From current knowledge, NS3 and NS5 are essential factors involved in flaviviral 5’-capping. It is a generally accepted notion that the cap is added at some stage during the positive strand RNA synthesis. In this section, I summarize my results obtained toward a better understanding of the 5’-capping mechanism although more work remains to be done.
Aims of this dissertation are as follows;

Aim 1: To characterize MTase activity \textit{in vitro}

Aim 2: To characterize GTase and GTPase activities \textit{in vitro}
Fig. 7.1 Flaviviral 5’-capping mechanism

pppA ----------- RNA  
           ↓ NS3  RNA triphosphatase
ppA- RNA  
           ↓ NS5  GTP  Guanylyltransferase
GpppA- RNA  
           ↓ NS5  SAM  Methyltransferase
7MeGpppA- RNA
GpppA2’OMe RNA
7MeGpppA2’OMe RNA
CHAPTER 2
MATERIALS AND METHODS II

2.1. Materials II

2.1.1. Molecular cloning reagents

All stock solutions of buffers and reagents were prepared using molecular biology grade chemicals and Milli-Q H₂O. RNase-free water was used in all assays involving RNA substrate. All reagents were stored at room temperature unless indicated otherwise. All recombinant DNA work was done following established protocols and regulations by the Institutional Biosafety Committee for handling biohazard materials such as recombinant DNA and plasmid-transformed *E. coli* waste disposal.

Phusion (High Fidelity) DNA polymerase was purchased from Thermo Scientific (Pittsburgh, PA). Oligodeoxynucleotides primers were purchased from Integrated DNA Technology (Table 8.1). Deoxyribonucleoside triphosphates mixture (10 mM each), 100 bp and 1 kb ladders, restriction enzymes (NdeI and HindIII-HF), and DH5α and BL21 (DE3) competent cells, were purchased from New England Biolab. T4 DNA ligase, Luria-Bertani (LB) powder, glucose, kanamycin, isopropyl-β-d-thiogalactopyranoside (IPTG) and other chemicals were purchased from Fisher Scientific.

2.1.2. Protein purification reagents and preparation

Protein purification reagents: Buffer A (50 mM Tris-HCl, pH 7, 300 mM NaCl, 10% glycerol) was prepared by mixing autoclaved 2X buffer-salt solution (100 mM Tris-HCl, pH 7, 600 mM NaCl) with 50% glycerol (5X) and deionized H₂O. Lysis buffer (50 ml per 1 L bacterial
culture) was freshly prepared from buffer A (50 ml) and 500 μL NP-40 (Nonidet P-40; octylphenoxypolyethoxyethanol) to the final concentration of 1%, a tablet or 500 μl of protease inhibitor cocktails, and 2-3 mg lysozyme. Imidazole (1 M in buffer A) was freshly prepared just before use. To make 500 mM imidazole elution buffer, the stock was diluted in buffer A (1:1). Wash buffers (buffer A with 5, 10, 15, 20, or 25 mM imidazole) were prepared by serial dilution of the imidazole stock. Protease inhibitor cocktails (without EDTA), Talon resin and a column were purchased from Fisher Scientific (Pittsburgh, PA). Dialysis buffer: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM MgCl₂, and 40% glycerol was prepared by mixing autoclaved buffer, salt and glycerol. DTT (1 mM at the final concentration) was added to the buffer just before the dialysis step. The dialysis membrane (10,000 Daltons cut-off) was prepared according to the manufacturer’s protocol. Quantification of nucleic acids (DNA and RNA) was done by spectrophotometry and confirmed by gel electrophoresis. The gel conditions for DNA and RNA were 0.7-2% native agarose electrophoresis, and 7 M urea-8% TBE (89 mM Tris-HCl, pH 8.3, 89 mM Boric acid, 2 mM EDTA) -polyacrylamide gel electrophoresis, respectively. Quantification of protein was done by Pierce BCA (bicinchoninic acid) protein assay kit, and confirmed by 10-12% SDS-PAGE.

2.1.3. RNA substrate preparation

The RNA substrate consists of the 5’- terminal 200 nucleotides of DENV2 genome. The cDNA was PCR amplified from DENV2 mini genome (pSY2) (You and Padmanabhan, 1999) using the following forward and reverse primers, respectively: D2_200_F and D2_200_R (Table 8.1). MEGAscript from Ambion (Life Technologies, Grand Island, NY) was used for T7
RNA polymerase-catalyzed *in vitro* transcription. Micro Bio-Spin 30 columns Bio-Rad (Hercules, CA) were used to remove unincorporated nucleotides. Acid Phenol/Chloroform (RNase free) was purchased from Invitrogen (Life Technologies). RNA clean and concentrator kits (TM-5, TM-25) were purchased from Zymo research.

### 2.1.4. MTase assay reagents

MTase assay: 10x MTase Buffer (500 mM Tris-HCl, pH 7.5, 100 mM KCl, 20 mM MgCl₂, and 10 mM DTT) was prepared with RNase-free water, and stored in aliquots (10 µL) at -20 °C. Radioactive \([\alpha-^{32}P]GTP\) was purchased from Perkin-Elmers. S-adenosylmethionine (SAM, 32 mM) was purchased from New England Biolab, dissolved in RNase-free water to give a stock solution of 0.8 mM, and stored in aliquots (10 µL) at -20 °C. ScriptGuard RNase inhibitor (40 U/µL) was purchased from CellScript (Madison, WI). Nuclease P1 and PEI cellulose plates were purchased from Sigma Aldrich. Nuclease P1 was diluted with RNase free water to the concentrations of 4 U/µL; and stored in aliquots at -20 °C.

Vaccinia virus capping enzymes: ScriptCap m7G capping system and ScriptCap 2’O methyltransferase kit were purchased from Cellscript (Madison, WI). Phosphoimager screen and the Storm 840 scanner were from Amersham Bioscience (Sunnyvale, CA).

### 2.1.5. GTase assay reagents

GTase assay: 10x GTase buffer (500 mM Tris-HCl, pH 7.5, 50 mM MgCl₂, and 50 mM DTT) was prepared with RNase-free water, and stored in aliquots (10 µL) at -20 °C. RNA substrate was prepared by *in vitro* transcription. Gel-loading buffer II (denaturing PAGE)
consisting of 95% Formamide, 18 mM EDTA, and 0.025% SDS, Xylene Cyanol, and Bromophenol Blue was purchased from Ambion (Life Technologies). Vaccinia virus capping enzyme (m7ScriptCap), and [α-32P]GTP were purchased as described above.

2.1.6. GTPase assay reagents

GTPase assay: 10x GTPase buffer (500 mM Tris-HCl, pH 7.5, 100 mM KCl, 20 mM MgCl₂, and 20 mM DTT) was prepared with RNase-free water, and stored in aliquots (10 µL) at -20°C. Calf intestinal alkaline phosphatase (CIP) was purchased from NEB.

2.2. Methods II

2.2.1. Construction of DENV2 NS5 MTase1-265aa and DENV2 NS51-405aa

The coding regions for DENV2 NS51-265aa and DENV2 NS51-405aa were amplified from a DENV2 NS5 expression plasmid (Ackermann and Padmanabhan, 2001) with the forward primer D2MT F TG, and the reverse primers D2MT R HindIII for the construction of DENV2 NS5-MTase1-265aa plasmid, and D2MTE1215 R HindIII for the construction of DENV2 NS51-405aa plasmid (Table 8.1). The PCR fragments were cloned into the pET28b vector using standard recombinant DNA techniques. *Escherichia coli* (E. coli) DH5α competent cells were transformed with the expression plasmids and the positive clones were identified and verified by DNA sequence analysis.
2.2.2. Expression and purification of the NS3 and NS5 proteins

_E. coli_ BL21(DE3) competent cells were transformed with DENV2 NS5-MTase1-265aa and DENV2 NS51-405aa recombinant plasmids. The transformed bacteria were grown in LB supplemented with 30 µg/ml kanamycin and 0.5% w/v glucose at 37 °C until an OD_{600nm} of 0.6 was reached. Cells were pelleted and suspended in LB containing 30 µg/ml kanamycin and 1 mM isopropyl-β-d-thiogalactopyranoside and incubated at 16 °C for 24 h. Cells were harvested by centrifugation at 5000 x g at 4 °C for 10 min, washed once with 50 mM Tris-HCl, pH 7.5 and 200 mM NaCl, centrifuged at 5000 x g 4 °C for 10 min, and stored at -80 °C until use. The pellets were suspended in lysis buffer (50 mM Tris-HCl, pH 7, 300 mM NaCl, 10% glycerol, 1% NP-40, 0.5 mM protease inhibitors cocktail VII (Calbiochem), 2-3 mg per 50 ml lysozyme and incubated on ice for 30 min. The cell membranes were disrupted by 15 cycles of a 10 sec ultrasonic burst with a 50 sec interval on ice (Misonix Sonicator Model 3000). The bacterial lysates were centrifuged at 18,000 x g at 4 °C for 60 min and the supernatants were collected and filtered through 0.45 µm filters (Millipore Corp.). The soluble fraction was incubated at 4 °C overnight with Talon resin (Clontech) which was pre-equilibrated with buffer A (50 mM Tris-HCl, pH 7, 300 mM NaCl, 10% glycerol). The protein bound resin was centrifuged at 500 x g 4 °C for 10 min, and washed with 3 volumes of 4 °C buffer A. Proteins were eluted with freshly prepared buffer A containing 500 mM imidazole and dialyzed at 4 °C overnight against 2 liters of dialysis buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 40% glycerol, and 1 mM DTT. The concentrations and purity were checked with BCA assay kits (Pierce) and SDS-PAGE (12%) gel, respectively.
DENV2 NS5_{FL} was expressed and purified as previously described (Manzano et al., 2011) except for the inclusion of an additional FPLC step. A deletion mutant encoding the RdRP domain alone at the C-terminal of NS5 was cloned by Dr. Tadahisa Teramoto (Georgetown University) expressed and purified as described for the pQE32-NS5_{FL} protein (Ackermann and Padmanabhan, 2001). The WNV NS5_{FL} was expressed and purified as described (Nomaguchi et al., 2004). The DENV3 NS5_{FL} protein was a gift from Dr. Kay Choi (The University of Texas Medical Branch).

DENV2 NS2B-NS3_{FL(H51A)} mutant was expressed and purified as described (Yon et al., 2005).

2.2.3. MTase assay

The G-capped RNA was generated using the vaccinia virus-encoded capping enzyme (m7ScriptCap) following the manufacturer’s protocol. Briefly, the reaction mixture containing 1 mM GTP, 1 µCi $[^{32}\text{P}]$GTP, 4 units RNase inhibitor in the buffer provided by the manufacturer and 1 µg RNA were incubated at 37 °C for 1 h. The G-capped RNA was recovered from the reaction by passage through a P-30 column (Bio-Rad), followed by concentration using the RNA Clean and Concentrator kit (Zymo research). The MTase assay was performed in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl$_2$, 1 mM DTT, 4 units RNase inhibitor 400 nM S-adenosylmethionine (SAM), 1 µg RNA, and 500 nM DENV2 NS5_{FL} or as specified. The reaction mixture was incubated at 37 °C for 1 h. The RNAs were extracted and treated with nuclease P1 (Sigma Aldrich). The products were fractionated by thin layer chromatography (TLC) on cellulose PEI plates, which were developed for ~90 min with 0.45 M
ammonium sulfate as the solvent. The plate was dried and exposed to a phosphoimaging screen. The resulting images were quantified using ImageJ. In later experiments, the concentration of NS5 was reduced to 50 nM/assay.

2.2.4. GTase assay

The RNA substrate consists of the N terminal 200 nucleotides of the DENV2 genome. The cDNA was PCR amplified from a DENV2 mini genome (pSY2) (You and Padmanabhan, 1999) using D2_200_F and D2_200_R as the forward and reverse primers, respectively (Table 8.1). The PCR fragment was used as a template for in vitro transcription catalyzed by T7 RNA polymerase (MEGAsortscript) at 37 °C for 6 h following the manufacturer’s protocol. The transcription reaction products were then digested with DNase to remove the DNA template. The RNA was quantified by spectrophotometry and stored in aliquots (1 µg) at -80 °C per tube. The GTase assay was started with pretreatment of 1 µg DENV2 RNA substrate with vaccinia virus-encoded capping enzyme (m7ScriptCap) at 37 °C for 1 h in 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 4 units of RNase Inhibitor (NEB, Ipswich, MA). The RNA was recovered using the RNA clean and concentrator kit (Zymo research) and incubated with 500 nM DENV2 NS5FL (in the same buffer containing 1 mM GTP, 1 µCi [α-32P]GTP, 4 units RNase Inhibitor at 37 °C for 1 h. The reaction mixture was then inactivated with gel loading buffer II (Ambion), heated to 95 °C for 5 min and fractionated by PAGE (8%) containing 7 M urea. The position of the RNA in the gel was detected using ethidium bromide staining and visualized under UV. Radiolabeled RNA was quantified by phosphoimaging and ImageJ software. Data was plotted using GraphPad Prism v5 software.
2.2.5. GTPase assay

The substrates, 1 mM GTP and 1 µCi [$\alpha$-$^{32}$P]GTP, were incubated with 500 nM DENV2 NS5$_{FL}$, 500 nM DENV2-NS2BH-NS3$_{FLH51A}$, 1 µg di or triphosphorylated RNA, 80 µM SAM in buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2.5 mM MgCl$_2$, 1 mM DTT, 4 units of RNase inhibitor. The reaction was incubated at 37 °C for 2 h and stopped by heating to 95 °C for 5 min. Guanosine species were fractionated by thin layer chromatography (TLC) on PEI cellulose plates, which were developed with 0.45 M ammonium sulfate as the solvent. Plates were dried, exposed and quantified as previously described in MTase assay.

In time-course analyses, 1 mM GTP, 1 µCi [$\alpha$-$^{32}$P]GTP, 50 nM DENV2 NS5$_{FL}$, 50 nM DENV2-NS2BH-NS3$_{FLH51A}$, and 80 µM SAM were incubated in 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2.5 mM MgCl$_2$, 1 mM DTT, 4 units of RNase inhibitor for 120 min at 37 °C. Samples were collected at 1, 5, 10, 15, 20, 25, 30, 60, 90, and 120 min time points by heating to 95 °C for 5 min. Guanosine species were analyzed as discussed above.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2MT F TG</td>
<td>5’-TGGGAACTGGCAACATAGGAGAGACGC-3’</td>
</tr>
<tr>
<td>D2MT R HindIII</td>
<td>5’-TAATAAAAGCTTGATGTTACGTTCCGCTTCC-3’</td>
</tr>
<tr>
<td>D2MTE1215 R HindIII</td>
<td>5’-TAATAAAAGCTTGCTTCACCTTCTTGTGAATTCTTCTCT-3’</td>
</tr>
<tr>
<td>D2_200_F</td>
<td>5’-CAGTAATACGACTCACTATTAGTTGTAGTCTACGTG-3’</td>
</tr>
<tr>
<td>D2_200_R</td>
<td>5’-AGTGAGAATCTCTTTGTCAG-3’</td>
</tr>
</tbody>
</table>
Fig. 8.1 Diagram of flaviviral NS5
Fig. 8.2 DENV2 RNA\textsubscript{nt1-200} substrate and its DNA template shown on 2% native agarose gel electrophoresis.
Fig. 8.3 DENV2 NS5_1-265aa (left panel) and DENV2 NS5_1-405aa (right panel) after His-tag purification shown on 12% SDS-PAGE.
Fig. 8.4 DENV2 NS5$_{FL}$ after His-tag purification (lane 2) and size exclusion by FPLC (lane 3) shown on 10% SDS-PAGE.
CHAPTER 3
CHARACTERIZATION OF FLAVIVIRAL MTASE ACTIVITY

3.1 MTase activity test

3.1.1 Identification of radiolabeled 5’-capped products

To detect the methylated species, [α-32P]GTP was used as the substrate for the vaccinia virus-encoded GTase-catalyzed addition of the guanosine cap to the diphosphorylated RNA prior to the two methylation steps. Gp*ppA—RNA (* represents [32P]-label) that is formed prior to the two methylations has an advantage of visualizing directly the unmethylated, mono- and di-methylated species (Gp*ppA, Gp*ppA2’OMe, 7MeGp*ppA, and 7MeGp*ppA2’OMe) by autoradiography using a phosphoimaging. The unmethylated capped RNA (GpppA—RNA) was treated with no enzyme (Fig. 9.1, lane 1), vaccinia virus N7 MTase (m7ScriptCap) (Fig. 9.1, lane 2), vaccinia virus 2’O MTase (ScriptCap 2’O) (Fig. 9.1, lane 3), and both vaccinia virus MTases (Fig. 9.1, lane 4). The RNAs were extracted and recovered as described in subheading 2.2.3. Thin layer chromatography (TLC) had been used to separate the unmethylated, monomethylated and dimethylated species (Rahmeh et al., 2009) using solvents such as 1.2 M LiCl or 0.4 M (NH4)2SO4 (Rahmeh et al., 2009). In this study, we used 0.45 M (NH4)2SO4 for separation of GpppA, GpppA2’OMe, 7MeGpppA, and 7MeGpppA2’OMe by TLC on PEI cellulose coated plates. The 5’-capped species formed in the reactions catalyzed by the vaccinia virus N7 and/or 2’O methyltransferases were used as mobility markers for detection of the corresponding species formed in the DENV2 NS5 MTase catalyzed reaction (Fig. 9.1).
3.1.2 Time-course of DENV2 NS5\textsubscript{1-265aa} N7 and 2’O methyltransferase catalyzed reactions

In previous studies, buffers at different pH and ionic strengths were used for the two methylation reactions, pH 7 for N7 and pH 10 for 2’O MTases. However, more recently, a pH 7.5 buffer with slight difference in ionic strength that was applicable to both methylation steps was described (Chung et al., 2010). In this study, we used a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl\textsubscript{2}, and 1 mM DTT for both 2’O and N7 methylations (replacing 20 mM NaCl with 10 mM KCl, 2 mM MgCl\textsubscript{2}). Construction of the expression plasmid encoding the DENV2 MTase domain (NS5\textsubscript{1-265aa}) was described in Methods section. The MTase domain was expressed in \textit{E. coli} and the protein was purified, as well as RNA substrate. The MTase activities of the purified proteins were tested using both unmethylated and N7 methylated capped RNA (Fig 9.2). The reactions were incubated for 90 min and samples were taken at 1, 5, 10, 15, 20, 25, 30, and 60 min time points.

With the modified buffer and unmethylated G*ppp-RNA substrate, the double methylated cap analog (\textsuperscript{7}MeG*pppA\textsubscript{2’OMe}) was detected as the final product, as well as the single methylated byproduct (G*pppA\textsubscript{2’OMe}). Both species were produced at similar rates and proportion. Moreover, the G*pppA\textsubscript{2’OMe} species was recognized for the first time as a product of DENV2 NS5 methyltrasnferase activity. Using the N7-methylated RNA substrate, we also observed the double methylated product (\textsuperscript{7}MeG*pppA\textsubscript{2’OMe}) generated by 2’O methyltransferase activity of DENV2 NS5\textsubscript{1-265aa}. In conclusion, this modified buffer, incubation, and conditions of fractionation of products yielded optimal results to further study DENV2 NS5 methyltransferase activity.
3.1.3 Validation of DENV2 MTase assay using a known inhibitor, S-adenosylhomocysteine (SAH)

In order to develop the assay for screening compound libraries we tested its sensitivity using a known inhibitor of MTase, SAH (Dong et al., 2007, Zhou et al., 2007). SAH was serially diluted (4-fold) and pre-incubated with DENV2 NS5_{1-265aa} and G*pppA-RNA for 15 min before addition of the SAM substrate. The assay was continued for 60 min and the cap analogs were analyzed as described. IC_{50} of SAH (33.06 ± 9.42 μM) was calculated from non-linear regression curve fit of quantitated methylated cap products (\(^{7}\text{Me}\)G*pppA_{2′OMe} and \(^{7}\text{Me}\)G*pppA) combined. IC_{50} of SAH derived from our assay was similar to that of a previous report (IC_{50} ~75 μM) (Chen et al., 2013). We conclude that the assay is suitable to screen methyltransferase inhibitors.

3.2 Comparison of the methyltransferase activities of DENV2 NS5_{FL} and DENV2 NS5_{1-265aa} in a time course experiment

Unmethylated capped RNA substrate was used as a substrate in this assay. The experiment was conducted using the buffers and conditions as described above. Time points were taken at 1, 5, 10, 20, 30, 60, and 90 min. The 0 min time point was a no-enzyme control. Guanine cap analogs were fractionated by TLC (Fig. 9.4, upper panel), and quantification of the final product (\(^{7}\text{Me}\)GpppA_{2′OMe}, or type I cap) (Fig. 9.4, lower left panel) and the byproduct (GpppA_{2′OMe}) (Fig. 9.4, lower right panel), were done using a phosphoimaging. DENV2 NS5_{FL} showed a higher level of the final type I cap product (\(^{7}\text{Me}\)GpppA_{2′OMe}) compared to the NS5 MTase domain alone (DENV2 NS5_{1-265aa}). In 10 minutes, DENV2 NS5_{FL} produced a saturating level of type I cap species (~40%), while DENV2 NS5_{1-265aa} produced only ~24%. The
byproduct, GpppA₂'OMe species, was also saturated (~49%) at 10 minutes in the DENV2 NS5₅FL-catalyzed reaction whereas only it took ~60 minutes to reach this level (48%) in the DENV2 NS5₁-265aa-catalyzed reaction.

3.3 Comparison of the methyltransferase activities of various flaviviral NS₅s

The experiment was conducted using the buffers and conditions as described in the preceding experiment (subheading 2.2.3). Guanine cap analogs generated from vaccinia virus species were used as markers; GpppA, GpppA₂'OMe, ⁷MeGpppA, and ⁷MeGpppA₂'OMe. Various NS₅s at 500 nM were used in MTase reactions as described in each lane (Fig. 9.5, upper panel). We noticed that NS₅₅FL regardless of the species of origin; DENV2 NS₅₅FL (Fig. 9.5, lane 1), WNV NS₅₅FL (Fig. 9.5, lane 5), DENV3 NS₅₅FL (Fig. 9.5, lane 6), chimeric DENV4 NS₁-270aa-DENV2 NS₅₂₇₁-₉₀₀aa (Fig. 9.5, lane 7), and chimeric DENV4 NS₁-270aa(K74I)-DENV2 NS₅₂₇₁-₉₀₀aa (Fig. 9.5, lane 8) were equally efficient in type I cap species (⁷MeGpppA₂'OMe) production. On the contrary, homologous NS₅ domains; DENV2 NS₁-2₆₅aa (Fig. 9.5, lane 2), DENV2 NS₁-₄₀₅aa (Fig. 9.5, lane 3), and DENV2 NS₅₂₇₁-₉₀₀aa (Fig. 9.5, lane 4), were less active in type I cap species (⁷MeGpppA₂'OMe) production. The radiolabeled cap analogs were measured and calculated as percentage of the total radiolabeled substrate used. The table (Fig. 9.5, lower panel) shows the percent of each species relative to that obtained with DENV2 NS₅₅FL (Fig. 9.5, lane 1) set at 100%. Results showed that type I cap species (⁷MeGpppA₂'OMe) were relatively higher in reactions by all heterologous NS₅₅FLs (Fig. 9.5, lane 5-8), but relatively lower in homologous DENV2 NS₅ domains; DENV2 NS₅₁-₂₆₅aa (Fig. 9.5, lane 2), and DENV2 NS₅₁-₄₀₅aa (Fig. 9.5, lane 3) (MTase₁-2₆₅aa and NS₅₁-₄₀₅aa containing the NS3 binding domain of 3₆₉-₄₀₅aa). The type
I cap species ($^{7}\text{MeGpppA}_{2}\text{OMe}$) was undetected in DENV2 NS5$_{271-900}$aa (RdRp domain) (Fig. 9.5, lane 4). We concluded that flaviviral MTase reactions were independent of the sources species and that intact full-length NS5s were more efficient in performing MTase activities than partial NS5s.

3.4 Discussion

Until now, the only HTS assay utilized $^{3}\text{H-SAM}$ as a radiolabeled substrate. However, a false positive occurring from internal methylation of the RNA substrate could interfere with the screening (Dong et al., 2012). The $^{32}\text{P}$-labeled capped RNA can be used as an alternative HTS of in vitro methyltransferase assay. This assay has an advantage on characterization of the cap analogs. However, it still requires slight modification to be suitable for HTS.

The N-terminal domain of NS5 consisting of 265 amino acids is sufficient for the methyltransferase activity. Our results clearly showed that DENV2 NS5$_{FL}$ was more efficient in producing the type I cap species than the DENV2 NS5$_{1-265}$ domain suggesting that the folding of the full length NS5 is optimum in presenting the catalytic center of the MTase enzyme.

Contrary to the previous claim that flaviviral capped RNA serves as a serotype specific template for methylation (Dong et al., 2007), we find that DENV2 RNA serves equally well as a methylation template for heterologous NS5s.

We also reproducibly found for the first time the monomethylated, GpppA$_{2}\text{OMe}$, species as a byproduct in our MTase assays. This particular cap species is uncommon since it has never been recorded in mammalian cells. The only document we found was in the mRNAs of insect
oocytes, which was proposed as the translational inactivation mechanism prior to switching to fertilization (Kastern and Berry, 1976).
Fig. 9.1 Guanine cap analogs on TLC analysis. The cap analogs generated from MTase reactions of GpppA—RNA with no enzyme (lane 1), vaccinia virus N7 MTase (ScriptCap m7G) (lane 2), vaccinia virus 2’O MTase (ScriptCap 2’O) (lane 3), and both vaccinia virus MTases (lane 4). Guanine cap analogs processed from nuclease P1 treated RNA were fractionated by TLC and visualized by phosphoimaging.
**Fig. 9.2** Time-course analysis of DENV2 NS5\(^{1-265}\text{aa}\) MTase activities. The G*pppA-RNA (left upper panel) and 7MeG*pppA-RNA (right upper panel) substrates (1 μg) were incubated in the reaction containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl\(_2\), 1 mM DTT, 4 units RNase inhibitor, 400 nM SAM, and 50 nM DENV2 NS5\(^{1-265}\text{aa}\) at 37 °C for 90 min. Samples were taken at 1, 5, 10, 15, 20, 25, 30, 60 min time points. Guanine cap analogs processed from nuclease P1 treated RNA were fractionated by TLC and quantified by phosphoimaging. Each cap species was identified by comparing their retardation factors (\(R_f\)) to those of the established markers. The 7MeG*pppA\_2′OMe product and G*pppA\_2′OMe byproduct from GpppA-RNA (left lower panel) and 7MeGpppA-RNA (right lower panel) substrates were quantified and plotted as percentage of the products over time.
**Fig. 9.3** SAH inhibition of DENV2 MTase *in vitro*. Four-fold serial dilutions of SAH (left panel, concentrations labeled at each lane) were co-incubated with 500 nM DENV2 NS51-265aa and 1 μg G*pppA-RNA in the buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 4 units RNase inhibitor for 15 min before addition of 400 nM S-adenosylmethionine (SAM) substrate. The reactions were incubated at 37 °C for 60 min. Guanine cap analogs processed from nuclease PI treated RNA were fractionated by TLC and quantified by phosphoimaging (left panel). Reduction of the level of methylated cap products (7MeG*pppA₂'OMe and 7MeG*pppA) in different [SAH] was referred to the effectiveness of SAH to inhibit the reaction. Data were plotted and IC₅₀ of SAH was calculated by nonlinear regression analysis (right panel).
Fig. 9.4 Time-course analysis comparing MTase activities of DENV2 NS5_{FL} and DENV2 NS5_{1-265aa}. The G*pppA-RNA (upper panel) was incubated in the reaction containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 4 units RNase inhibitor, 400 nM SAM, and 50 nM DENV2 NS5_{FL} or DENV2 NS5_{1-265aa} at 37 °C for 90 min. Samples taken at 0, 1, 5, 10, 20, 30, 60, and 90 min time points (labeled at each lane). Guanine cap analogs processed from nuclease P1 treated RNA were fractionated by TLC and quantified by phosphoimaging. Each guanine cap analog was identified by comparing their retardation factors (Rf) to those of the established markers. The {}^{7}\text{Me}\text{G*pppA}_{2'\text{OMe}} product (lower left panel) and the G*pppA_{2'\text{OMe}} byproduct (lower right panel) from DENV2 NS5_{FL} and DENV2 NS5_{1-265aa} MTase activities were quantified and plotted as percentage of the products over time.
Fig. 9.5 MTase activities of various flaviviral NS5s. Various flaviviral NS5s at 500 nM (upper panel, labeled at each lane) were incubated with the DENV2 G*pppA-RNA in the reaction containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 4 units RNase inhibitor, and 400 nM SAM at 37 °C for 60 min. Radiolabeled guanine cap analogs generated from vaccinia virus MTases were used as markers. Guanine cap analogs processed from nuclease P1 treated RNA were fractionated by TLC and quantified by phosphoimaging. Each guanine cap species was normalized as percentage of the total radiolabeled substrate, and compared to DENV2 NS5FL (set as 100%) (lower panel).
CHAPTER 4
CHARACTERIZATION OF FLAVIVIRAL GTASE AND GTPASE ACTIVITIES

4.1 GTase activity of DENV2 NS5

Guanylyltransferase is involved in the second step of flaviviral 5’-capping. The enzyme carries out two consecutive steps; (1) GTP hydrolysis to GMP and (2) transfer of GMP moiety to diphosphorylated RNA. These enzyme activities were reported to be part of the N-terminal domain of NS5 by Issur et al 2009 (Issur et al., 2009b). In the study, the N-terminal domain of NS5 was shown to be covalently linked to GMP via a specific lysine residue. Also, the authors demonstrated the transfer of GMP to diphosphorylated WNV RNA_{nt1-81} by WNV NS5_{FL}. This activity was shown to be enhanced by interaction with NS3. Moreover, NS5 preferred binding to diphosphorylated RNA with a 5’ adenine and in the presence of GTP (Henderson et al., 2011).

However, we could not demonstrate the guanylyltransferase activity of DENV2 NS5_{FL} under conditions described in the literature. We used the vaccinia virus capping enzyme (m7G ScriptCap) as the source of RNA triphosphatase. To generate a 5’-diphosphorylated RNA substrate, the DENV2 RNA_{nt1-200} from the 5’ terminus of DENV2 was treated with vaccinia virus capping enzyme in the absence of GTP and SAM. The RNA was extracted and incubated with no enzyme (1), DENV2 NS5 MTase_{1-265} (2), DENV2 NS5_{FL} (3) or vaccinia virus capping enzyme (4) in the presence of GTP. The reaction mixtures were treated with proteinase K and extracted with phenol/chloroform. RNA was purified using RNA clean and concentrator kit. RNA was characterized in 7 M urea 8% PAGE gel (Fig. 10.1). Each lane had 500 ng or 7.5 μM of RNA substrate (except lane 4 containing 200 ng RNA) and was confirmed by ethidium bromide staining. Radiolabeled RNA signals from reactions that contained DENV2 NS5_{1-265aa} (2)
or DENV2 NS5FL (3) were indistinguishable from that of the reaction buffer (1) (Fig. 10.1, lanes 1-3). Moreover, in the presence of NS3, most GTP added to the reaction as a substrate for GTase was hydrolyzed to GDP instead of GMP by the NS5 proteins.

4.2 GTPase activity of DENV2 NS3 and NS5

For a better understanding of GTase activity of NS5, we sought to characterize the activities of NS5 in GTP hydrolysis, the first step in the GTase reaction to produce GMP and PPi prior to the transfer of GMP to the 5'-end of diphosphorylated RNA. In parallel, we also studied the classic GTPase activity of NS3. NS3 can hydrolyze any of the four NTPs and produce NDP and Pi. Activity varies with the substrate, ATP and GTP being the best substrates compared to CTP and UTP (Li et al. 1999).

To characterize the GTPase activities of these two proteins, GTP was treated with calf intestinal alkaline phosphatase (CIP). The GTP hydrolysis products produced by the phosphatase after one minute incubation were fractionated by TLC (Fig. 10.2, lane 8). GDP and GMP were formed as expected and served as the mobility markers on the TLC for the analysis of the products formed by NS5- and NS3-catalyzed hydrolysis of GTP. We observed that NS3 completely hydrolyzed GTP to GDP within 2 h at 37 °C (Fig. 10.2, lane 3). However, we found only a trace amount of GMP product when NS5 alone was incubated with GTP (Fig. 10.2, lane 1). Co-incubation of NS3 and NS5 (Fig. 10.2, lane 5) with GTP resulted in GDP as the predominant product suggesting that GTP hydrolysis activity of NS3 outcompeted that of NS5 GTPase activity as it was shown to be very weak. GTase and MTase activities in capping could be concerted reactions and RNA template and SAM are also involved as substrates in these
sequential reactions. Therefore, we investigated the effect of SAM addition on the GTP hydrolysis by NS5. SAM had no effect on NS5 (Fig. 10.2, lane 2) and NS3-NS5 co-incubation (Fig. 10.2, lane 6). The GTPase activity of NS3 could be inhibited by SAM (Fig. 10.2, lane 4), but not by triphosphorylated RNA (Fig. 10.3, lane 1-2).

In the presence of diphosphorylated RNA (ppRNA), NS5 had increased GTPase activity to produce GMP (Fig. 10.4, lane 3-4). The ppRNA was prepared from pretreatment of pppRNA with NS3 which has the 5’-RNA triphosphatase activity. NS3 was later removed and RNA was recovered by RNA clean and concentrator kit. In a time-course experiment, we showed that formation of GMP increased with time of incubation with NS5FL (Fig. 10.5). In summary, we confirmed that NS5 was responsible for the first step of guanylyltransferase, GTP hydrolysis to GMP, and RNA was necessary for this activity. However, we are unable to demonstrate the transfer of GMP to the diphosphorylated RNA template produced by using either vaccinia virus 5’-RNA triphosphatase activity or that of DENV2 NS3. When we did the experiments in parallel using the vaccinia virus GTase (ScriptCap m7G), the transfer of GMP to the 5’-end of diphosphorylated RNA was very efficient (Fig. 10.1, lane 4).

4.3 Discussion

GTase structures and catalytic mechanisms are well conserved throughout all kingdoms of life with an N-terminal nucleotide transferase domain and a C-terminal oligonucleotide/oligosaccharide binding fold (OB fold) (Ferron et al., 2012). GTase has been difficult to identify in certain viruses since it is transiently formed (Orthoreovirus) or embedded within another protein (O. Mononegavirales). However, the conserved catalytic site always uses
a lysine residue in hydrolyzing GTP and forming a covalent link with GMP. Alignment of the DENV2 sequence with a known guanylyltransferase enzyme (CobY, PDB code 3rsb) revealed that the GTP binding domain of DENV2 could be within NS4A (2k region) and NS4B (hydrophilic region and trans-membrane domain 4) sequences (unpublished data). In future studies, we will explore the role of NS4B and NS4A as cofactors for the GTase activity of NS5.

Both flaviviral enzymes (NS3 and NS5) have GTP binding sites although they perform different enzymatic functions with different RNA and/or GTP substrates. Crystal structures have shown that NS5 amino acid F25 (DENV2) or F24 (WNV) is responsible for GTP/GpppA binding (Geiss et al., 2009, Yap et al., 2010). Also, the C-terminal domain of NS3 contains a DExH RNA helicase/NTPase catalytic triad and mutation at a K199E mutation attenuated the activities (Li et al., 1999). We believed that understanding the GTP hydrolysis pathway of NS3 and NS5 would give an insight to the capping mechanism.

The flaviviral NTPase activity of NS3 was stimulated by non-specific RNAs such as poly(A) and poly(U) (Li et al., 1999). On the contrary, the RNA triphosphatase (RTPase) activity was inhibited by ATP in a dose dependent manner due to competitive inhibition for the shared catalytic site (Bartelma and Padmanabhan, 2002). Moreover, the RTPase activity was enhanced by NS5 (Yon et al., 2005). We could not detect an RNA stimulated GTPase activity of NS3 using DENV2 RNAntl-200 (Fig. 10.3, lane 1-2). Detailed study of genomic RNA and NS3 GTPase activity is required in order to validate the finding. The NS3 GTPase activity (GTP to GDP) is undesirable in the capping reaction since it will deplete the GTP required for the capping enzyme. SAM inhibited NS3 GTPase only when NS3 alone was present in the system.
(Fig. 10.2, lane 4). It might be possible that SAM is a regulator of the GTPase/RTPase switch but more detailed study is also required to validate this conclusion.

We also found that NS5 was not a good GTPase since only a trace amount of GMP was formed when NS5 was incubated with GTP (Fig. 10.2, lane 1). However, the proportion of GMP produced increased in the presence of diphosphorylated RNA (ppRNA) formed by NS3 (Fig. 10.4, lane 3-4). Diphosphorylated RNA had the highest binding affinity for flaviviral NS5 (Henderson et al., 2011). We hypothesized that GTase activity could be enhanced with ppRNA by an induced fit mechanism of the other substrate (GTP). Detailed study will be done in the future.
Fig. 10.1 GTase activity of DENV2 NS5s. The diphosphorylated DENV2 RNA_{nt1-200} substrate was incubated with no enzyme (1), DENV2 NS5_{1-265aa} (2), DENV2 NS5_{FL} (3), and vaccinia virus capping system (m7ScriptCap) (4) in the reaction containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 1 mM GTP, 1 μCi [α-^{32}P]GTP, 4 units of RNase inhibitor at 37 °C for 1 h. The RNAs were characterized by denaturing PAGE and radioactive signals were quantified by phosphoimaging. Signals from radiolabeled DENV2 RNA_{nt1-200} were compared to DENV2 NS5_{FL} (3) set at 100%.
Fig. 10.2 GTPase activity of DENV2 NS3 and/or NS5 and the effect of SAM. The substrates, 1 mM GTP and 1 µCi [α-32P]GTP, were incubated with 500 nM DENV2 NS5 (lane 1, 2), 500 nM DENV2 NS3 (lane 3, 4), or both NS3 and NS5 (lane 5, 6), and 80 μM SAM (lane 2, 4, 6) in the buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2.5 mM MgCl₂, 1 mM DTT. The reaction was incubated at 37 °C for 2 h and stopped by heating to 95 °C for 5 min. Guanosine species were fractionated by TLC and quantified by phosphoimaging. The reaction with no-enzyme was used as a negative control (lane 7). CIP was used to generate radiolabeled guanosine species (lane 8).
Fig. 10.3 GTPase activity of DENV2 NS3 and the effect of triphosphorylated RNA. The substrates, 1 mM GTP and 1 µCi [α-32P]GTP, were incubated with 500 nM DENV2 NS3 (lane 1, 2) and 1 µg pppRNA_{nt1-200} (lane 1, 3) in the buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 4 units of RNase inhibitor. The reaction was incubated at 37 °C for 2 h and stopped by heating to 95 °C for 5 min. Guanosine species were fractionated by TLC and quantified by phosphoimaging. The reaction with no-enzyme was used as a negative control (lane 4). CIP was used to generate radiolabeled guanosine species (lane 5).
Fig. 10.4 GTPase activity of DENV2 NS5 and the effect of diphosphorylated RNA. The substrates, 1 mM GTP and 1 μCi [α-\(^{32}\)P]GTP, were incubated with 500 nM DENV2 NS5 (lane 1, 2, 3, 4), 80 μM SAM (lane 2, 4), and 1 μg DENV2 ppRNA\(_{nt1-200}\) (pre-treated with NS3) (lane 3, 4) in the buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2.5 mM MgCl\(_2\), 1 mM DTT, 4 units of RNase inhibitor. The reaction was incubated at 37 °C for 2 h and stopped by heating to 95 °C for 5 min. Guanosine species were fractionated by TLC and quantified by phosphoimaging.

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**Fig. 10.5 Time-course analysis of GTPase activity.** The substrates, 1 mM GTP and 1 µCi [$\alpha$-$^{32}$P]GTP, were incubated with 50 nM DENV2 NS5, 50 nM DENV2 NS3, 80 µM SAM, and 1 µg DENV2 pppRNA<sub>nt1-200</sub> in the buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 2.5 mM MgCl<sub>2</sub>, 1 mM DTT, 4 units of RNase inhibitor for 120 min at 37 °C. Samples were collected at 1, 5, 10, 15, 20, 25, 30, 60, 90, and 120 min time points by heating to 95 °C for 5 min. Guanosine species were fractionated by TLC and quantified by phosphoimaging (upper panel). GMP product was quantified and plotted over time (lower panel).
In antiviral research, the 5’-capping has become a novel drug target for its specificity and potency (Geiss et al., 2011, Li et al., 2013, Saeedi and Geiss, 2013). Flavivirus requires a cap to start its initial translation (Lindenbach et al., 2007) and to evade from innate immunity (Xagorari and Chlichlia, 2008). Flavivirus encodes its own capping enzymes. There are structural differences in the viral and cellular capping machineries that the viral capping enzymes could potentially be a good drug target (reviewed by (Bartenschlager et al., 2013)). Although DENV RNA is 5’-capped and the cap is required for translation, in the presence of an inhibitor of eIF4E (the cap-binding translation initiation factor). DENV can switch from cap-dependent to a non-canonical cap-independent translation mode (Edgil et al., 2006). However, it has been reported that inhibitors of flaviviral N7-MTase lead to a complete suppression of viral replication (Ray et al., 2006, Dong et al., 2008b). The guanine cap is essential for viral translation and N7-methylation enhanced translation efficiency by 25 fold (Ray et al., 2006). Therefore, considering both specificity and potency, the 5’-capping machinery is a promising drug target.

Several questions still remain to be answered. For example, does the 5’-capping and positive strand synthesis occur in a concerted and coupled manner? Are there any cofactors involved for the GTase activity of NS5 that would enhance its activity in transfer of GMP to the dephosphorylated RNA? At what step of the viral replication, the 5’-cap is added? The RdRP activity of NS5 and the helicase activity of NS3 have been implicated in both negative and positive strands RNA synthesis. NS5 and NS3 exist as a complex in DENV-infected cells (Kapoor et al., 1995); and their activities are required in both viral replication and 5’-capping in
virus-induced membranous organelles (Chu and Westaway, 1992, Salonen et al., 2005, Roosendaal et al., 2006, Mackenzie et al., 2007) (reviewed by Bartenschlager et al., 2013). Common host factors involving in positive strand RNA synthesis had been categorized by their functions as follows; 1) RNA binding proteins facilitating the viral RNA synthesis 2) proteins involving in membrane-bound replication complex; 3) lipid synthesis enzymes (such as FASN); and 4) chaperones facilitating the protein folding (such as HSP70) reviewed by Nagy and Pogany, 2012. We believe that the listed questions could be better demonstrated with the knowledge of components in replication complex, as well as their functions, contributions, and interactions with the viral RNA synthesis and the 5’-capping.
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