Several compounds have been recently identified which are effective against dengue virus, a tropical arbovirus highly prevalent in southeast Asia and central America. Initial intrinsic fluorescence characterization of the mechanism of action of compound B reveals binding affinity to the dengue capsid protein. A molecular beacon unwinding assay and malachite green ATPase assay indicate that compound C works to disrupt a component of the RNA-dependant helicase ATPase activity, possibly by competitively inhibiting RNA binding. These results are preliminary and further study is warranted to fully characterize the action of all three compounds.

Key Words: Dengue virus, antiviral, capsid, helicase, NS3

Corresponding e-mail address: Schneider.Christine.a@gmail.com
Investigations into the Mechanism of Action of Dengue Virus Inhibitor Compounds

By

Christine A. Schneider

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APPROVED:

Mentor, representing Microbiology

Committee Member, representing Molecular and Cellular Biology

Committee Member, representing Microbiology

Chair, Department of Biochemistry and Biophysics

Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Christine A. Schneider, Author
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To my family and friends— thank you for listening to my lamentations and tribulations over the past year. You all kept my going and helped renew my spirits.

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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Background</td>
<td>1</td>
</tr>
<tr>
<td>Project Description</td>
<td>5</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>6</td>
</tr>
<tr>
<td>Cell Culture Efficacy and Cytotoxicity</td>
<td>6</td>
</tr>
<tr>
<td>Helicase Unwinding Assay</td>
<td>7</td>
</tr>
<tr>
<td>Capsid Purification and Expression</td>
<td>9</td>
</tr>
<tr>
<td>Intrinsic Fluorescence</td>
<td>9</td>
</tr>
<tr>
<td>ATPase Assay</td>
<td>10</td>
</tr>
<tr>
<td>RESULTS/DISCUSSION</td>
<td>11</td>
</tr>
<tr>
<td>CONCLUSION</td>
<td>20</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>22</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Dengue Virus Life Cycle</td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td>Gene Map of Dengue Virus</td>
<td>6</td>
</tr>
<tr>
<td>3.</td>
<td>Hairpin Induced Quenching</td>
<td>8</td>
</tr>
<tr>
<td>4.</td>
<td>Molecular Beacon Helicase Assay</td>
<td>13</td>
</tr>
<tr>
<td>5.</td>
<td>Helicase Assay Results</td>
<td>14</td>
</tr>
<tr>
<td>6.</td>
<td>Lineweaver-Burke Plot— ATPase Activity</td>
<td>15</td>
</tr>
<tr>
<td>7.</td>
<td>Intrinsic Fluorescence Wild-Type Capsid</td>
<td>18</td>
</tr>
<tr>
<td>8.</td>
<td>Intrinsic Fluorescence Mutant Capsid</td>
<td>19</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cell Culture Efficacy and Cytotoxicity</td>
<td>12</td>
</tr>
<tr>
<td>2. Kinetic Parameters</td>
<td>16</td>
</tr>
</tbody>
</table>
Investigations into the Mechanism of Action of Dengue Virus Inhibitor Compounds

Introduction

It is easy to dismiss an arbovirus like dengue; the mosquito vector is only common in tropical regions and the disease typically presents relatively mild symptoms in the general infected population. Severe cases of dengue virus infection do occur and are associated with both significant morbidity and high mortality rates. Dengue virus is a major emerging infectious disease, necessitating an increase in research efforts to create both a vaccine and an effective treatment. The former presents substantial challenges and potentially deadly consequences while the latter has proven elusive. Progress on both fronts is being made but is fraught with challenges. An estimated 50-100 million cases of dengue are diagnosed each year resulting in roughly 25,000 deaths (8). Recent reports indicated dengue is endemic in over 100 countries (3).

Dengue is a mosquito-borne virus which uses the yellow fever mosquito, *Aedes aegypti*, as the primary vector, using the mosquito’s gut to initially replicate before transmission. The virus particles, following infection and replication, emerge from host cells fully infectious and are carried by the mosquito between hosts through blood meals. Dengue can also be carried by the Asian Tiger mosquito, *Aedes albopictus*, but this appears to be much less common (4). Dengue is classified as an emerging infectious disease because, due to shifts in global climate patterns, the range of *A. albopictus* is expanding to include portions of the southern United States while *A. aegypti* mosquitoes, which are endemic to the southern U.S. are expanding their range as well. For the first
time in 2009, locally acquired dengue cases were reported in Key West, Florida, representing the shift in A. aegypti territory (2). The outbreak, affecting 28 people, represents the first U.S. outbreak of dengue since 1945 and the first locally-acquired outbreak in Florida since 1934 (2).

Dengue virus consists of four serotypes which are very similar in DNA sequence homology. Initial infection with one serotype typically results in flu-like symptoms including fever, abdominal pains, muscle and joint pain, nausea, and headache (9). The muscle and joint pain gave dengue its alternate name—break-bone fever. Although painful, the majority of those infected recover. Dengue is particularly dangerous upon reinfection with one of the remaining three serotypes. Frequently this type of infection leads to dengue shock syndrome (DSS) or dengue hemorrhagic fever (DHF), both of which have a much higher mortality rate. DSS and DHF are much more common in children under 15 years of age for reasons still unknown (9). Symptoms of DSS and DHF include fever, decrease in platelet count, hemorrhaging, and vascular leakage (9). The only treatment met with mild success for DHF/DS patients is to supplement them with IV fluids in an attempt to replace blood volume lost due to vascular permeability resulting in plasma and blood loss. Shock symptoms will set in when sufficient loss of plasma from the blood occurs and patients usually perish or recover within 24 hours of shock onset (9).

Although infection with one serotype of dengue confers resistance for life to that strain there is evidence that infection with another serotype increases your chances of developing DHF or DSS. This is explained at least in part by the Antibody Enhanced Replication (AER) model. The existing antibodies to the heteroserotype serve to increase
viral replication in cells. AER substantially increases the viral load, or number of viruses present in each cell, leading to cell death and vascular permeability following assault brought on by cytokine cascade in the immune system. In addition, dendritic cells, the primary cellular targets for dengue infection, contain the FcγR-receptor necessary for viral entry and subsequent replication. The pre-existing dengue antibodies from the primary infection assist with infection of FcγR-receptor containing cells, resulting in more cells infected and thus increased viral replication (9).

Dengue is a single stranded positive sense RNA virus and member of the Flavivirus branch of the Flaviridae family which includes members like Yellow Fever virus, West Nile virus, and Japanese Encephalitis virus. The dengue life cycle proceeds through stages of decapsidation, uncoating, RNA translation, replication, virus assembly, and encapsidation/maturation. The infectious dengue virus particle enters the cell through a receptor-mediated process using the FcγR-receptor, among others. Once endocytosed into the cell, the viral capsid dissolves, exposing the single-stranded RNA genome which is carried to the endoplasmic reticulum for translation to produce a single polyprotein. The genome is then replicated on host cell membranes and packaged into a capsid coat on the outside surface of the ER which buds off to the golgi. The viral coat is immature as the capsid proteins must undergo cleavage in the Golgi to form tightly-packed capsid units and an infectious, mature virion (5). Mature viral particles are then exocytosed from the cell (Figure 1).
The polyprotein produced by dengue virus contains 10 proteins in total—3 structural proteins involved in forming the capsid, membrane, and viral envelope, and 7 non-structural proteins responsible for viral replication processes. Non-structural protein 3 (NS3) serves to process the viral polyprotein by acting as a protease while simultaneously acting as an RNA-dependant helicase driven by NTPase activity (8). Correct functioning of the NS3 protein is essential for efficient and effective viral proliferation. Without the protease activity of the NS3 region, the envelope protein surrounding the nucleocapsid core would not reach maturation. The nucleocapsid core is formed by the packing of viral RNA around capsid dimer and trimer structures (5). It has recently been demonstrated
that the dengue capsid protein formation relies on co-localization with neutral lipid droplets within the host cell, though the need for such an interaction is still unknown (7).

In the development of drugs to tackle dengue virus, two candidates were discovered designated compound A and compound C. These drugs were found to be effective against dengue virus in cell culture using Vero (Green Monkey Kidney) cells. Resistant viruses were generated against compound A and compound C by continuous passage of dengue-infected cells in the presence of low levels of each drug. The resistant viruses were then sequenced and unique mutations were identified. The mutations associated with resistant compound C virus were found in the NS3 region of the dengue virus genome which is responsible for encoding dengue helicase (Figure 2). It was hypothesized that compound C is affecting the unwinding function of the dengue helicase protein which necessitated quantifying helicase activity in the presence of compound C to provide conclusive evidence of mechanism of action. The compound A resistant virus mutations were found predominantly in the capsid region of the dengue genome. Recently it was discovered that proper viral capsid formation involves co-localization with lipid droplets (7). This was demonstrated by confocal co-localization study by fluorescent staining. Immature capsid protein does not associate with lipid droplets and cannot form mature infectious virions.
The working hypothesis was that compound A interacts with the capsid protein disrupting co-localization of dengue capsid with lipid droplets, potentially explained by binding of compound A to the capsid protein. This thesis attempts to provide evidence for the mechanism of action of these key viral inhibitors.

Materials and Methods

Cell Culture Efficacy and Cytotoxicity
EC50, EC90, and CC50 studies were performed by Kara Cardwell. Briefly, Vero cells were infected with dengue virus to an MOI of 0.1 pfu/cell in media containing titrated compound for 90 minutes. The media was then aspirated from the cells and replaced in virus-free media containing compound. At 48 hours, the supernatant was harvested and frozen at -80°C. The supernatants were then titered on Vero cells with agarose overlays for 7 days, fixed with gluteraldehyde and stained with crystal violet.

CC50 studies evaluate the concentration of compound to cause 50% cytotoxicity in cell culture. Each compound was tested in seven different cell lines, including multiple mammalian, invertebrate (mosquito), and human cell lines. Briefly, cells were seeded in
96-well cell culture plates and compounds are added in a serial dilution and allowed to proliferate for 2 days. Alamar blue was prepared in a 1:10 dilution in phosphate buffered saline solution and added to each well. Plates were returned to the 37°C incubator for 5 hours and $A_{570-600}$ was read on a spectrophotometer (Envision, Perkin Elmer).

**Helicase Unwinding Assay**

Cy-5 fluorescently labeled duplex DNA substrate was annealed by mixing 20μM Cy-5 labeled top strand DNA (Integrated DNA Technologies) with 20μM complimentary bottom strand DNA (Integrated DNA Technologies) in buffer containing 10mM Tris-HCl and 20mM NaCl. This was heated at 95 °C for 5 minutes and allowed to cool at room temperature for 1 hour. In addition, a control reaction was created by mixing 20μM labeled top strand in buffer containing 10mM Tris-HCl and 20mM NaCl. This was also heated at 95 °C for 5 minutes and allowed to cool slowly at room temperature for one hour. Substrates were stored at -20°C wrapped in foil to protect the fluorophore from degradation. To validate formation of duplex DNA complexes, 10nM of the duplex mixture was added to 25mM MOPS and 2mM MgCl$_2$ in black polystyrene 96-well plates. 10nM of the previously prepared top strand was also added to 25mM MOPS and 2mM MgCl$_2$ in separate wells. The plate was then read using a Wallac EnVision spectrophotometer (Perkin Elmer). The top strand mixture had a roughly 85% decrease in fluorescence over the duplex indicating that the top strand forms hairpins which induce quenching (see Figure 3).
Figure 3: Fluorescence values measured in arbitrary units (a.u.). Bottom strand hairpin contains no fluorophore and therefore functions as an assay background value. Hairpin formation on the top strand results in roughly 90% loss in Cy-5 fluorescence.

Wild-type dengue helicase was purified by Aldevron using cDNA constructs produced by Chris Harver. Enzyme was initially tested in the assay to demonstrate activity and was found to be active (data not shown). Reactions were carried out in quadruplicate. Each reaction contained 10 mM MOPS, 2 mM MgCl₂, 5 nM Cy-5 labeled substrate, and 7.25 nM NS3 helicase. Trials were performed using both dengue helicase and UvrD helicase from E.coli (BioHelix Corporation) to determine specificity of drug inhibition. Reactions were plated in white 96-well polystyrene plates from NUNC and read using a Wallac Envision Spectrophotometer (Perkin Elmer) in 40 second intervals. After the first 5 reads, ATP (Invitrogen) in ultra-pure water was added to a final concentration of 3 mM per well to the plates using a multi-channel pipet. The plate was continually read at 40 second intervals for 30-40 minutes.
**Capsid Purification and Expression**

Native and mutant dengue capsid protein was expressed and purified by David King. Briefly, PCR was used to amplify the capsid region from dengue-2 viral cDNA. Amplified DNA was ligated into PQE-30Xa plasmid and transformed into JM109 expression vector cells. Recombinant containing cells were verified through sequencing. Cells were grown to an OD of 0.6 in LB media with ampiciliin and kanamycin. Expression was induced with 1M IPTG and cells were harvested by centrifugation. Cells were frozen at -20° C overnight, then lysed using a microfluidizer, centrifuged to remove cellular debris, and filtered through a 0.45 μM syringe filter. A 5ml His-trap column was used to further purify the capsid protein and eluted in buffer containing increasing concentrations of imidazole, NaCl, and Na₂HPO₄.

**Intrinsic Fluorescence**

Fluorescence measurements were taken using a Fluorolog-3 fluorescence spectrophotometer (Jobin Yvon, Horiba) equipped with a mercury-arc lamp. A scan was performed at 295nm on protein samples diluted in PBS from 310nm to 380nm in the presence and absence of Compound A; Compound A active and inactive analogues; and DMSO, the drug vehicle. Capsid was diluted until the peak fluorescent count measured was between 1 and 2 million counts. Background PBS and drug fluorescence measurements were also performed and subtracted from each sample so that only the capsid behavior in each environment was examined. Samples were prepared as 500ul volumes for use in a quartz microcuvette. Each sample group was performed in triplicate. The cuvette was rinsed in between sample groups with ethanol and water and air dried. Samples were only measured once to minimize photobleaching the tryptophan. Data
analysis and collection was performed using DataMax software, version 2.20 installed on a SpectraAcq processor (Jobin Yvon, Horiba).

**ATPase Assay**

A malachite green assay was used to quantify the release of free phosphate from ATP hydrolysis per a previous protocol using HCV helicase (1). Briefly, dengue helicase (Aldevron) was diluted to 1nM concentration in enzyme dilution buffer (25mM MOPS, 0.2% Tween-20, 0.1mg/ml BSA, 1mM DTT). The reactions were carried out in phosphate free reaction buffer (25mM MOPS, pH 6.5, 5mM MgCl₂). Poly-uridine RNA substrate is added in varying concentrations to the reaction followed by 0.25 mM ATP. Amounts of RNA used were reported in μM of uridine monophosphate, assuming that each poly-U substrate has an average length of 1250 nt. Reactions were carried out in clear 96-well clear, flat bottom plates (Whatman). An HP digital titration instrument was used to dispense drug into wells from a 10mM stock solution diluted in DMSO. Reactions were initiated by adding enzyme between 10 and 15% of the reaction volume. At noted intervals of time, 20ul malachite green reagent (BioAssay Systems) was added to each reaction to halt ATPase activity and bind free phosphate in solution. The binding initiates a color change in the malachite which can be quantified using absorbance at 620 nm. The plates were allowed to develop for 30 minutes and read on a TECAN SpectraFluor Plus microplate reader. Standard curves were created by diluting phosphate buffer to known phosphate concentrations. Sample OD values were then fitted to the linear portion of the standard curve to extrapolate released molar phosphate in each sample. Phosphate free reaction buffer was used as a background and subtracted from
both the standard and the sample OD values. Reactions were repeated in quadruplicate and wells with a standard deviation exceeding 25% were excluded from data analysis.

**Results and Discussion**

EC50 studies evaluate the effective concentration of compound that reduces viral replication by 50%. Similarly, EC90 studies evaluate the concentration of compound that reduces viral replication by 90%. All three compounds were evaluated for their EC50 and EC90 values to determine potential as antiviral drug compounds and found to perform very well (see Table 1). Compound A and compound B are analogues of one another and are both very effective against dengue virus, requiring less than 100 nM concentration to eliminate 90% of virus replication. Compound C is less robust, requiring nearly 100 times the concentration to inhibit viral replication by 90%, however still has a low enough required concentration to be effective as a drug treatment. It is important to move forward with development of all three compounds as A and B have different targets from compound C. Having two antiviral targets more effectively addresses concerns of development of antiviral-resistant mutants becoming widely seroprevalent.

Cleavage of Almar Blue is used to quantify cellular activity and metabolic function of cells in live culture where the appearance of a red pigment produced is proportional to the number of living cells. This cytotoxicity assay yields a measurement known as a CC50 which helps determine cytotoxicity of potential antiviral compounds. All three compounds require very high concentrations to induce cytotoxic effects, levels far and above what is necessary for effective antiviral function (see Table 1). This characteristic
is highly desirable in antiviral compounds and reduces concerns of highly detrimental side-effects of using these compounds for treatment of dengue infection. In addition, the therapeutic index of the three compounds below, calculated by ration of CC50 to EC50, exceed 150 indicating there is a very large margin of error to reach toxic effects in vivo.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (uM)</th>
<th>EC90 (uM)</th>
<th>CC50 (uM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound A</td>
<td>0.021</td>
<td>0.075</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>Compound B</td>
<td>0.011</td>
<td>0.092</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>Compound C</td>
<td>0.326</td>
<td>9.27</td>
<td>&gt; 50</td>
</tr>
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Table 1: 50% and 90% effective concentration and cytotoxicity of each compound against dengue-2 determined via cell culture.

Helicase unwinding activity was measured using a molecular beacon assay (Frick et al.). A DNA duplex was formed using a 17 base pair DNA top strand labeled at the 5’ end with the fluorophore, Cy-5, and containing a fluorescence quencher at the 3’ end that was complementary to an unlabelled bottom strand. A set of inverted repeats are located at either end of the 17 base pair top strand facilitating hairpin formation when the top strand is unbound to the bottom strand. In the presence of active helicase, the top and bottom strands will be separated from one another resulting in hairpin formation of the top strand and measurable decrease in fluorescence as the fluorophore and the quencher are brought in close proximity (see Figure 4).
Figure 4: (a) DNA duplex containing Cy-5 fluorophore and fluorescence quencher molecule. (b) with addition of helicase, the two strands of the DNA duplex are unwound, allowing for hairpin formation in the strands. (c) The DNA strands separate, forming hairpins and inducing quenching of fluorescence in the Cy-5 fluorophore.

Employing this method, quantification of chemical effects on wild-type dengue helicase was possible. The dengue helicase is believed to be the target for compound C as described in the introduction. If compound C and its active analogues disrupt the unwinding activity of dengue helicase, addition of compound C to the helicase assay should result in inhibition of unwinding activity and no decrease in fluorescence over time. The addition of another drug with a different target should have no effect on the unwinding rate seen in wild-type dengue helicase, and the fluorescence is expected to decrease similar to untreated helicase. For this purpose, compound A was used as a negative control because it is believed to target the viral capsid. To test specificity of
compound C to inhibiting only the dengue helicase, commercially available UvrD helicase purified from *E.coli* (BioHelix Corporation) was tested in the assay as well; none of the drug compounds should inhibit UvrD helicase unwinding in this assay if compound C and its analogues are specific inhibitors of dengue helicase. Drugs were titrated into each reaction using an HP digital titration instrument at various concentrations to minimize DMSO concentrations in the assay.

No inhibition was observed in helicase unwinding activity following addition of any drug compounds in either dengue helicase or UvrD helicase reactions (see Figure 5).

This is not entirely unexpected as it was recently found that a compound which target HCV helicase protein, does not inhibit unwinding activity in vitro, but inhibits the RNA-dependent ATPase activity of the helicase protein by blocking access of the RNA substrate to the helicase (1). A malachite green assay was performed to assess the ability
of compound C to inhibit the RNA-dependent ATPase activity. While still preliminary, there appears to be competitive inhibition of RNA-dependent ATPase activity in the presence of compound C as evidenced by closely correlating km values obtained from a Lineweaver-Burke plot (see Figure 6).

**Lineweaver-Burke Plot-- ATPase Activity**

*Figure 6*: Lineweaver-Burke plot of results from malachite green phosphatase assay. Dengue helicase ATPase activity was measured in the presence and absence of drug at RNA concentrations ranging from 0 nM, 0.1 nM, 0.2 nM, 0.3 nM, 0.5 nM, and 5 nM. Reactions halted by malachite green reagent addition at intervals from 1 to 10 minutes.
The calculated Vmax (sec\(^{-1}\)) in the presence of compound C ranges from 0.028-0.033 sec\(^{-1}\), with the exception of 0.5 μM. The Km of the purified dengue helicase in the absence of compound was calculated to be 4.231 nM. This Km decreases as concentration of compound C increases, but not consistently (see Table 2 for additional kinetic parameters). These data are still preliminary given that the competitive inhibition does not appear fully dose dependant and at the time of the defense of this thesis, additional supporting data was not available.

<table>
<thead>
<tr>
<th>Compound C (μM)</th>
<th>Vmax (sec(^{-1}))</th>
<th>km (nM)</th>
<th>kcat (nM/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.028</td>
<td>4.231</td>
<td>0.028</td>
</tr>
<tr>
<td>0.015</td>
<td>0.031</td>
<td>1.502</td>
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<td>0.050</td>
<td>0.031</td>
<td>0.895</td>
<td>0.031</td>
</tr>
<tr>
<td>0.150</td>
<td>0.029</td>
<td>1.366</td>
<td>0.029</td>
</tr>
<tr>
<td>0.500</td>
<td>0.039</td>
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</tr>
<tr>
<td>5.000</td>
<td>0.033</td>
<td>1.062</td>
<td>0.033</td>
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Table 2: Kinetic parameters determined using a malachite green assay.

Sequencing data of the compound A resistant dengue virus provided evidence that compound A targets and disrupts some property of the dengue capsid protein. Binding of the drug to the capsid protein would likely disrupt proper capsid formation and assembly, resulting in non-infectious virus particles. When drugs bind to proteins, there is often a substantial change in protein properties or protein shape. Proteins with buried fluorescent amino acids such as tryptophan or tyrosine are useful for measuring shape changes and binding effects. Shielding effects from the amino acids surrounding the fluorescent amino acid alter the measured fluorescence and wavelength for which peak fluorescence is observed. Binding of a ligand (or drug in this case) induce shape changes observable using intrinsic fluorescence. The dengue capsid protein exists as dimers, both of which
have a buried tryptophan molecule making intrinsic fluorescence measurements a feasible way to measure drug/protein interactions.

Wild-type dengue capsid protein exhibited peak fluorescence at or near 338nm. Tryptophan existing in a completely hydrophobic environment, such as buried deeply inside a protein, exhibit fluorescence maxima of approximately 410-424 nm while surface tryptophans typically exhibit peak fluorescence around 450nm (6). Based on this range, the tryptophan within the dengue capsid protein is not completely buried in a hydrophobic environment but is not on the surface of the capsid dimer either. No shift in the wavelength of peak fluorescence was measured upon addition of drug up to 2.5μM, but a 19% reduction in peak fluorescence was observed indicating that a change was induced in the available quantum acceptors (Figure 7). This change suggests that the local environment surrounding the tryptophan has been altered by a shift in nearby amino acids. It appears that compound A and another active analogue, compound B, bind to the capsid protein in a dose-dependent fashion.
This experiment was repeated using purified mutant capsid protein from the compound A resistant virus. Compound A resistant mutant dengue capsid in the presence of compound A exhibits a 17% decrease in fluorescence in the presence of 2.5μM drug compared to 19% observed in the wild-type. In addition, capsid in the presence of compound B shows no decrease in mutant peak fluorescence compared to 11% in the wild-type (Figure 8).
If capsid binding produces the inhibitory effect observed in cell culture, it is expected that the compound A-resistant mutant capsid protein will not exhibit the same binding as the wild-type capsid if this is the mechanism of action for compound A. Compound B is a structural analogue of compound A so a change in binding affinity is neither expected nor unreasonable. Some cross resistance has been documented for compound A-resistant mutant virus against compound B in cell culture. The chemical structure of compound A and compound B contain different functional groups so it is possible that the mechanism
of action for each of these drugs is different and/or that the mechanism of action includes binding or association with the capsid protein in addition to cellular effects. The loss of binding affinity between mutant capsid protein and compound B provides evidence that the mechanism of action for compound B does involve a binding interaction; the very minimal decrease in binding affinity between compound A and mutant capsid protein indicates that more study is needed to pinpoint and characterize capsid protein interactions with this compound.

**Conclusions**

Preliminary evidence obtained from a molecular beacon helicase unwinding assay and a malachite green ATPase assay indicate that, as predicted from sequencing data from compound-resistant mutants, the target of compound C involves the NS3 protein. Specifically, compound C appears to influence the RNA-dependant ATPase activity of the NS3 helicase protein and has no effect on the unwinding activity.

The mechanism of action of both compound A and compound B was investigated using intrinsic fluorescence to probe compound binding to viral capsid protein. Both compound B and compound A bind to the native viral capsid protein, but only compound B loses this binding affinity with the compound A resistant mutant. This provides no concrete evidence of a mechanism of action but both compound A and compound B have been demonstrated to be post entry inhibitors. Compound A might potentially maintain binding activity to capsid subunits but not disrupt the uncoating or re-packaging activity of the dimers.
Additional studies are warranted to follow-up on the evidence gathered in this work. NMR characterization in the presence of compound is highly recommended to characterize binding interactions between each compound and its respective target protein. In addition, it is suggested that the ATPase assay be repeated with titration of ATP to characterize competitive inhibition between compound C and ATP.

To move forward with development of essential inhibitory compounds to combat viral infections like dengue it is crucial to characterize the mechanism of action for potential drug candidates. Without this necessary intermediate step, it is difficult to pursue further study of compound action including animal testing and eventual application to the FDA for approval. In the case of dengue, the development of antiviral compounds is in dire need, particularly with the expanding habitat range of mosquito carriers. The work presented herein marks the important first look into the mechanism of action of effective dengue inhibitors.
References


