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CHARACTERIZATION OF A NOVEL NUCLEOSIDE ANALOG FOR DENGUE VIRUS INFECTION

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By

Silvia D. Dimitrova, BS
Georgia State University, 2012

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ABSTRACT

CHARACTERIZATION OF A NOVEL NUCLEOSIDE ANALOG FOR DENGUE VIRUS INFECTION

By: Silvia D. Dimitrova

Dengue virus (DENV) causes a febrile disease prevalent in tropical and subtropical regions of the world that affects approximately 390 million persons annually. This mosquito-borne single-stranded RNA virus is a member of the family *Flaviviridae* and is classified into four serotypes, DENV 1-4. Currently, there are no approved antiviral agents that are available to treat dengue infections. The development of a therapeutic agent is necessary and could decrease morbidity and mortality rates among dengue-infected persons. One specific target for an antiviral agent is the DENV RNA-dependent RNA polymerase (RdRp) that replicates the viral genome and is essential for virus survival. An approach to inhibit the activity of the RdRp enzyme is to utilize ribonucleoside triphosphate (NTP) analog inhibitors that act as chain terminators during the synthesis of new genomic RNAs and prevent viral replication. Our laboratory previously discovered that the ribonucleoside analog 7-deaza-7-fluoro-2'-C-methyl-adenosine (DFMA) synthesized by our chemists inhibits dengue virus replication. The focus of this thesis work was to characterize the anti-DENV potency of DFMA utilizing both cell biological and biochemical assays. In addition, the phosphorylation of DFMA in the megakaryoblastic cell line Meg-01 was studied. Finally, the anti-DENV mechanism of action of DFMA was studied by selecting DFMA drug-resistant virus *in vitro* in a tissue culture system. This thesis work will advance anti-DENV drug development, as well as increase our knowledge with regards to mechanisms of DENV inhibition and viral resistance to ribonucleoside analog drugs.

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I would like to thank the faculty of the Microbiology and Molecular Genetics (MMG) Program for their mentorship and the Laney Graduate School, Division of Biological and Biomedical Sciences, for financial support and excellent tutelage. Lastly, I thank my mom, dad, and sister for the love and support they have given me.

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CHAPTER I: INTRODUCTION

Overview:

Global Burden of Dengue Disease

Worldwide, dengue fever constitutes a significant public health problem that needs to be addressed by therapeutic intervention[1]. The causative agent of dengue fever is dengue virus (DENV), a mosquito-borne single-stranded RNA virus. A project to determine the global distribution of dengue infections using clinical records and modeling derived an evidence-based map of dengue risk and estimated the apparent and inapparent dengue infections worldwide on the basis of the global population in 2010.The researchers estimated that DENV caused approximately 390 million infections globally in 2010 - there were 96 million apparent dengue infections and an additional 294 million inapparent dengue infections that were mild or asymptomatic and were undetected by the public health surveillance system in 2010[2]. Other studies cite that approximately 500,000 people develop the severe forms of dengue illness termed dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) and require hospitalization every year. Over 20,000 dengue related deaths occur annually worldwide[1]. The WHO has classified dengue as “the most important mosquito-borne viral disease in the world.” Dengue is also a significant problem in the United States: indigenous cases of dengue have been reported in Puerto Rico, Texas and Florida, and several outbreaks of dengue have occurred in Hawaii[3]**.** The WHO has established two sets of dengue case classification criteria. The 1997 criteria classified dengue into DF, DHF (Grades 1 and 2) and DSS (DHF Grades 3 and 4). The revised 2009 criteria

classify dengue according to levels of severity: dengue without warning signs; dengue with warning signs (abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy, liver enlargement, increasing haematocrit with decreasing platelets); and severe dengue (dengue with severe plasma leakage, severe bleeding, or organ failure). The 2009 classification into severity levels is considered to be more sensitive in capturing severe disease than the 1997 guidelines[4].

The Dengue Virus

DENV is classified in the family *Flaviviridae*, genus Flavivirus. The genome is composed of positive-sense single stranded RNA that is complexed with multiple copies of a small, basic capsid (C) protein to form the nucleocapsid. The nucleocapsid is enclosed within a lipid bilayer with two or more species of envelope (E) glycoprotein. Translation of the single open-reading frame (ORF) encoded in the DENV genome produces a large polyprotein that is cleaved by host and viral proteases into viral proteins. The genome organization of DENV includes 3 structural proteins (C, prM, and E) located in the N-terminal portion of the polyprotein and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) located in the remainder of the polyprotein. NS5 is a large, highly conserved, and multifunctional protein whose sequence is located in the last 900 codons of the DENV genome ORF at the C-terminal end of the polyprotein. NS5 is a phosphoprotein that possesses RNA capping and RNA-dependent RNA polymerase (RdRp) activities encoded within its N- and C-terminal regions, respectively[5].

The replication cycle of DENV begins when the enveloped virion enters the host cell through receptor-mediated endocytosis. DENV uses the clathrin-mediated endocytosis pathway for entry; this process involves the virus first associating with

clathrin-coated pits and then being delivered to endosomes, where the virus fuses with Rab7-positive late endosomes[6]. Following uncoating of the nucleocapsid, the RNA genome is released into the cytoplasm of the infected cell. The genome is infectious and can serve as mRNA for translation of viral proteins at the host's ribosomes. Progeny virions assemble by budding into an intracellular membrane compartment, most likely the endoplasmic reticulum, then traverse through the host secretory pathway and are released at the cell surface[5]. There are four dengue serotypes, DENV 1-4, which diverge approximately 30% across the genome[7]. The four DENV serotypes infect a variety of target cells; the virus in dengue subjects infects both megakaryocytes and hepatocytes[8–13]. For this reason, we will utilize Meg-01 (a megakaryoblast cell line) and Huh-7 (a hepatocyte cell line) in this project to study how a novel ribonucleoside analog functions to inhibit DENV infection.

Immune Responses to DENV

During dengue infection, both cellular and humoral immune responses are involved in controlling the virus. The DENV can infect both CD4+ T cells and CD8+ T cells[14]. Pattern recognition receptors such as toll-like receptors (TLRs), particularly TLR-3, TLR-7, and TLR-8, and intracellular sensors (such as RNA helicases) participate in the immune recognition of RNA viral genomes[15]. The primary TLR involved in dengue viral recognition, TLR-3, recognizes DENV RNA after endosomal acidification. Sensing of the viral genome leads to signaling cascades that activate the production of IFN α/β. IFN-α/β secreted by virus-infected cells triggers warning signals to adjacent cells that an infection is occurring, as well as an induction of cellular antiviral responses. IFN-α/β is a potent inhibitor of DENV infection[7,16]. RIG-I and MDA-5 also play a major role in

restricting dengue virus infection. RIG-I and MDA-5 are intracellular RNA virus sensors that recognize viral RNA and participate in the TLR-independent INF induction pathway. More specifically, RIG-I and MDA-5 are activated upon DENV-1 infection of Huh-7 cells, and knockdown of these two sensors results in enhancement of DENV replication in the Huh-7 cell line[17]. In regards to the humoral immune responses, an important aspect that contributes to severe dengue infection is antibody-dependent enhancement (ADE). During secondary dengue infection with a different serotype, cross-reactive antibodies from the previous infection bind to the DENV virion and form an immune complex that is recognized by FcγRs, which mediate uptake into the target cells of the myeloid lineage. Thus, DENV infection through ADE can be enhanced, resulting in decreased innate immune responses and increased viral replication[16]. DENV also antagonizes the innate immune response. The DENV NS2B3 protease complex cleaves human STING and antagonizes type I INF production in human but not mouse cells[18].

Vaccines

The development of a multivalent vaccine for dengue that provides protection against all four serotypes has proven difficult since the possibility of more severe disease upon secondary infection arises when a person possesses partial immunity to DENV. Thus, considerations regarding the efficacy of a vaccine against DENV 1-4 as well as protection against severe disease need to be addressed when designing and testing a DENV vaccine candidate. The vaccine that is furthest along in development is a recombinant, live-attenuated, tetravalent (LATV) DENV vaccine called CYD-TDV by Sanofi-Pasteur[19]. The vaccine is based on the yellow fever 17D backbone and includes the 4 strains CYD-1 (Thai strain PUO-359/TVP-1140), CYD-2 (Thai strain PUO-218),

CYD-3 (Thai strain PaH881/88) and CYD-4 (Indonesian strain 1228; TVP-980). It was shown to induce both cellular and humoral immune responses in humans against DENV 1-4. The tetravalent CYD vaccine is manufactured by using serum-free Vero cells for both viral and cell culture; thus, the vaccine does not contain any materials of animal origin, nor does it contain any preservatives, adjuvants, or antibiotics. A proprietary stabilizer is present in the finished vaccine product which contributes to overall good stability of the CYD-TDV: vaccine from phase III lots was stable up to 1 month at 25 ± 2°C. These facts can help address concerns from the general population about the safety of the vaccine ingredients and stability of the vaccine[20]. Efficacy studies have been completed including one phase IIb clinical trial and two phase III clinical trials. A meta-analysis conducted in 2014 to assess the safety, immunogenicity, and efficacy of CYD-TDV included 6678 patients randomized to receive the CYD-TDV (4586) or placebo (2092). In regards to safety, there was no significant difference between placebo and treated groups and approximately 5.5% of patients were withdrawn from the study. The levels of neutralizing antibodies were measured by weighted mean differences and were always higher in the vaccinated group for DENV1, DENV2, DENV3, and DENV4[21]. Another DENV vaccine candidate, developed by the National Institute of Allergy and Infectious Diseases, is the LATV dengue vaccine TV003 and TV005. A single dose of either TV003 or TV005 induced seroconversion to four DENV serotypes in 74–92% (TV003) and 90% (TV005) of flavivirus seronegative adults and elicited near-sterilizing immunity to a second dose of vaccine administered 6–12 months later[19].

Nucleoside Analog Inhibitors

One approach in the development of an antiviral agent against dengue is to target the DENV RNA-dependent RNA polymerase (RdRp) that replicates the viral genome and that is essential for virus survival[22]. A method to inhibit the activity of the RdRp enzyme is to utilize ribonucleoside analog inhibitors (NAIs) that act as chain terminators during the synthesis of new genomic RNA **(Figure 1).** NAIs comprise a large class of approved antiviral regimens for viral diseases such as herpesviruses, hepatitis B (HBV), hepatitis C (HCV) and human immunodeficiency virus (HIV)[23]. In general, NAIs can be phosphorylated into their corresponding 5'-mono, 5'-di, and 5'-triphosphates (NTP) by cellular kinases. Then, once phosphorylated inside the cell, the fraudulent NTPs compete with the natural substrate during the process of viral RNA-dependent RNA polymerization. NTP analogs interfere with viral polymerase activity by serving as chain terminators during RNA synthesis and halting viral replication[24,25]. NAIs offer several advantages when compared to other classes of antiviral agents such as entry and protease inhibitors. First, NAIs often target viral polymerase active sites that are conserved, and thus, the emergence of resistance to these inhibitors is relatively low due to the barrier posed by the conservation of the target sequence. In addition, the high barrier to NAI resistance is due to fitness deficits of mutant viruses[26]. Moreover, NAIs frequently display comparable potency against different serotypes/genotypes of a virus as well as among viruses in different families. For example, tenofovir-DF is an orally bioavailable adenosine nucleotide analog that is FDA approved for the treatment of both HIV and HBV[27,28]. Finally, NAIs have a long intracellular half-life allowing for less frequent dosing[29]. For this project, the viral protein that will be targeted for drug development is the NS5. The C-terminal portion of NS5 encodes the RNA-dependent-

RNA polymerase (RdRp) of DENV[30–33]. This project is focused on characterizing a putative inhibitor of the DENV RdRp and understanding the mechanisms of viral resistance to this inhibitor.

DFMA

The nucleoside analog 7-deaza-7-fluoro-2'-C-methyl-adenosine (DFMA) was initially identified as displaying anti-HCV activity in a study by Merck assessing the structure-activity relationship of heterobase-modified 2'-C-methyl ribonucleosides as inhibitors of hepatitis C virus RNA replication using a replicon assay with HB-1 cells[34]. DFMA also displays antiviral activity against DENV and is a putative inhibitor of the DENV RdRp**.** Dr. Leda Bassit in our laboratory originally tested a number of nucleoside analogs that were active against HCV to determine if they inhibit DENV replication in Vero cells. She identified the NAI β-D-2'-C-methyl-6-amino-7-fluoro-7-deazapurine ribose, referred to as DFMA, as displaying antiviral activity against DENV in Vero cells (EC_{50}=0.3-0.4 μM) (unpublished data). This compound is a modified adenosine analog with a methyl addition on the 2' carbon of the sugar and a fluorine addition at the seventh position of the base. It also lacks a nitrogen at position 7 of the adenosine base[34].

Aims and Goals of the Project

One aim of this thesis project was to characterize DFMA pharmacologically for DENV serotype 2. A second aim of this thesis project was to purify DENV3 NS5, the DENV enzyme which possesses RNA-dependent RNA polymerase (RdRp) activity, in order to use it in an *in vitro* polymerase activity assay to biochemically characterize DFMA. A third aim of this thesis project was to select for DENV with resistance to DFMA

in the cell lines Meg-01 and Huh-7 and characterize the resistance mutations genotypically for DENV2. In addition, the cellular pharmacology of DFMA in the Meg-01 cell line was investigated. The long-term goal of this thesis project is to develop a therapeutic agent to treat dengue infection and to decrease morbidity and mortality rates among dengue-infected persons, particularly children.

Figures

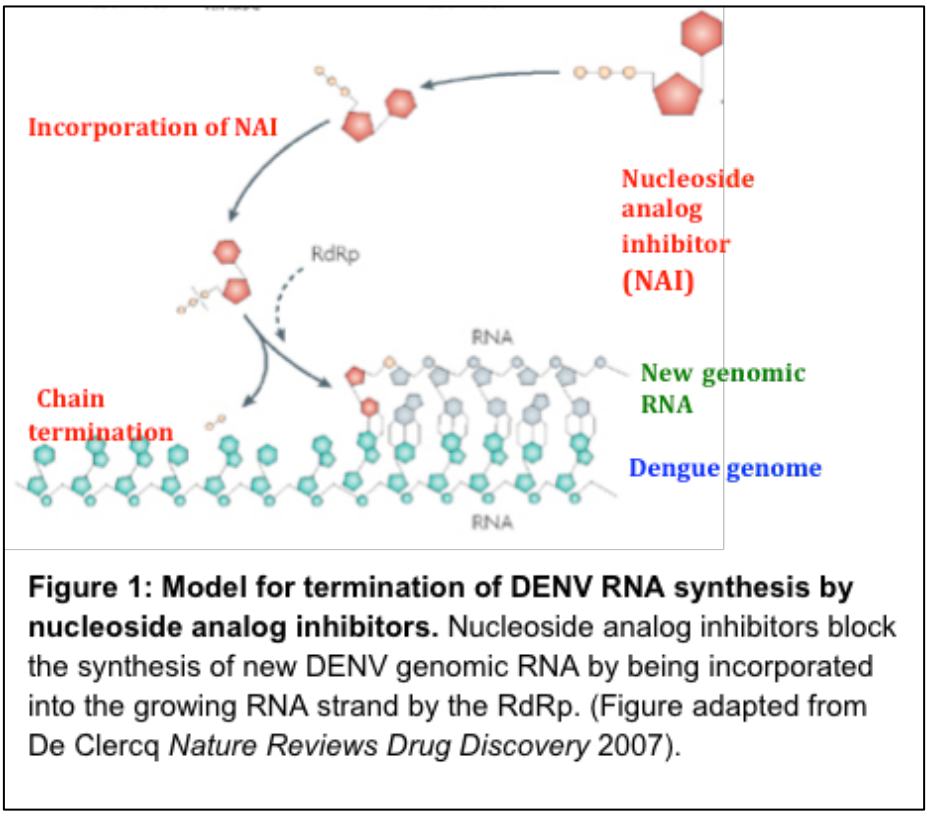


Figure 1: Model for termination of DENV RNA synthesis by nucleoside analog inhibitors. Nucleoside analog inhibitors block the synthesis of new DENV genomic RNA by being incorporated into the growing RNA strand by the RdRp. (Figure adapted from De Clercq *Nature Reviews Drug Discovery* 2007).

CHAPTER II: POTENCY OF DFMA IN THE MEGAKARYOBLASTIC CELL LINE MEG-01

Introduction

DFMA is a novel nucleoside analog that needs to be characterized in regards to dengue. Currently, a few nucleoside analogs have been identified and characterized in terms of inhibition of DENV replication; only one compound has been tested in a clinical trial. NITD008, an adenosine nucleoside analog, in its triphosphate form was found to inhibit the DENV RdRp *in vitro* and to suppress peak viremia in DENV-infected mice; however, adverse side effects were observed in rats and dogs after 2 weeks of an oral dosing regimen of NITD-008[35]. Balapiravir, a cytidine nucleoside analog, was not efficacious in a phase II clinical trial involving adult dengue infected persons[36]. Recently, the antiviral activity of 2'-C-methylcytidine against dengue has been characterized in both a DENV replicon Huh-7 cell line as well as a suckling mouse model[37]. Due to the limited availability of active and safe NAIs for dengue, no such drugs have entered phase III clinical trials. This proposal aims to study the new nucleoside analog DFMA in regards to dengue. DFMA was initially identified as an inhibitor of hepatitis C virus replication and displayed good potency (EC_{50}=0.07 μM) in a HCV replicon cell line, without any apparent cytoxicity (CC_{50} >100 μM)[34]. The mechanism of action of DFMA against dengue is unknown; in addition, the pharmacological properties of this compound in DENV-infected human cells have not been fully elucidated. Thus, part of this thesis was focused on characterizing the pharmacodynamics of DFMA in a human megakaryoblast cell line. Since megakaryocytes are major reservoirs for DENV in dengue-infected persons, it is imperative that antiviral drugs against dengue are confirmed to inhibit DENV replication in this cell type. To the best of our knowledge,

antiviral studies for dengue virus using megakaryocytes or megakaryocytic cell lines have never been previously performed.

Materials and Methods

Compound and Cell Line: Meg-01 (obtained from Dr. Francois Villinger, Yerkes National Primate Research Center) was maintained in HyClone RPMI-1640 (GE Healthcare) supplemented with 10% fetal bovine serum (+Penicillin/Streptomycin). All cell lines were maintained at 37°C/5% CO_2. The compound DFMA was synthesized in-house, dissolved in DMSO to a final concentration of 40 mM, and stored at -20°C[34].

Replication Kinetics of DENV2 in Meg-01 cells: Meg-01 cells (1×10^5) were infected with DENV2 strain 16681 (a clinical isolate that has been passaged in vitro, obtained from Dr. Oscar Perng) in a T_{25} flask at four different MOI's (0.1, 0.5, 1, and 2). The cells were harvested at days 3, 4, 5, and 6 post infection. The cells were separated from the media via centrifugation. Total nucleic acid isolation was performed with the MagNAPure LC Total Nucleic Acid Isolation Kit (Roche) using the MagNAPure LC Instrument. cDNA synthesis was performed with the SuperScript Vilo cDNA Synthesis Kit (utilizes M-MLV Reverse Transcriptase and random hexamers as primers). Two Step qRT-PCR was performed with the FastStart Universal Probe Master (Rox) Kit by Roche utilizing the hydrolysis probe detection format. The primers were specific for the 3'-noncoding sequence specific for DENV2 and the probe contained FAM as the reporter and TAMRA as the quencher [38].

Replication Kinetics of DENV2 in Meg-01 supernatants: The protocol for establishing the DENV kinetics in the Meg-01 supernatants was provided by Dr. Oscar Perng (National Cheng Kung University, Taiwan). Meg-01 cells (20×10^6) were infected

with DENV2 at an MOI of 0.1 in a 2 mL total volume for 2 hr/37°C. The supernatant media was washed to remove excess virus, and 1 x 10^6 cells were seeded per T25 flask. In total, seven flasks were seeded with Meg-01 cells without the addition of DFMA, and seven flasks were seeded with Meg-01 cells with the addition of 5 μM RS-574. Supernatant samples were collected on days 1, 2, 3, 5, 7, 10, and 14 post infection. At day 5 and 10, 2 mL of RPMI-10 media was added to the flasks. Viral RNA from all fourteen supernatant samples was extracted with the EZ1 Virus Mini Kit and EZ1 Advanced XL instrument (Qiagen) using 300 μL of supernatant lysed with 100 μL Buffer AVL (Qiagen). To quantify the DENV2 RNA in the supernatant, one-step qRT-PCR was performed using the LightCycler 480 RNA Master Hydrolysis Probe Kit and LightCycler 480 II Instrument (Roche) **(Figure 4)**. A standard curve was constructed with a pGEMTeasy plasmid containing a 153 bp insert of the 3' noncoding region of the DENV2 genome provided by Hui-Mien Hsiao[38]. The plasmid was serially diluted and used in qRT-PCR reactions; the plasmid copies/μL were calculated using the plasmid concentration and molecular weight. The standard curve was used to calculate the DENV2 RNA concentration in copies/μL in the Meg-01 supernatant samples.

Dose-Response of RS-574 in Meg-01 Cells: Meg-01 cells (2 x 10^6 cells) were seeded in a T_{75} flask and infected with DENV2 at an MOI of 0.1 in a 15 mL total volume for 2 hr. The infected Meg-01 cells were added to the wells of a 24-well plate for a final concentration of approximately 100,000 cells/well. DFMA was added to the wells of plate at a final concentration of 0.1, 1, and 10 μM. For controls, wells were seeded with uninfected Meg-01 cells and untreated Meg-01 cells (infected with DENV2 at an MOI = 0.1 without addition of DFMA). The uninfected, untreated, and treated cells were

collected at 6 days post infection. Total nucleic acid isolation was performed on 200 µL of sample (cells suspended in PBS) using the MagNA Pure LC Total Nucleic Acid Kit and Instrument (Roche). cDNA synthesis was performed using the SuperScript Vilo Kit (Invitrogen). cDNA was used in qRT-PCR reactions using FastStart Universal Probe Master (ROX) master mix (Roche) based on the hydrolysis probe detection format **(Figure 2)**. The difference in Ct values between the untreated and treated flask was calculated (ΔCt). The percent inhibition was determined using the formula for half-life: Percent Inhibition = (1-(e^(-0.693*ΔCt)))*100.

Results

In order to determine a time point for sample collection during the dose-response studies, we first established the replication kinetics of DENV2 in infected Meg-01 cells as well as in the supernatants of infected Meg-01 cells. The peak of DENV2 replication in the Meg-01 cells was at day 6 for an MOI of 0.1, 0.5, and 1, and 2 **(Figure 5)**. In order to measure the kinetics of DENV2 release into the supernatant of infected Meg-01 cells in the presence or absence of DFMA, we quantified the DENV2 RNA by qRT-PCR at 1, 2, 3, 5, 7, 10, and 14 days post infection. The release of DENV2 into the supernatant of infected Meg-01 cells peaked at day 10 **(Figure 6A)**. In addition, the viability of DENV2-infected Meg-01 cells in the presence or absence of RS-574 was measured using a trypan blue exclusion assay (using the ViCell XR Cell Viability Analyzer, Beckman Coulter). The cell viability was quantified at 1,2,3,5,7,10, and 14 days post infection. The Meg-01 cell viability for untreated and treated DENV2-infected Meg-01 cells showed a similar trend, peaking at day 3 and declining until day 7 to 10, and increasing until day

14. Thus, DFMA treatment did not appear to cause substantial cell death compared to the untreated Meg-01 cells when tested up to 5 μM **(Figure 6B)**.

We performed a dose-response study to determine the median effective concentration (EC_{50}) and EC_{90} values of DFMA in DENV-2 infected Meg-01 cells**.** The EC_{50} value of DFMA was calculated to be in the range of <0.1 to 0.91 μM (n = 2). The EC_{90} value of DFMA was calculated to be in the range of 2 to 6.7 μM (n = 2). The Meg-01 cell viability for each condition (uninfected, treated, 0.1 μM, 1 μM, and 10 μM DFMA) was measured using a trypan blue exclusion assay (using the ViCell XR Cell Viability Analyzer, Beckman Coulter). The cell viability did not decrease when compared to the untreated control for Meg-01 cells treated with 0.1 and 1 μM DFMA (n = 2), indicating that the antiviral effects of DFMA were not due to cell death. However, a decrease in cell viability was measured in DENV2-infected Meg-01 cells treated with 10 μM DFMA (n = 1), indicating that DFMA at this concentration could be toxic to Meg-01 cells **(Figure 3)**. The potency of DFMA in the Meg-01 cell line was comparable to the potency of the compound in Huh-7 cells, exhibiting an EC_{50} of 4 μM and EC_{90} of 9.6 μM **(Table 1).**

Discussion

The nucleoside analog DFMA inhibited DENV2 replication in Meg-01 cells and supernatants. The potency of DFMA against DENV2 was determined to be <0.1-0.91 μM (EC_{50}) and 2-6.7 μM (EC_{90}) in Meg-01 cells. DFMA inhibited DENV2 release into the supernatant of infected Meg-01 cells at days 1, 2, 3, 5, 7, 10, and 14 post infection. Further independent experiments need to be performed to determine a reproducible EC_{50} and EC_{90} value of DFMA in DENV2-infected Meg-01 cells, and lower

concentrations of DFMA such as 0.01 µM can be tested. In addition, plaque assays with BHK cells can be performed using the supernatant samples from the untreated and DFMA treated Meg-01 cells to show inhibition of DENV viral titer by DFMA.

Figures

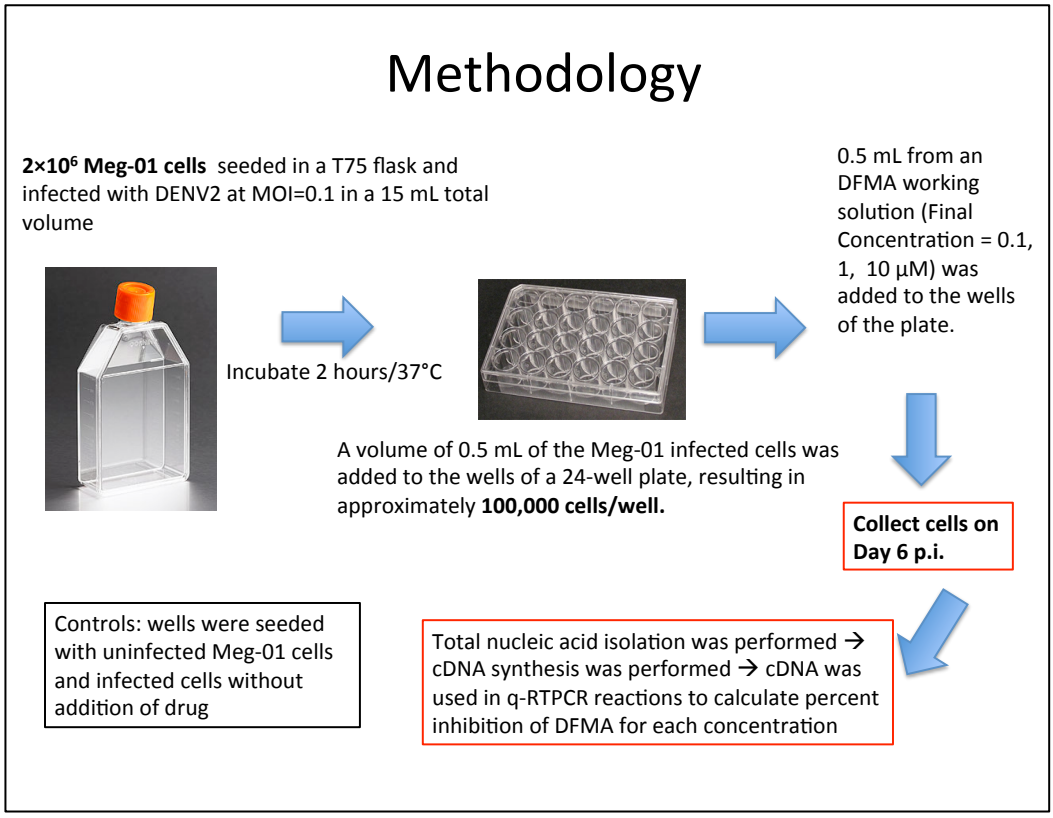


Figure 2: Methodology for dose-response studies of DFMA in Meg-01 cells. Meg-01 cells were cultured in a 24-well plate with or without the addition of DFMA (0.1, 1, and 10 µM). After 6 days post infection, cells were harvested and viral nucleic acids were isolated. The percent inhibition of viral replication for the untreated and treated Meg-01 cells was calculated using qRT-PCR and DENV2 specific primers and probe using the hydrolysis probe detection format.

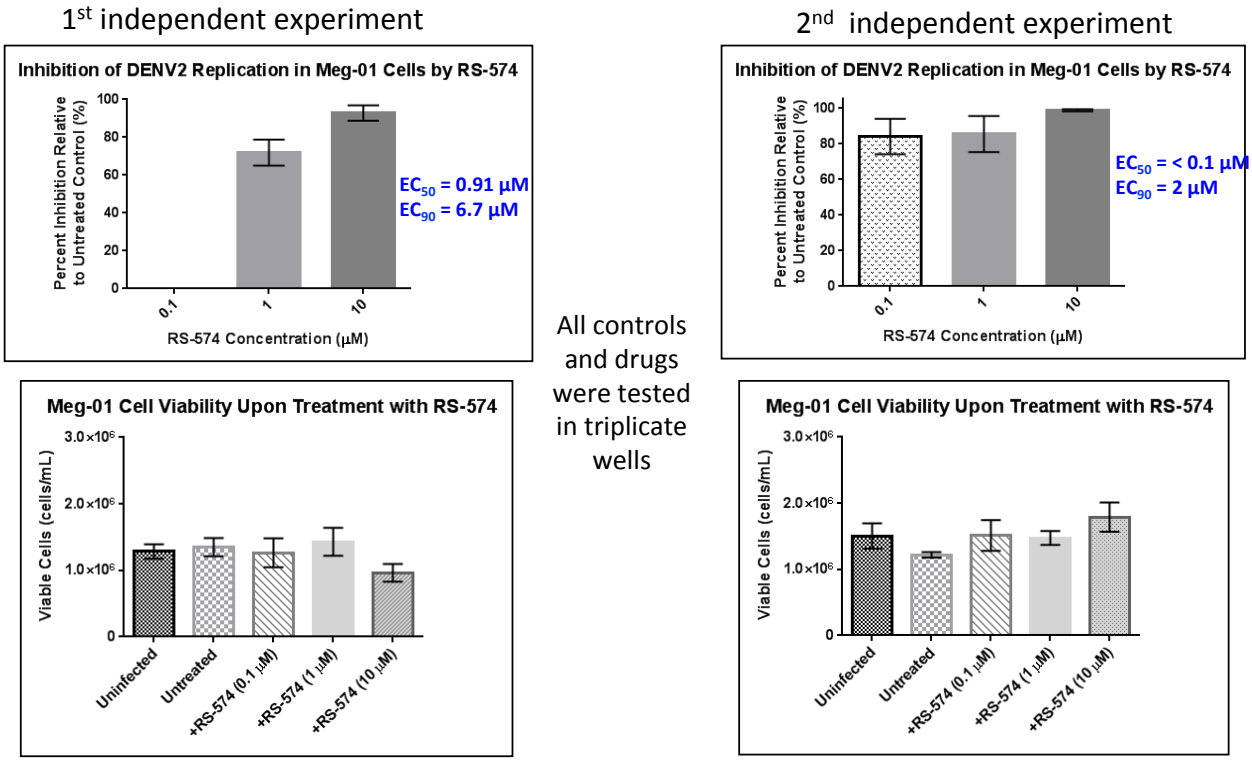


Figure 3: The dose-response study of DFMA in Meg-01 cells and determination of EC_{50}/EC_{90} values. Meg-01 cells were cultured in a 24-well plate with or without the addition of DFMA (0.1, 1, and 10 µM). After 6 days post infection, cells were harvested and viral nucleic acids were isolated. The percent inhibition of viral replication for the untreated and treated Meg-01 cells was calculated using qRT-PCR and DENV2 specific primers and probe using the hydrolysis probe detection format. The EC_{50} and EC_{90} values were calculated using the formula for half-life to determine the percent inhibition relative to the untreated Meg-01 cells. The EC_{50} value of DFMA was calculated to be in the range of <0.1 to 0.91 µM (n = 2). The EC_{90} value of DFMA was calculated to be in the range of 2 to 6.7 µM (n = 2). The Meg-01 cell viability for each condition (uninfected, treated, 0.1 µM, 1 µM, and 10 µM DFMA) was measured using a trypan blue exclusion assay (ViCell XR Cell Viability Analyzer, Beckman Coulter). The cell viability did not decrease when compared to the untreated control for Meg-01 cells treated with 0.1 and 1 µM DFMA (n = 2), indicating that the antiviral effects of DFMA were not due to cell death "RS-574" refers to DFMA in the figures.

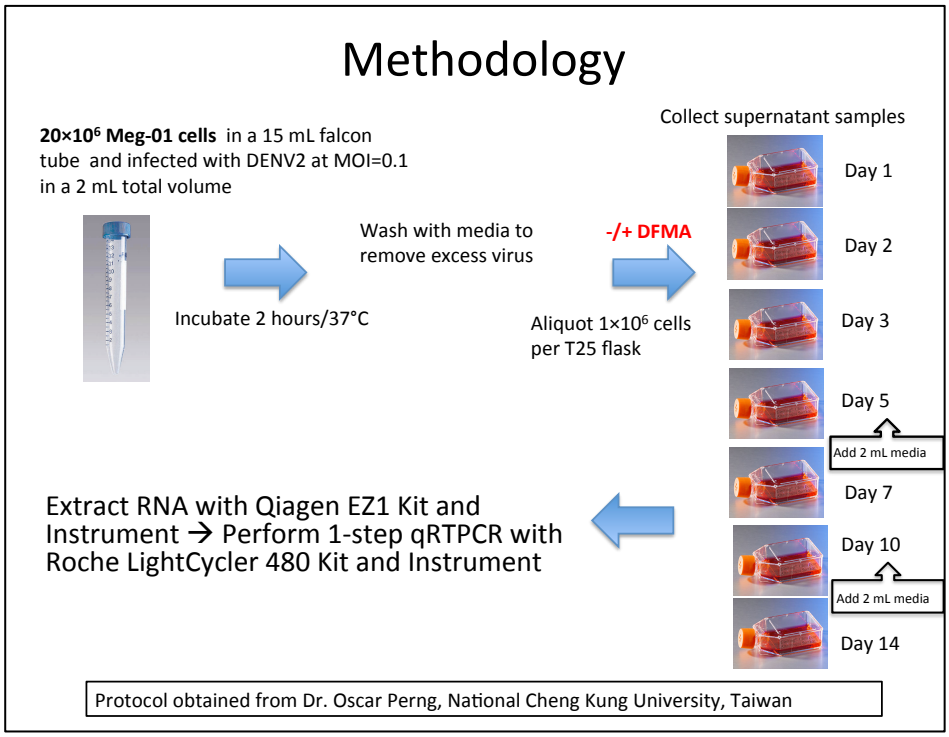


Figure 4: Methodology for studying replication kinetics of DENV-2 in Meg-01 supernatants and testing DFMA inhibition of DENV2 replication in supernatants of infected Meg-01 cells. In order to measure the kinetics of DENV2 release into the supernatant of infected Meg-01 cells in the presence or absence of DFMA, we cultured 1x10^6 Meg-01 cells in T25 flasks and quantified the DENV2 RNA by qRT-PCR in the supernatant at 1, 2, 3, 5, 7, 10, and 14 days post infection with or without the addition of DFMA (5 μM). The protocol for this method was obtained from Dr. Oscar Perng, National Cheng Kung University, Taiwan.

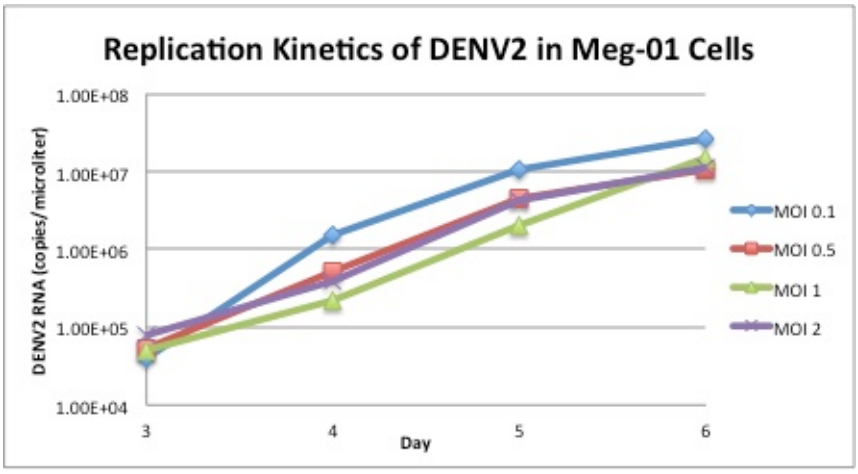
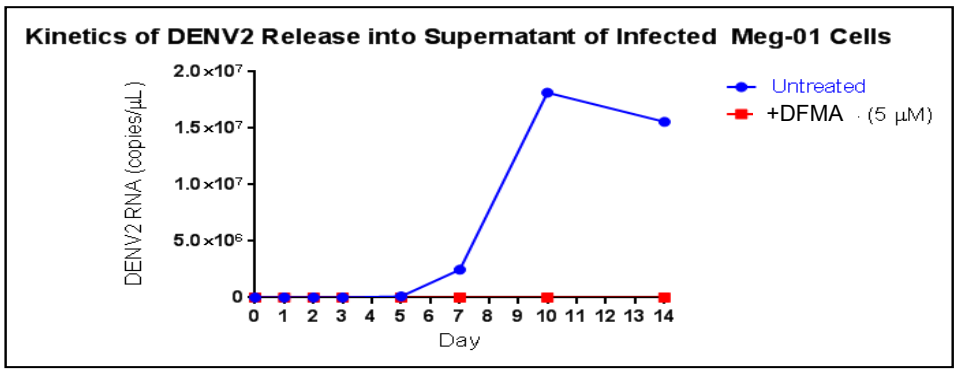


Figure 5: Kinetics of DENV2 Replication in Meg-01 cell line. Viral load in Meg-01 cells infected with DENV2 16681 at an MOI of 0.1, 0.5, 1, and 2 was measured using qRT-PCR to detect expression of a portion of the 3' noncoding region of the DENV genome and quantify the amount of DENV2 RNA present. The peak of DENV2 replication in the Meg-01 cells was at day 6 for an MOI of 0.1, 0.5, and 1, and 2.

A



B

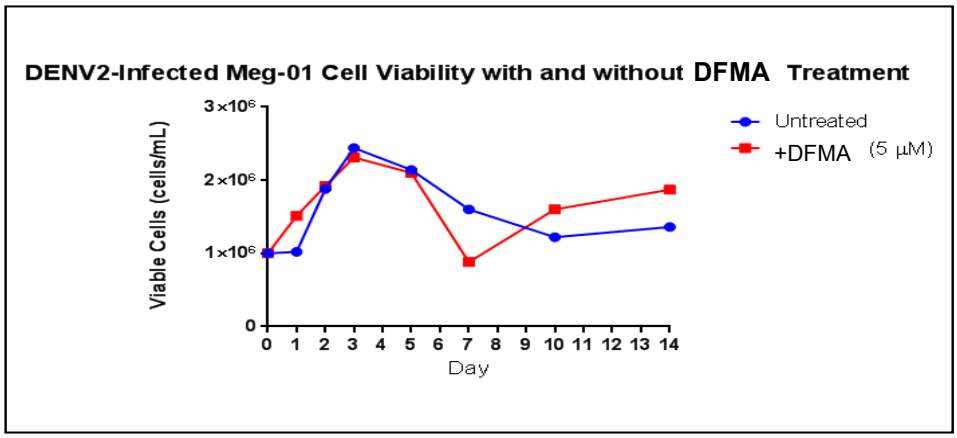


Figure 6: A: The kinetics of DENV2 release into the supernatant of infected Meg-01 cells in presence or absence of DFMA (5 µM). Meg-01 cells were infected with DENV2 at an MOI of 0.1 and cultured. The DENV2 RNA was quantified by qRT-PCR in the supernatants of DENV2-infected Meg-01 cells at 1,2,3,5,7,10, and 14 days post infection. The release of DENV2 into the supernatant of infected Meg-01 cells peaked at day 10. B: The viability of DENV2-infected Meg-01 cells in the presence or absence of DFMA (5 µM) was measured using a trypan blue exclusion assay (ViCell XR Cell Viability Analyzer, Beckman Coulter) at 1, 2, 3, 5, 7, 10, and 14 days post infection. The Meg-01 cell viability for untreated and treated DENV2-infected Meg-01 cells showed a similar trend, peaking at day 3 and declining until day 7 to 10, and increasing until day 14. Thus, DFMA treatment did not appear to cause substantial cell death compared to the untreated Meg-01 cells when tested up to 5 µM.

	DFMA Activity for DENV2	
	EC_{50} (µM)	EC_{90} (µM)
Huh-7 Cell Line	4	9.6
Meg-01 Cell Line	<0.1-0.91 (n=2)	2-6.7 (n=2)

Table 1: Comparison of DFMA potency against DENV2 in the cell lines Meg-01 and Huh-7. The potency of DFMA in the Meg-01 cell line was comparable to the potency of the compound in Huh-7 cells, exhibiting an EC_{50} of 4 µM and EC_{90} of 9.6 µM.

CHAPTER III: CELLULAR PHARMACOLOGY OF DFMA IN THE CELL LINE MEG-01

Introduction

In order for a nucleoside analog to achieve antiviral activity in infected cells, it must first be converted to the tri-phosphate nucleotide by cellular nucleoside/nucleotide kinases. Since different cell types have different expression levels of the nucleoside/nucleotide kinase, the conversion efficiency of the nucleoside to the nucleotide tri-phosphate form directly affects the intracellular concentration of the nucleoside tri-phosphate, leading to different antiviral EC_{50} values of the compound in different cell types. The kinetics of intracellular nucleoside tri-phosphate formation are also important in regards to dengue infection, particularly because dengue infection is acute, a nucleoside analog that treats dengue should be fast-acting to meet the narrow time of treatment window[39]. The goal of this part of the thesis project was to determine the intracellular concentration of DFMA mono-, di-, and triphosphate metabolites in Meg-01 cells, and to determine an optimal time point for the conversion of DFMA to its tri-phosphate form in the model cell line.

Materials and Methods

Compound and Cell Lines: The original Meg-01 cells (Dr. Villinger) were obtained from Dr. Francois Villinger at Yerkes National Primate Research Center. The Meg-01 cell line referred to as "ATCC" was obtained from ATCC (CRL-2021)[40]. Both cell lines were maintained in HyClone RPMI-1640 (GE Healthcare) supplemented with 10% fetal bovine serum (+Penicillin/Streptomycin). All cell lines were maintained at 37°C/5% CO_2. The compound DFMA was synthesized in-house, and a stock solution was prepared in DMSO to a final concentration of 40 mM, and stored at -20°C.

Cellular Pharmacology: The following protocol was based on a protocol in our lab used to examine the cellular pharmacology of HIV-1 nucleoside analogs in primary macrophages[41]. On day 1 of the experiment, 1×10^6 Meg-01 cells were seeded per well in the wells of a 12-well plate. DFMA from a 200 µM working solution was added to the treated wells of the plate for a final concentration of 10 µM and 50 µM**.** Both Dr. Villinger's and the ATCC Meg-01 cells were used in the cellular pharmacology experiments, and one 12-well plate was used for each cell line. The two plates were incubated for 4 hours/ 37°C/ 5%CO_2. The cells were collected by using a micropipette to transfer the 1mL total well volume to a centrifuge tube. The final cell counts were 0.99 x 10^6 viable cells/mL (Dr. Villinger's Meg-01 cells at passage 64) and 0.91 x 10^6 viable cells/mL (ATCC Meg-01 cells at passage 7) as measured with the ViCell XR (Beckman Coulter). The samples were centrifuged, and the media was removed completely before flicking the pellet. Then, 1mL ice cold 1X phosphate-buffered saline (PBS) (Thermo Fisher Scientific) was added to the samples. The supernatant was removed after centrifugation. The PBS wash step was repeated, and the residual PBS was removed completely as it can interfere with LC-MS/MS. 1 mL of 70% methanol containing the internal standard ddATP was added to each sample to extract the metabolites from the cells, and the samples were stored at -80°C overnight. On Day 2, all the cell samples were vortexed, centrifuged, and dried with a CentriVap Centrifugal Vacuum Concentrator (Labconco). The dried samples were stored at -20°C until ready for LC-MS/MS analysis.

LC-MS/MS Analysis of Metabolites: Mass spectrometry analysis was performed by Sijia Tao (Instructor at Emory University, Department of Pediatrics). Structurally similarly

compounds to DFMA were used to construct calibration curves to quantify the intracellular concentration of DFMA in Meg-01 cells. Dr. Tao used standards 2'-Me-DMP and 2'-Me-DTP to make calibration curves for quantification of 2'-Me-7-F-AMP and 2'-Me-7-F-ATP. 2'-Me-7-F-ADP was quantified based on the calibration curve of 2'-Me-DTP. The Triple quadrupole mass spectrometry (TSQ) instrument (Thermo Scientific) was used for LC-MS/MS analysis. Dr. Tao used the SRM (selected reaction monitoring) mode to detect the compound. The principle of this method is to apply collision energy for the molecule to break down into a parent (m/z is [M+1] in positive mode) and a product m/z (the fragment ion)[42]. The parent and product m/z values for the metabolites DFMA (2-Me-7-F-Adenosine) and the mono-, di-, and tri-phosphate forms of DFMA are shown in **Figure 8**.

Results

Since DFMA displays potency against DENV2 in Meg-01 cells, we reasoned that this nucleoside analog must be phosphorylated in these cells in order to convert the parent compound to the active triphosphate form. In order to confirm the results with Dr. Villinger's cells, we obtained a new lot of Meg-01 cells (Number CRL-2021) from ATCC. However, differences in cell morphology were observed between the two cell lines; the Meg-01 cells from ATCC at passage 1 were predominantly suspension cells that had a lymphoid morphology while the adherent cells adopted a fibrous morphology where cell protrusions were visible **(Figure 7)**. In contrast, the Meg-01 cells from Dr. Villinger were entirely suspension cells with a lymphoid morphology. We hypothesized that these differences in cell characteristics would lead to a difference in the DFMA mono-, di-, and tri-phosphate levels between the two cell lines.

LC-MS/MS analysis showed that the parent nucleoside DFMA was phosphorylated to the mono-, di- and tri- phosphates in both the Dr. Villinger and the ATCC Meg-01 cell lines **(Table 2)**. After treated with the same concentration of DFMA, cells from ATCC generated a lower amount of 2'-Me-7-F-ATP than those from Dr. Villinger **(Figure 9)**. Interestingly, there was a 4.7 fold difference in the 2-Me-7-F-ATP concentration between the Dr. Villinger and ATCC Meg-01 cells treated with 10 μM DFMA. There was a 3.3 fold difference between the Dr. Villinger and ATCC Meg-01 cells treated with 50 μM DFMA.

Discussion

The results indicated that when the parent nucleoside DFMA is used to treat Meg-01 cells extracellularly, the nucleoside is phosphorylated by cell kinases to the mono-, di-, and tri-phosphate forms. Thus, DFMA is able to be converted to the active triphosphate form and could function as a substrate for the DENV RdRp enzyme. The differences in the intracellular levels of 2'-Me-7-F-ATP could be due to differences between the morphology and growth rate of the Dr. Villinger and ATCC Meg-01 cells. The cells from ATCC (used at passage 7) grow much slower than those from Dr. Villinger (used at passage 64). When Dr. Tao monitored the intracellular natural NTP levels in both cell types, she found that cells from ATCC have a lower level of TTP and UTP than the Meg-01 cells from Dr. Villinger (unpublished data).

Figures

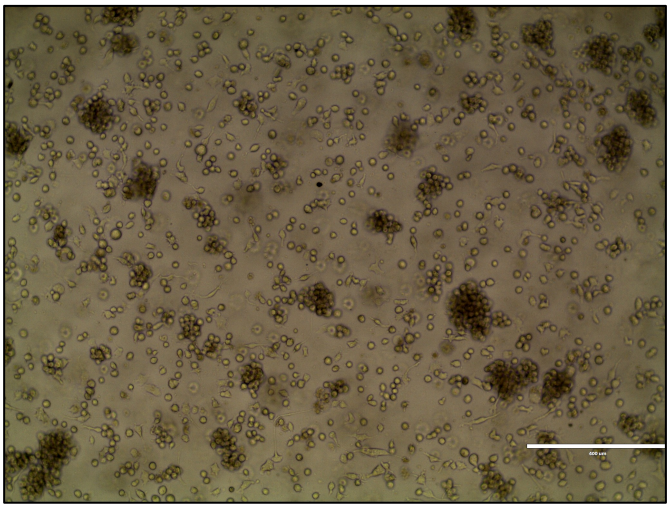


Figure 7: Meg-01 cells (ATCC, CRL-2021) at passage 1. Suspension cells have a lymphoid morphology while adherent cells adopt a fibrous morphology where cell protrusions are visible. Image was obtained with EVOS XL Cell Imaging System (Life Technologies). Scale bar represents 400 µm.

Drug/ metabolite	Parent m/z > product m/z
DFMA	299.1 > 153
2'-Me-7-F-AMP	379 > 153
2'-Me-7-F-ADP	459 > 153
2'-Me-7-F-ATP	539 > 153

Figure 8: Representative m/z values for the metabolites DFMA (2-Me-7-F-Adenosine) and the mono-, di-, and tri-phosphate forms of DFMA in Meg-01 cells treated with DFMA for 4 hours. The Triple quadrupole mass spectrometry (TSQ) instrument (Thermo Scientific) was used for LC-MS/MS analysis. Dr. Tao used the SRM (selected reaction monitoring) mode to detect DFMA as well as the mono-, di-, and tri-phosphate metabolites of DFMA in Meg-01 cells treated with DFMA. This method applies collision energy so the molecule breaks down into a parent (m/z is [M+1] in positive mode) and a product m/z (the fragment ion). The parent and product m/z values for the metabolites DFMA (2-Me-7-F-Adenosine) and the mono-, di-, and tri-phosphate forms of DFMA ranged from 299.1 to 539 for the parent m/z and 153 for the product m/z. "RS-574" refers to DFMA. (Dr. Sijia Tao, Emory University).

Metabolites (pmol/10^6 cells)	ATCC		Dr. Villinger	
	10 µM (n = 2)	50 µM (n = 1)	10 µM (n = 3)	50 µM (n = 3)
2'-Me-7-F-A	31.3 ± 2.3	113	5.51 ± 0.84	14.3 ± 3.0
2'-Me-7-F-AMP	21.9 ± 2.3	76.5	49.5 ± 6.8	118 ± 25
2'-Me-7-F-ADP	18.3 ± 0.1	102	39.8 ± 3.2	138 ± 20
2'-Me-7-F-ATP	12.8 ± 0.3	66.3	59.6 ± 5.6	220 ± 22

Table 2: Quantitative results for DFMA in Meg-01 cells. The concentration of the 2'-Me-7-F-Adenosine, 2'-Me-7-F-Adenosine mono- phosphate, 2'-Me-7-F-Adenosine di-phosphate, and 2'-Me-7-F-Adenosine tri-phosphate metabolites in Meg-01 cell lines treated with 10 or 50 RS-574 (2'-Me-7-F-Adenosine) was measured using LC-MS/MS analysis by utilizing the Triple quadruple mass spectrometry (TSQ) instrument Structurally similarly compounds to DFMA were used to construct calibration curves to quantify the intracellular concentration of DFMA in Meg-01 cells. Dr. Tao used standards 2'-Me-DMP and 2'-Me-DTP to make calibration curves for quantification of 2'-Me-7-F-AMP and 2'-Me-7-F-ATP. 2'-Me-7-F-ADP was quantified based on the calibration curve of 2'-Me-DTP. Analysis showed that the parent nucleoside DFMA was phosphorylated to the mono-, di- and tri- phosphates in both the Dr. Villinger and the ATCC Meg-01 cell lines. The number following the metabolite concentration indicates the standard deviation. (Dr. Sijia Tao, Emory University).

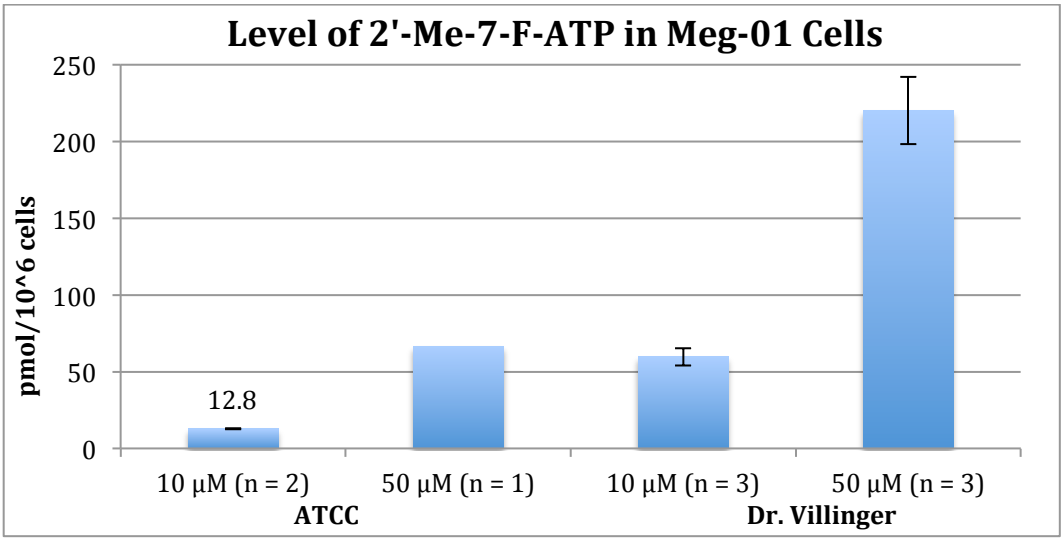


Figure 9: The level of 2'-Me-7-F-ATP (the triphosphate form of RS-574) in Meg-01 cells from ATCC versus Dr. Villinger. The concentration of the 2'-Me-7-F-ATP metabolite was measured with LC-MS/MS. After treatment with the same concentration of DFMA, cells from ATCC generated a lower amount of 2'-Me-7-F-ATP than those from Dr. Villinger. There was a 4.7 and 3.3 fold difference in the 2-Me-7-F-ATP concentration between the Dr. Villinger and ATCC Meg-01 cells treated with 10 µM and 50 µM DFMA. The number of replicate wells in the 12-well plate for each concentration tested is listed next to the compound concentration in parentheses. Error bars indicate standard deviation. (Dr. Sijia Tao)

CHAPTER IV: DENGUE VIRUS RESISTANCE TO DFMA

Introduction

Knowledge is lacking in regards to the mutations in the DENV genome that contribute to nucleoside analog resistance. Only one study has focused on selecting and characterizing DENV resistant to Brequinar, an inhibitor of an enzyme in the pyrimidine biosynthesis pathway that blocks dengue RNA synthesis by depleting intracellular pyrimidine pools. Interestingly, this study identified the mutation E802Q in the priming loop of NS5 as contributing to Brequinar resistance, and determined that E802Q confers resistance through enhancement of viral RNA synthesis[43]. To fill the knowledge gap in the mechanisms of dengue resistance to nucleoside analogs, this thesis work focused on selecting dengue virus resistant to DFMA and characterizing the mutations genotypically. This also provided information about the time frame for emergence of dengue virus resistant to DFMA, thus recapitulating the process by which DENV will evolve in nature once this drug is administered in a patient. Nucleoside analogs have been regarded as posing a high barrier to resistance, and this has been shown for another virus in the family *Flaviviridae*, hepatitis C – although resistant virus can be selected *in vitro*, in humans the virus is highly unfit and thus does not easily become resistant to nucleoside analogs compared to other classes of drugs[44]. However, the barrier to nucleoside analog resistance has not been studied for dengue *in vitro* or *in vivo*.

Materials and Methods

Virus, Compound, and Cell Lines: The virus used for all experiments was DENV2 Strain 16681 (Titer = 3.2 x 10^6 ffu/mL), a clinical isolate from Thailand, kindly obtained

from Kristina Clark at Yerkes National Primate Research Center. Meg-01 (obtained from Dr. Villinger) was maintained in HyClone RPMI-1640 (GE Healthcare) supplemented with 10% fetal bovine serum (+Penicillin/Streptomycin) and was infected with DENV2 at an MOI of 0.1 at passage 1. Huh-7 was maintained in HyClone DMEM supplemented with 10% fetal bovine serum (+Penicillin/Streptomycin) and was infected with DENV2 at an MOI of 0.5 at passage 1. All cell lines were maintained at 37°C/5% CO_2. The compound DFMA was synthesized in-house, dissolved in DMSO to a final concentration of 40 mM, and stored at -20°C.

Generation of DENV2 Resistant to RS-574 in Cell Culture: The protocol for generating resistant DENV2 was based on the principle of increasing the selective pressure of the nucleoside analog during the passaging of the virus in tissue culture. More specifically, the protocol was derived from studies on the selection of HIV-1 with resistance mutations to nucleoside analogs in cell culture from Dr. Raymond Schinazi's laboratory and Dr. Mark Wainberg's laboratory[45,46]. DENV2 resistant to DFMA was generated by passaging the virus on Huh-7 and Meg-01 cells in the presence of DFMA. For each round of passaging, Huh-7 and Meg-01 cells seeded in T_{25} flasks were pre-treated with DFMA for 1 hr in 5 mL (for passage 1) or 2 mL (for subsequent passages) of media. The pre-treated cells were infected with DENV2 for 2 hr by the addition of 4-5 mL of the previous passage's supernatant to the culture. As a control, untreated Huh-7 and Meg-01 cells were infected with 4-5 mL supernatant from the previous passage for 2 hr and incubated in tandem with the treated flasks. Finally, media was added to each of the untreated and treated flasks to bring the total volume of the culture to 10 mL. For each passage, viral supernatants were harvested 4-5 days post infection and were used

to start a new passage **(Figure 10)**. To determine the percent inhibition of viral replication for each passage, 300 µL of supernatant was collected from the untreated and treated flasks the day prior to passaging. Isolation of DENV2 RNA was performed with the EZ1 Virus Mini Kit and EZ1 Advanced XL instrument (Qiagen) on 300 µL of supernatant lysed with 100 µL Buffer AVL (Qiagen). To quantify the percent inhibition of viral replication, qRT-PCR was performed using the LightCycler 480 RNA Master Hydrolysis Probe Kit and LightCycler 480 II Instrument (Roche) with the following reaction conditions: 5 µL of the RNA and 15 µL of a mastermix containing the LightCycler mix, primer/probe, activator, and water. The qRT-PCR reactions were performed in a LightCycler 480 Multiwell Plate 96 (Roche). The difference in Ct values between the untreated and treated flask was calculated (ΔCt). The percent inhibition was determined using the formula for half-life: Percent Inhibition=(1-(e^(-0.693*ΔCt)))*100.

DENV2 genome isolation and NS5 amplification: cDNA synthesis was performed with the SuperScript VILO MasterMix kit (Invitrogen) using the EZ1 extraction total nucleic acid as a template. NS5 was amplified from the cDNA with primers Fwd-DENV2-BamHI NS5-ME and Rev-DENV2-XhoI-NS5-ME and with Phusion High-Fidelity DNA Polymerase (New England Biolabs). PCR products were purified with the QiaQuick PCR Purification Kit (Qiagen). Adenosine overhangs were added with TAQ Polymerase. A TOPO cloning reaction was performed with the NS5 PCR product and pCR2.1 vector following specification of the TOPO TA Cloning Kit (Invitrogen). XL1-Blue Supercompetent cells (Agilent Technologies) were transformed with the pCR2.1-NS5 plasmid. Overnight cultures were inoculated with 59 colonies, and plasmid DNA was

purified with the QiaPrep Spin MiniPrep Kit (Qiagen). The 59 colonies were screened for the presence of the NS5 insert by restriction digest with BamHI/NotI or BamHI/XhoI.

Sequencing of NS5 from DENV2 Resistant to DFMA: The Untreated Clone 10 pCR2.1-NS5 and Treated Clones 4, 7, and 9 pCR2.1-NS5 plasmids were sent for sequencing with M13 Reverse Primers and the internal primers DENV2 NS5 16681 seq 5-7 **(Table 3)**. The NS5 gene region from the untreated and treated clones was sequenced via Sanger sequencing through GeneWiz.

Results

In the Huh-7 cell line, quantifying the percent inhibition of DENV2 RNA between the treated and untreated Huh-7 cultures for each passage was used to monitor development of DFMA DENV2 resistant virus. Resistance selection was terminated at Passage 33 (P33). During the selection process, the Huh-7 cells were used at passage 12 to 48 and were 40-90% confluent in the T_{25} flask on the day the DENV2 supernatant from the previous passage was added to the culture. The DENV2 virus derived during the selection process was able to replicate at 12 μM DFMA (3X the EC_{50} concentration) at P30-P32 **(Figure 11)**. The phenotype of this virus indicates that it may harbor mutations that render it resistant to DFMA since the DENV2 RNA quantity in the Passage 32 12 μM DFMA treated culture was 2.71×10^5 copies/μL.

In the Meg-01 cell line, development of DFMA resistance was also monitored by quantifying the percent inhibition of DENV2 RNA between the treated and untreated Meg-01 cultures. Resistance selection was terminated at P18. During the selection process, the viability of the Meg-01 cells was 85-97% (measured with ViCell XR Cell Viability Analyzer), with the exception of Passage 2 (81.6% viability). The Meg-01 cells

were used at passage 17 to 54. The DENV2 virus derived during the selection process was able to replicate at 4 μM RS-574 at Passage 7 and 5 μM DFMA at Passage 15, and the phenotype of this virus indicates that it may harbor mutations that render it resistant to DFMA **(Figure 12)**.

The Untreated Clone 10 pCR2.1-NS5 and Treated Clones 4, 7, and 9 pCR2.1-NS5 plasmids were sent for sequencing with M13 Reverse Primers at GeneWiz. Unfortunately, the sequence for clone 9 of pCR2.1-NS5 contained nonspecific results in the trace data and thus, these results were not used further **(Figure 13)**. The Untreated Clone 10 and Treated Clones 4 and 7 sequences and chromatograms were aligned to identify any mutations in the DENV2 NS5 gene. However, no reliable mutation was found in the NS5 sequence since the untreated and treated pCR2.1-NS5 sequences were identical. A reason why an NS5 mutation was not identified in the clones selected is that a viral quasispecies exists in the resistant DENV2 populations that was selected in the Huh-7 culture. In order to verify that other DENV2 viruses in the population do not harbor NS5 mutations, more pCR2.1-NS5 clones can be screened and sequenced.

A mutation attributed to RS-574 resistance can be present in the remaining portion of the DENV2 NS5 from pCR2.1-NS5. In addition, the remaining portion of the Untreated Clone 10 pCR2.1-NS5 was sent for sequencing with custom designed primers based on the DENV genome **(Table 3)**. To conclude the sequencing study, the remaining portion of the Treated Clones 4 and 7 pCR2.1-NS5 will be sent for sequencing with the custom designed primers to compare the untreated and treated NS5 sequences.

Discussion

The next step for this part of the project is to phenotypically characterize the DFMA resistant DENV2. To do this, plaque morphologies of wildtype versus mutant DENV2 can be compared after performing plaque assays on BHK cells. In addition, the fold increase in DFMA resistance of the mutant DENV2 virus can be determined, and the $TCID_{50}$ of the wildtype versus mutant DENV2 virus can be compared. Reverse genetics can be utilized to introduce the mutations identified as contributing to DFMA resistance in cell culture into an infectious clone of the DENV2. The full-genome-length cDNA clone, called pD2/IC-30P, was constructed from three intermediate clones by ligating the separate nucleotide regions into a modified pBR322 plasmid[47]. Site-directed mutagenesis can be performed with the QuikChange site-directed mutagenesis kit (Agilent Technologies). The presence of the desired mutations can be confirmed through sequencing, and the mutant molecular clones can be transfected into Huh-7 cells for the production of mutant virus. Drug susceptibilities of the mutant viruses can be tested using qRTPCR to determine the EC_{50} and EC_{90} of DFMA against the mutant viruses. While the current selected population may harbor resistant mutation(s) against DFMA, it is plausible that the continuous culture of the current resistant population with high drug concentrations may select viral population with even higher resistance to DFMA.

To biochemically confirm that mutations selected for in cell culture contribute to DFMA resistance, we will demonstrate that DFMA has reduced potency against purified DENV mutant NS5 enzyme with the *in vitro* polymerase activity assay. The mutations present in NS5 that were identified to confer resistance to DFMA will be introduced into

the pET28a expression vector containing the NS5 gene of DENV 1-4 via site-directed mutagenesis. The NS5 mutant proteins will be expressed in *E. coli* BL21 (DE3) cells and purified. Susceptibility of the mutant NS5 enzyme to DFMA will be assessed by calculating the inhibitory potency (IC_{50}) of RS-574 against the mutant DENV NS5 enzyme in *in vitro* polymerase assays[48,49].

Figures

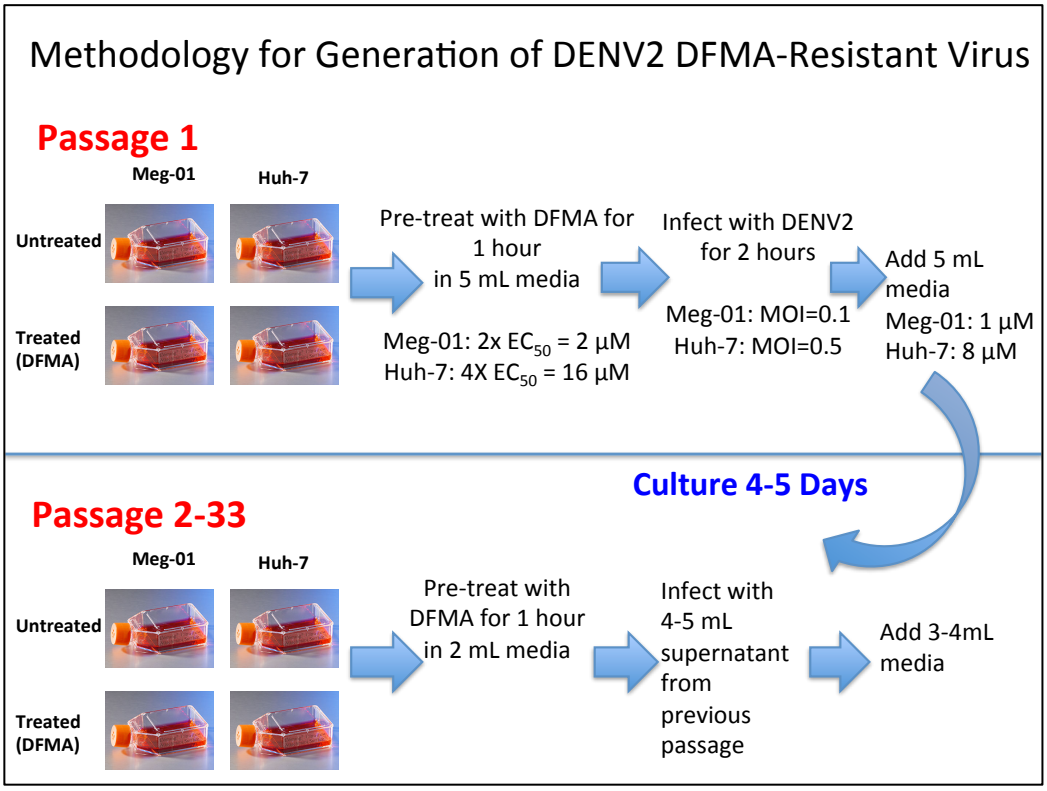


Figure 10: Scheme for selection of RS-574 resistant DENV2. For each passage, Meg-01 and Huh-7 cells were pre-treated with DFMA for 1 hr. Then, the cells were infected with DENV2 for 2 hr, and the total culture volume was brought up to 10 mL. For Huh-7 cells, resistance selection was terminated at P33, after 6 months of passaging. For Meg-01 cells, resistance selection was terminated at P18, after 3 months of passaging.

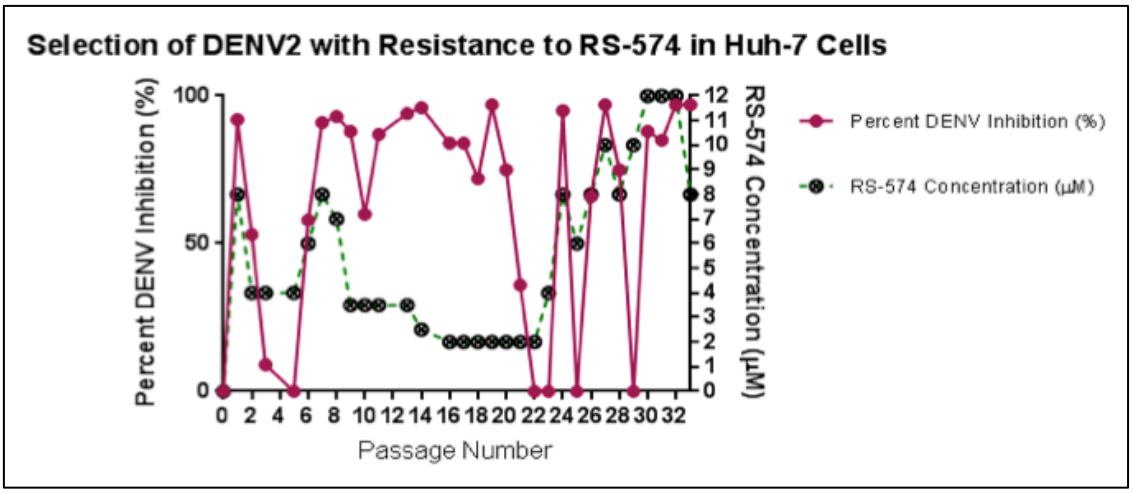


Figure 11: Selection of DENV2 16681 with Resistance to DFMA in Huh-7 cells. For each passage, development of DFMA resistance was monitored by quantifying the percent inhibition of DENV2 RNA between the treated and untreated Huh-7 cultures via qRT-PCR. Resistance selection was terminated at P33. "RS-574" refers to DFMA.

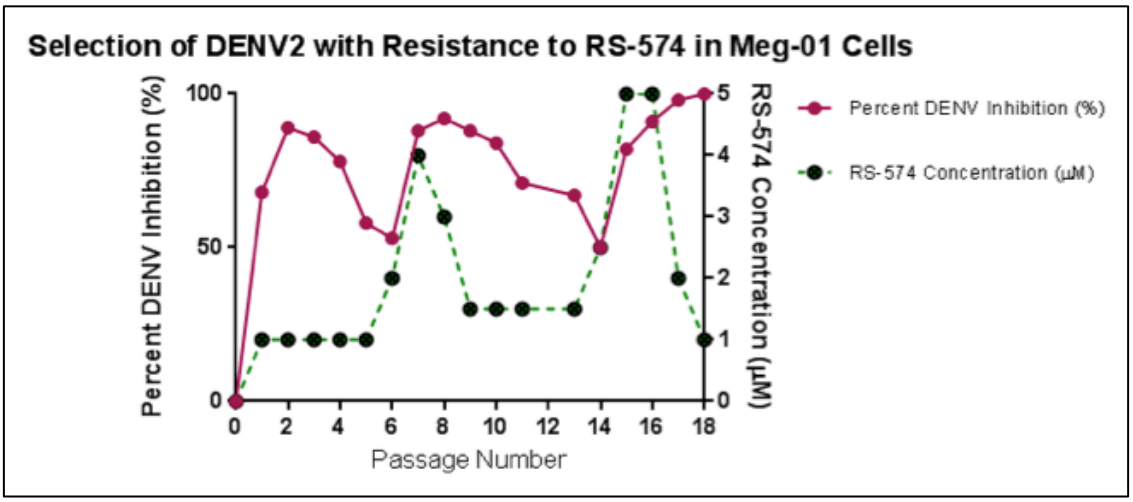


Figure 12: Selection of DENV2 16681 with Resistance to RS-574 in Meg-01 cells. For each passage, development of DFMA resistance was monitored by quantifying the percent inhibition of DENV2 RNA between the treated and untreated Meg-01 cultures via qRT-PCR. Resistance selection was terminated at P18. "RS-574" refers to DFMA.

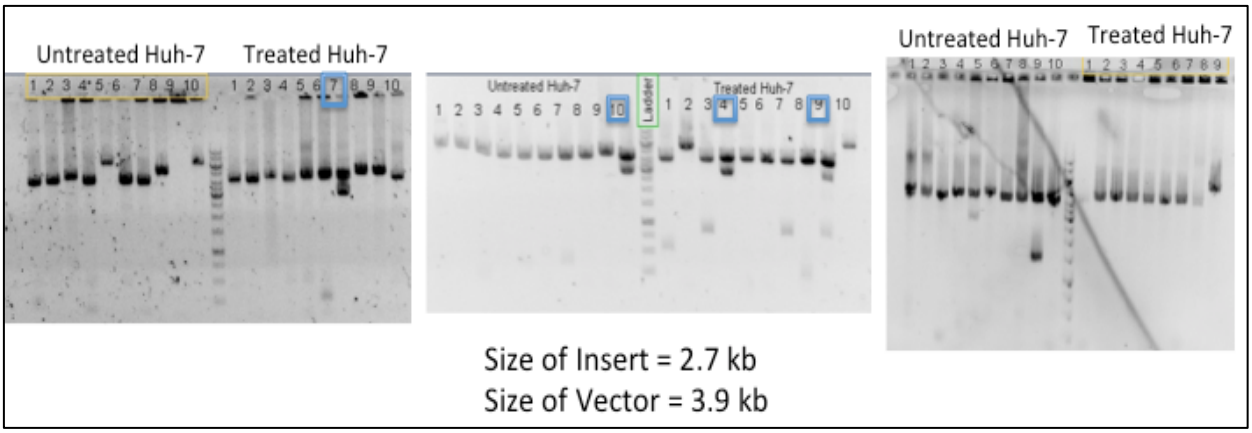


Figure 13: Analysis of *Escherichia coli* XL-1 Blue colonies transformed with pCR2.1-NS5 plasmid containing NS5 from DENV2 infected Huh-7 cells. The 59 colonies of XL1-Blue cells containing the pCR2.1-NS5 plasmid were screened for the presence of the NS5 insert by restriction digest with BamHI/NotI or BamHI/XhoI. Results indicate that Untreated pCR2.1-NS5 clone 10 and Treated pCR2.1-NS5 clones 4,7, and 9 contained the NS5 insert. The size of the NS5 insert was 2.7 kilobases (kb) and the size of the pCR2.1 vector was 3.9 kb.

Name	Sequence 5'- 3'
DENV2 NS5 16681 seq 5	5' AACTGGCAACATAGGAGAG 3'
DENV2 NS5 16681 seq 6	5' GGACGAACACTCAGAGTCCTTAAC 3'
DENV2 NS5 16681 seq 7	5' CAGCATATTGACGCTGGGAAAG 3'

Table 3: Internal primers used for sequencing NS5 from the resistant DENV2 16681 virus. The remaining portion of the Untreated Clone 10 pCR2.1-NS5 was sent for sequencing at GeneWiz with custom designed primers based on the DENV genome.

CHAPTER V: PURIFICATION AND ACTIVITY OF DENGUE VIRUS NS5 PROTEIN

Introduction

The mechanism of action of the nucleoside analog DFMA is unknown, although we suspect it inhibits DENV replication by interacting with the viral polymerase. Thus, we hypothesized that DFMA in its 5'-triphosphate form inhibits DENV replication by targeting the viral protein NS5 and serving as an inhibitor of this enzyme. Therefore, part of this thesis was geared towards utilizing biochemical approaches to test if DFMA inhibits the dengue RdRp, leading to termination of viral RNA synthesis. The first step of this process was to purify DENV3 NS5 enzyme that possessed RNA-dependent RNA polymerase (RdRp) activity. We chose to focus on purifying full-length NS5 because it has been demonstrated that the N-terminal methyltransferase domain of NS5 regulates RNA synthesis initiation and elongation by the C-terminal polymerase domain[50]. This chapter is focused on purifying DENV3 NS5 from bacterial cells and testing if the purified NS5 has RNA-dependent RNA-polymerase catalytic activity using an *in vitro* polymerase assay to quantify primer-extension of a primer-template hybrid.

Materials and Methods

Cloning DENV3 NS5 into the expression vector pET28a: DENV Serotype 3 NS5 Accession Number AY662691 was ordered from IDT through Custom Gene Synthesis[32]. Codon optimization for the gene was performed for *Escherichia coli.* Alignment of NS5 versus the NS5 codon optimized sequence showed a 74.8% nucleotide sequence identity and 100% amino acid sequence identity. BamHI and SacI were unique restriction sites not present in codon optimized DENV3 NS5. Thus, a BamHI site was added to the N-terminus of NS5, and a SacI site was added to the C-

terminus of NS5. 3 µg of the pUC-IDT-NS5FL plasmid (carrying an Ampicillin resistance marker) and 3 µg of the pET28a plasmid (carrying a Kanamycin resistance marker) were digested with FastDigest BamHI and SacI (ThermoScientific) in the same reaction. A phenol-chloroform extraction followed by an ethanol precipitation was performed to isolate pure, digested plasmid DNA. The digested plasmid DNA was ligated with T4 DNA Ligase (Invitrogen) overnight at 21°C. The ligation reaction was used to transform XL1-Blue Supercompetent cells (Agilent Technologies), and the transformants were plated on kanamycin plates to select for colonies that harbored the pET28a plasmid containing the NS5 insert. Bacterial colonies were used to start overnight cultures, and plasmid DNA was isolated from 10 cultures using the QIAprep Spin Miniprep Kit (Qiagen) **(Figure 14)**. In order to confirm the presence of the NS5 insert, miniprepped plasmid DNA from 10 clones was digested with FastDigest BamHI and SacI (ThermoScientific) and analyzed on a 0.8% agarose gel **(Figure 15)**.

Expression of DENV3 NS5: In order to obtain pure, active NS5 for DENV3 with RdRp catalytic activity for enzymatic assays, we used the pET Expression System (Novagen) to express recombinant His-tagged NS5 *in Escherichia coli* BL21(DE3) cells. OneShot BL21(DE3) cells (Invitrogen) were transformed with the pET28a-NS5-clone10 plasmid. A colony was picked and grown in 250 mL LB (+30 µg/mL Kanamycin) overnight. Two 1L LB flasks (+30 µg/mL Kanamycin) were inoculated with 50 mL of the *E. coli* overnight culture. The culture was grown to log phase of growth with shaking for 2 hours. The final OD_{600} of Flask 1 was 0.722 and of Flask 2 was 0.664. The two flasks were left at 4°C for 4 hours to allow the cultures to cool. The cultures were induced with 0.4 mM IPTG (with the addition of 50 µM $MgCl_2$ and 50 µM Zinc Acetate) at 16°C overnight[51]. The cells

were pelleted by centrifugation at 17 hours and 21 hours post induction. The cell pellets were stored at -80°C.

Purification of DENV3 NS5: In order to purify DENV3 NS5 protein from the BL21(DE3)-pET28a-DENV3 NS5 pellets, Talon resin (Clontech) was utilized. First, Lysis Buffer (50 mM Hepes pH 7.2, 10% glycerol, 500 mM NaCl, 0.2% IGEPAL, 10 mM imidazole, 3.25 mM $MgCl_2$) was added to the pellets in addition to lysozyme, PMSF, and cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) and rotated for 30 min at 4°C. Then, the cultures were sonicated (10 cycles at 13 amp and 3 cycles of 20 amp) and centrifuged at 12,000 rpm for 40 min in a Beckman centrifuge (rotor =JA25.5) to clarify the lysate. The supernatant was added to 4 mL of Talon Resin (+Protease Inhibitor + PMSF) and rotated for 1 hour at 4°C. All subsequent steps were performed at 4°C. The resin and supernatant slurry was transferred to a chromatography column (BioRad) for gravity purification. The flow through was collected, and the column was washed with Wash Buffer (50 mM Hepes pH 7.2, 10% glycerol, 500 mM NaCl, 0.2% IGEPAL, 10 mM imidazole). The NS5 protein was eluted with Elution Buffer (50 mM Hepes pH 7.2, 10% glycerol, 500 mM NaCl, 0.2% IGEPAL) in sequential steps with buffer containing 25 mM, 50 mM, 100 mM, 250 mM, 500 mM imidazole. The protein was dialyzed overnight in 4L Storage Buffer (20 mM Hepes pH 7.2, glycerol 10%, 250 mM NaCl, 1 mM DTT, 0.1% CHAPS (w/v)). The NS5 protein was concentrated with Amicon Ultra Centrifugal Filter Device 50K (EMD Millipore). For purification 1, the final total protein concentration was 7.18 mg/mL in a 15 mL Total Volume of Storage Buffer (measured with NanoDrop 1000, Thermo Scientific). For purification 2, the final total protein concentration was 8.45 mg/mL in a 15 mL total volume of Storage Buffer

(measured with NanoDrop 1000, Thermo Scientific). The DENV3 NS5 protein was aliquoted in a 20 µL or a 500 µL volume (in storage buffer) into centrifuge tubes. All samples were flash frozen in liquid nitrogen and stored at -80°C.

Activity of pure DENV3 NS5: We tested activity of DENV3 NS5 by a filter-binding assay to measure the incorporation of ^{32}P alpha ATP into the P13/T30 primer-template hybrid **(shown in Figure 18)**. The reaction conditions were as follows: 20 mM Tris Buffer pH 7.5, 25 mM KCl, 0.5 µM P13/T30 Primer-Template Hybrid Cold, 0.20 µM ^{32}P α ATP (from 3.33 µM/250 µCi stock), Super RNAse Inhibitor (0.2 µL), 3 mM DTT, 2 mM $MgCl_2$, 2 mM $MnCl_2$, NTP Mix (400 µM CTP, UTP, GTP + 12 µM ATP), and 10 µM NS5 Enzyme (from Purification 2 at 80 µM stock concentration). As a negative control, one reaction did not contain NS5 enzyme. The reactions were incubated 2 hr at 30°C and 37°C. At 1 and 2 hr post the initiation of the reactions, samples were collected and blotted on DE81 (DEAE) paper. The DE81 paper was washed with distilled water, sodium phosphate (Na_2HPO_4), and ethanol and then dried. The ^{32}P radioactivity was measured by quantifying the counts per minute (cpm) for 3 replicates of each reaction with TopCount (PerkinElmer).

Sequencing of DENV3 NS5 FL: The pET28a-DENV3-NS5FL plasmid was used as a template for sequencing reactions with the six primers DENV3 NS5 FL seq 1-6 that spanned the 2.7 kb gene **(Table 4)**. The full length NS5 gene was sequenced via Sanger Sequencing at GeneWiz.

Results

DENV3 NS5 was cloned into the expression vector pET28a as shown in **Figure 14**. Analysis of the pET28a-DENV3-NS5FL plasmid DNA from 10 clones by gel

electrophoresis was performed to check for presence of NS5 insert. The plasmid DNA from the 10 clones was digested with BamHI and SacI and analyzed by on a 0.8% agarose gel. As a negative control, an unligated digestion reaction of the pET28a and DENV3 NS5 plasmids was used to transform XL1-Blue cells. The plasmid DNA was isolated and digested with BamHI and SacI. Results indicated that all 10 clones contained the 2.7 kb DENV3 NS5 insert **(Figure 15)**. DENV3 NS5 was purified from BL21(DE3)-pET28a-NS5-clone10 pellets. Denaturing SDS-PAGE analysis of the purification fractions (sonicate, flow-through, wash, and elutions) showed that NS5 eluted at 25 mM (Elution 1), 50 mM (Elution 2), 100 mM (Elution 3), 250 mM (Elution 4), and 500 mM imidazole (Elution 5). The purity of NS5 was approximately 60% full-length protein and 40% of smaller molecular weight bands that could be degradation products **(Figure 16)**. The Elution 2(E2) to Elution 5 (E5) fractions for both purifications were combined and dialyzed overnight in Storage Buffer. The RNA-dependent RNA polymerase catalytic activity of the purified NS5 was assessed by measuring the incorporation of radioactive ATP within the primer-template hybrid complex shown in **Figure 17.** The purified DENV3 NS5 exhibited approximately a 2.2 to 2.3-fold increase in RdRp activity compared when compared to the negative control **(Figure 18)**.

The full length NS5 from the pET28a-DENV3-NS5FL plasmid was sequenced with GeneWiz in order to assure that no nucleotide changes were present in the cloned NS5 gene. The pET28a-DENV3-NS5FL plasmid was used as a template for sequencing reactions with the six primers shown in **Table 4**. No nucleotide changes were found in the cloned DENV3-NS5FL sequence compared to the IDT NS5 sequence Accession Number AY662691.

Discussion

DENV3 NS5 was successfully purified, and the pure NS5 enzyme exhibited an increase in RdRp activity compared to a negative control. Additional optimization of NS5 RdRp activity is needed, including optimizing for manganese, magnesium, and enzyme concentration. In order to determine the accurate NS5 enzyme concentration, the extinction coefficient can be determined and that value can be used to determine the accurate concentration by reading the absorbance at 280 nm with NanoDrop 1000 (ThermoScientific). The purity of the NS5 prep can be re-analyzed after the AmiconUltra (Millipore) concentration step by SDS-PAGE, and the protein concentration can be re-calculated. It is expected that the Amicon column removed the bands that were less than 50 kDa; however, SDS-PAGE analysis could show a similar gel pattern as in **Figure 16**. If this occurs, Fast Protein Liquid Chromatography (FPLC) can be used to further purify NS5 from pooled 500 µL enzyme aliquots. Once higher purity of the NS5 enzyme prep is achieved, inhibition studies with DFMA-TP can be performed.

The next step in order to establish this filter-binding assay for analyzing inhibition of the DENV RdRp by DFMA is to characterize known NS5 inhibitors such as 2'-methyl-GTP and 3'-dGTP[32,39]. In addition, it would be beneficial to make active site mutants for DENV3 NS5 using the pET28a-DENV3-NS5 FL as the template for mutagenesis to confirm that purified NS5 is not contaminated with exogenous protein. Thus, if the purified active site mutant NS5 enzyme loses activity, it would indicate that the majority of the enzyme prep consists of DENV3 NS5 and not exogenous protein.

Figures

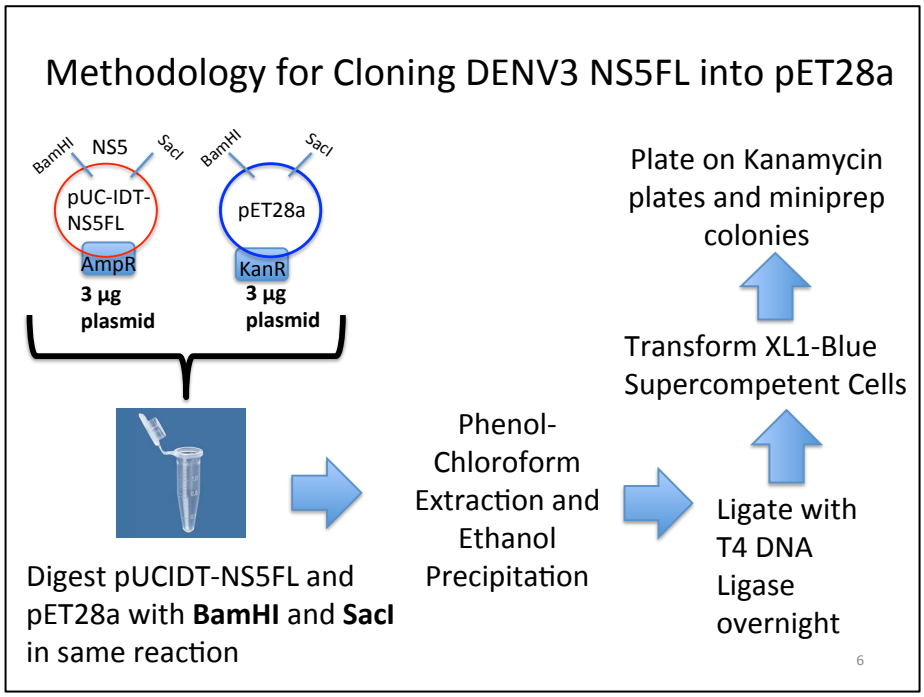


Figure 14: Schematic representation of the cloning strategy used to clone DENV3 NS5 Full Length into the pET28a expression vector. DENV Serotype 3 NS5 Accession Number AY662691 was ordered from IDT through Custom Gene Synthesis. Codon optimization for the gene was performed for *Escherichia coli.* BamHI and SacI were unique restriction sites not present in codon optimized DENV3 NS5. Thus, a BamHI site was added to the N-terminus of NS5, and a SacI site was added to the C-terminus of NS5. 3 µg of the pUC-IDT-NS5FL plasmid (carrying an Ampicillin resistance marker) and 3 µg of the pET28a plasmid (carrying a Kanamycin resistance marker) were digested with FastDigest BamHI and SacI (ThermoScientific) in the same reaction. A phenol-chloroform extraction followed by an ethanol precipitation was performed to isolate pure, digested plasmid DNA. The digested plasmid DNA was ligated with T4 DNA Ligase (Invitrogen) overnight at 21°C. The ligation reaction was used to transform XL1-Blue Supercompetent cells (Agilent Technologies), and the transformants were plated on kanamycin plates to select for colonies that harbored the pET28a plasmid containing the NS5 insert.

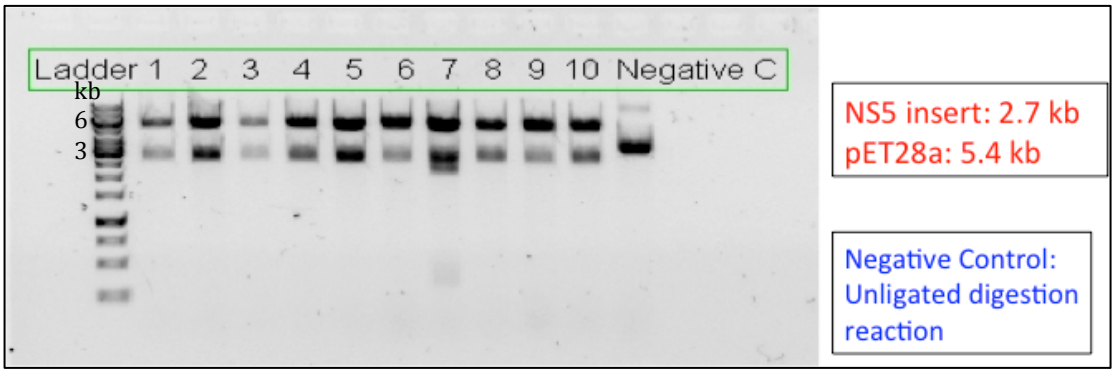


Figure 15: Analysis of pET28a-DENV3-NS5FL plasmid DNA from 10 clones by gel electrophoresis to check for presence of NS5 insert. The plasmid DNA from the 10 clones was digested with BamHI and SacI and analyzed by on a 0.8% agarose gel. As a negative control, an unligated digestion reaction of the pET28a and DENV3 NS5 plasmids was used to transform XL1-Blue cells; the plasmid DNA was isolated and digested with BamHI and SacI. Results indicated that all 10 clones contained the 2.7 kilobase (kb) DENV3 NS5 insert.

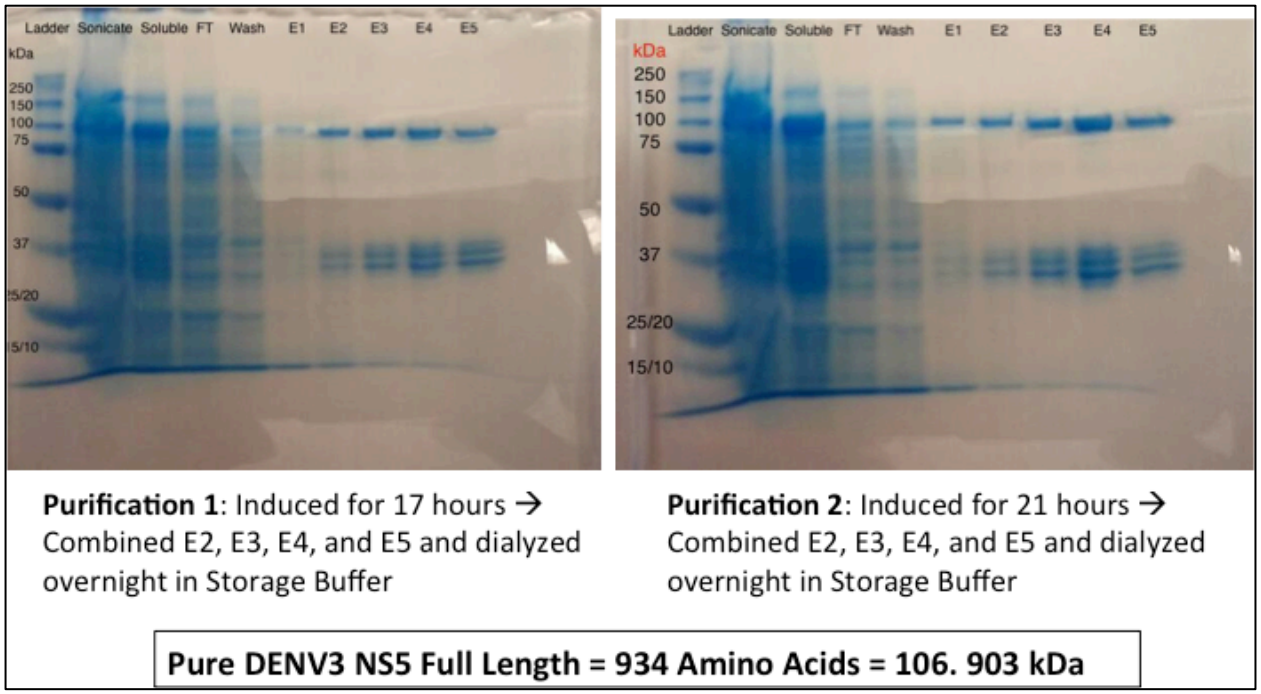


Figure 16: SDS-PAGE analysis of the purification fractions. 10 µL of the sample was added to 10 µL Sample Buffer (Novex) and 2 µL beta-mercaptoethanol (BME). The samples were heated at 95°C for 5 minutes. The samples were loaded on a 12% Tris-Glycine Gel (Novex) and analyzed under denaturing conditions. FT denotes flow-through; E1 to E5 denote Elution 1 to Elution 5, in that order.

*ATP → 3 TOTAL can be incorporated

P13 3’ C AGA CUA ACA ACU 5’

T30 5’ CAG AUA CAG UCA GAU GCG UCU GAU UGU UGA 3’

Figure 17: The sequence of the primer RNA (P13) and template RNA (T30) used in primer-extension assays with purified DENV3 NS5. Schematic representation of incorporation of radioactive ATP by pure DENV3 NS5 within the primer-template hybrid complex. The primer sequence consisted of 13 ribonucleotides (P13), and the template sequence consisted of 30 ribonucleotides (T30). The incorporation sites of the radioactive ATP within the template sequence are colored red.

	no NS5	NS5/30°C/1hr	NS5/37°C/1hr	NS5/30°C/2hrs	NS5/37°C/2hrs
Background	70	68	75	38	36
Replicate 1	1352	2670	2635	2982	3017
Replicate 2	1345	2659	2780	3250	2746
Replicate 3	1477	2380	2475	3096	3107
Replicate 1 (-Background)	1282	2602	2560	2944	2981
Replicate 2 (-Background)	1275	2591	2705	3212	2710
Replicate 3 (-Background)	1407	2312	2400	3058	3071
Average CPM	**1321**	**2502**	**2555**	**3071**	**2921**
STDEV	**74**	**164**	**153**	**134**	**188**

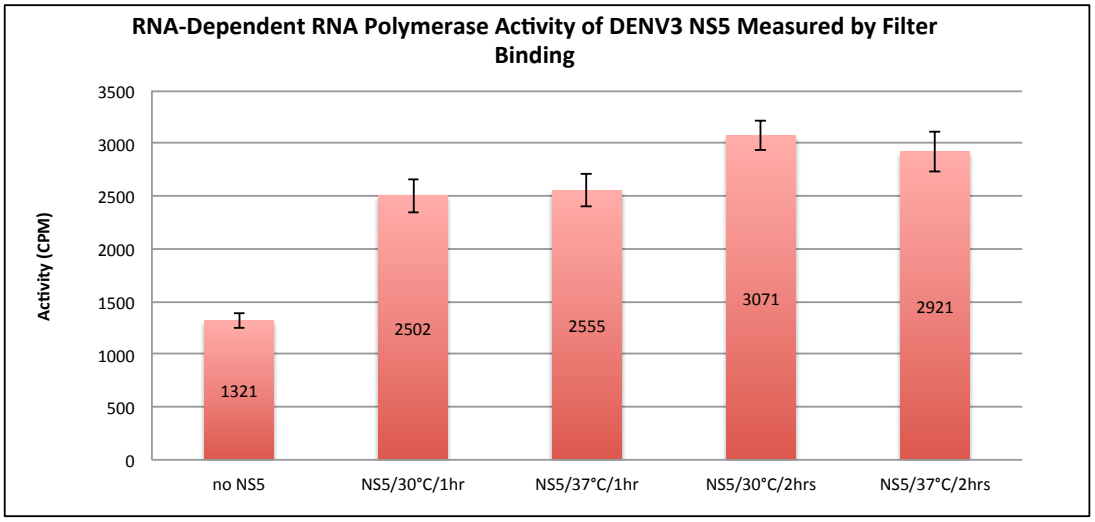


Figure 18: Filter binding assay measuring RNA-dependent RNA polymerase activity of the purified DENV3 NS5. A filter-binding assay was used to measure the incorporation of radiolabeled ATP into a primer-template hybrid by pure DENV3 NS5 enzyme. The pure NS5 enzyme was incubated with the P13-T30 hybrid at 30°C or 37°C for 1 or 2 hours. Samples from the reactions were blotted onto DE81 paper. The table shows the counts per minute (cpm) values obtained for 3 replicates for each reaction condition as measured by TopCount (PerkinElmer). The bottom panel shows a graphical representation of the average cpm values for the reaction conditions tested. Results indicated that the purified DENV3 NS5 exhibited approximately a 2.2 to 2.3-fold increase in RdRp activity compared when compared to the negative control The error bars represent the standard deviation of 3 replicates.

Name	Sequence 5'- 3'
DENV3 NS5 FL seq 1 AY662691	5' TACGGGCAGCCAAGGCGAAAC 3'
DENV3 NS5 FL seq 2 AY662691	5' AACCGTGGCTGAAAAACAATC 3'
DENV3 NS5 FL seq 3 AY662691	5' TGACGGACACAACGCCGTTTG 3'
DENV3 NS5 FL seq 4 AY662691	5' TGGGTTATATCTTGCGTGAC 3'
DENV3 NS5 FL seq 5 AY662691	5' AAGTGCGTAAAGACATTCCGC 3'
DENV3 NS5 FL seq 6 AY662691	5' ACGTGGGCGCAGAATATTCCG 3'

Table 4: Primers for sequencing DENV3 from pET28a-DENV3-NS5FL. The pET28a-DENV3-NS5FL plasmid was used as a template for sequencing reactions with the six primers DENV3 NS5 FL seq 1-6 that spanned the 2.7 kb gene. The full length NS5 gene was sequenced via Sanger Sequencing at GeneWiz.

CHAPTER VI: CONCLUSION

Future Directions

Characterizing the antiviral properties and mechanisms of resistance for DFMA will advance anti-dengue drug development. Assessing the pharmacological properties and mechanism of action of DFMA will generate new knowledge on a novel NAI for dengue as well as the mechanisms of viral resistance to this NAI. Furthermore, understanding the function of DFMA can lead to examining the structure-activity relationship (SAR) of other 2'-methyl modified nucleoside analogs such as Sofosbuvir (brand name Sovaldi) for dengue. Cocrystallization studies of nucleoside analogs such as DFMA with the NS5 enzyme will be beneficial to understand the SAR of 2'-methyl modified NAIs. Drug modifications can be included in the DFMA molecule to make the nucleoside analog more active. Synthesizing phosphate prodrugs of DFMA and other 2'-methyl modified NAIs will bypass the first phosphorylation step when these compounds are administered to infected cells or animals[52]. In addition, varying the lipophilicity of the compounds by adding one or more halogens to the sugar can make them more active in DENV-infected cells. This thesis project serves as the foundation to improve and characterize nucleoside analogs as therapeutic agents to treat dengue infection and to investigate mechanisms of resistance to these compounds.

Screening for anti-DENV drugs is essential in the quest to develop an FDA-approved antiviral drug for dengue. Since nucleoside analogs such as NITD-008 and DFMA will not be developed clinically due to inherent cytotoxicity, new leads are needed for biochemical and pharmacological studies in regards to DENV infection both *in vitro* and *in vivo*. An application of this thesis work is that the Meg-01 cell line can be utilized

for obtaining relevant pharmacological data regarding NAI drugs for dengue as well as screening anti-DENV drugs, since these cells are thought to be infected in humans. Currently, a Vero cell line (African green monkey kidney cells) and a BHK-DENV replicon cell line (baby hamster kidney cells) have been used to evaluate the anti-DENV activity of compounds[53–55]. Since these cell lines are not human-derived, it is problematic to test drug efficacy and cytotoxicity in these systems and then relate the results to humans. Subsequently, screening for anti-DENV drugs in Meg-01 cells will allow for better lead compound identification since these human cells have been shown to be primarily infected by DENV in the bone marrow of humans and are thus physiologically relevant[8–11]. This research project will contribute to increasing the number of anti-DENV candidate drugs that are in the pipeline.

Concluding Remarks

There are many considerations that have to be taken into account in regards to drug-development work on NS5. An important consideration for enzymatic assays aimed at demonstrating inhibition of RdRp activity by an NAI using pure NS5 is the post-translational modification of NS5. The NS5A/NS5 proteins of viruses from several general of the family Flaviviridae are phosphorylated by serine/threonine kinases[56]. DENV NS5 is phosphorylated by Protein Kinase G (PKG) at a strictly conserved serine/threonine phosphoacceptor site present in the genomes of the mosquito-borne flaviviruses. This phosphorylation of NS5 by PKG also modulates DENV replication in cell culture as a PKG activator and inhibitor was able to increase and decrease DENV replication in HEK293T cells, respectively[57]. In addition, DENV2 NS5 interacts with NS3 *in vivo*, and this interaction mediates the differential phosphorylation of NS5. NS5 is

present in its phosphoprotein form in the nucleus, while NS5 interacts with NS3 in the cytoplasm of cells[58].

An additional consideration in the search for a nucleoside analog drug for dengue infection is the timing of NAI administration. It is feasible that two scenarios of NAI administration are possible. The first scenario involves prophylaxis for people in dengue endemic regions or those living in areas with an ongoing dengue outbreak. The FDA-approved nucleoside analog could be administered to healthy people who are at risk for contracting dengue in order to prevent dengue infection as well as to prevent the spread of the virus throughout the local population. Prophylaxis with NAIs is a concept that has been studied for viruses such as norovirus and HIV. Prophylactic treatment of sentinel AG129 mice with 2'-C-methylcytidine (an NAI with anti-DENV activity) was able to provide protection against infection with genotype V (murine) norovirus[59]. Additionally, the combination drug Truvada® (emtricitabine and tenofovir disoproxil fumarate) has been FDA approved for pre-exposure prophylaxis to reduce the risk of HIV-1 infection in high-risk adults who are HIV-negative[60]. Conceivably, the prophylaxis concept can be applied to reducing the risk of dengue infection among people who are at higher risk for acquiring DENV. The second scenario for administration of an approved NAI is to dose the drug early during the dengue illness, in the first 24-48 hours after onset of symptoms. This approach for NAI administration was used in the first phase II clinical trial testing the effectiveness of Balapiravir in adult dengue patients. During the clinical trial, Balapiravir was administered to DENV-positive patients who experienced symptoms for <48 hours prior to initial dosing[36]. Thus, this approach of NAI dosing can

be taken in the future when setting guidelines for the timing of NAI delivery to dengue-infected persons.

Despite the significant advances in identifying NAIs with anti-DENV activity and understanding the functions of those NAIs, challenges remain to the nucleoside antiviral approach for dengue. First, NAIs often exhibit unpredictable *in vivo* toxicity. It is difficult to foresee what side effects a nucleoside analog will incur when administered to a person using *in vitro* cell culture assays; therefore, many NAIs have failed in clinical trials. Toxicity of NAIs is attributed to inhibition of mitochondrial polymerases. Second, renal toxicity can occur after NAI administration because NAIs are predominantly excreted through the kidney due to their polar nature. In addition, toxicity can arise due to the blocking of nucleoside triphosphate degradation inside cells. However, despite all these challenges to the development of nucleoside analog drugs for DENV, this class of drugs represents an attractive approach for combating dengue disease due to the numerous advantages this class of drugs offers[39].

Addendum

After this thesis work was completed, an article was published in MBio entitled "Suppression of Drug Resistance in Dengue Virus." The authors (Roberto Mateo, Claude Nagamine, and Karla Kirkegaard) selected DENV2 strain 16681 resistant virus to the nucleoside analog 2'-C-methyl-7-deaza-adenosine (also termed MK-0608, or 7-DMA). The DENV2 virus was passaged onto BHK-21 cells in the presence of increasing concentrations of MK-0608. The two viral pools passaged in the presence of MK-0608 acquired mutations in the NS5 coding region: A60T in pool 1 and Y201H in pool 2. Both of these mutations mapped to the methyltransferase domain of the NS5 protein. Thus, it is likely either that MK-0608 inhibits methyltransferase activity of DENV NS5, and can possible serve as a competitive inhibitor of the methyltransferase domain[61].

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