A CYTOPATHIC EFFECT-BASED HIGH-THROUGHPUT SCREENING ASSAY IDENTIFIED TWO NOVEL COMPOUNDS THAT INHIBIT DENGUE INFECTION: STREPTOVITACIN A AND NAGILACTONE C

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Kevin Dylan McCormick, M.S.

University of Pittsburgh, 2011

Dengue is an emerging infectious disease and is spreading world-wide at exponential levels. Two billion people in over 100 countries are at risk for infection from one of the four serotypes of the dengue virus. Those infected with dengue may develop diseases such as dengue fever and dengue hemorrhagic fever (DHF) and of the 500,000 cases that progress to DHF each year, more than 22,000 will result in fatality. Discovering new antivirals to treat DHF is essential to reducing this disease burden. Here, we have developed a cytopathic effect-based high-throughput screen (HTS) to discover possible inhibitors of Dengue viral infection of hepatocytes *in vitro*. Dengue virus infection of hepatocytes induces massive cell death, "cytopathic effect (CPE)", which we converted into a screening assay whereby inhibitors of Dengue infection prevent cells from dying. In this assay, the viral induced CPE is quantitated by monitoring cellular ATP levels, which positively correlates with cellular viability. ATP in the cell culture will drive the oxidation of luciferin resulting in the emission of light that is quantitated using a luminometer. The assay is simple and highly reproducible yielding a screening window coefficient, Z- factor, of 0.78±0.12 between plates. The Z-factor is a statistical parameter commonly accepted as an assay quality assessment and is reported as a value 0 to 1 and anything over 0.5 is considered excellent quality. This assay is advantageous to current

methodology as it simultaneously screens possible inhibitory compounds while controlling for any unwanted toxicity triggered by these drugs. Our initial HTS of a 288 small compound library yielded a total of eleven hits that prevented the CPE of dengue infection. Further evaluation with an immunofluorescence assay showed that two of these compounds, Streptovitacin A and Nagilactone C, are highly potent inhibitors of dengue infection. At effective inhibitory doses, they did not appear to be cytotoxic, and therefore both of these compounds are possible antivirals and could be used to elucidate various cellular mechanisms utilized during the dengue life cycle. The discovery of these two inhibitors demonstrates the efficacy of our newly developed assay and the public health significance of this project.

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I would also like to thank my loving wife, Kathryn McCormick. Without her support I am not sure if I could have made it this far. Special thanks to our cat Kiki lala (RIP 04.19.2011) she will always be remembered.

LIST OF ABBREVIATIONS

ATP, Adenosine-5'-triphosphate

Baf A, Bafilomycin A

C, Capsid Protein

CC_{50}, 50% Cytotoxicity Concentration

CD, Cluster of Differentiation

CPE, Cytopathic Effect

DAPI, 4',6-diamidino-2-phenylindole

DC-SIGN, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

DS-RNA, Double-Stranded Ribonucleic acid

DEN, Dengue Virus

DV, Dengue Virus Serotype

E, Envelope Protein

ER, Endoplasmic Reticulum

FBS, Fetal Bovine Serum

GFP, Green Fluorescence Protein

HCV, Hepatitis C Virus

HCVcc, Cell Culture derived Hepatitis C Virus

HTS, High-Throughput Screening Assay

HSP, Heat Shock Protein

IC_{50}, 50% Inhibitory Concentration

IFA, Immunofluorescence Assay

IFN-α, Interferon α

LARII, Luciferase Assay Reagent II

LUC, Luciferase

M, Membrane Protein

MOI, Multiplicity of Infection

MYA, Mycophenolic Acid

NCI, National Cancer Institute

NEAA, Non-Essential Amino Acids

NDV, New Castle Disease Virus

NS, Non-Structural Protein

PI, Post Infection

prM/E, Pre-Membrane/Envelope Protein

RDRP, RNA-dependent-RNA-polymerase

RLU, Relative Light Units

RLUexp, luciferase counts obtained from inhibitor treated wells

RLUmax, luciferase counts obtained from uninfected wells

RLUmin, luciferase counts obtained from untreated infected wells

SARS, Severe Acute Respiratory Syndrome Coronavirus

SS-RNA, Single-Stranded Ribonucleic acid

StDev, Standard Deviation

$TCID_{50}$, 50% Tissue Culture Infectious Dose

YPLL, Years of Potential Life Lost

1.0 INTRODUCTION

1.1 DESCRIPTION OF THE PROBLEM

Two billion people in over 100 countries are at risk for infection from one of the four serotypes of Dengue virus (DEN) making it the most prevalent mosquito-borne virus worldwide (10, 23). Those infected by this virus may develop diseases ranging from the self-limited febrile illness dengue fever (DF) to the more severe dengue shock syndrome (DSS) and dengue hemorrhagic fever (DHF). Of the 500,000 cases that progress to DHF each year, more than 22,000 will result in fatality (31). Unfortunately there are currently no vaccines or antiviral therapies approved for the treatment of infection. Novel therapeutics, therefore, are in urgent need to fight the infection.

1.1.1 Dengue Virus

DEN is an arthropod-borne virus (arbovirus) and is transmitted to humans by the imbibing of a mosquito, more specifically the *Aedes* genera of mosquito and most often from *Aedes aegypti* (31). It is an enveloped positive sense single-stranded RNA virion that belongs to the genus Flavivirus, family Flaviviridae. The 10.7kb genome of DEN is enclosed in the structural proteins, capsid (C), membrane (M) and envelope (E) forming the ~50um spherical lipid-enveloped particles (23).

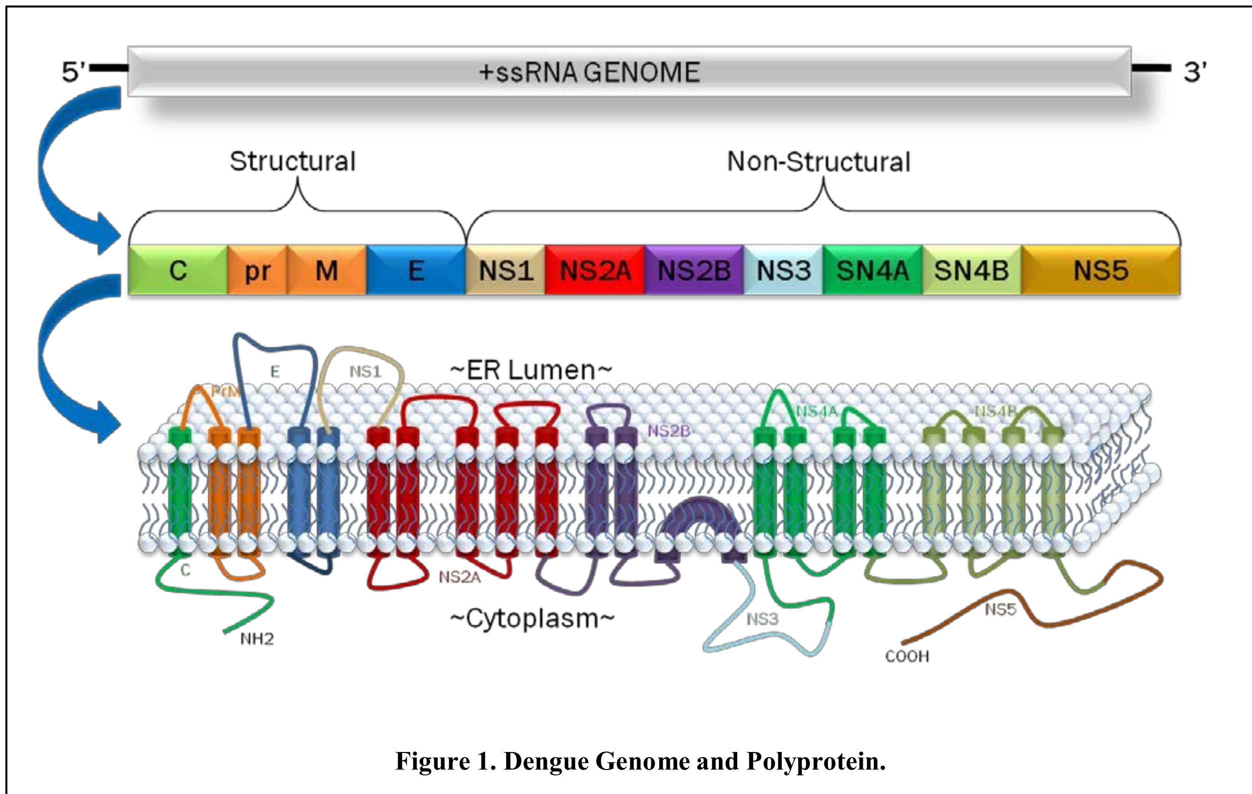


Figure 1. Dengue Genome and Polyprotein.

The positive-sense single-stranded genome of DEN is translated into a polyprotein containing three structural and seven nonstructural proteins. This polyprotein contains several transmembrane regions and is integrated into the lipid bilayer of the ER where it is cleaved by both host and viral proteases (Figure 1) (1, 24). Virus assembly occurs in the ER after cleavage and the virions are exocytosed through the Golgi secretary vesicle system (24).

It has not yet conclusively been shown what the primary sites of infection are by the virus given that viral antigens are found in many host cell lines during infection (8). In reality, dengue has abroad cellular tropism as it uses a number of ubiquitous receptors and cellular factors for entry including DC-SIGN, manose receptor, heparan sulfate, GRP78/BiP, CD14, Hsp90, and a 37/67-kDa high affinity laminin receptor (3, 4, 13, 18-20, 23, 28, 29). After the virion has attached to the required cellular receptors it is thought to migrate to pre-existing clathrin coated pits on the host cell before it enters via clathrin-mediated endocytosis (30). Once inside the

endosomes, a reduction in pH triggers fusion of the E protein with the lipid membrane and the releasing of the viral nucleocapsid into the cell cytosol (1, 23). The positive-sense RNA is translated into a polyprotein which is cleaved by both viral and host proteases and RNA-dependent-RNA-polymerase (RDRP) will replicate the viral genome of DEN.

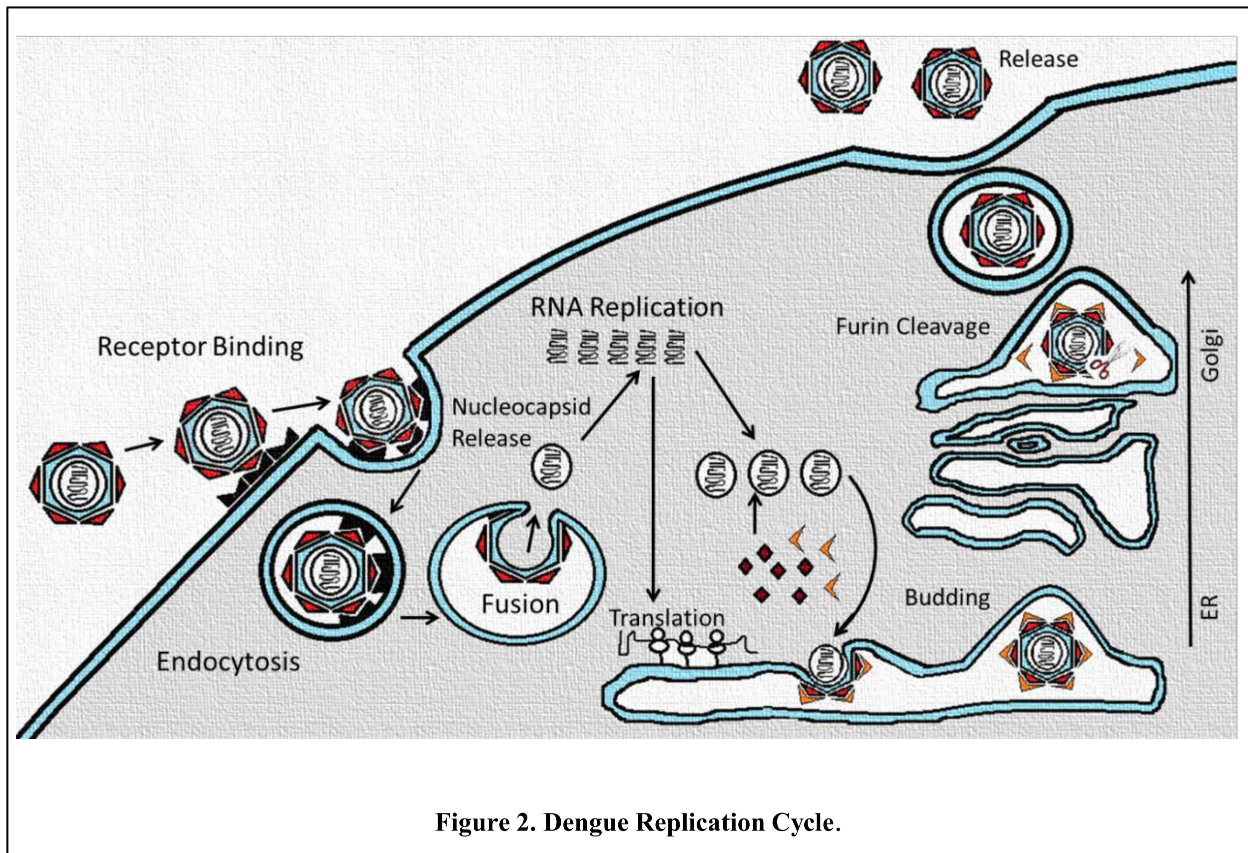


Figure 2. Dengue Replication Cycle.

1.1.2 Dengue Pathogenesis

DEN is now endemic to tropical and sub-tropical regions and over the past 20 years has vastly increased in both cases and countries infected (9). The regions that are most susceptible to infection are the Asia–Pacific, the Americas, the Middle East and Africa (11). In addition, recently there has been a 4 fold increase in dengue cases reported in the Americas (12). Since many of these regions lack dengue surveillance, these shocking statistics are probably an under

estimate to the true problem. There are four serotypes (DV1-4) all of which share the same structural and pathogenic characteristics but have distinct genetic features. It is thought that DV2 and DV3 contribute most to the severity of disease (11).

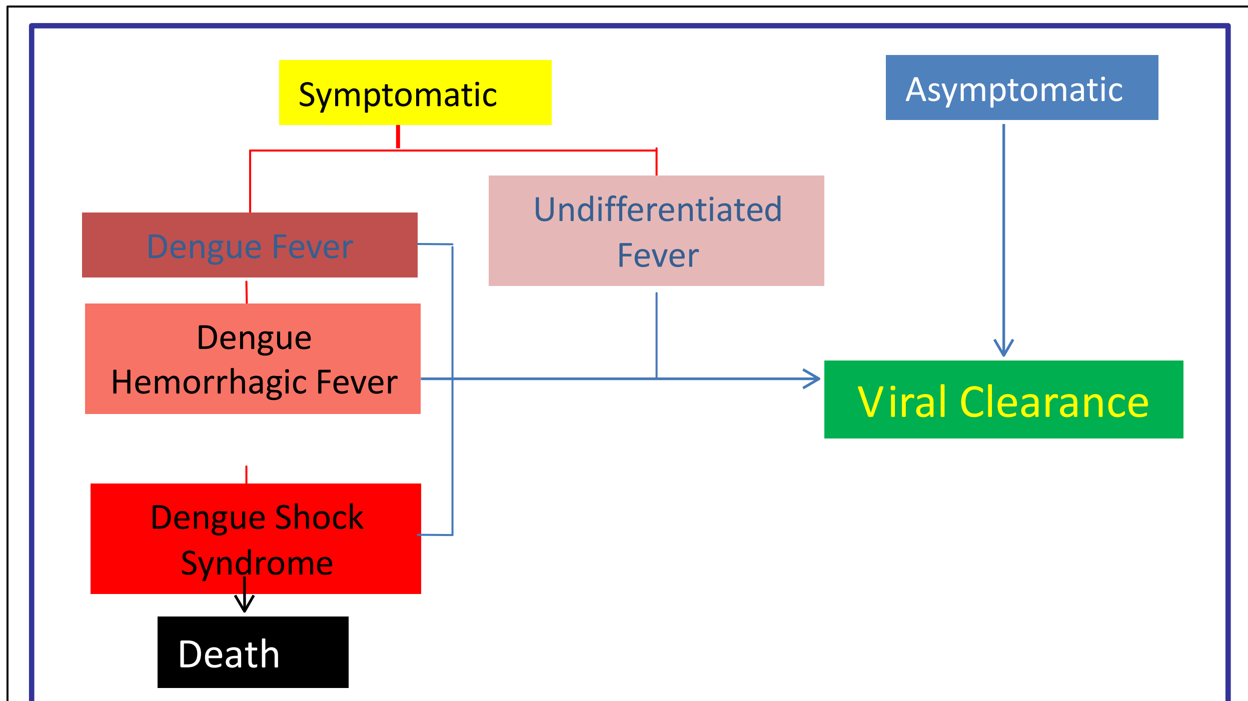


Figure 3. Dengue Pathogenesis flow chart. Once infected individuals will either develop symptoms or remain asymptomatic before viral clearance. For those who become symptomatic, they could either develop "dengue fever" or maintain an undifferentiated fever. Some will progress from dengue fever to dengue hemorrhagic fever and dengue shock symptom which may lead to death.

Generally, those infected by this virus may develop diseases ranging from the self-limited febrile illness DF to the more severe DSS and DHF (Figure 3). Of the 500,000 cases that progress to DHF each year, more than 22,000 will result in fatality (31). Symptoms for DF generally resemble a severe flu but can vary between cases and also according to age. For example, young children are often asymptomatic or have undifferentiated fever whereas the disease in older children and adults the disease is more severe (11). However the majority of deaths related to dengue infection are in young children, increasing the years of potential life lost (YPLL) and overall disease burden.

The overall pathogesis of dengue infection is poorly understood. For example, it has not conclusively been shown what the primary sites of infection are and exactly what cells are involved in disease progression. However, recent data indicates that dengue may infect liver sinusoidal endothelial cells and hepatocytes *in vivo* suggesting that the liver may play a key role in dengue pathogenesis (34).

1.1.3 Dengue Prevention and Treatment

One might imagine an obvious solution to preventing this global disease could be to eliminate the mosquito vector; however mosquito control measures have failed on numerous occasions and attempts often create more problems (12). For example although eradicating mosquitoes will reduce the amount of infections that occur, it also limits the number of people who possess antibodies to fight the infection. This limitation in herd immunity may cause detrimental effects if the mosquitoes have a chance to multiply at exponential levels for example after a natural disaster.

Although a few compounds, ribavirin and mycophenolic acid, have shown to be potent inhibit Dengue infection *in vitro,* there are no antiviral therapies approved for the treatment of infection (27). Since there is no vaccine to prevent infection novel therapeutics are in urgent need to treat the disease. High-throughput screening assays are a rapid, easy, cost-efficient way to evaluate large libraries of compounds to find possible inhibitors of infection from viruses. One such system, the CPE-based high-throughput assay, has proven to be robust and reproducible systems to screen inhibitors for a number of viruses including influenza, hepatitis C, bluetongue, human rhinovirus and severe acute respiratory syndrome coronavirus (SARS) (5, 16, 21, 22, 26). Because we have observed a similar cytopathic effect in DEN, we hypothesized

that novel inhibitors of DEN infection could be identified through a similar CPE-based larger scale screening assay.

2.0 STATEMENT OF THE PROJECT

While propagating the DV2, we noticed that it induces rapid cell death in the human hepatoma cell line, HuH7.5.1. This virus-induced cell death (cytopathic-effect) can be converted into a screening assay in which inhibitors of Dengue infection will prevent cells from dying. In this assay, viral induced cytopathic effects are quantitated by monitoring cellular ATP levels, which positively correlate with cellular viability. ATP in the cell culture will drive the oxidation of luciferin resulting in the emission of light that is quantitated using a luminometer. Since this assay measures overall cellular viability, it simultaneously screens for inhibitory compounds while controlling for unwanted cytopathic effects caused by the drugs (drugs that are toxic will add to the viral educed cytopathic effect). We hypothesize that novel inhibitors of DEN infection can be identified through a cytopathic effect-based larger scale screening. To test the hypothesis, our objectives were:

Specific Aim 1 : The development and optimization of a CPE-based high-throughput screening assay to identify novel inhibitory compounds of Dengue infection of hepatocytes.

Specific Aim 2: Screening a small compound library and the characterization of two novel inhibitors Streptovitacin A and Nagilactone C.

We believe that this high-throughput screen will contribute to developing "lead" compounds of novel antivirals and could be used to elucidate mechanisms of the dengue life-cycle.

3.0 MATERIALS AND METHODS

3.1 PROPAGATION AND IDENTIFICATION OF PROTOTYPE VIRUS

3.1.1 Cell lines and Reagents

Both Vero and C6/36 cells were purchased from ATCC and maintained as instructed by the manufacturer. The Huh7.5.1 line was generated from a cured HCV replicon and was provided by Dr. Francis Chisari (Scripps Research Institute). All cell lines were maintained in DMEM supplemented with 5% Penicillin and streptomycin, 1% NEAA and 10% fetal bovine serum (FBS) (Gemini Bio-Products).

Antibodies were produced from hybridoma cells purchased from ATCC (HB-112 and HB-114). Both of these cell lines were maintained as instructed by the manufacturer. Secondary antibodies were purchased from molecular probes (Invitrogen). M ycophenolic acid and Bafilomycin A were purchased from Sigma.

3.1.2 Propagation of Virions

Prototype Dengue strains were propagated in either Vero or C6/36 cells depending on their passage in previous cell lines. Cells were seeded 24 hour s prior to infection in a T175 cell culture flask. When the cells had reached ~60-80% confluence they were infected with virus at

an MOI of 0.01. The cells were incubated for 7 days or until they began to die from the cytopathic effect caused by the virus. After propagation, the virus containing media was collected and centrifuged at 1500g for 5 minutes to remove extracellular debris. The supernatant was then passed through a 0.22um filter syringe and alloquated before it was frozen at -80C for long term storage.

3.1.3 Western Blot Assay

Cell Lysates (25µl) were subjected to electrophoresis through a 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to nitrocellulose membranes by using the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Richmond, Calif.) in transfer buffer (15.6mM Tris base, 120mM glycine). The membrane containing transferred proteins was blocked with 5% skim milk in Wash Buffer at room temperature for 10 m inutes. T he membranes were incubated for 1h at room temperature with either a monoclonal antibody produced by hybridoma cell line HB-114 (2H2) or HB-112 (4G2) against and actin at a dilution of 1:10and 1:500 in 5% skim milk in Wash buffer, respectively. After incubation for 2 h at room temperature, membranes were incubated with horseradish peroxidase-conjugated mouse anti-goat IgG1:500 in 5% skim milk in Wash buffer for 1 h at room temperature. Finally, the signal was developed using the ECL.

3.1.4 Immunofluorescence Assay

All cells were seeded 24 hours before infection to reach an 80% confluence. T he cells were washed for 5 minutes 3 times with 1X PBS. After washing the cells, they were fixed using a 2%

paraformaldehyde and permeablized using 0.2% Triton or fixed and permeablized and fixed with 100% methanol. The dengue envelope proteins were detected using a 1:10 dilution of the 4G2 or 2H2 primary antibody isolated from a hybridoma culture and 1:500 of a HRP secondary antibody. Nuclei were stained using Draq5 or DAPI as indicated.

3.1.5 Virus Tittering (Reed & Muench method)

Viruses were tittered using a Reed & Muench Tissue Culture Infectious $Dose_{50}$ Assay ($TCID_{50}$/ml) system. An example of the application of this assay is demonstrated in Figure 6. Cells were seeded so that they will reach 80% confluence in 24 hours. On the day of the experiment, serial dilutions of virus are made in media and a total of 8 wells were infected with each serial dilution of the virus. After 48 hours incubation, an IFA was completed for all of the used wells. The wells were then examined under an epifluorescence for any positive wells. Anything greater than one positive cell indicated a positive infected well. Interestingly though where one cell is infected there is a small grouping of infected cells surrounding forming a foci.

3.2 LUCIFERASE-BASED HIGH THROUGHPUT SCREEN

3.2.1 Cytopathic Effect - Luciferase Reagent

The CPE Luciferase reagent was prepared by combining a 1ml of a 5X D-Luciferin Stock Solution (1mM D-luciferin, 25mM Glycylglycine, 10mM DTT), 1ml luciferase enzyme (1mg/ml), 0.5ml 250mM Glycylglycine and 3.5ml H_2O in 9ml Luciferase Assay Buffer

(25mMGlycylglycine pH 7.8, 15mM Potassium Phosphate pH 7.8, 15mM $MgSO_4$, 4mM EGTA in H_2O). The solution was then aliquoted into 1.8ml eppendorf tubes and kept at -80 C for long-term storage.

3.2.2 CPE-based high-throughput screening assay.

Huh7.5.1 cells are counted with a hemacytometer and $1.8*10^4$ cells are seeded per well in a 96 well format 24 hours prior to the experiment. After cells had reached an 80% confluence they were infected with DV2 in the presence or absence of a compound for two hours. After the infection the virus was replaced with fresh compound containing media. After a set duration, the CPE solution was thawed at room temperature just before the assay. All media was removed from the cells. A 5X passive lysis buffer was diluted to 1X in 1X PBS and then 70µl of the 1X lysis buffer was added to each well. The plates were incubated in the 37 degree incubator for 10 minutes and then 50µl of the lysate was transferred to a luminometer plate. At least 50µl of the CPE buffer was then quickly added to all wells in the luminometer plate and the luciferase reading was quantified with an Auto Lumat luminometer.

3.2.3 Cell Culture Derived (HCVcc) Luciferase Assay

Production procedure of HCVcc (JFH-1 strain) expressing firefly luciferase that was inserted between NS5A and NS5B (sequence available upon request) was described elsewhere (17, 32, 33). A 5X passive lysis buffer was diluted to 1X in 1X PBS and then 70µl of the 1X lysis buffer was added to each well. The plates were incubated in the 37 degree incubator for 10 minutes and then 50µl of the lysate was transferred to a luminometer plate. At least 50µl of the LARII

luciferin buffer was then quickly added to all wells in the luminometer plate and the luciferase reading was quantified with an Auto Lumat luminometer.

3.2.4 New Castle Disease Virus-Green Fluorescence Protein Assay

NDV-GFP virus production- NDV-GFP virus was a generous gift from Dr. Chris Basler (Mount Sinai School of Medicine, NYC) and was produced in 10-days old embryonated eggs (Charles River Laboratories International, Inc. Wilmington, MA). The inoculated eggs were incubated for two days at 37 ^{o}C and the allantoic fluid containing virus was harvested, filtered and stored in -80°C until use. The use of this virus has been described elsewhere [48].

NDV infection assay- $1.8*10^{4}$Huh7.5.1 cells were seeded in 96 well plates. Cells were washed with PBS and 20 µl (MOI 1) of NDV-GFP virus was added in 180 µl of PBS (with Ca and Mg) and left at 37 ^{o}C for 1 hour. Cells were then washed with PBS and imaged. GFP signal started to appear after 12 hours but peaked at 24hours post-infection. DAPI was added to stain nuclei.

3.2.5 High-Throughput Screening Assay Data Analysis

The percentage of inhibition was calculated using a formula: % inhibition= (RLUexp-RLUmin)/(RLUmax-RLUmin)*100 where RLUexp represents the luciferase counts obtained from inhibitor treated wells, and RLUmax typically comes from uninfected wells whereas RLUmin comes from DEN infected and treated with DMSO.

Since signal-to-background does not report any information on data variation, a screening window coefficient, called "Z- factor," was used here to show the reproducibility and robustness of the assay (35). The Z' factor = $1-((3SdDev_S+3\ SdDev_N)/(|Mean_S - Mean_N|))$ where S is the luciferase counts from uninfected wells and N is the counts from infected wells. The Z' values are calculated taking luciferase counts in triplicate from within the same experiment. The signal-to-background was calculated as a ratio between the RLUmax/RLUmin. Our data showed an average Z-factor of 0.78±0.12 between plates.

4.0 RESULTS

We hypothesize that novel inhibitors of DEN infection can be identified through a larger scale screening. To test the hypothesis, our objectives were:

Aim 1: Propagation and tittering of prototype strains of Dengue virus.

Aim 2 : The development and optimization of a CPE-based high-throughput screening assay to identify novel inhibitory compounds of Dengue infection of hepatocytes.

Aim 3: Screening a small compound library and the characterization of two novel inhibitors Streptovitacin A and Nagilactone C.

4.1 SPECIFIC AIM 1 RESULTS

Aim 1: Propagation, detection and tittering of prototype strains of Dengue virus.

Until recently our lab was primarily focused on hepatitis C virus work. However we recently decided to branch out and begin working on Dengue, another RNA virus that that belongs to the Flavivirus family. We obtained all four serotypes of prototyped virus as a gift from Dr. Ernesto Torres De Azeved Marques Jr. The following experiments outline the steps we took to propagate the newly obtained virus using Vero and C6/36, two cell lines that were new to our lab, and methods we utilized to confirm the infection of our cell lines. In addition these

experiments show that we successfully produced antibodies from hybridoma cells received from ATCC.

4.1.1 Experimental Scheme of Dengue propagation and detection

Because we obtained only a small quantity of DV2 (<1ml) we first needed to amplify the virus to obtain enough to complete several experiments. The DV2 Thailand strain was propagated as illustrated in Figure 4. Briefly, C6/36 cells were seeded 24 hours prior to infection in a T175 cell culture flask. When the cells had reached 80% confluence they were infected with virus at an MOI of 0.01. The cells were incubated for 7 days before the media was collected. Several 1 ml aliquots were then prepared to prevent the loss of viable virus from repeated freeze-thaws. After harvesting the virus we infected Vero and C6/36 cells to determine if we could distinguish infection with our hybridoma produced antibodies on an IFA and western blot.

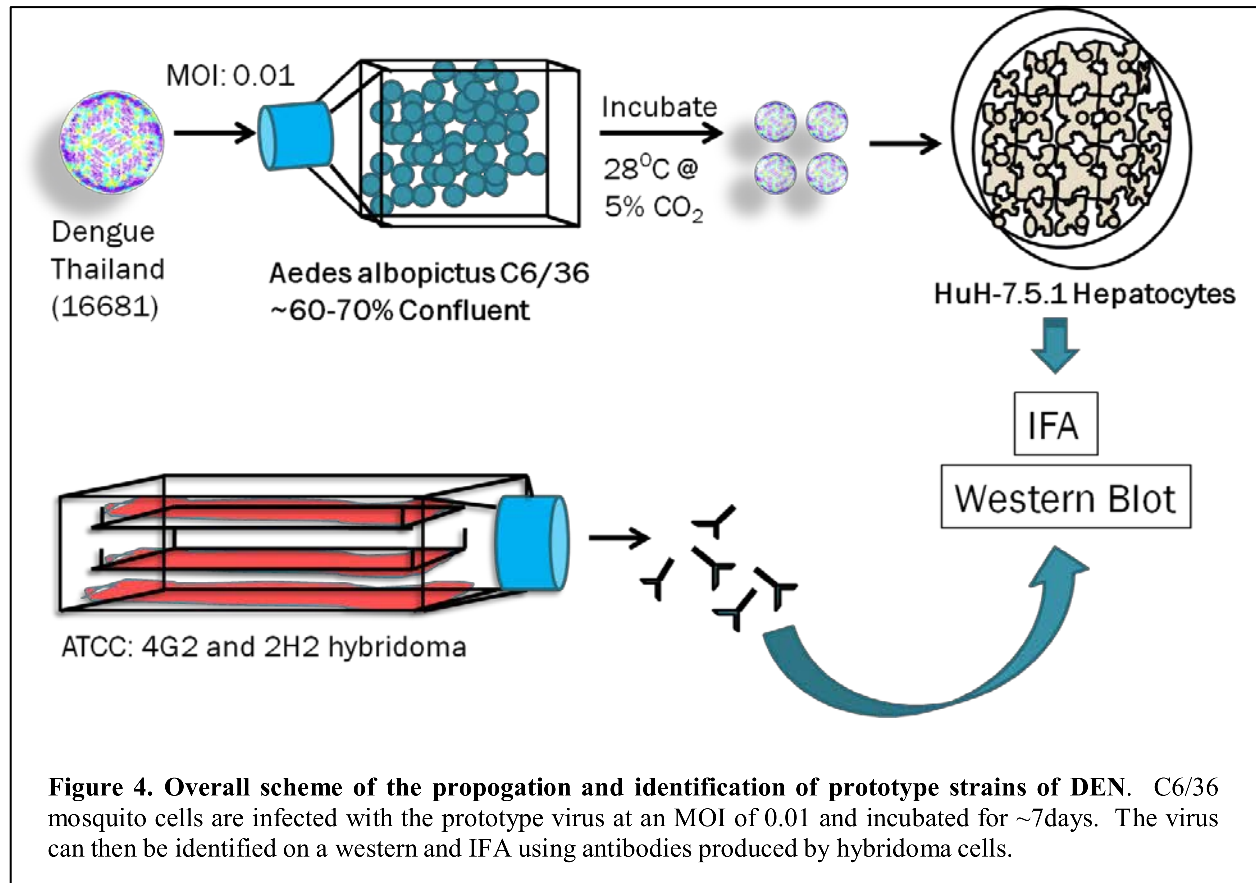
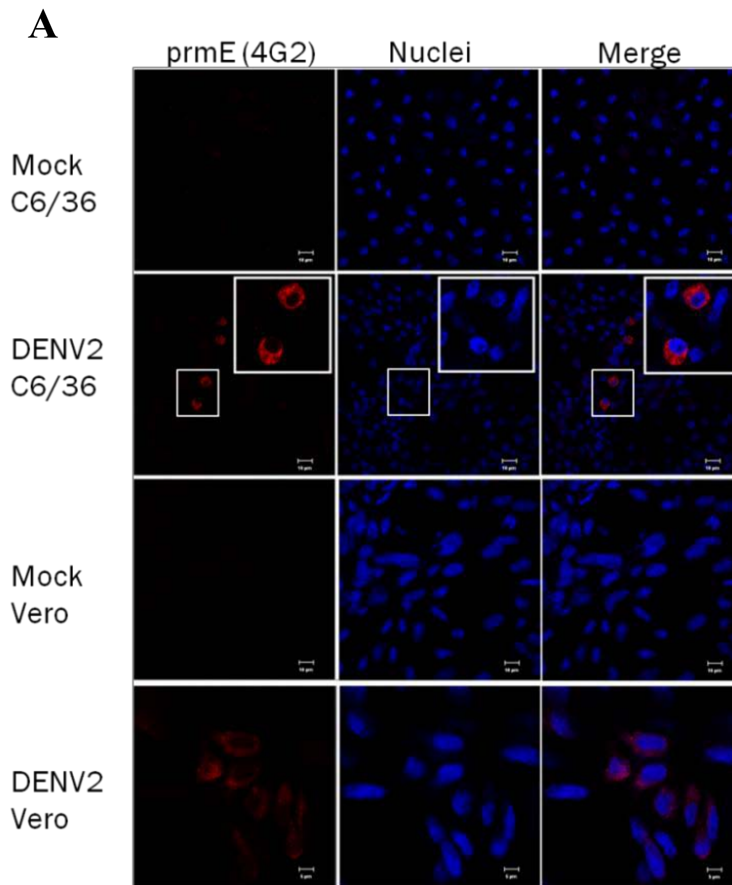


Figure 4. Overall scheme of the propogation and identification of prototype strains of DEN. C6/36 mosquito cells are infected with the prototype virus at an MOI of 0.01 and incubated for ~7days. The virus can then be identified on a western and IFA using antibodies produced by hybridoma cells.

4.1.2 Detection of the DV2 Infection of Vero and C6/36

We were able to detect infection from the newly propagated virus using both an IFA and western blot (Figure 5). As shown in Figure 5A there was no prM/E signal in the mock (media) infection for both C6/36 and Vero cells, however there are still a large number of viable cells present as indicated by the blue DAPI nucleus stain. Conversely, in the cells infected with the DV2 strain we see a distinct red signal surrounding the nucleus (cytoplasm) of the cell which is a distinct characteristic for RNA viruses such as Dengue. Incubation of the western blot (Figure 5B) with the hybridoma produced prM/E antibodies indicated a band in both positive lanes, but not the negative lanes of C6/36 and Vero cells. B-actin was included as a loading control.



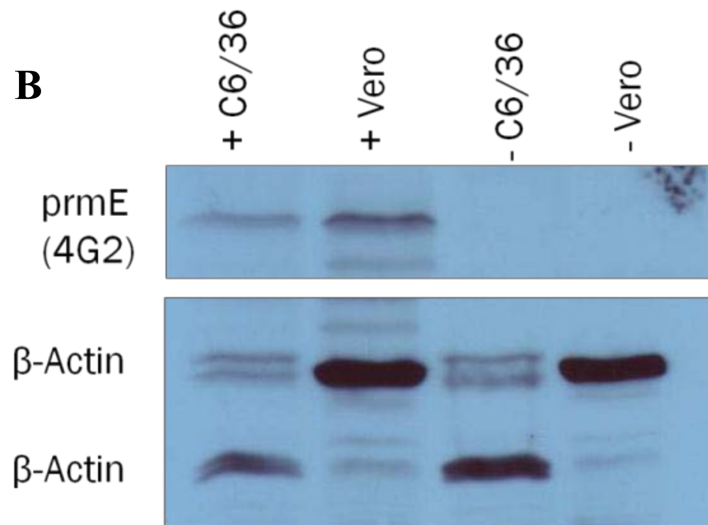


Figure 5. The infection of propagated virus in C6/36 and Vero cells. (A) IFA of C6/36 and Vero cells infected or uninfected (Mock) with the DEN2 virus. The prM/E was detected with the 4G2 primary antibody conjugated with Alexa Fluor 568 as a secondary. (B) Western blot of C6/36 and Vero cells infected (+) of uninfected (-) with the DV2 clinical isolate virus. The prM/E was detected in both of these infected cell lines with the 4G2 primary antibody, but not in the uninfected. β-Actin was used as a loading control.

4.1.3 Tittering of DV1-4 using the Reed and Muench method to calculate TCID50/ml.

Since we had shown that we could detect infection of DV2 on an IFA, we attempted to propagate and titer all 4 serotypes of DEN. To this aim we used the Reed and Muench method to calculate a TCID50/ml as demonstrated in Figure 6. Briefly, serial dilutions are made of the virus and then used to infect 8 wells of Vero cells. The wells are then counted as infected or uninfected and these values are extrapolated into a virus titer.

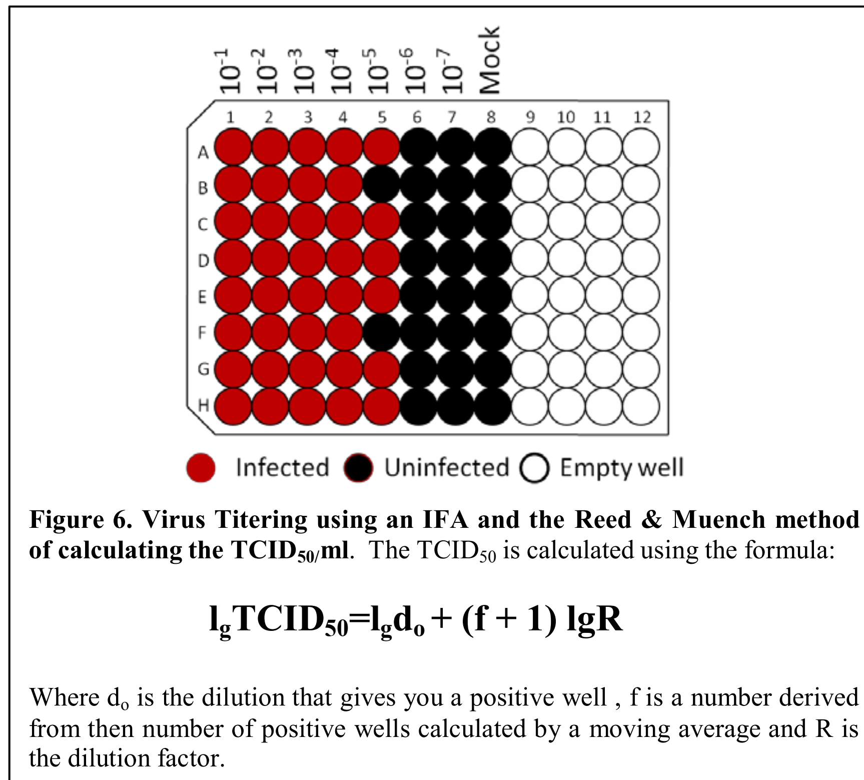


Figure 6. Virus Titering using an IFA and the Reed & Muench method of calculating the $TCID_{50}$/ml. The $TCID_{50}$ is calculated using the formula:

$$\mathbf{l_g TCID_{50} = l_g d_o + (f + 1)\ lgR}$$

Where d_o is the dilution that gives you a positive well , f is a number derived from then number of positive wells calculated by a moving average and R is the dilution factor.

As shown in Table 1, we obtained similar concentration to the initial for all viruses except the Vero propagated DV1 Hawaii strain. Only the DV2 serotype caused a cytopathic effect during the propagation procedure the remaining strains were propagated for a total of 7 days before harvesting.

Table 1. $TCID_{50}$/ml of each strains of propagated prototyped virus. Initial concentrations as well as newly propagated concentrations are indicated. The virus propagation was continued using the same cell lines passed by the previous lab.

Serotype	Strain	Original Concentration (FFU/ml)	New Concentration (TCID50/ml)	Cell Line propagated
DENV1	HAWAII	2.67E+06	1.00E+04	Vero
DENV2	Thailand 16681	4.20E+06	3.16E+06	C6/36
DENV3	H-87	7.60E+04	1.00E+06	C6/36
DENV4	H-241	6.40E+04	6.31E+04	C6/36

4.1.4 The Infection of Human Hepatoma Cell Lines Huh7.5.1 and HepG2

After we had determined the titer for each virus we then attempted to infect human hepatoma cell lines Huh7.5.1 and HepG2 with the DV2 serotype. In this experiment it seems Huh7.5.1 cells (Figure 7A left panel) are either more susceptible or permissive for infection from DEN than the HepG2 cells (Figure 7A right panel). Similar to the infection of Vero and C6/36 cells, we observed viral antigen isolated in the cytoplasmic region surrounding the nucleus which is a signature for RNA virus. We confirmed the infection of Huh7.5.1 cells in the IFA by a western blot (Figure 7B). We also tested increasing dilutions of the antibody to determine the minimal amount necessary to show strong results.

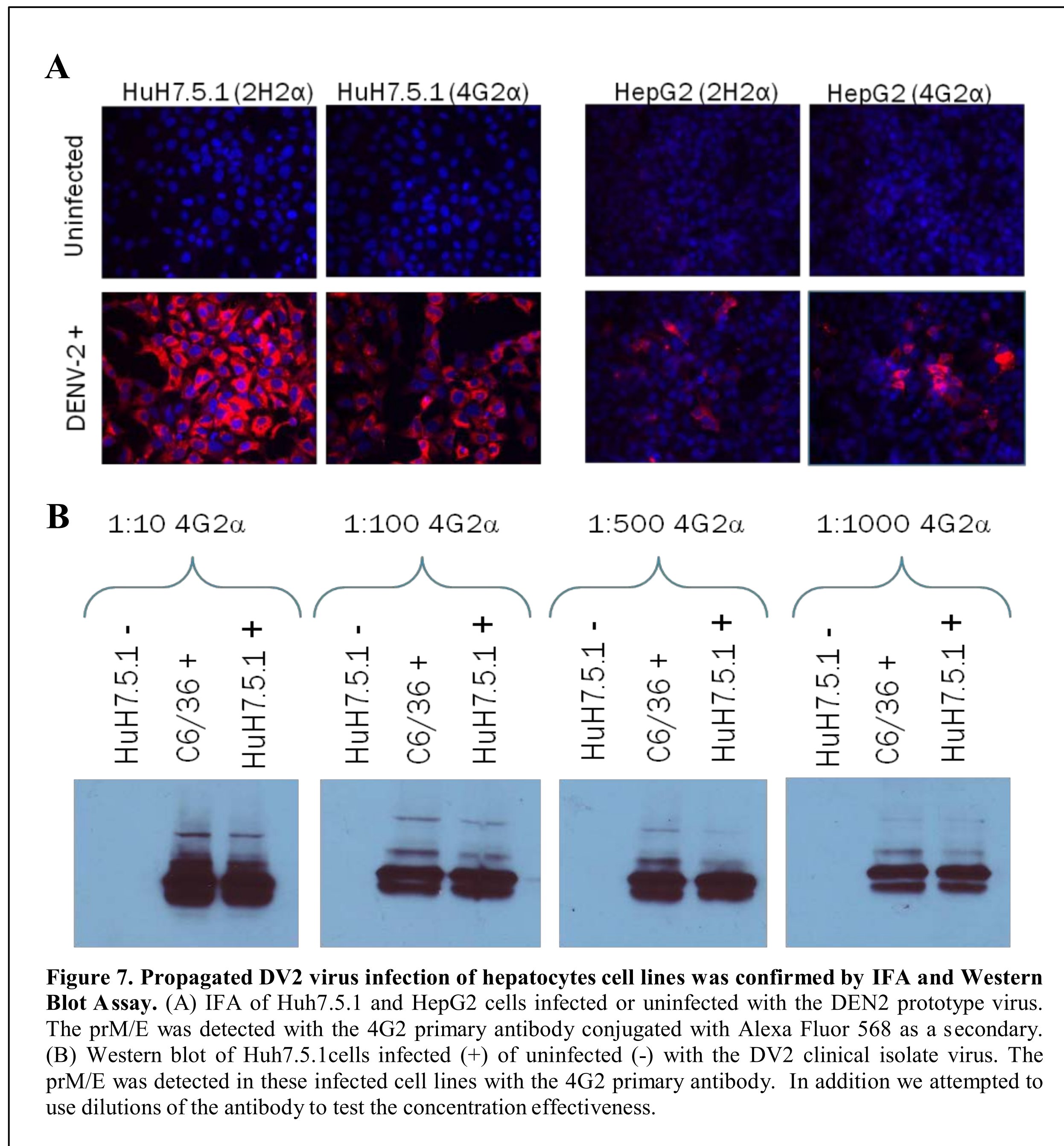


Figure 7. Propagated DV2 virus infection of hepatocytes cell lines was confirmed by IFA and Western Blot Assay. (A) IFA of Huh7.5.1 and HepG2 cells infected or uninfected with the DEN2 prototype virus. The prM/E was detected with the 4G2 primary antibody conjugated with Alexa Fluor 568 as a secondary. (B) Western blot of Huh7.5.1cells infected (+) of uninfected (-) with the DV2 clinical isolate virus. The prM/E was detected in these infected cell lines with the 4G2 primary antibody. In addition we attempted to use dilutions of the antibody to test the concentration effectiveness.

4.2 SPECIFIC AIM 2 RESULTS

Aim 2 : The development and optimization of a CPE-based high-throughput screening assay to identify novel inhibitory compounds of Dengue infection of hepatocytes.

While propagating the DV2 virus, we noticed that there is a cytopathic effect (Figure 8A). The cells were stained 24 and 72 hours post infection (PI) with trypan blue, a die that will easily defuse into cells that have a damaged cellular membrane but not cells that are viable. Both the Mock and DV2 infected cells appear >95% viable after 24 hours, however at 96 hours PI, the DV2 infected wells have lost over 80% of the total monolayer.

4.2.1 Experimental Scheme of the Cytopathic-Based High-Throughput Screening Assay

This virus-induced cell death (cytopathic-effect) was converted into a screening assay in which inhibitors of Dengue infection will prevent cells from dying. In this assay, viral induced cytopathic effects are quantitated by monitoring cellular ATP levels, which positively correlate with cellular viability. ATP in the cell culture will drive the oxidation of luciferin resulting in the emission of light that is quantitated using a luminometer. As shown in Figure 8B, there is a window of high luciferase readings in uninfected cells compared to low levels in infected known as the signal-to-background ratio. If a compound (compound A) prevents the infection of dengue, then we expect to see luciferase readings significantly higher than the noise. However if the compound (compound B) causes additional cytotoxicity we will see readings at or below that of the untreated infected cells.

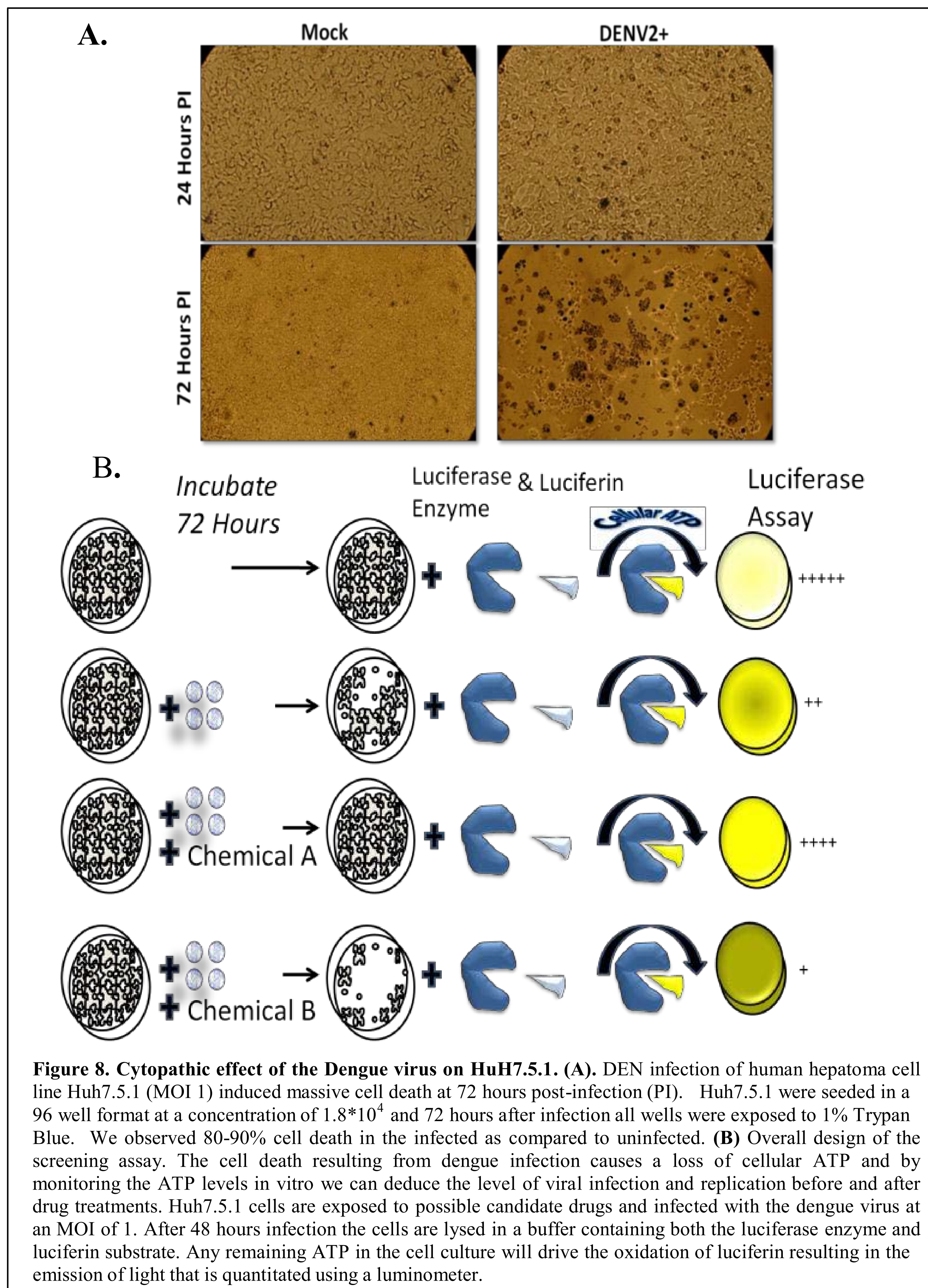


Figure 8. Cytopathic effect of the Dengue virus on HuH7.5.1. (A). DEN infection of human hepatoma cell line Huh7.5.1 (MOI 1) induced massive cell death at 72 hours post-infection (PI). Huh7.5.1 were seeded in a 96 well format at a concentration of $1.8*10^4$ and 72 hours after infection all wells were exposed to 1% Trypan Blue. We observed 80-90% cell death in the infected as compared to uninfected. **(B)** Overall design of the screening assay. The cell death resulting from dengue infection causes a loss of cellular ATP and by monitoring the ATP levels in vitro we can deduce the level of viral infection and replication before and after drug treatments. Huh7.5.1 cells are exposed to possible candidate drugs and infected with the dengue virus at an MOI of 1. After 48 hours infection the cells are lysed in a buffer containing both the luciferase enzyme and luciferin substrate. Any remaining ATP in the cell culture will drive the oxidation of luciferin resulting in the emission of light that is quantitated using a luminometer.

4.2.2 Optimization of Multiplicity of Infection and Duration of the Cytopathic Effect-Based HTS Assay.

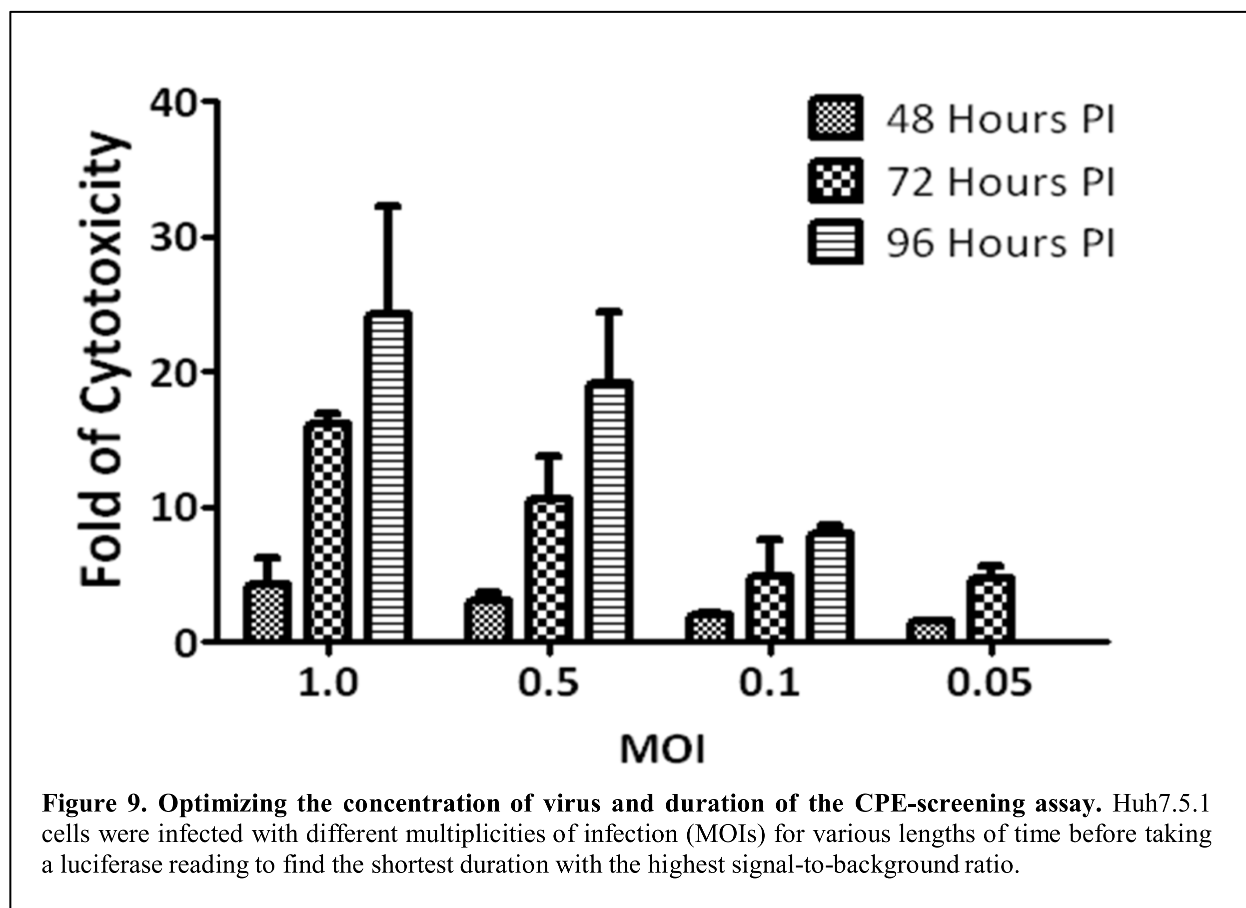


Figure 9. Optimizing the concentration of virus and duration of the CPE-screening assay. Huh7.5.1 cells were infected with different multiplicities of infection (MOIs) for various lengths of time before taking a luciferase reading to find the shortest duration with the highest signal-to-background ratio.

Figure 9 shows the optimization of this screening assay to find the MOI with the broadest signal-to-background ratio in the shortest duration. Having a broad signal-to-background will increase the overall sensitivity of our assay and allow us to find hits that have a high probability of inhibiting infection. The duration is important to optimize as well since too short of an infection will not cause cell death and too long of a period will cause the chemicals to degrade and lose effect. We therefore reasoned that infecting cells with an MOI of 1 for a total of 72 hours is an

optimal condition. Although the signal/noise ratio is >16, which is somewhat lower than the 22 fold at 96 hours, it is still a much broader window than at 48 hours or at any of the other MOIs.

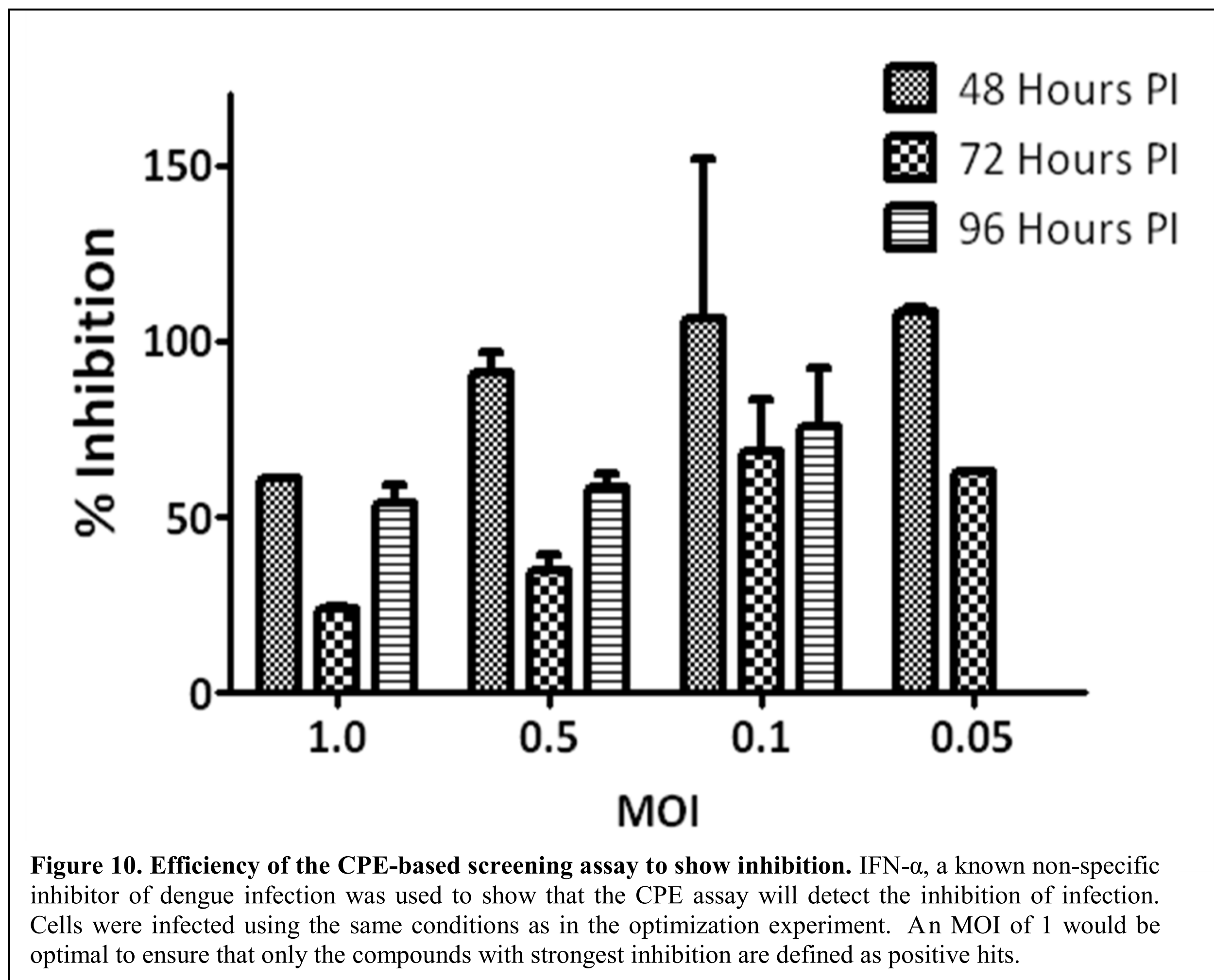


Figure 10. Efficiency of the CPE-based screening assay to show inhibition. IFN-α, a known non-specific inhibitor of dengue infection was used to show that the CPE assay will detect the inhibition of infection. Cells were infected using the same conditions as in the optimization experiment. An MOI of 1 would be optimal to ensure that only the compounds with strongest inhibition are defined as positive hits.

The optimization experiment from Figure 9 was repeated using IFN-α, a known inhibitor of dengue infection, to show that the assay will in fact detect inhibition (Figure 10). Although the inhibition was lowest at an MOI of 1 and duration of 72 hours, we reasoned that this demonstrated the robustness of the assay, as it will only show positive hits that inhibit infection more than a known non-specific inhibitor.

Since this assay measures overall cellular viability, it simultaneously screens for inhibitory compounds while controlling for unwanted cytopathic effects caused by the drugs (drugs that are toxic will add to the viral educed cytopathic effect). This is demonstrated with Bafilomycin A in figure 11. In a $TCID_{50}$/ml assay (Figure11A), Baf A inhibited the infection of nearly 100%, however Baf A causes immense toxicity to the hepatocytes (Figure 11B). Since our assay measures total cellular viability after treatment with compounds and virus, this drug would have been screen out (Figure 11C).

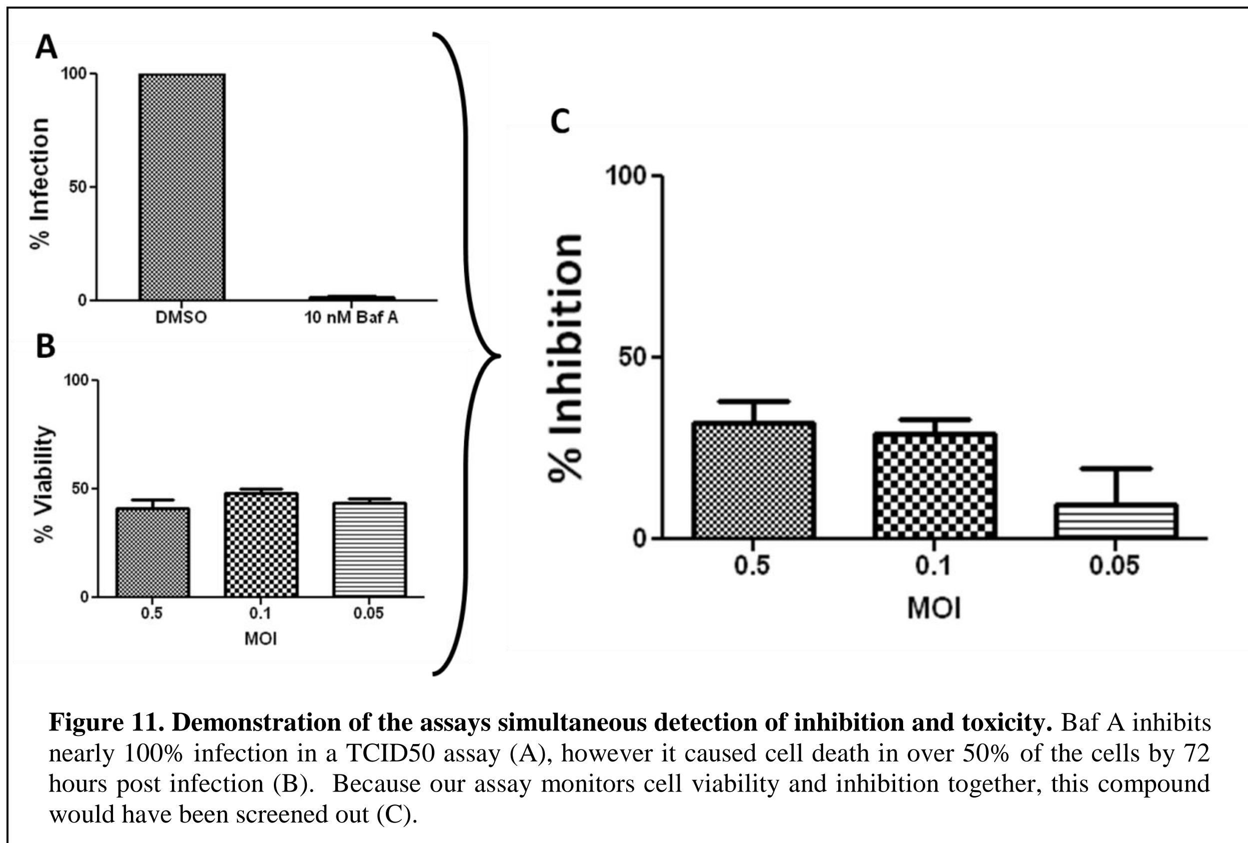


Figure 11. Demonstration of the assays simultaneous detection of inhibition and toxicity. Baf A inhibits nearly 100% infection in a TCID50 assay (A), however it caused cell death in over 50% of the cells by 72 hours post infection (B). Because our assay monitors cell viability and inhibition together, this compound would have been screened out (C).

4.3 SPECIFIC AIM 3 RESULTS

Aim 3: Sreening a small compound library and the characterization of two novel inhibitors Streptovitacin A and Nagilactone C.

4.3.1 Screening a Small Compound Library

Results from the initial CPE-based high-throughput screen on the National Cancer Institute (NCI) 264 compound library are shown in Figure 12. A total of 11 compounds inhibited the infection over our 50% cut off value. The compounds that fell below 0% mark are as such because of the high level of toxicity. Two of the eleven compounds, Streptovitacin A and Nagilactone C, were shown to inhibit infection with an IFA and were therefore further characterized in the following experiments.

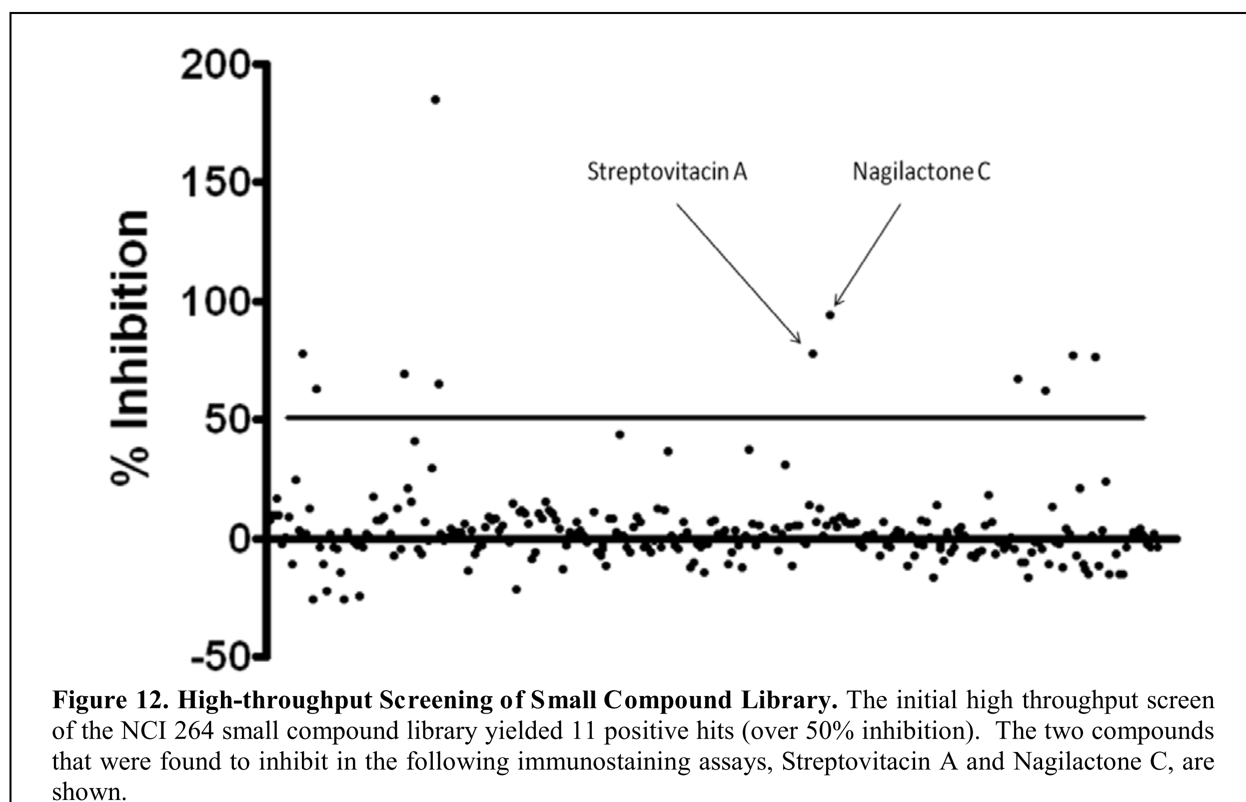


Figure 12. High-throughput Screening of Small Compound Library. The initial high throughput screen of the NCI 264 small compound library yielded 11 positive hits (over 50% inhibition). The two compounds that were found to inhibit in the following immunostaining assays, Streptovitacin A and Nagilactone C, are shown.

4.3.2 Validation of the Positive Hits with an Immunofluorescence Assay

We attempted to validate the positive hits from the high-throughput screen using an IFA. Light microscopy images (Figure 13 left) were taken to show the cell viability and morphology after exposure to these chemicals. Untreated cells infected with media (mock) or DV2 48 hours prior to staining were used as a control. There is no fluorescent signal in the mock infected cells, however the DV2 cells show a high level of fluorescence that is localized to the cytoplasm surrounding the cell nucleus. Compound 377 B 11 (streptovitacin A) inhibited infection at all concentrations tested and 3977 G 11 (nagilactone C inhibited at all except the lowest 1μM concentration. W e included results from one of the other compounds tested (3978 G8) as a control as it did not inhibit infection at any of the concentrations.

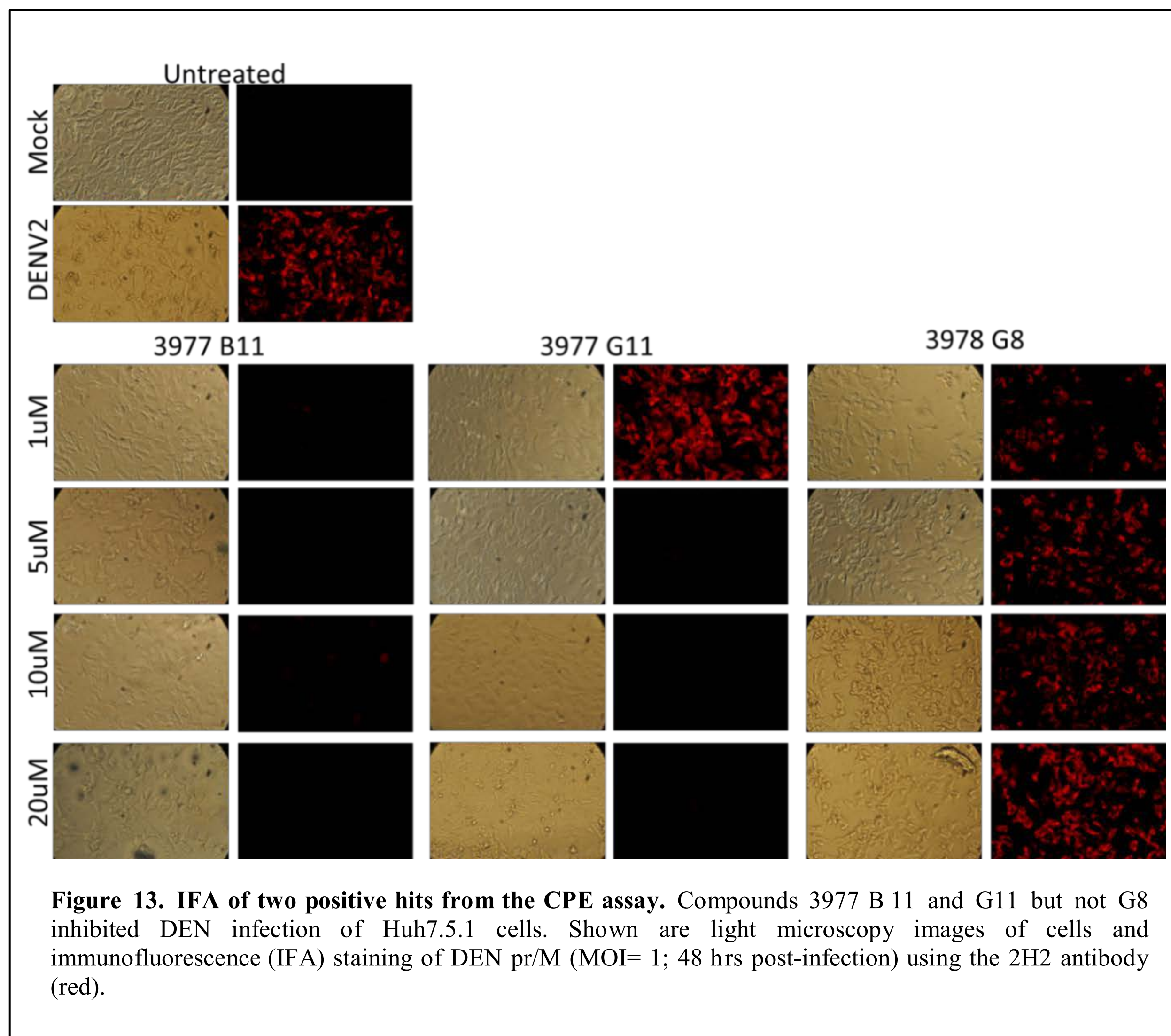


Figure 13. IFA of two positive hits from the CPE assay. Compounds 3977 B 11 and G11 but not G8 inhibited DEN infection of Huh7.5.1 cells. Shown are light microscopy images of cells and immunofluorescence (IFA) staining of DEN pr/M (MOI= 1; 48 hrs post-infection) using the 2H2 antibody (red).

4.3.3 Calculating an IC_{50} and CC_{50} for the Two "Lead" Compounds.

After confirming that Streptovitacin A and Nagilactone C inhibited the infection of Huh7.5.1 cells by means of IFA, we then attempted to further characterize these compounds by creating inhibitory curves to calculate the IC_{50} and CC_{50} values (Figure 14). To construct these curves we infected cells in the presence of serial dilutions of compounds. Mycophenolic acid was used as a control for this experiment and the IC_{50} value that we obtained from the curve (0.8uM Figure 14A) is analogous to the value provided in previous research (7). Streptovitacin A had a similar

dose response as mycophenolic acid and therefore a similar IC_{50} (Figure 14B). Even though the IC_{50} value was slightly higher for Nagilactone C (~2μM as we expected from the previous IFA experimental results), it is still in proximity to the mycophenolic acid. The CC_{50} value for streptovitacin A was 2.23uM, but we could not determine a CC_{50} value for nagilactone C since there was no cytotoxicity at 10μM, our maximum possible concentration.

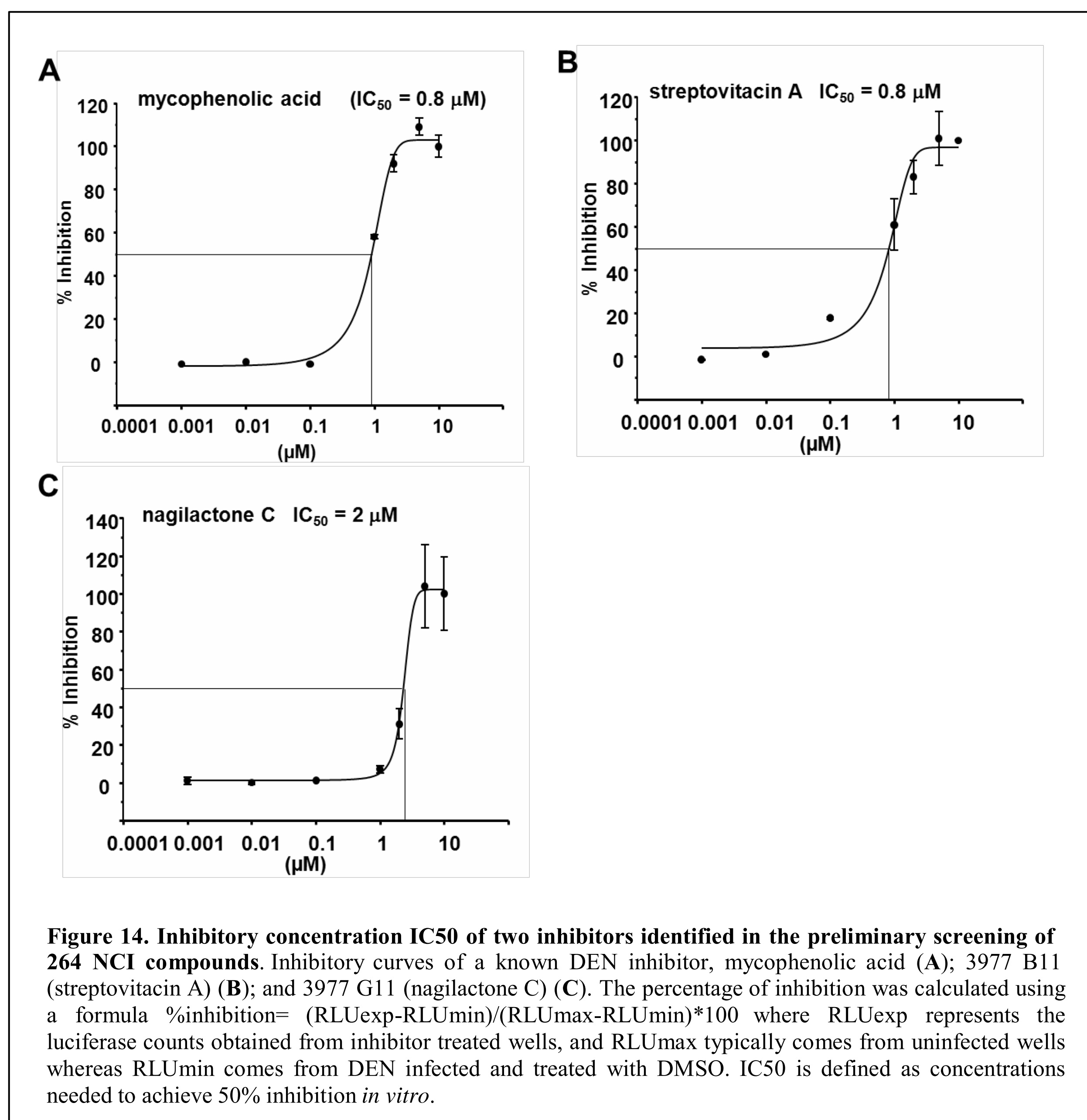


Figure 14. Inhibitory concentration IC50 of two inhibitors identified in the preliminary screening of 264 NCI compounds. Inhibitory curves of a known DEN inhibitor, mycophenolic acid (**A**); 3977 B11 (streptovitacin A) (**B**); and 3977 G11 (nagilactone C) (**C**). The percentage of inhibition was calculated using a formula %inhibition= (RLUexp-RLUmin)/(RLUmax-RLUmin)*100 where RLUexp represents the luciferase counts obtained from inhibitor treated wells, and RLUmax typically comes from uninfected wells whereas RLUmin comes from DEN infected and treated with DMSO. IC50 is defined as concentrations needed to achieve 50% inhibition *in vitro*.

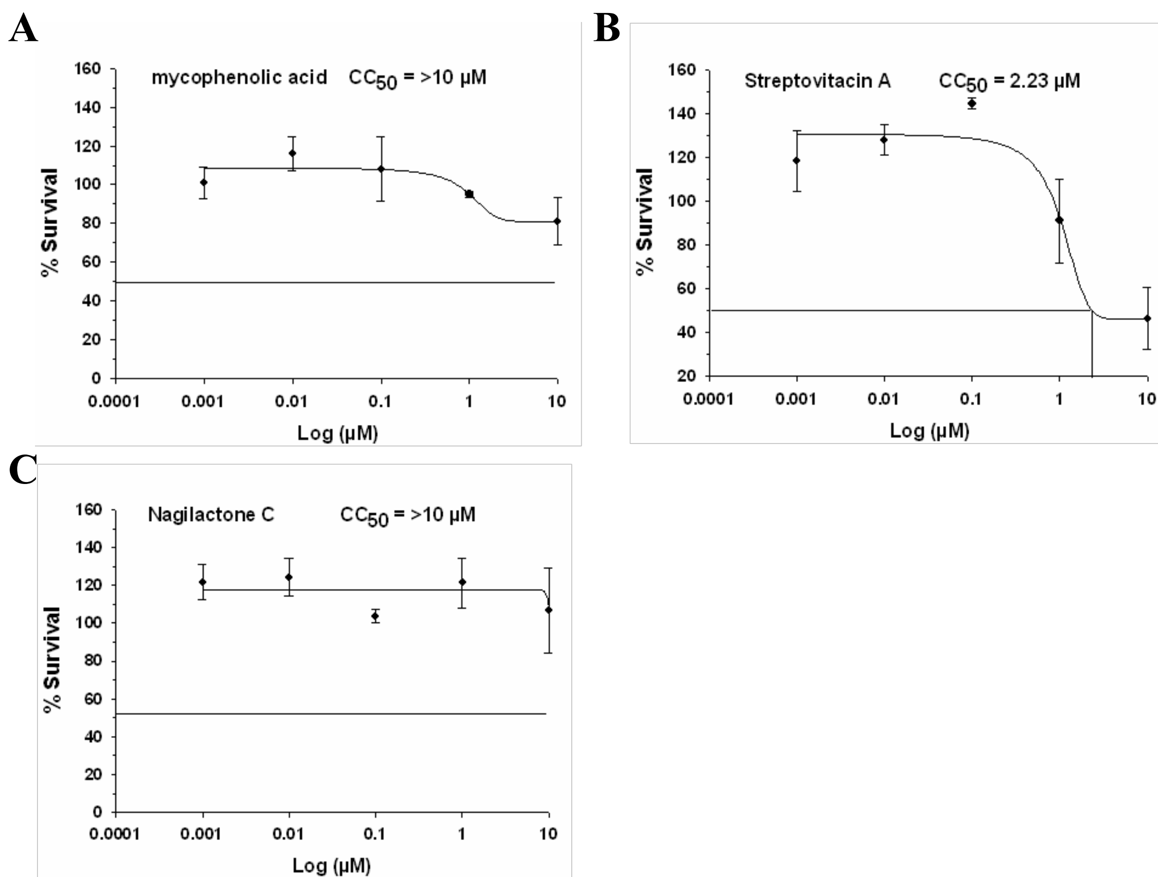


Figure 15. Cytotoxicity concentration $CC_{50}$of the two inhibitors identified the a preliminary screening of 264 NCI compounds. % Survival curves of a known DEN inhibitor, mycophenolic acid (**A**); 3977 B11 (streptovitacin A) (**B**); and 3977 G11 (nagilactone C) (**C**). The percentage of survival was calculated using a formula %survival= RLUexp/(RLUmax*100 where RLUexp represents the luciferase counts obtained from inhibitor treated wells, and RLUmax typically comes from DMSO treated wells. CC_{50} is defined as concentrations needed to achieve 50% survival*in vitro*. CC_{50} is indicated with each compound.

Now that we had determined the IC_{50} and $CC_{50}$of these inhibitory compounds we decided to further characterize by evaluating their inhibitory properties on the other three serotypes of DEN. As shown in Figure 15, 2 μM of both streptovitacin A and mycophenolic acid and to a lesser extent nagilactone C were able to inhibit the infection of DV2, 3 and 4. Interestingly DV1 did not show the same inhibition, however, the cells in the untreated IFA did not show as strong of a signal as did the other three serotypes.

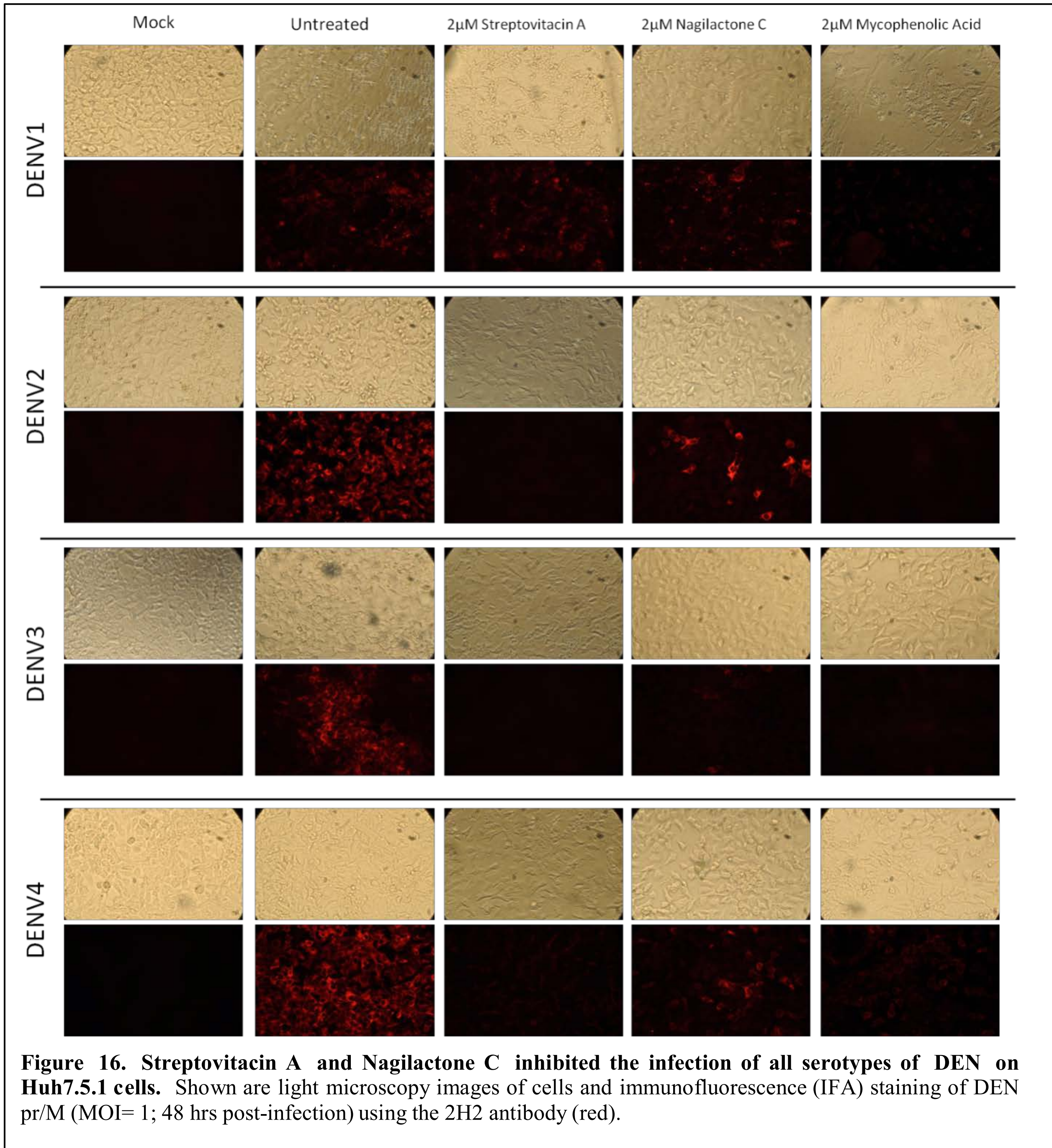
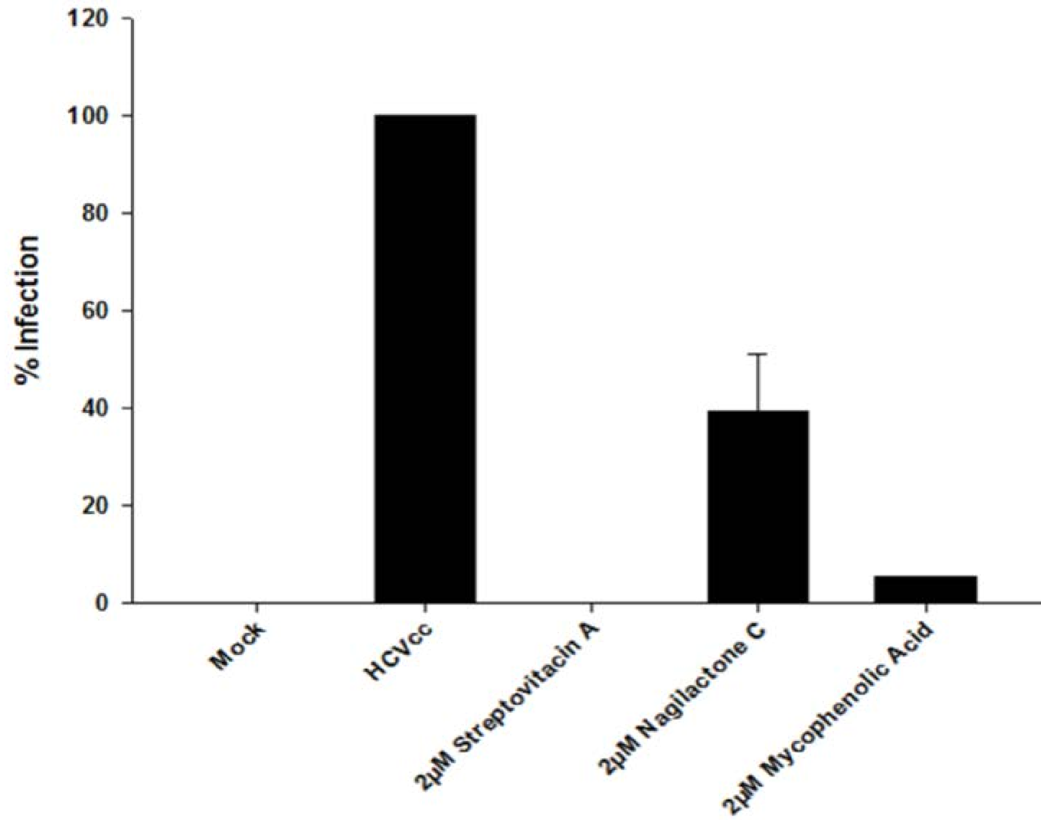


Figure 16. Streptovitacin A and Nagilactone C inhibited the infection of all serotypes of DEN on Huh7.5.1 cells. Shown are light microscopy images of cells and immunofluorescence (IFA) staining of DEN pr/M (MOI= 1; 48 hrs post-infection) using the 2H2 antibody (red).

In addition to evaluating the inhibitor effect on all four serotypes of DEN, we also characterized the ability of streptovitacin A and nagilactone C to block the infection of two other RNA viruses, hepatitis C (HCV) and New Castle Disease Virus (NDV). The HCV clone that we used to infect has a luciferase gene insert that will be expressed during infection and similarly the NDV virus has a GFP insert (Figure 17). The inhibitory results from both of these viruses are comparable to those observed in the previous experiment, streptovitacin A and mycophenolic acid inhibited infection almost completely whereas nagilactone C inhibited but to a lesser extent.

A.



B.

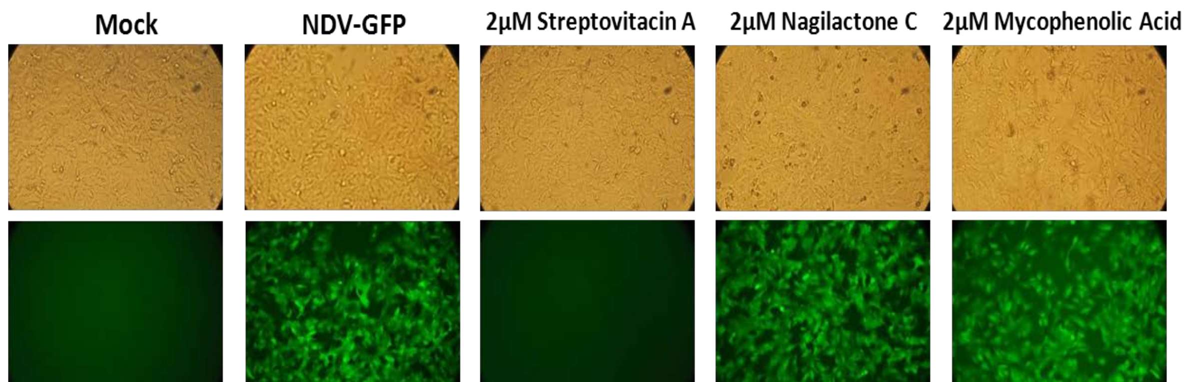


Figure 17. Streptovitacin A and Nagilactone C inhibited the HCVcc and NDV-GFP infection of Huh7.5.1 cells.
(A) Huh7.5.1 cells were infected with a hepatitis C luciferase expressing clone in the presence or absence of compounds. (B)Huh7.5.1 cells were infected with a New castle virus – green fluorescence expressing clone in the presence or absence of compounds. Mycophenolic acid, a known inhibitor of DEN infection was used as a control.

5.0 DISCUSSION

Despite the severe disease burden of dengue infection each year, no vaccines or antivirals have been found to treat this disease. In this study, we have successfully established a robust technological high-throughput screening assay to find compounds that prevent the cytopathic effect of dengue on hepatocytes. We hypothesized that novel inhibitors of DEN infection could be identified through this larger scale screening assay. These inhibitory compounds could be used to elucidate cellular pathways that DEN may utilize for establishing its infection and possibly in the long term as new therapeutics to combat the infection.

We have optimized the CPE-based assay using a range of virus MOIs for various durations to find the largest signal-to-background ratio. Having a broad window for signal-to-background permitted us to indentify compounds that have a potent effect in preventing this cytopathic effect. At a MOI of 1 and a duration of 72 hours we observed the largest signal-to-background ratio (>16) in the shortest amount of time. Using IFN-α as a positive control under these experimental conditions, we see that there is less that 50% inhibition of cell death. We reasoned that this would be an appropriate MOI and time period to demonstrate the potency of the compounds as a strong inhibitor of dengue infection like IFN-α could not inhibit at this concentration.

This assay is advantageous to current methodology because it simultaneously filters out compounds that inhibit infection but at the same time are toxic to cells. This simultaneous effect

is demonstrated with Bafilomycin A in figure 11. In a $TCID_{50}$/ml assay previously carried out on Baf A showed almost complete inhibition of infection, however because it is somewhat toxic it would be screened out in our assay. One obvious limitation to this assay is that rather than the compounds directly inhibiting virus infection, they could be acting on and inhibiting a pathway or mechanism required for cell death. However, data validation is crucial in modern high-throughput screening. By setting a relatively stringent criterion, we anticipate to only characterize those compounds that potently inhibit DEN infection without high levels of cytotoxicity. The proposed functional characterizations include IFA-based detection of viral protein pr/M, determination of CC_{50} and IC_{50} as well as therapeutic window.

We screened a 264 compound library and found a number of hits that inhibit at least 50% of this cytopathic effect. Further evaluation of these positive hits with an IFA showed that two compounds, streptovitacin A and nagilactone C, block dengue infection as is demonstrated in Figure 9. W e then did a dose response curve to find the IC_{50} for streptovitacin A and nagilactone C and found the IC_{50} for each was comparable to mycophenolic acid, a known inhibitor of dengue (7). Unfortunately were not able to determine the CC_{50} value as our highest concentration available for each compound did not cause cytotoxicity.

Streptovitacin A and nagilactone C have been extensively studied for their use as possible antitumor reagents. The chemical structures for both of these compounds are provided in Figure 18. Streptovitacin A is a hydrolated form of cycloheximide, a strong antifungal inhibitor compound produced by the bacterium *Streptomyces griseus*, and also functions as an inhibitor of protein biosynthesis (25). However unlike its parental compound, streptovitacin A does not cause a depression of DNA or RNA synthesis and has low cytotoxicity and thus it was previously studied as a possible antitumor agent. In addition this compound was used to study

the life cycles of other SS-RNA viruses including poliovirus and coxackievirus B (15). The effects of this compound was also demonstrated on the vaccinia DNA virus and reovirus, a DS-RNA virus (6)

Nagilactone C is a natural plant compound produced by the genus *Podocarpus* and has been shown to have antifungal properties (14), inhibit plant growth and display insecticidal properties(2). In 2004, Chan *et al.* elucidated the inhibitory properties of nagilactone C using HeLa cells. T hey found that this potent protein synthesis inhibitor acts by blocking eEF-1α-dependent loading of aminoacyl-tRNA into the ribosomal A site, as well as peptidyl transferase(2). Interestingly when they added nagilactone C to HeLa cells protein synthesis was decreased by 95%, however when they removed the compound and replaced the media they observed a reversal in the inhibition.

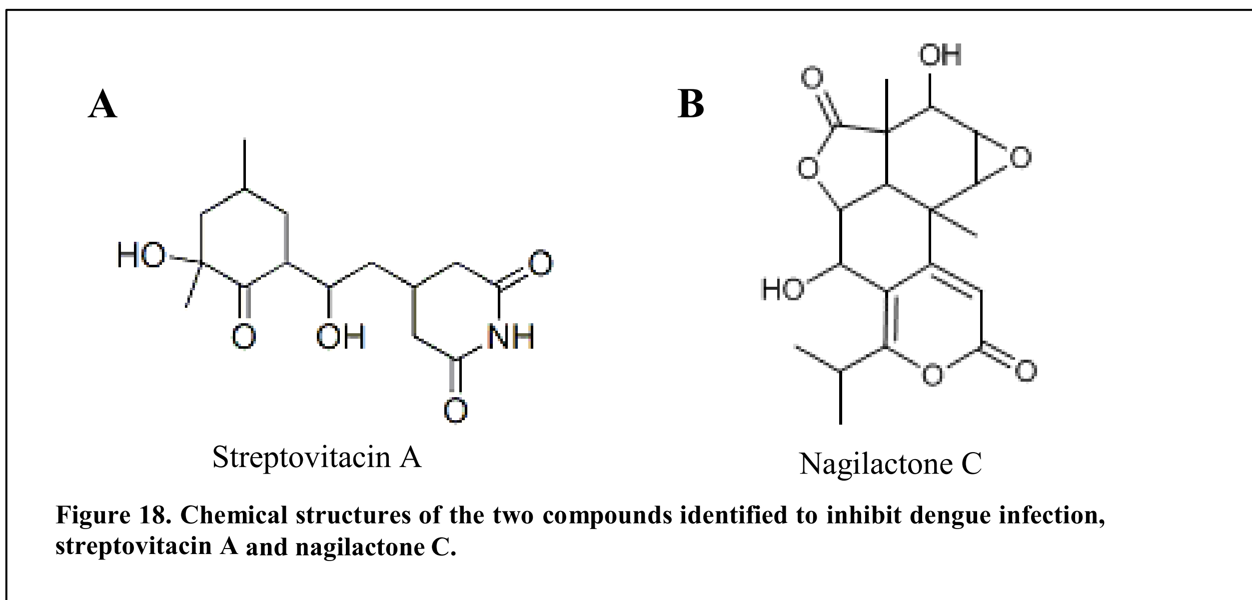


Figure 18. Chemical structures of the two compounds identified to inhibit dengue infection, streptovitacin A and nagilactone C.

Our preliminary study has demonstrated that the CPE-based screening assay we developed is highly robust and highly reproducible yielding a Z' of nearly 0.9 within the same experiment. The Z-factor is a statistical parameter commonly accepted in HTS to show the ability of an

assay to identify "hits". The Z-factor is reported as a number 0 to 1 and anything over 0.5 is considered excellent (35). We therefore reason that this method can be used to identify novel inhibitors of DEN infection. Unfortunately, the current study is of limited scope given that we only performed the initial screening using a 264 compound library. To prove this assay is adaptable to a high-throughput manner, in the future we plan to screen a natural product library from TimTec (700 compounds, gift of Dr. Nicholas Sluis-Cremer) and a homemade mini pathway inhibitor library (100 inhibitors) comprising inhibitors of commons kinases, cell trafficking, cell death). The first library will provide an excellent non-biased library to verify the assay robustness. The goal of screening the mini-pathway inhibitor library is to identify cellular pathways that DEN may utilize for establishing its infection.

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