กลไกการออกฤทธิ์ต่อต้านการติดเชื้อไวรัสเดงกี่ในระดับเซลล์ของอนุพันธ์ฟลาโวนอยด์

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Cellular Mechanism of Flavonoid Derivatives Inhibiting Dengue Virus Infectivity

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A Thesis Submitted in Partial Fulfillment of the Requirements
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อภิญญา สุเริงฤทธิ์ : กลไกการออกฤทธิ์ต่อต้านการติดเชื้อไวรัสเดงกี่ในระดับเซลล์ของอนุพันธ์ฟลาโวนอยด์ (Cellular Mechanism of Flavonoid Derivatives Inhibiting Dengue Virus Infectivity) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร. พญ.ศิวะพร บุณยทรัพยากร, 105 หน้า.

ไวรัสเดงกี่เป็นไวรัสชนิดหนึ่งที่ทำให้เกิดโรคไข้เลือดออก ซึ่งถือเป็นปัญหาสาธารณสุขทั่วโลกและประเทศไทย ถึงแม้ว่าในปัจจุบันจะมีวัคซีนต้านไวรัสเดงกี่ แต่ด้วยราคาและข้อกำหนดการให้วัคซีนยังมีข้อจำกัดอยู่มาก จึงทำให้การศึกษาหายาต้านไวรัสเพื่อใช้ในเชิงการรักษาเพิ่มมากขึ้น โดยงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาค้นคว้าหาสารประกอบในกลุ่มฟลาโวนอยด์ที่มีประสิทธิภาพสูงต่อการต้านไวรัสเดงกี่ทั้ง 4 ซีโรไทป์และไวรัสซิก้า อีกทั้งศึกษากลไกการออกฤทธิ์ต่อวงจรชีวิตของไวรัสเดงกี่ โดยจากการศึกษาฤทธิ์การยับยั้งไวรัสเบื้องต้น พบว่า มีสารประกอบสองชนิดจากสิบชนิดที่มีเปอร์เซ็นต์การยับยั้งไวรัสมากกว่า 90 คือ สารประกอบไคซิน-โบรมีน (6,8-dibromo-5,7-dihydroxyflavone, FV13) และสารประกอบไคซิน-ไอโอดีน (6,8-diiodo-5,7-dihydroxyflavone, FV14) จึงนำไปศึกษาประสิทธิภาพ (EC_{50}) ต่อไวรัสเดงกี่ซีโรไทป์ที่ 1-4 และไวรัสซิก้า สายพันธุ์ sv0010/15 ซึ่ง ค่าประสิทธิภาพ (EC_{50}) ของ FV13 เท่ากับ 2.30±1.04, 1.47±0.86, 2.32±1.46, 1.78±0.72 และ 1.65±0.86 ไมโครโมลาร์ และค่าประสิทธิภาพ (EC_{50}) ของ FV14 เท่ากับ 2.30±0.92, 2.19±0.31, 1.02±0.31, 1.29±0.60 และ 1.39±0.11 ไมโครโมลาร์ ตามลำดับ โดยสารประกอบ FV13 และ FV14 มีค่าความเข้มข้นของสารที่มีความเป็นพิษต่อเซลล์ร้อยละ 50 (CC_{50}) เท่ากับ 44.58 ± 2.99 และ 44.51 ± 2.58 ไมโครโมลาร์ ตามลำดับ และเนื่องจากสารประกอบทั้งสองเป็นไคซิน-ฮาโลเจน จึงเลือกศึกษา กลไกการออกฤทธิ์ของสารประกอบ FV13 ที่ความเข้มข้น 10 ไมโครโมลาร์ เป็นตัวแทนของสารประกอบฮาโลเจนต่อไวรัสเดงกี่ ซีโรไทป์ที่ 2 ซึ่งพบว่า สารประกอบมีการออกฤทธิ์ในช่วงต้นของวงจรชีวิตไวรัส โดยจากการทดสอบเพื่อหาเป้าหมายของสารพบว่า สารประกอบ FV13 มีผลยับยั้งไวรัสหลังจากที่ไวรัสจับกับตัวรับบนผิวเซลล์ไปแล้ว โดยมีเปอร์เซ็นต์การยับยั้งอนุภาคไวรัส เท่ากับ 64.97±1.18% และยับยั้งอาร์เอ็นเอของไวรัสเท่ากับ 59.96±4.56% อย่างมีนัยสำคัญทางสถิติ ซึ่งสามารถยืนยันได้จากการทดสอบในเซลล์ที่มีการแสดงออกของอาร์เอ็นเอของไวรัส พบว่าสารประกอบ FV13 มีเปอร์เซ็นต์การยับยั้งการเพิ่มจำนวนของอาร์เอ็นเอของไวรัส เท่ากับ 75.38 ± 7.88% โดยเทียบเท่ากับไรบาวิริน ซึ่งเป็นยาต้านไวรัสที่มีฤทธิ์ในการแทรกแซงการสังเคราะห์ RNA มีค่าเท่ากับ 75.80 ± 8.62% ดังนั้นงานวิจัยนี้จึงเป็นงานวิจัยแรกที่ค้นพบว่า สารประกอบไคซิน-ฮาโลเจน เป็นสารประกอบที่มีประสิทธิภาพสูงในการต้านไวรัสเดงกี่และไวรัสซิก้า โดยออกฤทธิ์ต่อการเพิ่มจำนวนของไวรัส ซึ่งเป็นสารที่เหมาะสมแก่การนำไปพัฒนาโครงสร้างและการออกฤทธิ์เพื่อเป็นยาต้านไวรัสต่อไปในอนาคต

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: DENGUE VIRUS, ANTI-VIRAL COMPOUNDS, FLAVONOIDS, CHRYSIN

APHINYA SUROENGRIT: Cellular Mechanism of Flavonoid Derivatives Inhibiting Dengue Virus Infectivity. ADVISOR: SIWAPORN BOONYASUPPAYAKORN, M.D.,Ph.D., 105 pp.

Dengue virus infection is a global public health threat where specific treatment has not been established. Chrysin and flavanone derivatives were previously reported as potential anti-flaviviral inhibitors. We explored ten flavones, flavanones, chalcone, and anthraquinone derivatives that extracted and modified from natural products.Two chrysin derivatives showed inhibitory effects against DENV1-4 and ZIKV SV0010/15 infectivities with the EC_{50} values of 2.30±1.04, 1.47±0.86, 2.32±1.46, 1.78±0.72 and 1.65±0.86 µM of FV13 and 2.30±0.92, 2.19±0.31, 1.02±0.31, 1.29±0.60 and 1.39±0.11 µM of FV14 respectively. The CC_{50}s to LLC/MK2 of FV13 and FV14 were 44.58±2.99 and 44.51±2.58 µM, respectively. Time of addition assay revealed that the primary target was at early after infection. We observed that the compound did not interfere with the viral attachment, but rather showed its highest efficacy at post-attachment with viral titer inhibition of 64.97±1.18% and viral RNA inhibition of 59.96±4.56%. To confirm the replication inhibition, we tested with BHK-21/DENV2 replicon cells. We found that viral replication inhibition of 10 µM FV13 was 75.38 ± 7.88 %. This report demonstrated for the first time as a potential candidates to inhibit the dengue and Zika infectivities with high efficacy at micromolar level that could be developed as a broad-spectrum anti-flaviviral drug. This study also provided insights in cellular toxicity, stability, and suggested possible drug targets for further optimization.

Field of Study: Medical Science Student's Signature .....

Academic Year: 2016 Advisor's Signature .....

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LIST OF ABBREVIATIONS

ATCC	=	American Type Culture Collection
CC_{50}	=	50% Cytotoxicity concentration
Co-att	=	Co-attachment
CPE	=	Cytopathic effect
DDW	=	Deionized distilled water
DENV	=	Dengue virus
DMSO	=	Dimethyl sulfoxide
dpi	=	Day-post infection
EC_{50}	=	50% Efficacy concentration
EDTA	=	Ethylenediaminetetraacetic acid
FBS	=	Fetal bovine serum
Gln	=	Glutamine
h	=	Hour
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpi	=	Hour-post infection
MEM	=	Minimum essential medium
MES	=	2-(N-morpholino) ethanesulfonic acid
min	=	Minute
ml	=	Milliliter
MM	=	Maintenance medium
mM	=	Milimolar
M.O.I	=	Multiplicity of infection
neg	=	Negative
nm	=	Nanometer
NS	=	Non-structural protein
^{o}C	=	Degree Celsius
PBS	=	Phosphate buffer saline
PFU	=	Plaque forming unit
pos	=	Positive
Post-att	=	Post-attachment
Pre-att	=	Pre-attachment
RT-qPCR	=	Reverse transcriptase-quantitative polymerase chain reaction
RNA	=	Ribonucleic acid
rpm	=	Revolutions per minute
TOA	=	Time of drug addition
ZIKV	=	Zika virus

μg	=	Microgram
μl	=	Microliter
μM	=	Micromolar

CHAPTER 1

INTRODUCTION

1.1 Rationale

Dengue infection has been a major public health threat with the infected population estimated at 390 million every year (Bhatt et al., 2013). Around one of the thousands of infected population develops severe dengue or serious illness and death (WHO, 2017). In Thailand, the statistic from Bureau of Vector Borne Disease-Department of Disease Control, Ministry of Public Health has reported 63,310 cases in 2016, and 0.1% mortality of those cases. Moreover, the outbreaks occur once every two to three years (Bureau of Vector Borne Disease, 2016). Dengue virus, the causative agent, could lead to life-threatening conditions such as dengue hemorrhagic fever and dengue shock syndrome (Guzman et al., 2013). The virus is a member of the family *Flaviviridae* consisting of 4 serotypes (DENV1-4) and closely related to several human pathogens such as West Nile virus (WNV), Yellow fever virus (YFV), Japanese encephalitis virus (JEV) and Zika virus (ZIKV) (Lindenbach et al., 2013; Acheson, 2011). The virus transmits to human by mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. The flaviviral virion consists of a single-stranded, positive sense RNA covered with icosahedral capsid and lipid envelope containing viral membrane and envelope proteins. The virus attached to host cell receptors and internalized by clathrin-mediated endocytosis. The virus escapes from endosome after acidification triggering viral envelope fusion. Translation and replication occur in the cytoplasm and assembly occur at the endoplasmic reticulum (ER). The nascent virion travels and

matures through trans-Golgi network before budding out of the infected cell. The virus is capable of entering the circulatory system (viremia) and goes to its preferential sites are liver, monocytes, and organs in reticulo-endothelial system. Severe hemorrhage and shocks happen when the immune responses to the previous heterotype instead of the current infection. (Hasan et al., 2016; Diamond and Pierson, 2015; Acheson, 2011; Gubler, 1998,). Such strong but nonspecific immunological responses created pathological events like endothelial leakage and hemorrhagic shock. Current treatment is still limited to supportive with adequate fluid replacement.

Specific treatments for prophylaxis and therapeutics have been developed with great effort (Whitehorn et al., 2014). The first tetravalent dengue vaccine (Denvaxia®, CYD-TDV) was recently approved and used in 5 countries (Villar et al., 2015). However, the vaccine was not fully protective to all serotypes and even cast concerns about possible vaccine-mediated antibody-dependent enhancement (ADE). In parallel to prophylaxis, anti-dengue drugs for therapeutics has also become actively investigated to search for a potential small molecule that interferes with the viral replication thus decreasing viremia and disease progression (Lim et al., 2013). Potential drug targets can be originated either from the virus or host proteins involving in the viral life cycle (Krishnan and Garcia-Blanco, 2014; Lim et al., 2013; Sampath and Padmanabhan, 2009).

A flavonoid is a group of natural compounds found in fruits and vegetables which can be categorized into subgroups based on their functional moieties. Many compounds in the group were proven bioactive such as anti-inflammation and anti-viral infectivity (Ross and Kasum, 2002; Kumar and Pandey, 2013). Examples of active flavonoids are

quercetin (EC_{50} of 118.12 µM) (Zandi et al., 2011), fisetin (EC_{50} of 192.15 µM) (Zandi et al., 2011), baicalein (EC_{50} of 23.90 µM) (Zandi et al., 2012), Naringenin (EC_{50} of 17.97 µM) (Frabasile et al., 2017) and Baicalin (EC_{50} of 30.24 µM) (Moghaddam et. al., 2014). Note that flavone and flavanone derivatives were among the frequently reported anti-DENV compounds (Senthilvel et al., 2013; Lorena Ramos Freitas de Sousa et al., 2015). Preliminary results (Srivarangkul et. al., in preparation) suggested that two halogenated flavone derivatives showed high inhibition to DENV2 infectivity during a screening of the flavonoids. In this study, we focus on exploring the broad spectrum efficacy and cytotoxicity of these compounds against all DENVs and ZIKV and investigate for the potential part in the viral life cycle that the compounds specifically inhibited.

1.2 Objective

Although previous reports described the potentials of flavonoid derivatives as anti-dengue inhibitors *in vitro*, none of the compounds were reported with less than 20 µM efficacy. Also, no previous work has ever studied chemically modified flavonoid derivatives with cell-based system. Moreover, the mechanism of action study at the cellular level is still not established. In this study, we described two halogenated flavonoid derivatives with the anti-dengue and anti-Zika efficacies and explored the mechanism of drug action in the flavivirus life cycle.

CHAPTER 2

LITERATURE REVIEW

2.1 Virology

Dengue virus is a member of the Family *Flaviviridae* according to International Committee on Taxonomy of Viruses (ICTV) (Viralzone, 2011). Other human pathogens belong to this family include Japanese Encephalitis Virus (JEV), West Nile Virus (WNV), Zika virus (ZIKV), Yellow fever virus (YFV), and Hepatitis C Virus (HCV) (Acheson, 2011). Dengue Virus (DENV) consists of 4 distinct serotypes (DENV1-4) which are recently established in the human within a few hundred years. According to evolutionary genetics DENV4 was the first serotype to diverge, followed by DENV2, and the final split was DENV-1 and DENV-3 (Holmes and Twiddy, 2003). Dengue genome is a single-stranded, positive-sense RNA at approximately 11 kilobases (Poh et al., 2009), encoding 3411 amino acids. The genome is decorated with a type I cap (m7GpppAm) at the 5' end and without a poly(A) at the 3' end (Fig. 3.1). The viral particle is spherical, enveloped at the diameter about 50 nm (Byrd et al., 2013; Lim et al., 2013; Schmidt et al., 2012; Acheson, 2011). The external surface is decorated with membrane proteins (M-protein) and envelope proteins (E-protein) arranged into an icosahedral-like symmetry.

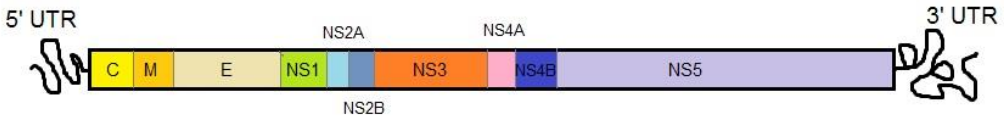


Figure 2.1 Schematic of dengue genome

2.2 Viral life cycle

The flavivirus life cycle began with viral glycoprotein, an envelope-protein (E-protein), attaching to the host cell receptors before internalization into the cells by clathrin-mediated endocytosis (Fig. 3.1) (Moller-Tank and Maury, 2014). The primary receptors are structurally diversified attachment factors including DC-SIGN, C-type lectins, mannose receptor, and immunomodulatory proteins (TIM/TAM receptors). *In vivo*, the target of DENV includes monocytes, macrophages, dendritic cells, mast cells, hepatocytes and endothelial cells. (Diamond and Pierson, 2015). After internalization, the clathrin-coated endosome delivered the virion into the cytosol. Envelope proteins are driven by mildly acidic condition (pH ~ 5-6) to rearrange conformation to trimer and ready for fusion and nucleocapsid release to the cytosol. The initial translation occurs at ER-bound ribosomal machinery and the polyprotein is translated and cleaved to three structural (C, prM/M, and E protein) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).

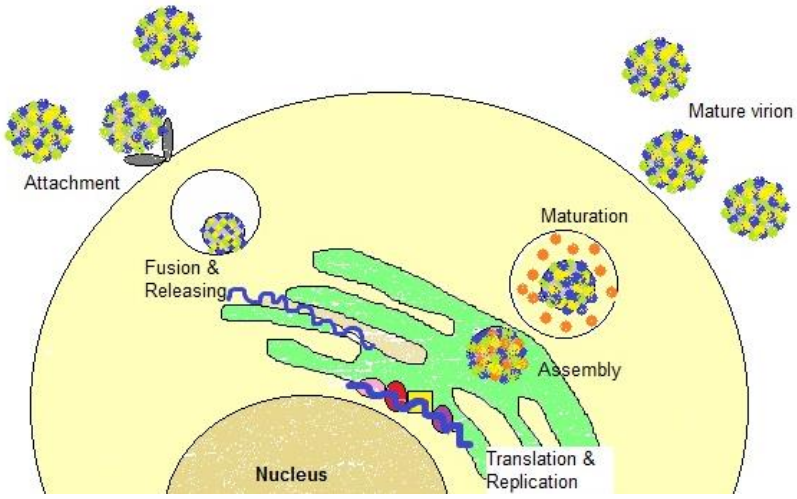


Figure 2.2 Schematic of dengue life cycle

Insights into functions and properties of viral proteins are necessary for antiviral drug discovery. Capsid (C) protein is a major protein for encapsidation or assembled with the viral genome to form nucleocapsid (Ma et al., 2004; Acheson, 2011). Membrane (M) protein plays an important role in arranging conformation of the surface proteins and indicates the status of immature (‘spiky') or mature (‘smooth') virion. (Perera and Kuhn, 2008). Envelope (E) protein is a glycoprotein to bind with host cell receptors for entry and fusion to release viral RNA (Kaufmann and Rossmann, 2011). The non-structural (NS) 1 protein is a soluble protein detected in patients' blood circulation and is a member of the replication complex (Gutsche et al., 2011; Acheson, 2011). NS2A is a hydrophobic protein spanning on endoplasmic reticulum that is generated in the endoplasmic reticulum (ER) lumen (Xie et al., 2013). NS2B acts as a NS3 co-factor to form serine protease activity (Niyomrattanakit et al., 2004; Acheson, 2011). NS3 is an essential, multifunctional protein that its N-terminus is a serine protease and C-terminus contains ATPase/helicase activity for an unwinding of the double-stranded RNA (dsRNA) and RNA

5′-triphosphatase activity for viral RNA 5′-capping reaction (Tay et al., 2015; Acheson, 2011). This encoding region is conserved in all dengue serotypes (Billoir et al., 2000). NS4A/NS4B is a member of replication complex but the actual function is still unclear (Acheson, 2011). Finally, NS5 is a largest and highly conserved sequence among flaviviruses (Billior et al., 2000). This protein has two functions, methyltransferase activity (MTase) at N-terminal domain and RNA-dependent RNA polymerase (RdRp) at the C-terminal domain (Lim et al., 2013). The viral replication takes place by viral replication complex (VRC) (Fig. 3.3) in the cytoplasm where endoplasmic reticulum invaginates into spherules. The positive single strand RNA (+ssRNA) is a template for synthesis of dsRNA, which is subsequently served as a template for replication of the new +ssRNA genome. Positive-strand RNA is packaged with capsid proteins and lipid envelope containing prM and E proteins derived from the ER. The virion progeny travels through ER-Golgi network while furin, a host protease, cleaved the pr-peptide from the virion to form mature progeny prior release by exocytosis (Diamond and Pierson, 2015; Murrel et al., 2011; Lindenbach et al., 2013).

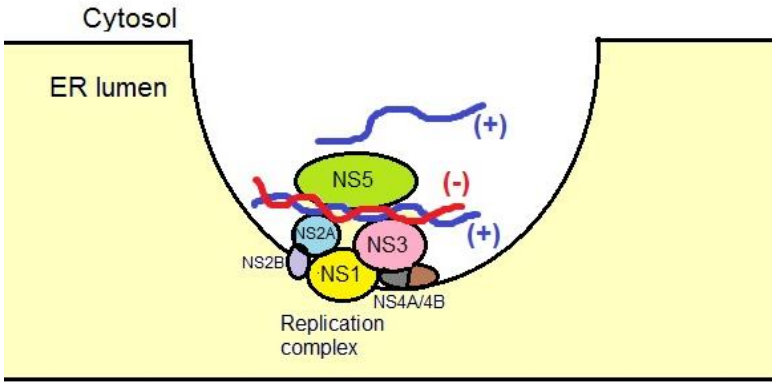


Figure 2.3 The viral replication complex with viral protein interaction

2.3 Epidemiology and pathology

Currently, dengue is the fastest spreading viral disease in the world covering at least 100 countries located in tropical and sub-tropical zones as its endemic area (Murray et al., 2013). The World Health Organization estimates that 50 to 100 million apparent infections occur annually (WHO, 2017; CDC, 2014). The disease widespread occurred in two major events; the World War II (WWII) and the modern transportation (Wilder-smith et al., 2010).

Like all subtropical countries, South East Asia (SEA) encounters the high incidence of dengue infection for half a decade (Murray et al., 2013; CDC, 2014) In Thailand, Bureau of the Vector - Borne Diseases, Department of Disease Control, Ministry of Public Health reported 63,310 hospitalized patients and 0.1% of those patients died. The clinical manifestations of dengue infection are categorized into three phases: febrile, critical, and recovery phases (WHO, 2009). The viral incubation period was usually around 4-7 days. Critical manifestations like severe plasma leakage, multiple organs failure, or shock (WHO, 2017) is mainly caused by immunopathogenic response to the secondary heterotypic dengue infection (Guzman et al., 2013; Halstead, 1979) creating massive cytokine storm. The primary prevention of dengue hemorrhagic disease is a personal protection from mosquito bites by using a mosquito repellent or vector control.

2.4 Treatment: vaccine and drug development

2.4.1 Development of dengue vaccine

Recently, Sanofi Pasteur launched the first dengue vaccine, Dengvaxia®, licensed for the prevention. The vaccine has been registered to use in Mexico, Philippines, Brazil, El Salvador, Costa Rica, Paraguay, Guatemala, Peru, Indonesia, Thailand and Singapore (Villar et al., 2015). WHO recommended the vaccine be administered to the endemic 9-45 years old population (WHO, 2017). Moreover, there are about six vaccine candidates surrently under clinical development (Vannice et al., 2016). For example, DENV-1-LVHC and TDENV-LAV developed by U.S. Army Medical Research and Materiel Command and DENVax by Takeda is currently verified in Phase I clinical trials. TV003/TV005 by Butantan Institute and CYD-TDV by Sanofi Pasteur were verified at Phase II and is currently at Phase III. (ClinicalTrials.gov, 2017; Vannice et al., 2016).

2.4.2 Development of dengue drug

Generally, the drug discovery and development process require more than ten years to complete the steps of target selection, proof-of-concept study, testing drug candidates in preclinical and clinical phases, and approval by the Food and Drug Administration (FDA). Several candidate compounds or inhibitors failed to be "druggable" because of two major reasons like low efficacy and high toxicity (Roses, 2008). However, drug discovery and development is important for finding cures to DENV infection since there is still no specific treatment for the already infected patients. Currently, certain drugs and chemical compounds under clinical trials listed as Table 3.1 and follows;

3.4.2.1 Ivermectin, an anti-parasitic drugs, was identified as a competitive inhibitor of dengue NS3 protease and other flaviviral protease including Japanese encephalitis virus, yellow fever virus and tick-borne encephalitis viruses. It also inhibited helicase activity *in vitro* with the EC_{50} in sub-nanomolar range (Lim et al., 2013; Mastrangelo et al., 2012). In the clinical trial, the efficacy and safety of ivermectin at a dose of 200 - 400 µg/kg once daily for 2 days was verified in children and adults with dengue infection by Mahidol University in 2015 (ClinicalTrials.gov, 2017).

3.4.2.2 Chloroquine, an anti-malarial drug, significantly inhibited dengue replication *in vitro* and *in vivo* with reduction of the duration of viremia and viral load (Farias et al., 2015; Farias et al., 2013). The nontoxic dose was reported at 50 µg/ml. However, with this dose, the duration of viremia in Vietnamese dengue patients was not reduced (Tricou et al., 2010). In 2009, University of Sao Paulo evaluated the effect of chloroquine in the treatment of dengue patients (ClinicalTrials.gov, 2017).

3.4.2.3 Balapiravir, a polymerase inhibitor with HCV, was studied for dengue and a report showed that the drug inhibited DENV (Nguyen et al., 2013). The drug is currently evaluated for safety, tolerability, and efficacy in dengue infection patients in 2016 by Hoffmann-La Roche (ClinicalTrials.gov, 2017).

3.4.2.4 Celgosivir, a bicyclic iminosugar, was an inhibitor of endoplasmic reticulum-resident α-glucosidase that was necessary for DENV replication. The inhibition was confirmed in an animal model and currently evaluated in Phase Ib clinical trials in Singapore (ClinicalTrial.gov, 2017; Sayce et al., 2016).

In summary, potential dengue drug candidates listed above have been proven with their efficacies in preclinical studies. However, many

drugs failed to retain its efficacy in clinical trials. New candidates are therefore required to be discovered to feed into the drug discovery pipeline making the field wide open and active for novel discoveries.

2.5 Flavonoids and derivatives inhibit dengue virus

Most natural compounds extracted from plants equipped with antimicrobial activities including bacteria, virus, or fungus. Flavonoid is a plant secondary metabolite containing the 15-carbon skeleton of two phenyl ring and one heterocyclic ring (Kumar and Pandey, 2013) (Figure 3.4). These compounds are found in fruits, vegetables, grains, bark, roots, etc., (Nijveldt et al., 2001) under various environmental stimulation. Six major subclasses of flavonoids include the (i) flavones, (ii) flavonols, (iii) flavanones, (iv) flavanols, (v) anthocyanidins, and (vi) isoflavones (Kiat et al., 2006; Ross and Kasum, 2002).

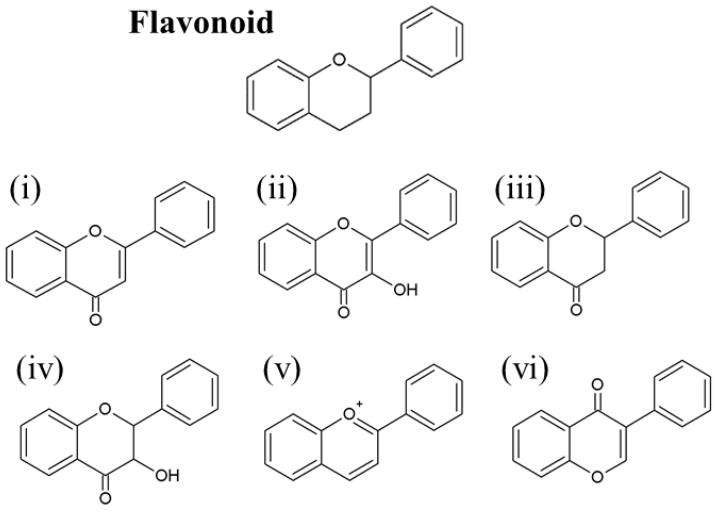


Figure 2.4 The structure of flavonoid subclass

This set of compounds has been reported with several biological activities as anti-cancer, anti-microbial, anti-inflammation. Some flavonoids can prevent the cellular injury from free radical by reacting and stabilizing the reactive oxygen species (Pietta, 2000). Interestingly, antiviral activities were found in all flavonoid subclasses (Table 3.2). In addition, multiple biological effects were reported such as anti-tumor, anti-cancer, anti-ischemia, metal-chelating activity, vasodilation, and anti-lipid peroxidation and anti-platelet aggregation (Prochazkova et al., 2011). Member of flavone, flavonol, and chalcone subclasses were previously reported as inhibitors of dengue virus as follows;

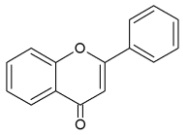
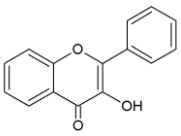
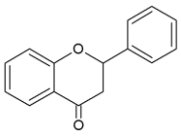
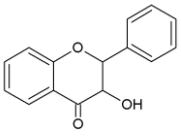
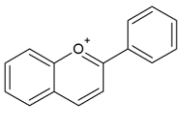
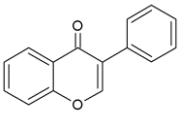
Previous report suggested quercetin, a flavonol derivative, inhibited DENV2 replication with reduction of viral RNA levels by 67% at concentration of 50 μg/ml in Vero cells (Zandi et al, 2011). Similarly, another report showed that quercetin in *Carica papaya* inhibited NS2B/NS3 protease with the highest binding energy from docking study (Senthilvel et al., 2013). Moreover, the quercetin from leaves of *Byrsonima coccolobifolia* Kunth showed a moderate DENV2-NS2B/NS3 inhibition activity while the agathisflavone, a flavone derivative, showed high DENV2-NS2B/NS3 inhibition activity (Lorena Ramos Freitas de Sousa et al., 2015). Fisetin possessed a dose-dependent anti-DENV replication and reduced viral RNA more than 65% at the concentration of 50 μg/ml with an unclear mechanism of action (Zandi et al, 2011). Naringenin showed a direct virucidal effect but were highly cytotoxic. (Zandi et al, 2011). In addition, baicalein, a flavone derivative, was demonstrated with a significant inhibition of DENV2 replication and showed a direct virucidal activity in Vero cells (Zandi et al, 2012). Furthermore, some of the flavonoid derivatives were evaluated for safety and efficacy in clinical

trials. For example, quercetin has been tested in hepatitis C patients in Phase I study for safety and tolerability by the University of California, Los Angeles in 2015. Moreover, the flavonoids have also been evaluated for efficacy and safety in other diseases and cancer (ClinicalTrials.gov, 2017). Based on the previous findings, the flavonoid derivatives are potential candidates for further exploration.

Table 2.1 The viral target and efficacy of compounds

Targets	Inhibitors	EC_{50} (μM)	CC_{50} (μM)	Action	Ref.
E-protein	NITD-448	9.8	48.7	Bind at βOG pocket	(Poh *et al.*, 2009)
	1662G07	16.9	>100	Interaction between E stem and E trimer	(Schmidt *et al.*, 2012)
	Peptide DN59	10	>30	Induced nucleocapsid ejection	(Lim *et al.*, 2013)
	Peptide 10AN1	7	>50	Interferes with virus binding to cells	
C-protein	ST-148	0.016	>100	Bind with C-protein	(Byrd *et al.*, 2013)
M-protein	MLH40 peptide	24-31	-	Block attachment to cell by 80%	(Panya *et al.*, 2015)
NS3 protease	anthraquinone (ARDP0006)	4.2	69	Bound to active site of viral protease	(Chu *et al.*, 2015, Lim *et al.*, 2013)
	Ivermectin	ND	ND		
	Benzethonium chloride	ND	ND		
NS3 helicase	ST-610	0.272	>100	Inhibit viral helicase unwinding activity	(Byrd *et al.*, 2013)
NS4B	NITD-618	1	>40	Interrupt the NS3-NS4B complex formation	(Lim *et al.*, 2013)
	CCG-3394	1.48	31	-	
	CCG-4088	0.4	13	-	
NS5 RdRp	NITD-008	0.64	>100	Inhibit viral replication	(Lim *et al.*, 2013)
	Balapiravir	1.3-11	ND	Viral polymerase inhibitor	(ClinicalTrials.gove, 2017, Wang, 2014)

Table 2.2 Antiviral activity of flavonoids and derivatives

Subclasses	Example	Antiviral activity	Ref.
Flavones 	Apigenin Luteolin Chrysin	Herpes simplex virus type, Auzesky virus, SARS-CoV, Dengue virus, Coxsackievirus B3 and Enterovirus 71	(Du *et al.*, 2016, de Sousa *et al.*, 2015, Wang, 2014)
Flavonols 	Quercetin Myricetin	Dengue virus type 2, Rabies virus, Herpes virus, Parainfluenza virus, Polio virus, Pseudorabies virus and SARS-CoV	(Yu *et al*, 2012, Zandi *et al.*, 2011)
Flavanones 	Naringenin Hesperidin	Hepatitis C virus Respiratory syncytial virus	(Goldwasser *et al.*, 2011)
Flavanols 	Epicatechin Gallocatechin	Hepatitis C virus Herpes simplex virus Enterovirus 71	(Lim *et al.*, 2013, Ho *et al.*, 2009)
Anthocyanidins 	Cyanidin Pelargonidin	Poliovirus type 1, Coxsackievirus B1, Human respiratory syncytial virus A2 and Influenza virus A/H3N2	(Nikolaeva-Glomb *et al.*, 2014)
Isoflavones 	Genistein Daidzein	Adenovirus, Herpes simplex virus, Human immunodeficiency virus, Respiratory syndrome virus, and Rotavirus	(Andres *et al.*, 2009)

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell cultures

3.1.1 Vero cells

Vero cells (ATCC® CCL-81) derived from a kidney of adult African green monkey (*Cercopithecus aethiops*) were continuously propagated in Laboratory of Virology, Department of Microbiology, Faculty of Medicine, Chulalongkorn University. The cells were maintained in Medium 199, Earle'salts (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 I.U./ml penicillin (Bio Basic Inc., Canada), and 100 μg/ml streptomycin (Bio Basic Inc., Canada), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma-Aldrich, USA) and 10% $NaHCO_3$ (Sigma-Aldrich, USA) at 37 ^oC under 5% CO_2. The cells were sub-cultured at three days intervals with split-ratio of 1:5 with growth medium (see in appendix B) using pre-warmed 0.05% trypsin-EDTA (see in appendix B).

3.1.2 LLC/MK2 cells

LLC/MK2 cells (ATCC® CCL-7) derived from a kidney of adult rhesus monkey (*Macaca mulatta*) were continuously propagated in laboratory of Virology, Department of Microbiology, Faculty of Medicine, Chulalongkorn University. The cells were maintained in minimal essential medium (MEM) (Gibco, USA) supplemented with 10% FBS, 100 I.U./ml penicillin, and 100 μg/ml streptomycin, 10 mM HEPES and 10% $NaHCO_3$

at 37 ºC under 5% CO_2. The cells were sub-cultured at three days intervals with split-ratio of 1:5 with growth medium (see in appendix B).

3.1.3 HepG2 cells and HEK-293 cells

HepG2 cells (ATCC® HB-8065) derived from liver hepatocellular carcinoma were a gift from Assist.Prof. Chanchai Boonla, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University. HEK-293 cells (ATCC® CRL-1573) derived from human embryonic kidney cells were a gift from Assoc.Prof. Parvapan Bhattarakosol, Department of Microbiology, Faculty of Medicine, Chulalongkorn University. Both cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% FBS, 100 I.U./ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES and 10% $NaHCO_3$ at 37 ºC under 5% CO_2. Cells were sub-cultured at three days intervals with split-ratio of 1:5 with growth medium (see in appendix B).

3.1.4 THP-1 cells

THP-1 Cell (ATCC® TIB202) were maintained in RPMI 1640 (Gibco, USA) supplemented with 10% FBS, 100 I.U./ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 10% $NaHCO_3$, 1% (v/v) Pyruvate (Gibco, USA) and 1% (v/v) GlutaMAX (Gibco, USA) at 37 ºC under 5% CO_2. Cells were sub-cultured at five days intervals with split-ratio of 1:5 with growth medium (see in appendix B).

3.1.5 C6/36 cells

C6/36 cells, a continuous mosquito cell line was derived from a larva of *Aedes albopictus*. C6/36 cell was a gift from Assist. Prof. Chutitorn Ketloy, Department of Clinical Pathology, Faculty of Medicine, Chulalongkorn University. Cells were maintained in MEM medium (Gibco, USA) supplemented with 10% FBS, 100 I.U./ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES (and 10% $NaHCO_3$ at 28 ^oC. The monolayer cell was subcultured by resuspension in growth medium (see in appendix B).

3.2 Virus stock preparation

3.2.1 DENV propagation

Four serotypes of DENV were reference laboratory strain including DENV1 strain 16007, DENV2 New Guinea C strain (NGC), DENV3 strain 16562, were a gift from Assoc. Prof. Padet Siriyasatien, M.D., Ph.D., and DENV4 strain C0036 was a gift from Prof. Kiat Ruxrungtham, M.D., and Assist. Prof. Chutitorn Ketloy, Ph.D., Chulalongkorn University. DENV were propagated in Vero cells. The monolayer cells in T25 cell culture flask were infected with DENV at 37 ^oC under 5% CO_2 with rocking every 15 min for 1 hour and maintenance medium (MM) was added (see in appendix B) before incubating at 37 ^oC under 5% CO_2. The cytopathic effect (CPE) of infected cells were observed under microscope after three to five day post infection (dpi). Supernatant was harvested to infect another Vero monolayer and the process was repeated for three to four passages to yield high viral titer. Supernatant was collected and centrifuged to remove cell debris at 1,500 rpm, 4 ^oC, 5 minutes before addition of FBS to 20%

FBS stored as aliquots at -70 ^{o}C. The viral titer was quantified by plaque titration assay.

3.2.2 ZIKV propagation

ZIKV strain SV0010/15 was a gift from Armed Forces Research Institute of Medical Sciences (AFRIMS), and Department of Disease Control, Ministry of Public Health, Thailand. ZIKV was propagated in C6/36 cells using MEM maintenance medium and incubated at 28 ^{o}C. The cytopathic effect (CPE) of infected cells were observed under microscope after five to seven dpi. Supernatant was harvested to infect another C6/36 monolayer and the process was repeated for three to four passages to high viral titer yield. Supernatant was collected and stored as aliquots as previously described. The viral titer was quantified by plaque titration assay.

3.3 Compound synthesis, purification and stability testing

3.3.1 Compound synthesis and purification

Eight flavonoid derivatives consisting of five flavones (FV), one flavanone (FN), and two chalcones (CH), as well as two anthraquinone derivatives (HD), were selected as group representatives for primary screening. FV2 (chrysin) was purchased from Sigma-Aldrich, USA. FV4 was extracted and purified from *Kaempferia parviflora*. FV6 was chemically modified from FV2 by methyl modification. FN2 was extracted and purified from *Boesenbergia rotunda*. CH1 and CH2 were purchased from Sigma-Aldrich, USA. HD1 and HD2 were a natural compounds from *Eleutherine palmifolia*. The purity and identity of each compound was

verified by 1H-NMR Tensor 37 infrared spectrometer (Bruker, USA) (data not shown).

The compound FV13 (6,8-dibromo-5,7-dihydroxyflavone) was synthesized and purified according to the established protocol (Do et al., 2009). Briefly, chrysin (5,7-dihydroxyflavone) (Sigma-Aldrich, USA) and NaBr (Sigma-Aldrich, USA) were simultaneously dissolved in acetones-water (5:1) (RCI labscan, Thailand). The suspension was incubated at room temperature overnight with a continuous stir. FV13 was identified by thin-layer chromatography (Merck, USA) with hexane-ethylacetate (3:2) solvent system and subsequently crystalized using absolute methanol (RCI labscan, Thailand).

The compound FV14 (6,8-diiodo-5,7-dihydroxyflavone) was synthesized and purified according to the established protocol (Park et al., 2005). Briefly, chrysin dissolved in glacial acetic acid and NaI (May& Baker, UK) dissolved in CH_2Cl_2 were mixed together. All compounds were stored as solid powder at room temperature. The stock solutions were prepared using dimethyl sulfoxide (DMSO) (Merck, USA) that as a universal drug solvent (Konstantin et al., 2006; Santos et al., 2003) to 50 mM concentration and stored as aliquots at -20 ^{o}C before use.

3.3.2 Stability testing

The FV13 was tested for the stability after dissolving in DMSO using 1H-NMR. The 4 mM FV13 solution was analyzed after dissolving in DMSO and incubated at room temperature for 24, 48, and 120 h. The solvent was sampled using 1H-NMR Tensor 37 infrared spectrometer. This experiment was assisted by Krongkarn Kingkaew, Graduate program, Department of Chemistry, Faculty of Science, Chulalongkorn Univeristy.

3.4 Plaque titration of virus

3.4.1 24-well plate plaque titration assay for dengue and zika virus

LLC/MK2 cells were seeded into 24-well plate at the concentration of $5x10^4$ cells/well and incubated at 37 ^oC under 5% CO_2. Supernatant or virus stock was 10-fold serially diluted in maintenance medium before infecting the cells at 100 ul/well. The plate was incubated at 37 ^oC under 5% CO_2 with gentle rocking every 15 min for 1 h. The experiment was done in duplicate and maintenance medium was used as a mock infection. Cells were washed with 1X PBS and semisolid overlayer medium, 0.8% gum tragacanth medium (see in appendix B), was introduced. The plate was incubated until plaque was visualized under microscope. Cells were fixed and stained with 10% formaldehyde (Carlo Erra, Italy), 5% isopropanol (Merck, Germany), and 1% crystal violet (Merck, Germany) (see in appendix B) for 1 h. The number of plaque forming units PFU/ml was determined manually and calculated with formula.

$$\text{PFU/ml} = \text{plaque no. per dilution} \times \text{Dilution factor} \times 10$$

3.4.2 Simplified dengue microwell plaque assay

The simplified dengue microwell plaque assay was adapted from Boonyasuppayakorn et al., 2015. Briefly, supernatant or virus stock (50 µl per well) of DENV1-4 was 10-fold serially diluted as previously described and simultaneously mixed 1:1 with LLC/MK2 cells (50 µl per well) at the concentration of 2.5×10^5 cells/ml. The experiment was done in triplicate and maintenance medium was used as a mock infection. The LLC/MK2-virus mixture was incubated at 37 °C under 5% CO_2 for 3 h before addition of 0.8% gum tragacanth medium (100 µl per well). The plate was incubated

until plaques became visually apparent under microscopy and cells were fixed, stained, counted, and calculated as described.

3.5 Initial compound screening

Eight flavonoid derivatives and two quinone derivatives with predicted anti-dengue activity were obtained from computational chemistry and natural product research units, Department of Chemistry, Faculty of Science, Chulalongkorn University. The compounds were dissolved in DMSO to the stock concentration of 50 mM and stored as aliquots in -20 ^{o}C until use. LLC/MK2 cells were seeded at the concentration of $5x10^{4}$ cells/well and infected with DENV2 at the M.O.I of 0.1 as previously described. The compounds were dissolved in DMSO to the final compound concentration of 10 and 25 µM in 1% DMSO. DMSO was used as a mock treatment with 100% infectivity rate. Cells were incubated at 37^{o}C for 5 days. Supernatants were harvested and viral titers were quantified by plaque titration. The compounds in which inhibited the viral titer no less than 90% were identified as active compounds. This experiment was studied by Wanchalerm Yuttithamnon, Department of Biology, Faculty of Science, Chulalongkorn University.

3.6 50% Efficacy concentration (EC_{50}) study

Active compounds from primary screening were diluted to eleven different concentrations (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µM) and added to DENV1-4 and ZIKV (M.O.I of 0.1) infected LLC/MK2 cells. Cells were incubated at 37^{o}C for 5 days before supernatants were harvested. Viral titers were quantified by plaque titration in the simplified method for DENV1-4 and the standard method for ZIKV. Effective

concentrations were calculated from non-linear regression plot of log concentration of compounds and the viral titer. Results were confirmed by at two independent experiments.

3.7 Cytotoxicity assay

Cells were seeded at 10^4 per well in 96-well plates and incubated overnight at 37°C 5% CO_2. The compounds were prepared at thirteen different concentrations (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250 and 500 µM) and added to LLC/MK2 cells. DMSO concentration of 1% was used as a 100% cell viability control that be proven non-toxic to the cells. The cell viability was accessed after 48 hour incubation using 10 µl CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, USA) according to manufacturer's protocol. The plate was incubated at 37 ^oC 4 h and read at the A_{450} nm with microplate reader model: VICTORTM X3 (PerkinElmer, USA). Cytotoxicity was calculated from non-linear regression plot of log concentration of compounds and the percent cell viability. Results were confirmed by two independent experiments.

3.8 Time of drug addition (TOA)

This study was designed to preliminary examine the mechanism of compound inhibition to the viral life cycle. LLC/MK2 cells were seeded at $5x10^4$ cells/well in 24-well plates and infected with DENV2 (M.O.I of 0.1). FV13 was added to the concentration of 10 µM of compounds in DMSO at various time points by dividing into two experiments; early time points (-1, 0, 2, 4, 6, 8, 10, and 12 h post infection) and late time points (12, 14, 16, 20, and 24 h post infection) and then incubated for 5 days. DMSO was added in all time-points in parallel. Supernatants were collected to

determine the viral titer by plaque titration assay and cell lysates were collected to determine viral RNA by RT-qPCR. Results were confirmed by at least two independent experiments.

3.9 RNA extraction and RT-qPCR

Total RNAs were extracted from cell lysates by TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The 700 µl aqueous phases were loaded into the column of Direct-zol™ RNA MiniPrep (Zymoresearch, USA) according to the manufacturer's protocol. Total RNAs were stored at -70°C until used.

RNAs were quantified by spectrophotometer (Eppendorf, USA) and adjusted to 0.1 µg/µl. The RT-qPCR was performed using 1×Power SYBRGreen PCR Master Mix, 400 nM each of C-protein primers (Shu et al., 2003) or 250 nM of NS1 primers (Manzano et al., 2011) (see in appendix C) and 0.1 µg of total RNA with Step-OnePlus Real-Time PCR System (Applied Biosystems, USA). The reactions were cycled at 48 ^{o}C 30 min and 95 ^{o}C 10 min, followed by 45 cycles of 95 ^{o}C for 20s (denaturation), 55 ^{o}C for 30s (annealing), 72 ^{o}C for 30s (extension). Results were collected in cycle threshold (Ct) and analyzed by absolute quantification. Each sample was analyzed in triplicated and results were confirmed with two independent experiments.

3.10 Functional inhibition assay

3.10.1 Anti-attachment assay

The assay protocol was adapted from Lani et al.,2016 and Jin et al.,2015. Briefly, LLC/MK2 cells were seeded in 24-well plates and incubated as previously described. Cells were then adsorbed by DENV2 (M.O.I. of 1) diluted in maintenance medium at 4 ^{o}C for 1 h with continuously gentle rocking. FV13 at 10 µM was added to DENV2 virus preparation for 1 h before adsorption (pre-attachment), during adsorption (co-attachment), and after adsorption plus three times washing with cold PBS (post-attachment). Cells were incubated in maintenance medium at 37 ^{o}C, under 5% CO_2 for 2 days before supernatants and pellets collection. DMSO-treated samples were used as no-inhibition control. Pictures were taken using Eclipse TS100 Inverted Routine Microscope (Nikon, USA). Results were confirmed by two independent experiments.

3.10.2 Fusion inhibition assay

The protocol was adapted from Ichiyama et al., 2013; Poh et al., 2009; Randolph and Stollar, 1990. DENV-infected C6/36 cells were fused under acidic pH from E–protein mediated fusion called syncytium formation (Randolph and Stollar, 1990; Summer et al., 1989). Briefly, C6/36 cells were seeded at $2x10^5$ cells/well in 24-well plate and incubated at 28 ^{o}C and DENV2 were infected at the M.O.I of 0.02 into a monolayer of the C6/36 simultaneously with 10 and 25 µM FV13. DMSO was used as a no-inhibition control and 4G2 antibody was used as a 100% inhibition control. The plate was incubated at 28 ^{o}C for two days before addition of 0.5 M 2-(N-morpholino) ethanesulfonic acid (MES) (pH 5.0) (Sigma-Aldrich, USA). Fused cells were observed under Eclipse TS100 Inverted

Routine Microscope (Nikon, USA). Results were confirmed by three independent experiments.

3.10.3 Replicon inhibition assay

BHK-21 cells expressing DENV2 replicon (BHK-21/DENV2) Boonyasuppayakorn et al., 2014 were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), and 0.3 mg/ml G418 (Bio Basic Inc., Canada). Briefly, cells ($5x10^4$/well) were seeded into 24-well plate and were incubated for 1 day at 37 °C under 5% CO_2 followed by addition of the compounds in 1% DMSO at final concentrations as indicated. DMSO (1%) alone was used as the no-inhibitor control (0% inhibition) and the reference compound was ribavirin (TargetMol, USA) at final concentrations as indicated. Cells were incubated at 37 °C for 72 h and lysed to quantified DENV2 replicon by RT-qPCR. Data were reported as percent inhibition compared with ribavirin, as a reference compound and DMSO, as a 0% inhibition. Results were confirmed by two independent experiments.

3.10.4 *In vitro* Protease assay

Assays were performed in triplicate in a 96-well half area black plate (Greiner Bio-One, USA). The reaction mixture (100 µl) contained 200 mM Tris-HCl, pH 9.5, 30% glycerol, 0.1% CHAPS, 1% DMSO, 50 nM DENV2 NS2BH-(QR)-NS3pro enzyme (Yon et al., 2005), 10 µM fluorogenic tetrapeptide substrate, Bz-Nle-Lys-Arg-Arg-AMC, and designated concentrations of compounds in DMSO (concentration of 1%). The compound-enzyme mixture was pre-incubated for 15 min at room temperature before addition of the substrate. The reaction was continued at

37 ^{o}C for 30 min. The release of AMC from the substrate was recorded every 1.5 min at 380 nm excitation and 460 nm emission in a SpectraMax Gemini EM spectrofluorometer (Molecular Devices, CA). DMSO alone (concentration of 1%) was used as the no-inhibitor control (100% protease activity) and the bovine pancreatic trypsin inhibitor (BPTI, also known as aprotinin), which has a Ki of 26 nM against the DENV2 protease, was used at 5 µM in DMSO concentration of 1% as a positive control (0% protease activity). Data were plotted and reported as percent inhibition of each compound to protease activity. This experiment was examined by Anuradha Balasubramanian, Department of Microbiology and Immunology, Georgetown University Medical Center.

3.11 Computational docking study

3.11.1 Envelope protein docking

The computational study was a protein-ligand interaction study that wildly studies in a field of drug discovery. Briefly, the target protein is 1OKE.pdb was downloaded from the Protein Data Bank. Flavone ligand library was constructed and optimized by Guassview software and was saved in Mol2 format. The ligand of flavone compounds was shown in a three-dimension structure. Molecular docking was used CDOCKER software in Accelrys program package. The experiment was examined by Kowit Hengphasatporn, Department of Biochemistry, Faculty of Science, Chulalongkorn University.

3.11.2 NS2B/NS3 protease docking study

Briefly, the structure of the compound was drawn by using HyperChemTM (Hypercube, Inc.) and optimized with molecular

mechanics method for geometry optimization. For the NS2B-NS3 protease structure was solved by the crystallized structure of crystal structure of DENV3 NS2B-NS3 in complex with aldehyde inhibitor Bz-nKRRR-H (pdb: 3U1I) was used as representative of DENV protease. The structure of the protein was visualized by Visual Molecular Dynamics software (VMD) and used Autodock4 (The Scripps Research Institute, US.) to perform molecular docking. The result was obtained in sets of binding and estimated free energy. The experiment was examined by Saran Pankaew, Department of Biology, Faculty of Science, Chulalongkorn University.

CHAPTER 4

RESULTS

4.1 Compound purification and stability

4.1.1 Compound purification result

Eight flavonoid and two anthraquinone derivatives were purified and verified by 1H-NMR Tensor 37 infrared spectrometer, the 1H-NMR result of FV13 and FV14 was a representative; FV13 (6,8-dibromo-5,7-dihydroxyflavone): pale green solid (68%), 1H-NMR at (400 MHz, DMSO-d6) was δ 13.65 (s, 1H), 8.05 (d, J =7.4 Hz, 2H), 7.56 (d, J =7.5 Hz, 3H), 7.07 (s, 1H) and FV14 (6,8-diiodo-5,7-dihydroxyflavone): pale yellow solid (77%), 1H NMR at (400 MHz, DMSO-d6) was δ 8.18 (d, J = 7.4 Hz, 2H), 7.60 (d, J = 8.7 Hz, 3H), 7.17 (s, 1H).

The structure of ten derivatives were shown in Fig. 5.1 and characteristic of two representatives: FV13 looked like a spongy solid and FV14 looked like a flaky solid (Fig. 5.2). All of the compounds were completely dissolved in DMSO.

4.1.2 Stability testing results

FV13, as a representative compound, also underwent stability test by NMR with dissolving the compound in DMSO and sampling the solution at 24, 72, and 120 h for 1H NMR analysis. The result was shown in Fig. 5.3 that we noticed the signal of compound was stable throughout 120 h. We concluded that no decay occurred to the compound at room temperature.

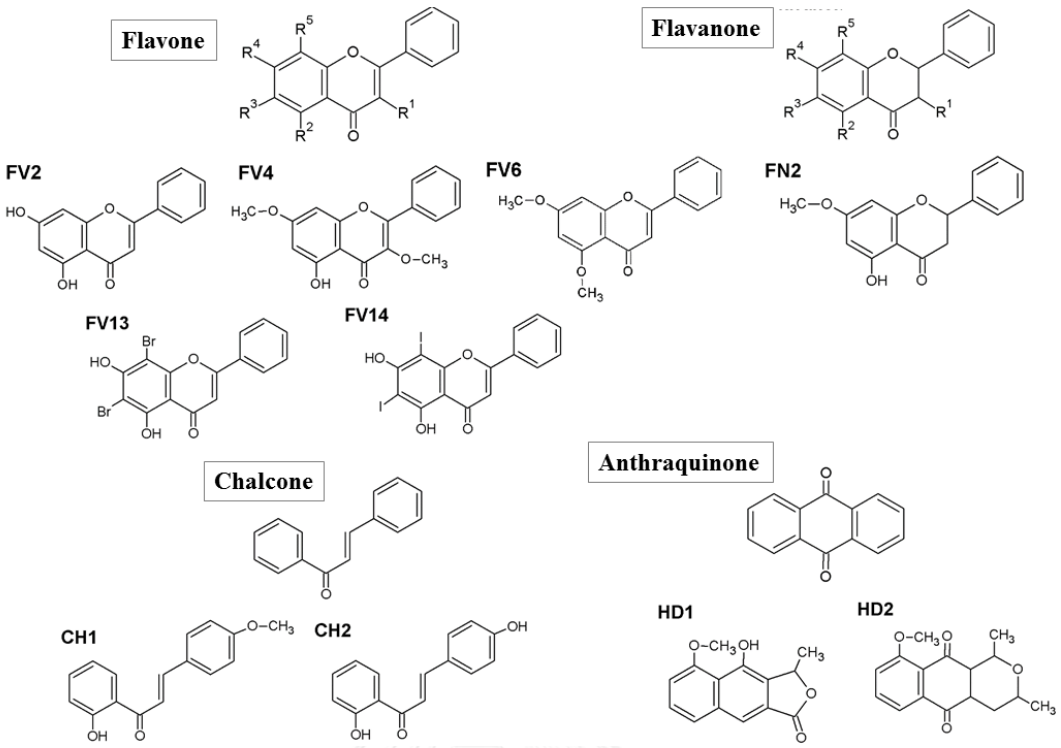


Figure 4.1 The subclass and structure of compounds

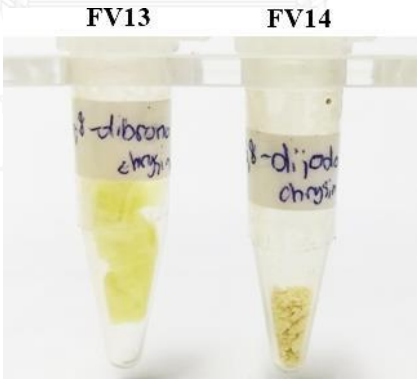


Figure 4.2 Characteristic of representative compound, FV13 and FV14

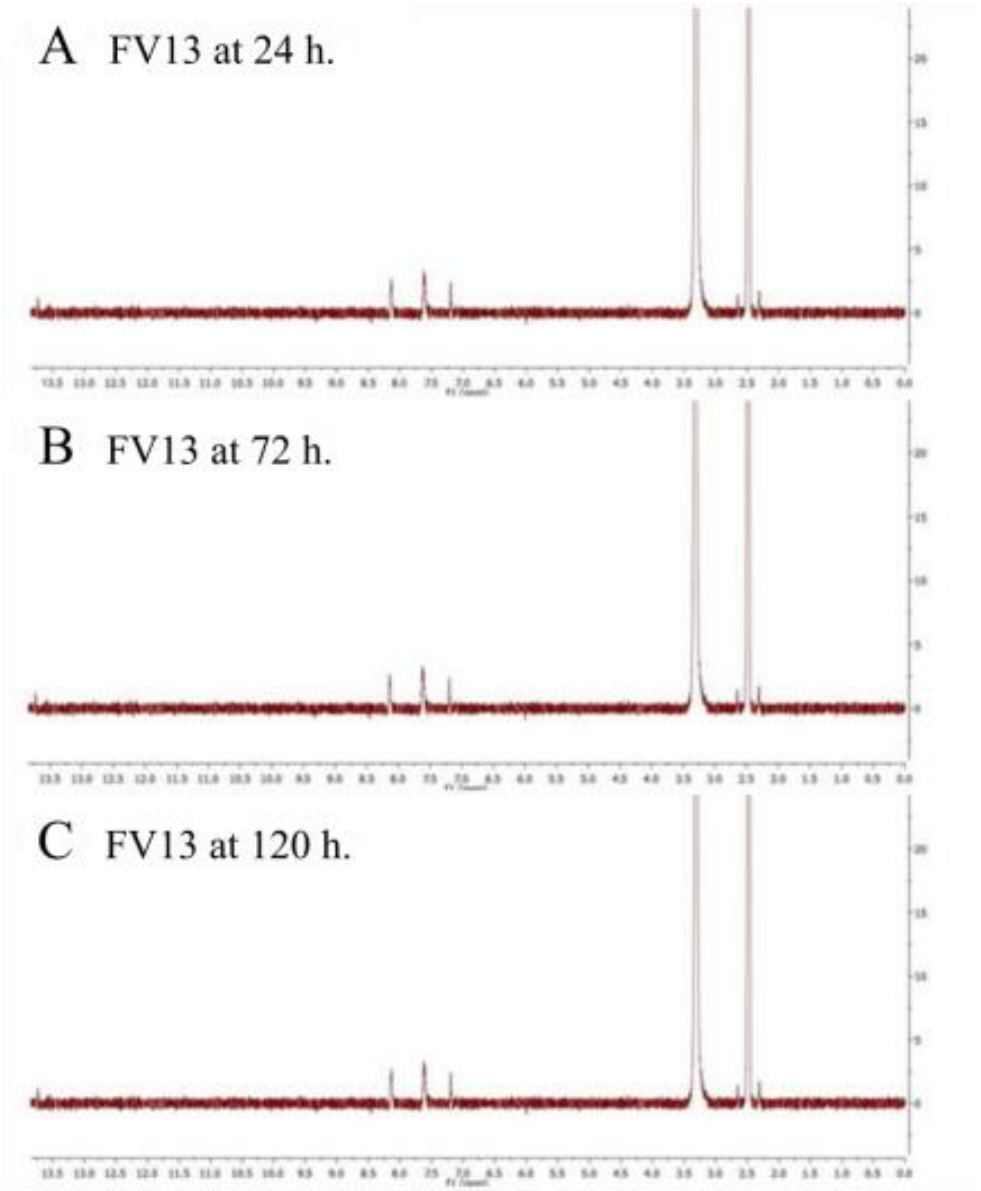


Figure 4.3 Stability of FV13 detected at (A) 24, (B) 72, and (C) 120 h after dissolving in DMSO.

4.2 Initial screening compounds with DENV2 in LLC/MK2 cells and cytotoxicity in mammalian cells and human cells

Previous studies suggested flavones, flavanones and flavonols are promising flaviviral inhibitors (Senthilvel et al., 2013; Zandi et al., 2012; Zandi et al., 2011; Kiat et al., 2006). We screened eight flavonoid and two anthraquinone derivatives (Fig. 5.1), from Asst. Prof. Warinthorn Chavasiri, Ph.D., Laboratory of organic chemistry compound's library, by adding the compounds at 10 μM and 25 μM to DENV2 infected LLC/MK2 cells (M.O.I. of 0.1) and the viral inhibition was identified by plaque titration assay of supernatants. The plaque formation was shown in white circles that represented infected cell area surrounded by uninfected cells (Fig. 5.4). Plaques were counted and calculated to percent inhibition by using DMSO as a no-inhibition control. The result showed two halogenated chrysins (FV13 and FV14), and one chalcone derivative (CH1) reduced plaque formation in both concentrations at approximately 99 % (Table. 5.1 and Fig. 5.4). Therefore, we chose three compounds for further investigations.

Cytotoxicity of selected compounds were tested in the LLC/MK2 cells to confirm the inhibition. Percent cell viability of 10 and 25 μM FV13 were 81.00 ± 2.69% (p-value < 0.05, paired t-test) and 59.40 ± 2.42% (p-value < 0.01, paired t-test), respectively. FV14 and CH1 showed lower percent cell viability (p-value < 0.05, paired t-test) (Table. 5.2 and Fig 5.5). CH1 was then excluded from further efficacy study. Moreover, we examined cytotoxicity of selected compounds in the THP-1, HEK-293, Vero and HepG2 cells at 48 h of 10 and 25 μM by percent cell viability of selected compounds (Table. 5.2 and Fig 5.5). Results suggested that human-derived cell lines, THP-1, HEK-293 and HepG2, were less

submissive to the compounds' cytotoxicity. Vero, in contrast, expressed the highest cytotoxic profile above all tested cell lines. From this accumulative data, we decided to further explore the efficacy of FV13 and FV14 as potential candidates of flaviviral inhibitors in LLC/MK2 cells.

Table 4.1 Primary screening result in LLC/MK2 cells

Compounds	**Primary Screening[a] (Mean ± SEM) % DENV2 inhibition in LLC/MK2**	
	Concentrations	
	10 µM	**25 µM**
FV2	43.00 ± 3.00	92.80 ± 3.06
FV4	38.00 ± 6.48	61.00 ± 3.00
FV6	55.00 ± 3.00	76.00 ± 7.35
FV13	99.45 ± 0.48	99.65 ± 0.41
FV14	99.48 ± 0.43	99.63 ± 0.03
FN2	62.00 ± 2.45	85.00 ± 3.00
CH1	99.78 ± 0.08	99.75 ± 0.15
CH2	52.00 ± 8.49	76.00 ± 4.24
HD1	78.00 ± 2.45	74.00 ± 2.45
HD2	54.00 ± 2.45	95.40 ± 0.24

[a] Cells were infected with the M.O.I. of 0.1 and treated with two concentrations during viral adsorption and post-infection. Supernatants were harvested at 120 hour post infection (hpi), and titrated by plaque assay.

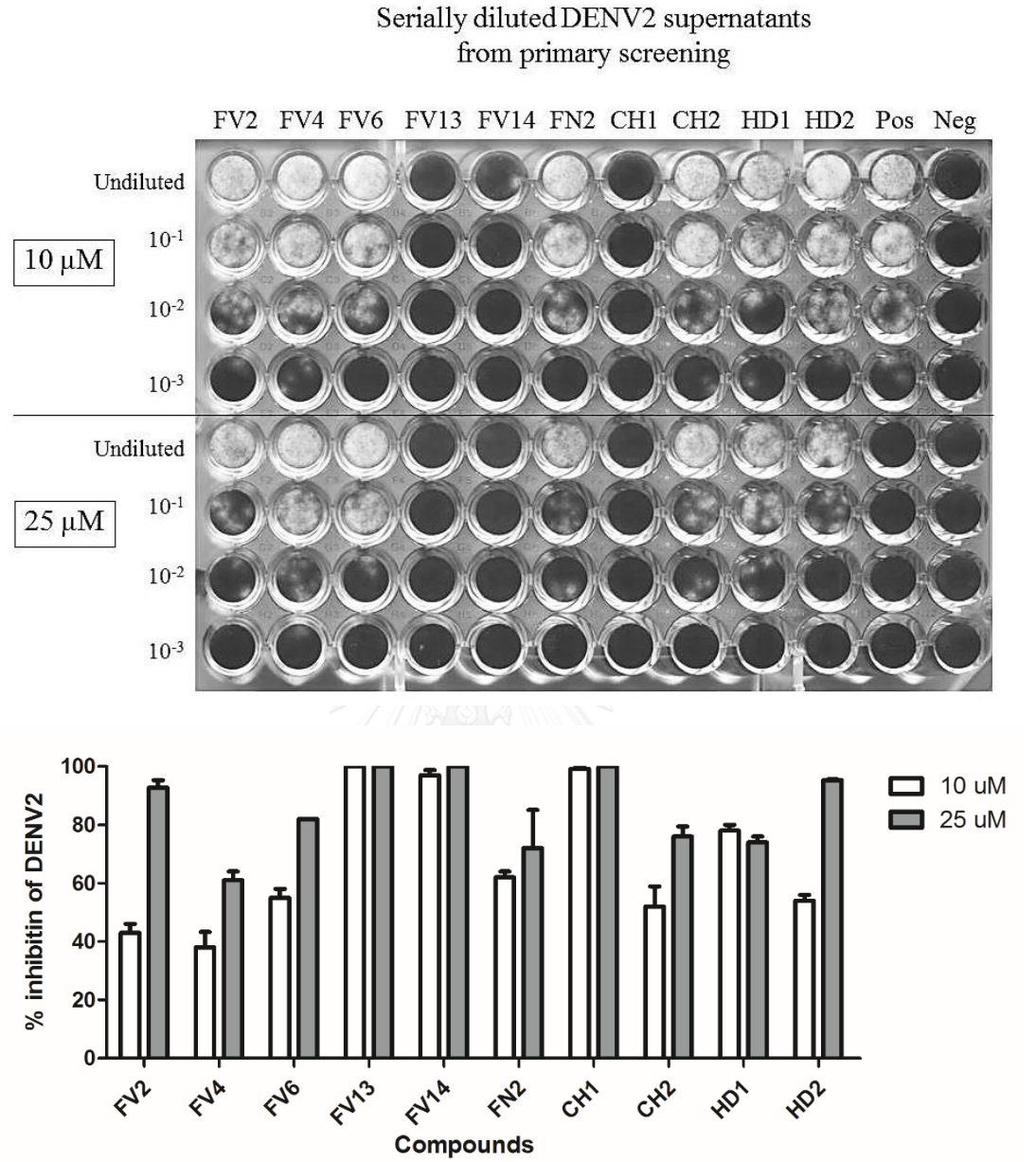


Figure 4.4 Plaque formation and percentage of screening result of ten derivatives in 10 μM (white bars) and 25 μM (gray bars) with DENV2 in LLC/MK2 cells.

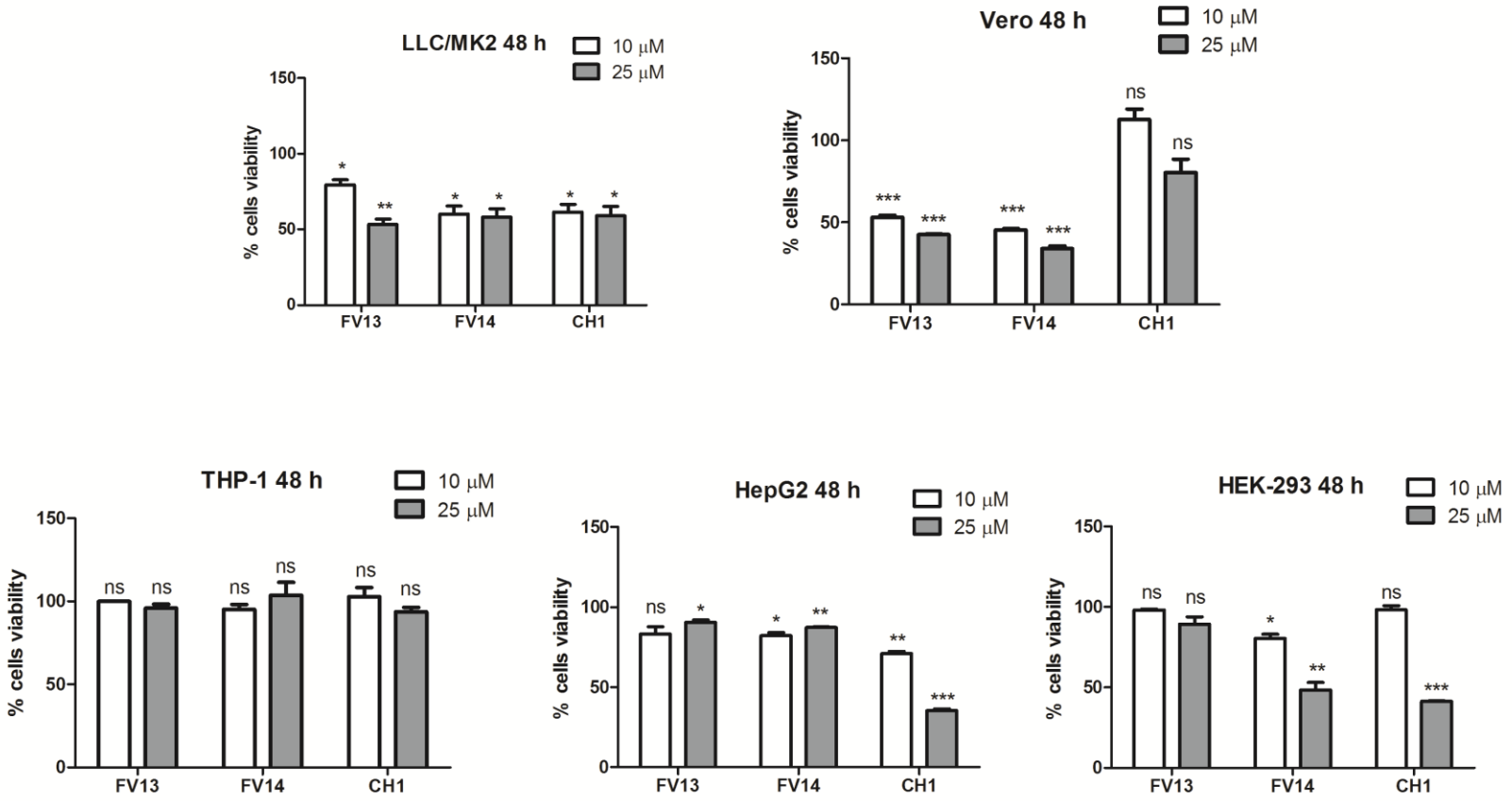


*Figure 4.5 Toxicity of active compounds in various cell lines, 10 µM (white bars) and 25 µM (gray bars) of selected compounds. Means ± standard error of mean (SEM) from two-independent experiments were reported, * = p-value < 0.05, ** = p-value < 0.01, ***p- value < 0.005, ns = not significant versus 1%DMSO treated, paired t-test*

Table 4.2 Percent cell viability of selected compounds to cell lines.

Compounds		% Cells viability[a] (Mean±SEM)				
		LLC/MK2	Vero	THP-1	HepG2	HEK-293
FV13	10 µM	81.00±2.69	53.10±1.71	>100	83.18±5.51	98.10±0.72
	25 µM	59.40±2.42	42.57±0.57	95.99±2.85	90.47±1.74	89.43± 5.60
FV14	10 µM	60.24±3.31	45.44±1.10	95.13±3.74	82.25±2.28	80.47±3.18
	25 µM	60.86±3.57	34.08±2.04	>100	87.28±0.57	48.36±5.76
CH1	10 µM	61.47±6.43	>100	>100	70.97±1.71	98.35±2.95
	25 µM	59.08±7.42	80.44±9.81	93.72±3.22	35.31±1.34	41.26±0.54

[a] All of the cells were tested with two concentration of compounds at 48 h, the cell viability was detected by using MTS assay. The data are mean ± standard error mean (SEM) from two independent experiments.

4.3 Efficacy study of FV13 and FV14 against all dengue serotypes and Zika virus

The two potential candidates of flaviviral inhibitors (FV13 and FV14) were examined the efficacy with all serotypes of dengue virus (DENV1-4) and Zika virus. Compound at various concentrations was added to virus infected LLC/MK2 cells at the M.O.I. of 0.1 and viral inhibition was accessed by plaque titration assay of the supernatant as previously described. We observed different plaque morphology from each serotype; for example, DENV1 showed small-sized plaque, DENV2 showed clear, pin-pointed plaque formation, in contrast, DENV3 and DENV4 showed large-sized plaque (see in appendix D) and then plotted to dose-response curve to calculate the EC_{50} analysis (pfu/ml). The dose-response curve from the EC_{50} experiment with DENV1-4 and ZIKV were shown in Fig. 5.6 and Fig. 5.7. Additionally, the EC_{50} values of both compounds against all tested viruses were within 1-2 µM (Table 5.3).

Based on these results, the anti-flaviviral activity of halogenated chrysins were conserved among DENV1-4 and ZIKV. Selectivity index represents the safety range when the compound is effectively inhibiting the virus but not toxic to the cells.

Table 4.3 Selectivity of halogenated compounds

Serotypes	Selectivity index (CC_{50}/EC_{50}) Mean±SEM			
	FV13		FV14	
	EC_{50} (µM)[a]	S.I.	EC_{50} (µM)[a]	S.I.
DENV1 (16007)	2.30 ± 1.04	19.42	2.30 ± 0.92	19.39
DENV2 (NGC)	1.47 ± 0.86	30.43	2.19 ± 0.31	20.37
DENV3 (16562)	2.32 ± 1.46	19.22	1.02 ± 0.31	43.64
DENV4 (c0036)	1.78 ± 0.72	25.12	1.29 ± 0.60	34.50
ZIKV (SV0010/15)[b]	1.65 ± 0.86	27.02	1.39 ± 0.11	32.14

[a] EC_{50} were determined by plaque assay in 96-well plates.
[b] EC_{50} was determined by plaque assay in 24-well plates.
The result showed mean±standard error mean (SEM) from two-independent experiments

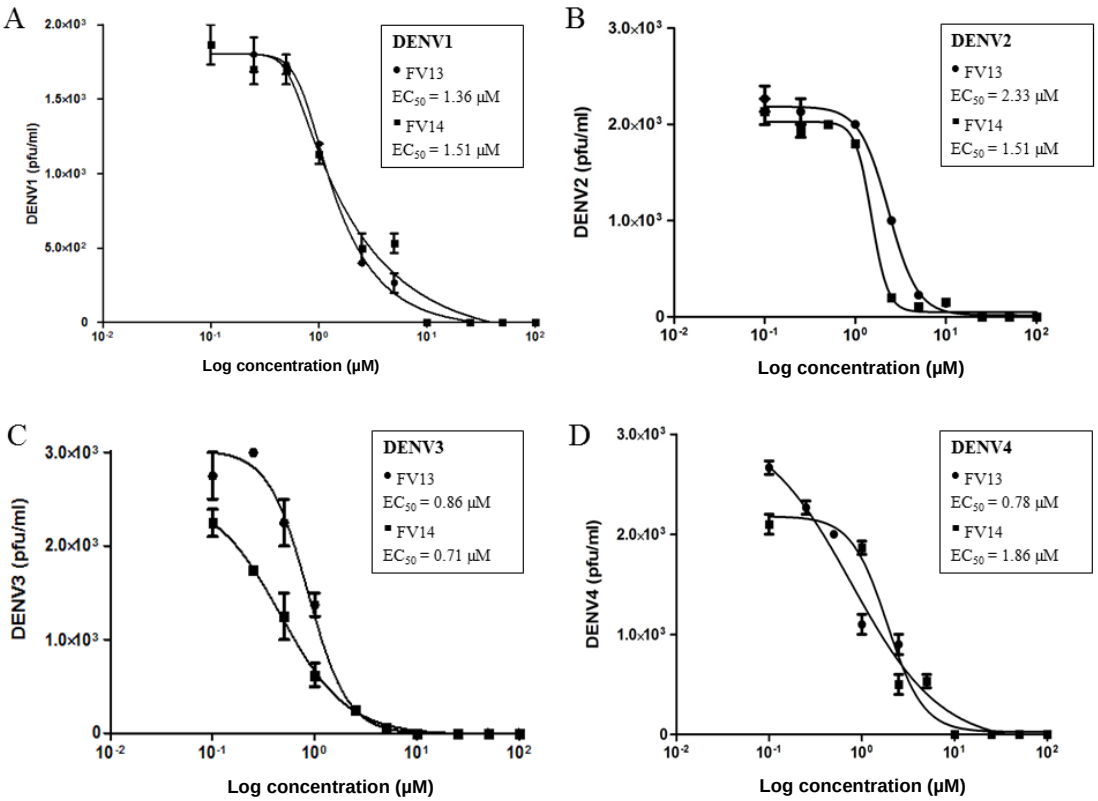


Figure 4.6 Dose-response curves of EC_{50} halogenated chrysins with (A) DENV1, (B) DENV2, (C) DENV3, and (D) DENV4 with representative values.

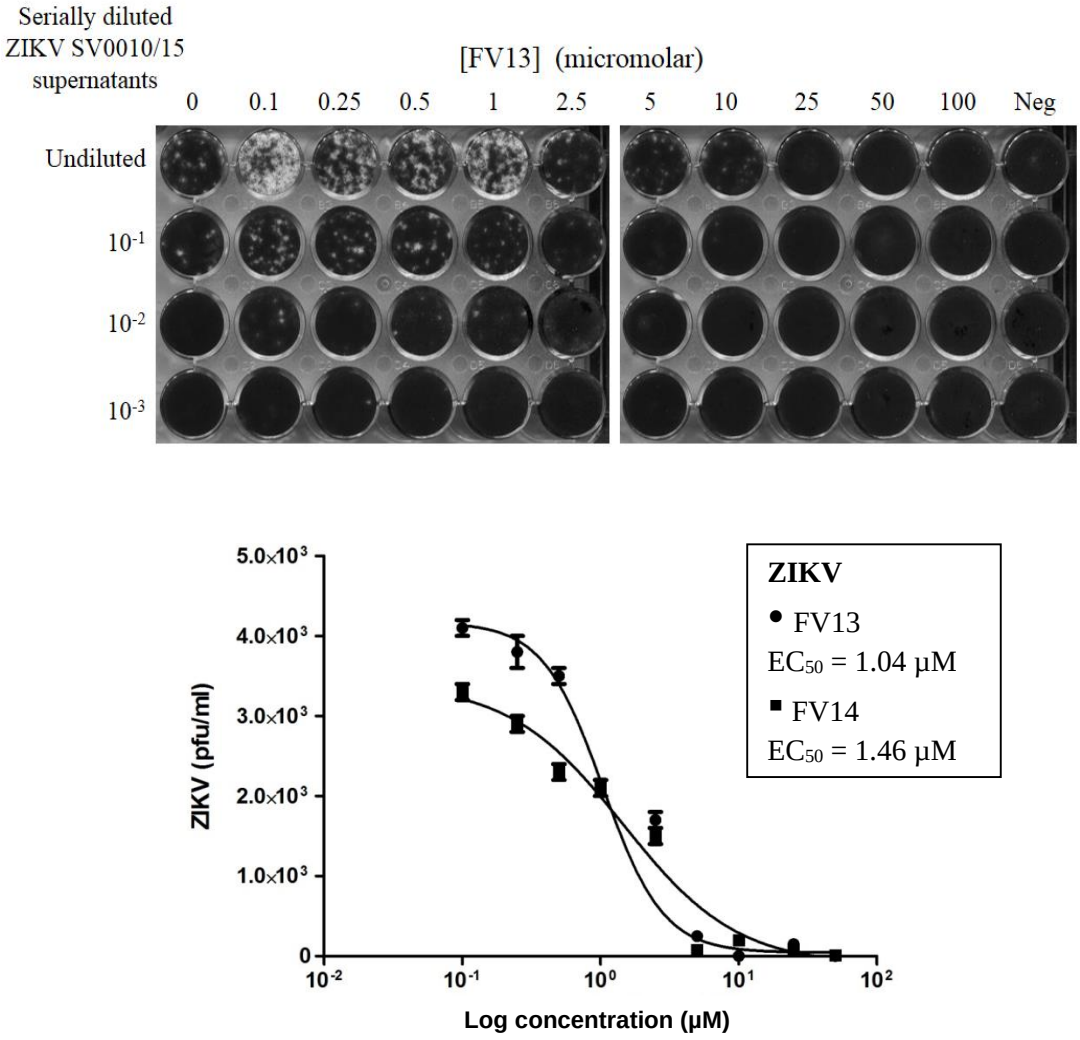


Figure 4.7 The plaque formations and dose-response curves of EC_{50} ZIKV experiments with representative values.

The safety of compound was evaluated by 50% cytotoxic concentration assay (CC_{50}), the results of FV13 and FV14 were 44.58 ± 2.99 μM, and 44.51 ± 2.58 μM, respectively (Fig. 5.8), in 48 h suggesting similar cytotoxicity in both compounds in the short-term incubation period. Moreover, two concentrations (5 and 10 μM) of FV13 and FV14 were further incubated for 120 h to study long-term cytotoxicity and corresponding to the incubation period of the efficacy (Fig. 5.9). Culture medium was refreshed once at 72 h along with the designated concentrations of the compounds. Results suggested that FV13 were similarly non-cytotoxic in both 48 and 120 h; whereas FV14 showed the higher cytotoxic effect in 120 h incubation. From the results, we decided to continue studying FV13 as a representative of the halogenated compounds.

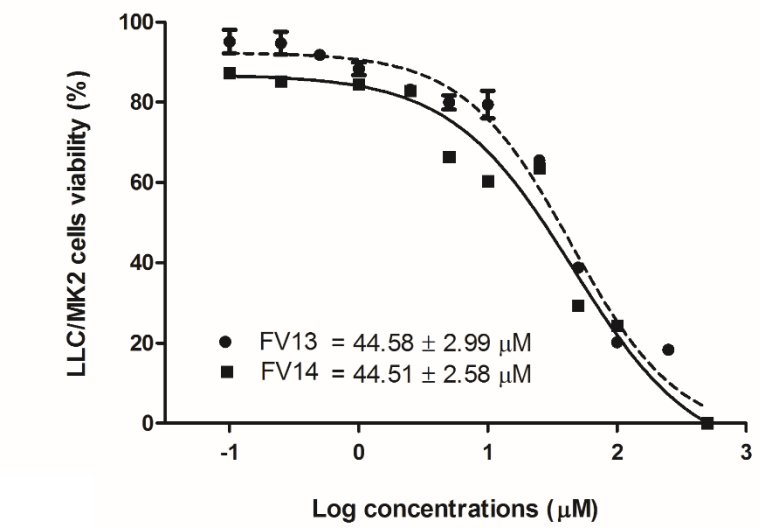


Figure 4.8 Non-linear regression curve and calculated cytotoxicity concentration (CC_{50}s) of two halogenated chrysins, FV13 (dash line) and FV14 (black line), in LLC/MK2 cells at 48 h from two-independent experiments.

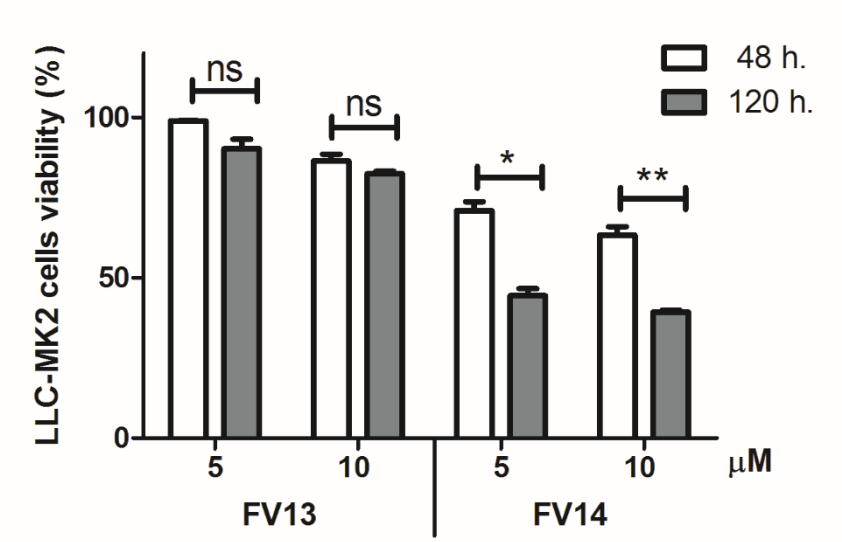


*Figure 4.9 LLC/MK2 cell viability (%) at 48 h. The values were replotted from (A) (white bars) and 120 h (gray bars). Means ± standard error of mean (SEM) from two-independent experiments were reported, * = p-value < 0.05, ** = p-value < 0.01, ns = not significant, paired t-test.*

Moreover, we explored the halogenated chrysin efficacy against DENV2 (NGC) infectivity to human-derived cell lines that were a representative targeted cell. HepG2 was tested for effective and cytotoxic concentrations of both compounds using the methods previously described in 4.6 and 4.7. The 50% efficacy of FV13 and FV14 were 11.55 ± 1.02 μM and 8.13 ± 0.94 μM, respectively from two-independent experiments. The dose-response curves were shown in Fig. 5.10. We noticed that FV14 showed higher efficacy than FV13 in HepG2 cells. But the exact cytotoxic concentrations (CC_{50}) of both FV13 and FV14 in HepG2 could not be calculated because the cells were >50% viable at 100 μM. Therefore, the selectivity index of FV13 and FV14 in HepG2 was not completely evaluated.

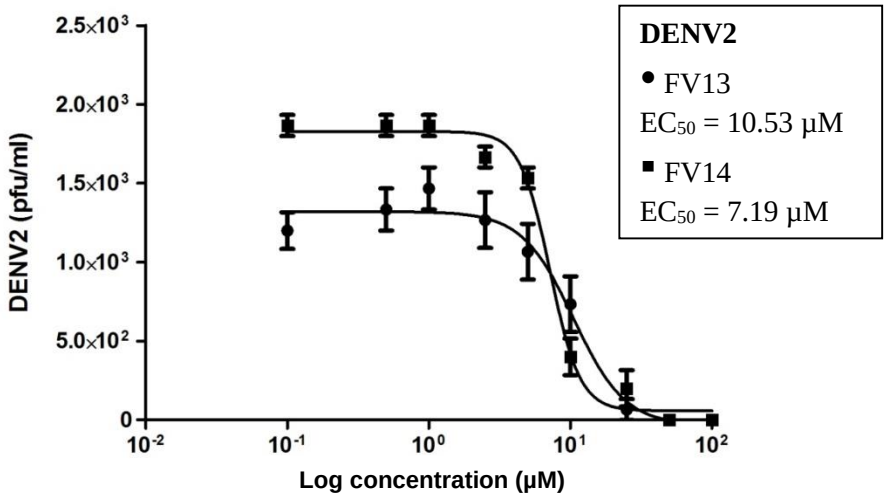


Figure 4.10 Dose-response curves of EC_{50} DENV2 experiments in HepG2 cells with representative values.

4.4 Compound inhibited multiple targets of viral life cycle

4.4.1 Initial screening mechanism: Time of drug addition

Both FV13 and FV14 are compounds containing two halogen atoms, thus we expected the similar mechanism of action of these compounds. FV13 showed the superior inhibitory effect in cell culture system probably due to less steric hindrance created by bromine. We studied time-of-addition assay to preliminary locate the possible targets in viral life cycle. Briefly, FV13 at the sub-lethal dose (10 µM) was added to DENV2 infected LLC/MK2 cells at various time points (Fig. 5.11), as previously described. DMSO was added to as a controlled treatment.

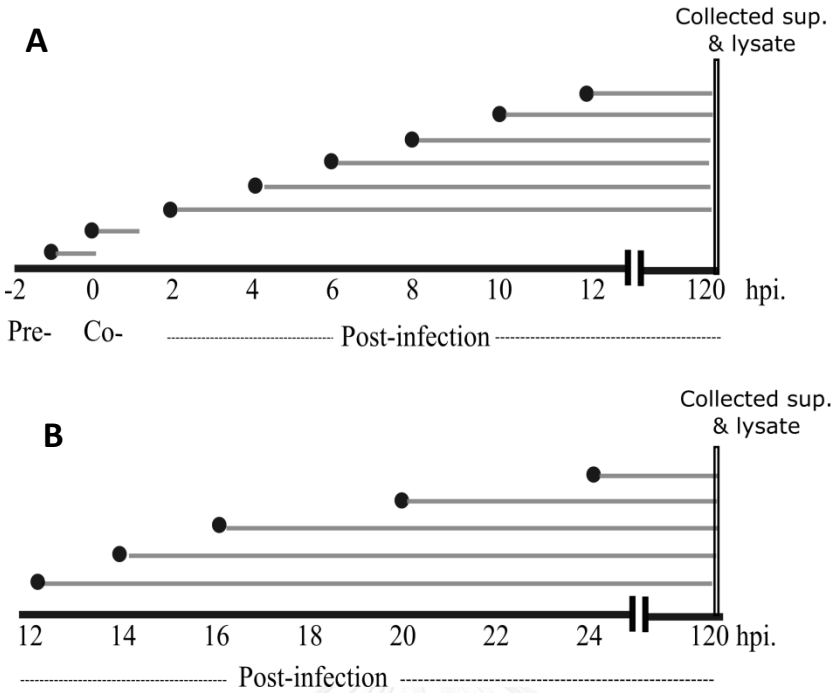


Figure 4.11 The TOA diagram, FV13 (black circles) was added to DENV2-infected cells at (A) early time points and (B) late time points. The compound was in maintenance medium (gray line) until 120 hpi, supernatant and cell lysate were collected. 1%DMSO was used for control in each time point.

The plaque titration results from compound-treated cells showed the abrupt 2-log10 decrease of the plaque titers at 2-10 hpi from DMSO-treated baseline titers and subsequently recovered to 1-log10 decrease at 12 hpi. The titer was stable from 12 to 24 hpi at 1-log10 decrease (Fig. 5.12 and Fig. 5.13). We also analyzed the intracellular viral RNA by RT-qPCR and observe the 1-log decrease at 2-12 hpi and recovered to the DMSO-treated baseline titers at 12 hpi (Fig. 5.12 and Fig. 5.13). From this result, we concluded that FV13 inhibited viral infectivity and genome replication with at least two targets in viral life cycle. The first target could possibly interact with FV13 early after infection (2 hpi). The other target could locate at the late post-replication step because of a 1-log discrepancy between 12-24 hpi plaque and RT-qPCR titers. Possible targets of late inhibition observed in plaque titration could be at the viral assembly, maturation, or release. Further investigations will reveal insights into the possible targets.

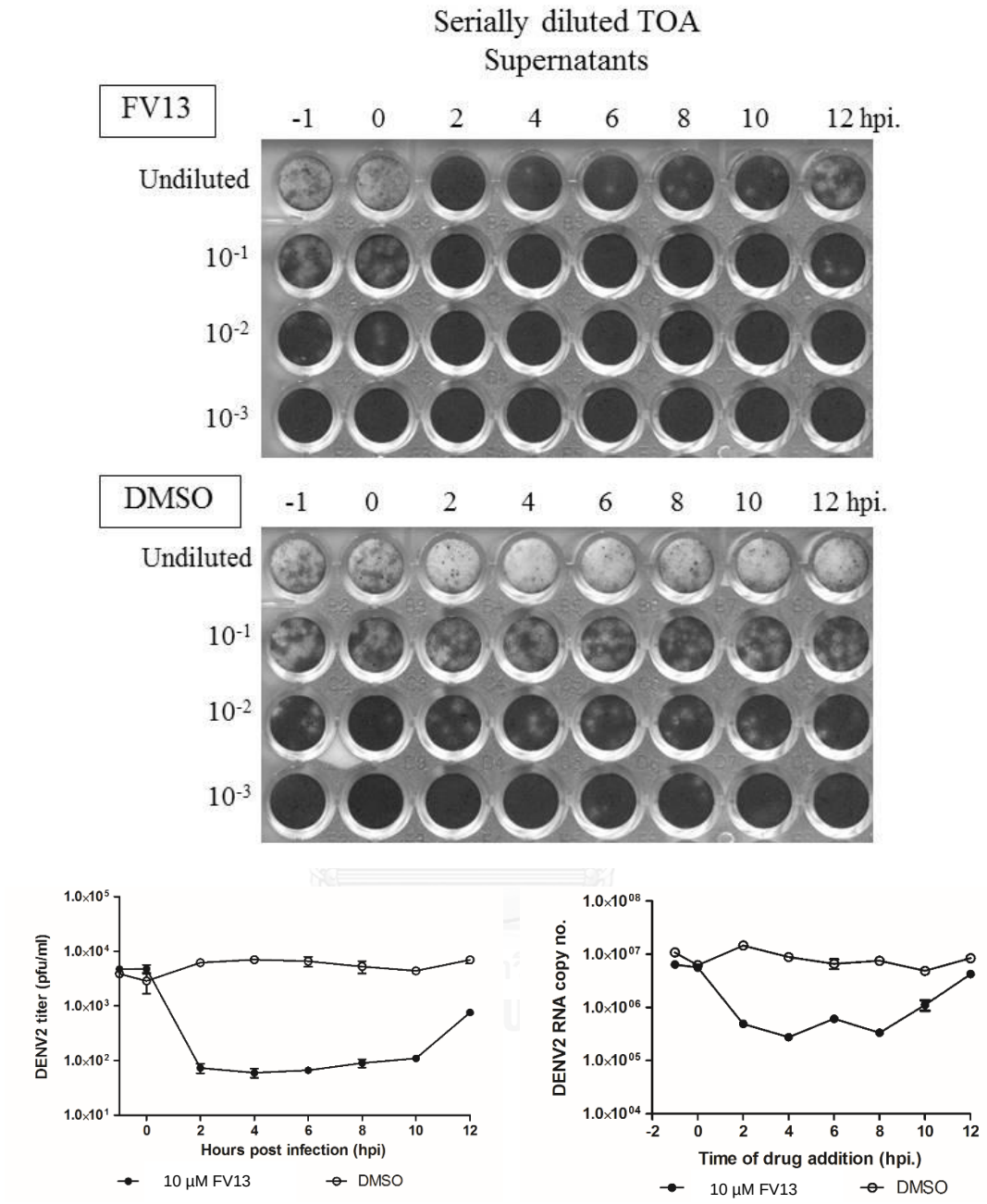


Figure 4.12 Plaque formations from TOA supernatants (early time points) in 96-well plate and graph of viral titer and viral RNA from two-independent experiments.

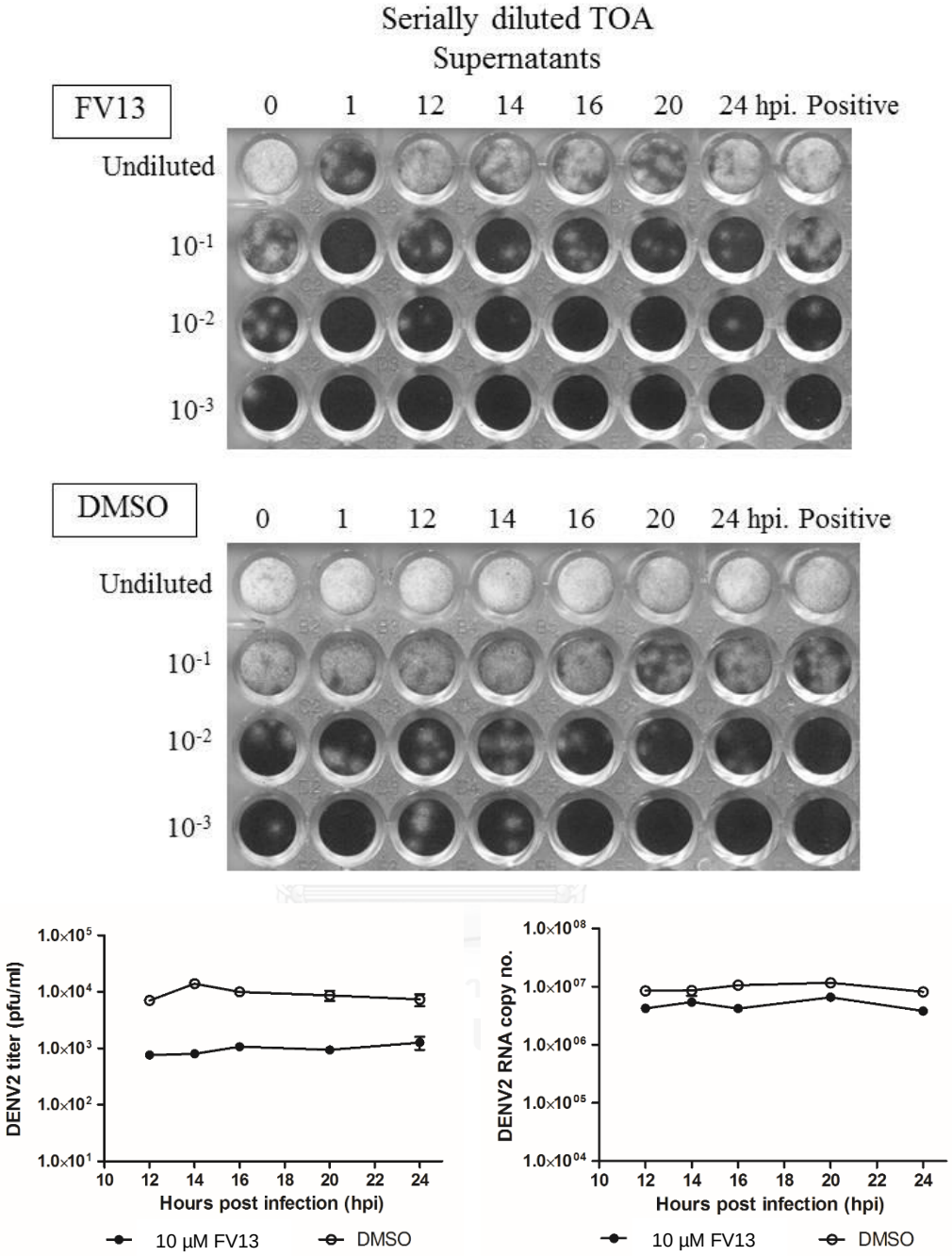


Figure 4.13 Plaque formations from TOA supernatants (late time points) in 96-well plate and graph of viral titer and viral RNA from two-independent experiments.

4.4.2 The search for possible targets at early steps

From the TOA result, we hypothesized that the compound could affect any early event in virus life cycle such as attachment, endocytosis, fusion, or translation (Pietta, 2000; Ross and Kasum, 2002). We primarily explored the neutralization and attachment by addition of FV13 to DENV2 before, during, and after infecting cells (M.O.I. of 1) (Fig. 5.14). At 48 hpi, the cytopathic effect was observed under the microscope. We noticed that the FV13-treated CPE at post-attachment wells reduced drastically, but this CPE reduction was not obvious in pre-, or co-attachment wells (Fig. 5.15). We concluded that the compound did not interfere with the viral attachment but rather showed its highest efficacy at post-attachment with viral titer inhibition of 64.97±1.18% (p-value < 0.01, paired t-test) and viral RNA inhibition of 59.96±4.56% (p-value < 0.05, paired t-test) (Fig. 5.16). Therefore, the compound could possibly inhibit post-attachment steps such as fusion or translation.

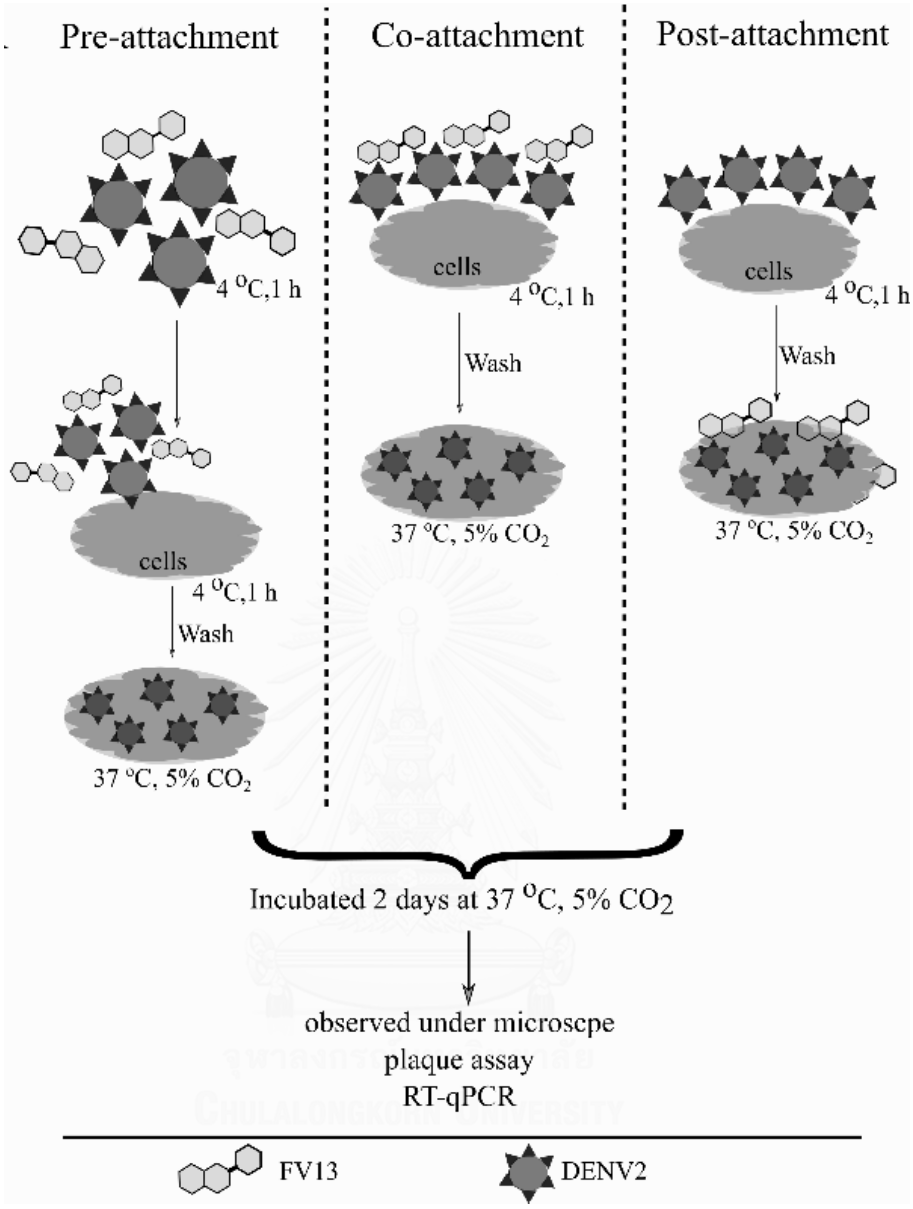


Figure 4.14 Attachment inhibition procedure diagram.

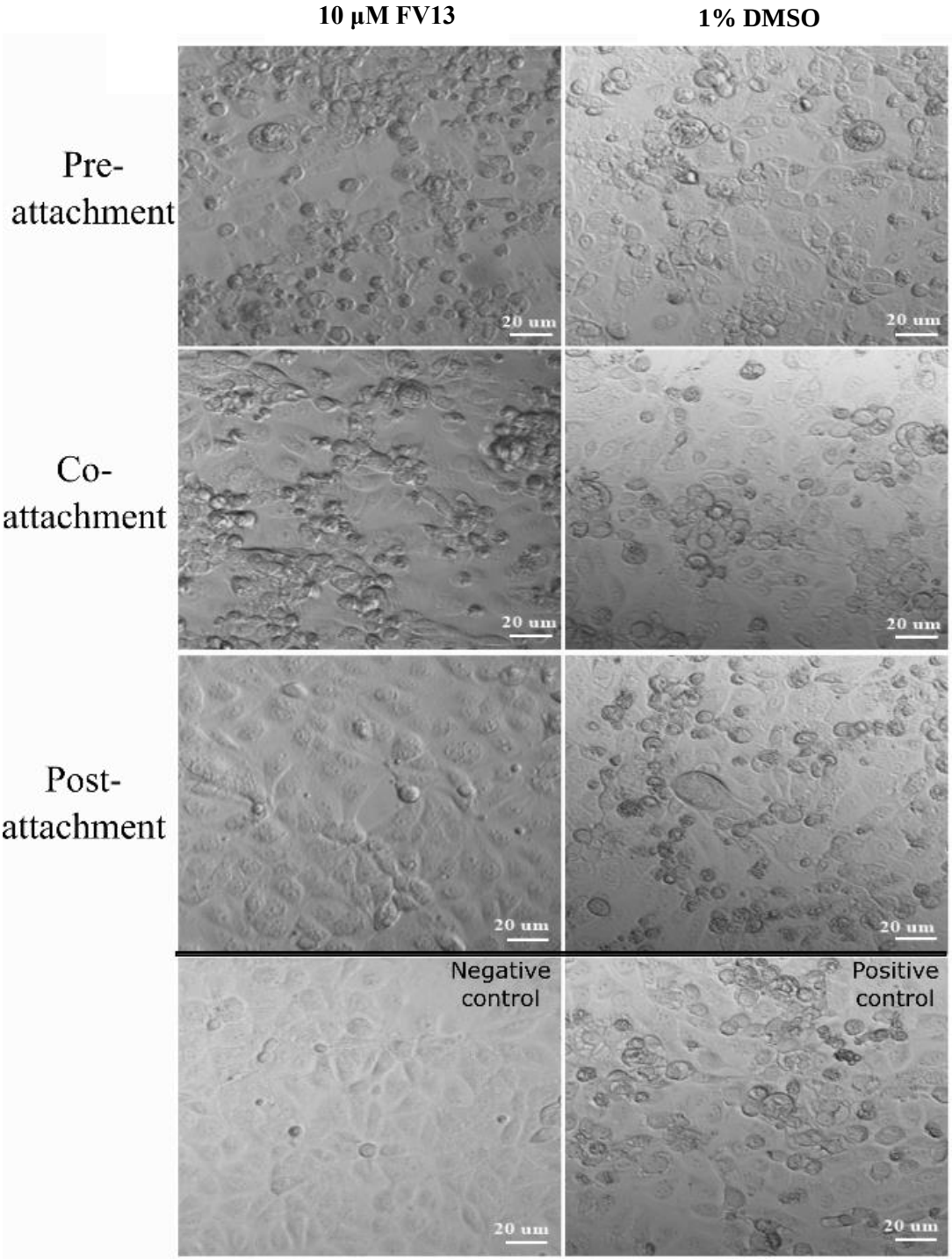


Figure 4.15 The cytophatic effect at 48 hpi, DENV2 infected LLC/MK2 cells were treated with 10 µM FV13 and 1%DMSO. Scale bars was represented to 20 micrometers.

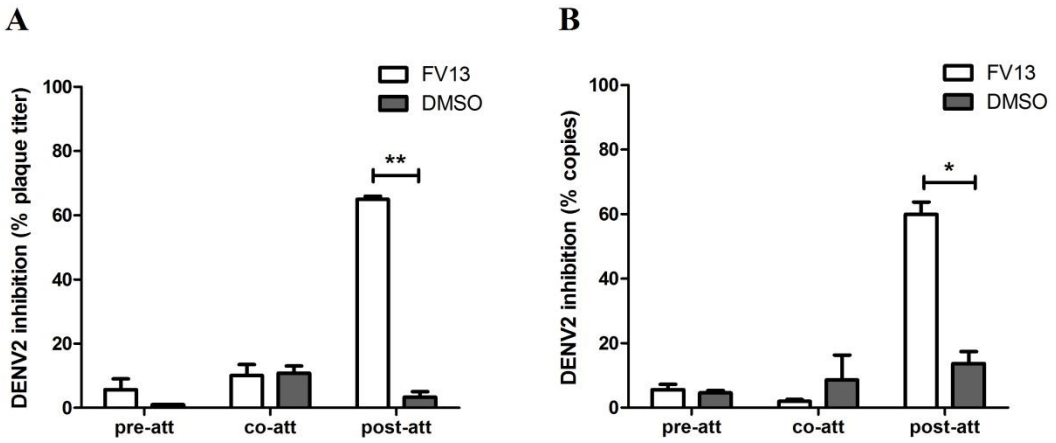


*Figure 4.16 Attachment inhibition study. (A) Percent inhibition of plaque titers from 48 h supernatants, and (B) DENV2 RNA copies from 48 h cell lysates of DENV2 infected LLC/MK2 cells treated with 10 µM FV13 or 1% DMSO at 3 conditions. Means ± standard error of mean (SEM) from three-independent experiments were reported, * = p-value < 0.05, ** = p-value < 0.01 versus 1%DMSO treated, paired t-test.*

Next, the fusion inhibition was explored. The method was adapted from Ichiyama et al., 2013; Poh et al., 2009; Randolph and Stollar, 1990 that 10 and 25 µM FV13 in DENV2 infected C6/36 cells (M.O.I. of 0.02) for 48 h before inducing acidic environment with MES addition. The antibody 4G2 that recognized fusion loop of DENV envelope and prevented syncytial formation (Summers et al., 1989), was used as a positive inhibition control. The fused cells or syncytial phenomenon were observed in the FV13-treated sample and the DMSO-treated control, whereas the inhibition was observed in the 4G2-treated sample (Fig. 5.17). Molecular docking results from Kowit Hengprasartporn Department of Biochemistry, Chulalongkorn University showed that FV13 interacted

with β-OG pocket at envelope protein with H-bond at Gln200-024, -026 and Gln271-H25 with an energy of -31.5 kcal/mol, FV14 bond at Gln200-024, -026 and Gln271-0E1 with an energy of -33.7 kcal/mol (Fig. 5.18). The energy of reference compound was -43 kcal/mol. Therefore, results from cell-based and docking suggested that FV13 did not inhibit fusion.

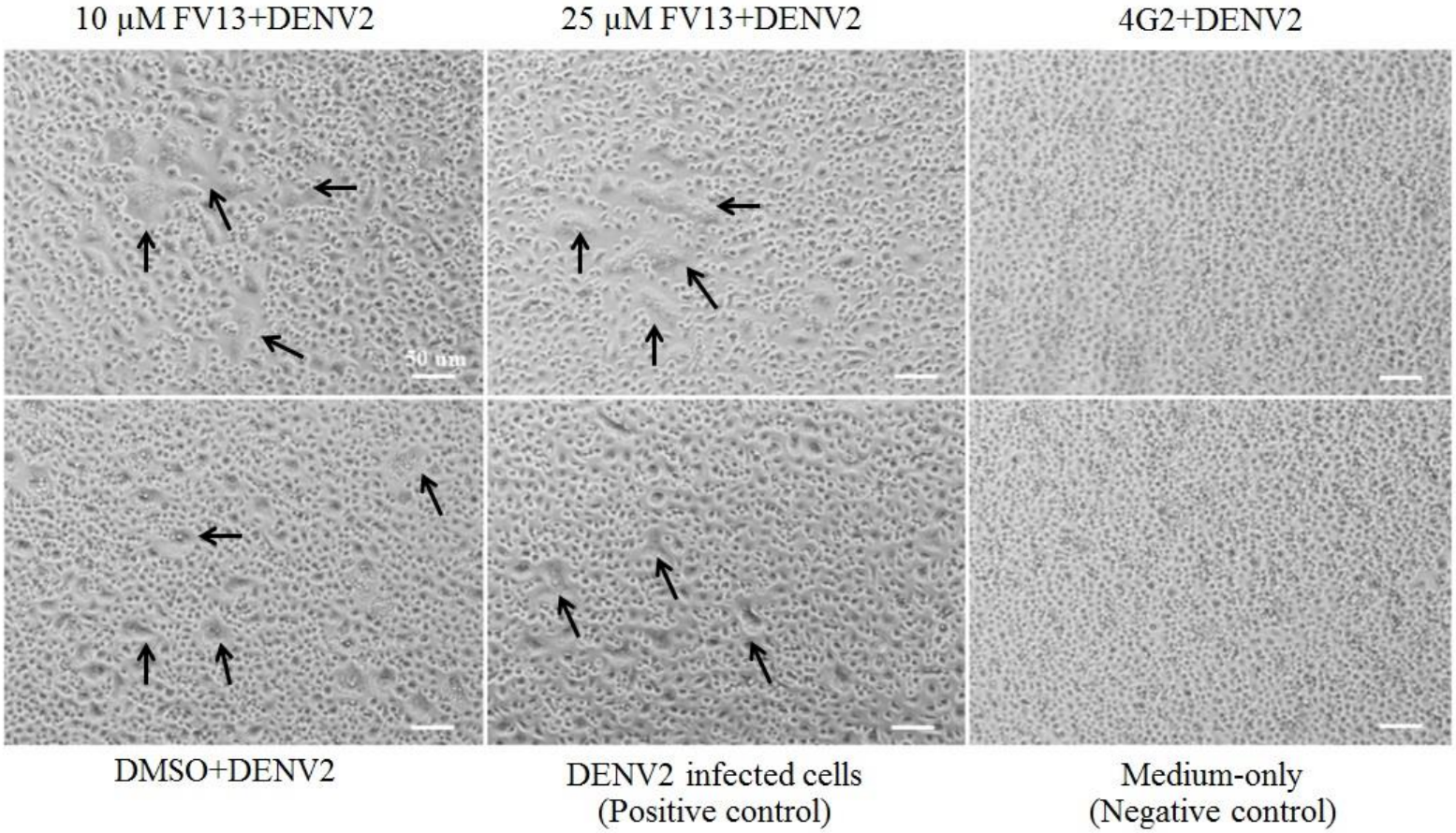


Figure 4.17 Morphology of DENV-infected C6/36 cells that were treated and induced fusion by MES, fused cells (arrows), scale bars was represented to 50 micrometers.

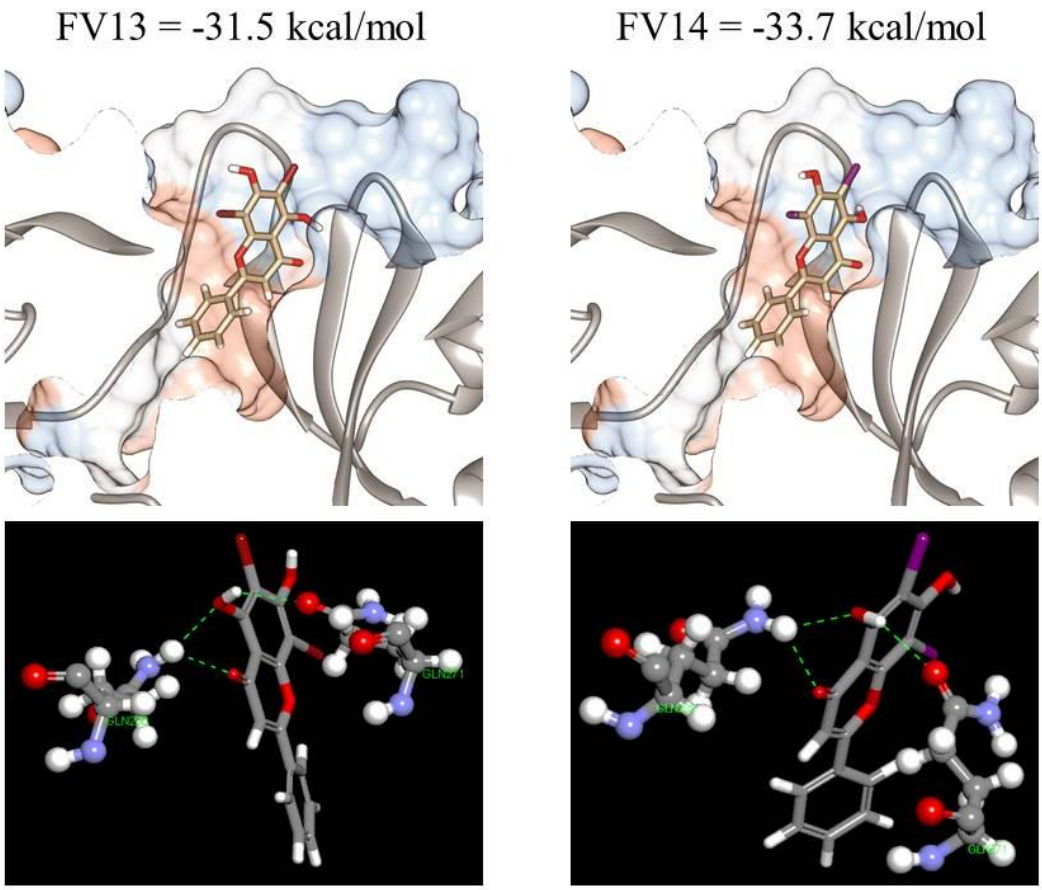


Figure 4.18 Schematic of the DENV E-protein (PDB# 1OKE), the β-OG site (gray stick) was docked with halogenated chrysin and H-bond between FV13 and FV14 with amino acid of E-protein.

Next, we studied FV13 inhibition in replicon replication system to investigate whether non-structural activities of flavivirus, mainly translation and replication, were affected. The viral replication inhibition was verified by using DENV2-replicon cells, a cell that containing all non-structural proteins and luciferase reporter stably expressed in BHK-21 cells (BHK-21/DENV2). First, the replicon cells were treated with 5 and 10 µM FV13 and incubated for 72 h before measuring replication efficiency by replicon RNA quantification. Ribavirin, a known dengue virus replication inhibitor (Takhampunya *et al*., 2006), was used as a positive inhibition control. We found that 5 µM and 10 µM FV13 efficiently inhibited DENV2 replicon replication at 72.99 ± 7.54% and 75.38 ± 10.80%, respectively (Fig. 5.19A). The inhibitory effects were similar to ribavirin that showed 72.87 ± 8.91 % and 75.80 ± 8.62% at 5 µM and 10 µM, respectively (Fig. 5.19A). Cytotoxicity was measured by MTS reagents and more than 99 % of all replicon cells were viable (Fig. 5.19B). Results from this study implied that viral translation and replication could be the target of FV13 as the replicon replications were inhibited.

Moreover, we tested the compounds for DENV2 NS2B/3 protease inhibition suggested by previous reports (Lorena Ramos Freitas de Sousa et al., 2015; Senthilvel *et al*., 2013). The molecular docking was studied by Saran Pankaew, Department of Biology, Chulalongkorn University showed interaction energy of FV13 and FV14 was -6.81 kcal/mol and -7.72 kcal/mol respectively (Fig. 5.20), suggesting a weak interaction between ligand and enzyme. Also, the results from *in vitro* protease assay were tested by Anuradha Balasubramanian, Department of Microbiology and Immunology, Georgetown University Medical Center. The enzyme was pre-incubated with FV13 and other compounds at 5, 10, and 25 µM

for 15 min before fluorogenic tetrapeptide substrate addition. The activity was measured and compared with DMSO-treated and aprotinin-treated controls as 0 and 100 percent inhibition. FV13 at 25 µM, the highest concentration, still inhibited protease activity less than 30% suggesting that the protease enzyme was unlikely the major target (Fig. 5.21).

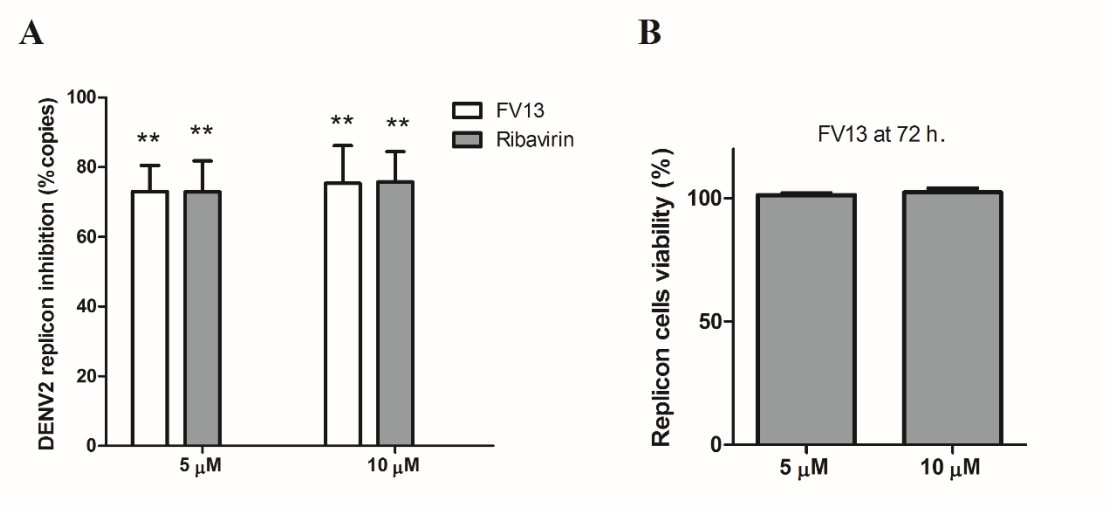


*Figure 4.19 Replicon inhibition study. (A) Percent DENV2/BHK-21 replicon inhibition with FV13 (white bars) and ribavirin (gray bars) treatment for 72 h. (B) Percent DENV2/BHK-21 replicon cell viability with 5 and 10 µM FV13 treatment for 72 h. Means ± standard error of mean (SEM) from two-independent experiments compared with 1%DMSO were reported, ** = p-value < 0.01, paired t-test.*

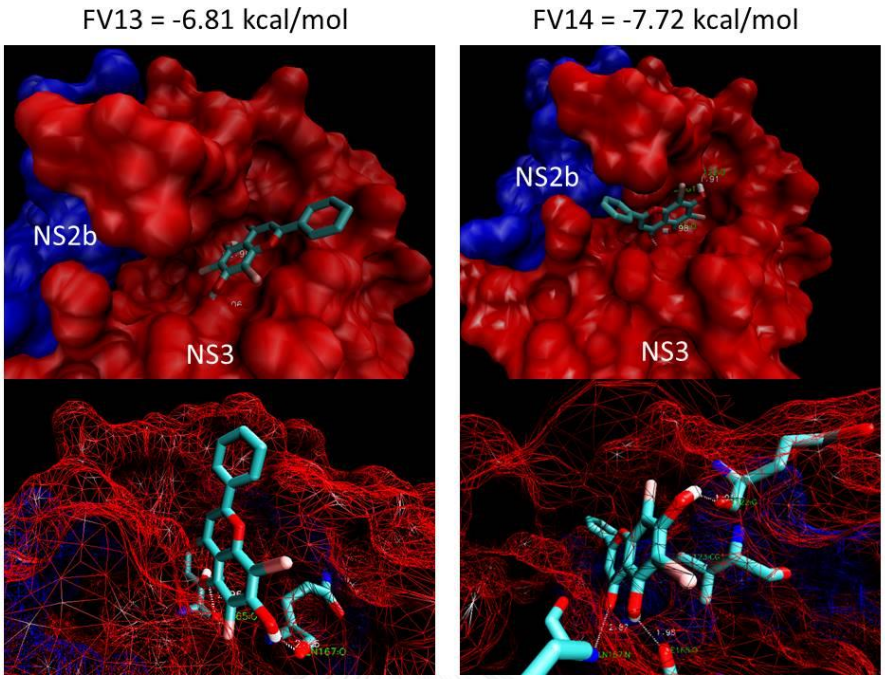


Figure 4.20 Schematic of the DENV2 NS2B/3 protease, the allosteric site of viral protease was docked with FV13 and FV14.

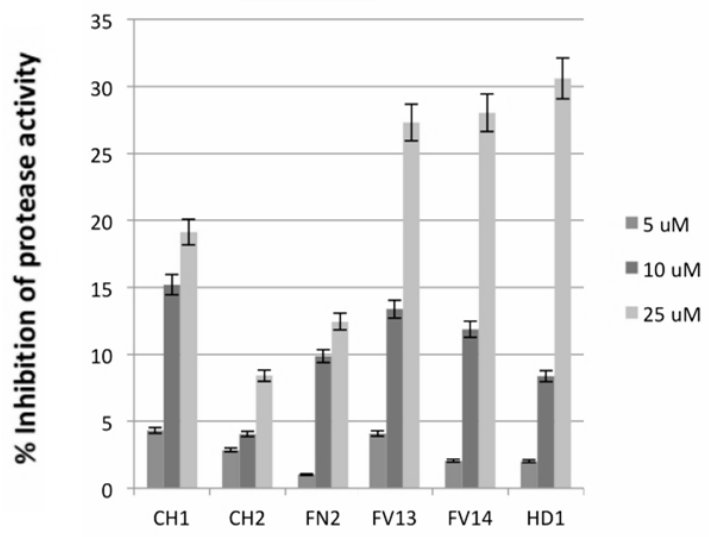


Figure 4.21 The in vitro enzymatic protease assay. The results obtained are represented in the bar diagram below with DMSO control represent as 100% active and percentage inhibition of protease activity.

4.5 Escape mutant study

DENV2 strain NGC was cultured in maintenance medium in the presence of sub-lethal dose of FV13 (5 μM). The infection and incubation were generated by 10 serial passages of the virus. The viral RNA were extracted and planned for genetic mutation analysis using whole genome sequencing. We experienced technical difficulties in amplifying PCR products for whole genome sequencing. Therefore, this experiment was not pursued to completion.

Until now, the exact molecular target of FV13 has not been identified but viral translation and replication were supposed to be the key steps of compound inhibition. From current data, we explored attachment, fusion, protease activity and none of them were targets of FV13. Hundreds of viral and cellular proteins are involved in translation and replication, therefore, we plan to discover the exact molecular target by developing the affinity-tagged compound for immunoprecipitating the protein(s) and analysis by LCMS/MS proteomic technology.

CHAPTER 5

DISCUSSION

In this study, we found that the halogenated chrysins were potential candidates against dengue and Zika virus infectivity. We noticed that both FV13 and FV14 could be re-crystallized when dissolved at high concentrations over time weeks/months. We then freshly diluted the compounds prior to use and stored the rest as aliquots in -20 ^{o}C. The stability of compound was examined and we found that the compound showed a consistently specific signal throughout 120 hours in DMSO solvent indicating no decay over the period.

This is the first report of two halogenated chrysins, FV13 and FV14, showing strong anti-flaviviral efficacies towards the unmodified, naturally derived flavonoids. Previously reported active compounds with anti-flaviviral (DENV2) infectivities in cell-based assay were quercetin (EC_{50} of 118.12 µM) (Zandi et al., 2011), fisetin (EC_{50} of 192.15 µM) (Zandi et al., 2011), baicalein (EC_{50} of 23.90 µM) (Zandi et al., 2012), Naringenin (EC_{50} of 17.97 µM) (Frabasile et al., 2017) and Baicalin (EC_{50} of 30.24 µM) (Moghaddam et. al., 2014). Therefore, FV13 and FV14 were obviously 20-100 times more potent than previously reported flavonoids. The compounds also showed broad spectrum activities against all dengue serotypes and a Zika virus (Table 5.3) making them a strong candidate for further drug development.

Moreover, the compounds showed selectivity indices at around 20-40 suggesting applicable safety range which is also suitable for further toxicity study in the animal model. Our study also included the cytotoxic

concentrations of FV13, FV14 and CH1 to human-derived THP-1, HEK-293, and HepG2 cell lines, representing common target tissues of DENV systemic infection. Results showed that FV13 and FV14 were non-cytotoxic since at least 80 % of all cells were viable (Table 5.2). It is possible that both halogenated chrysins would be similarly non-toxic like most naturally-derived flavonoid counterparts.

Previous pharmacokinetic studies of chrysin reported with low oral bioavailability in animal and human (Noh *et al*., 2016, Michael *et al*., 2016, and Zhang *et al*., 2009,). However, we expected to administer the drug in intravenous route as a supplement to standard intravenous fluid supportive treatment. Therefore, further *in vivo* toxicity and efficacy studies will focus on detecting toxic metabolites and half-life of the drug excretion by monitoring liver and kidney functions.

Mostly, the efficacy of lead compounds was related to their chemical structure and functional group. The halogens at the R3 and R5 positions in structure (Fig. 1) convey strong biological activities against flaviviral replication. Moreover, we noticed that a naturally purified flavone, FV2, actively inhibited DENV2 at 10 and 25 μM with 43.00 % and 92.80% respectively which we noticed that two hydroxyl groups (R2 and R4 positions) were preserved in most active compounds, FV2, FV13, FV14, as well as previously reported quercetin, fisetin, baicalein, luteolin, apigenin etc. This observation suggested that the hydroxyl groups at R2 and R4 could possibly have a crucial biological activity towards inhibiting DENV. Therefore, the further structural modification would need to add high EN groups at R3 and R5, along with hydroxyl groups at R2 and R4 positions.

In search of molecular targets and mechanisms, TOA result showed at least two targets located at early and late steps of infection. Viral titers at early (Fig. 5.12) and the late (Fig. 5.13) stages of viral life cycle were inhibited, but only intracellular RNA at early (Fig. 5.xx) was inhibited. It is likely that the affected late stages were assembly, maturation, or exocytosis, those occurred after intracellular replication. Moreover, a discrepancy between DMSO-treated viral titers (10^4 pfu/ml) and intracellular viral RNA (10^7 copies/ml) can be explained by the nature of detection methods. Plaque titration detected infectious virion in supernatants and RT-qPCR detected whole intracellular viral genomes those usually differ by a few logs depending on the type of virus. In this study, we noticed that the compound significantly inhibited at post-attachment and fusion was not the target from cell-based functional assay and docking study. Our results were in contrast to the previous study (Ismail and Jusoh, 2016) that described the Ile4, Gly5, Asp98, Gly100 and Val151 residues were the important amino acid residues of the E protein.

Our results showed that replicons RNA were inhibited by FV13 treatment. Translation and replication were the two major steps responsible for maintaining flaviviral replicon replication. In other words,replicon was a self-replicating flaviviral RNA sustainable by flaviviral nonstructural proteins within replication complex and cellular translation machinery like heat shock proteins (HSP), poly pyrimidine tract binding protein (PTB), calnexin, PDI and CLIMP63 (Junjhon *et al*., 2014; Khachatoorian *et al*., 2014; Sofia *et al*., 2010) or host signaling pathway or immune response including IFN, TNFα, NF-κB, IL-8, MIP-1α, MIP-1β, RANTES (Fink *et al*., 2007; Clyde *et al*., 2006; Warke *et al*., 2003). Since multiple proteins are expected to be targets of these compounds, further target identification

will require chemical affinity-tag purification and identification such as chloro-alkane tagging, azide-alkyne streptavidin-biotin system and liquid chromatography mass spectrophotometry system for protein identification.

In summary, we demonstrated for the first time that halogenated chrysin candidates inhibited the dengue and Zika virus infectivities with high efficacy at the micromolar level and less cytotoxicity. Organohalogens are approximately responsible for 30% of content in drug development (Xu *et al*., 2014). Therefore, halogenated chrysins could be novel drugs which predominantly performed to exploit their effects and influence several processes (Xu *et al*., 2014; Marcelo *et al*., 2010). This result also provided insights in cellular toxicity, stability, and suggested possible drug targets of halogenated chrysin for further optimization and development.

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APPENDIX

APPENDIX A

REAGENTS, MATERIALS AND INSTRUMENTS

Reagents

Absolute methanol	(Merk, Germany)
Crystal violet	(Merk, Germany)
Dimethyl sulfoxide	(Merk, Germany)
Direct-zol™ RNA MiniPrep	(Zymoresearch, USA)
EDTA	(Bio Basic Inc., Canada)
Fetal bovine serum	(GIBCO, USA)
Formaldehyde	(CARLO ERRA, Italy)
Geneticin (G418)	(Bio Basic Inc., Canada)
GlutaMax	(GIBCO, USA)
Gum tragacanth	(Sigma aldrich, USA)
HEPES	(Bio Basic Inc., Canada)
Isopropanol	(Merk, Germany)
M199 medium	(GIBCO, USA)
MEM medium	(GIBCO, USA)
Methanol	(Merk, Germany)
MES (N-morpholino ethanesulfonic acid)	(Sigma aldrich, USA)
MTS reagent	(Promaga, USA)
$NaHCO_3$	(Sigma aldrich, USA)
Penicillin G	(Bio Basic Inc., Canada)
Potassium chloride	(Merk, Germany)
Potassium phosphate	(Bio Basic Inc., Canada)
Power SYBRGreen PCR Master Mix	(ABI7500, USA)
Pyruvate	(GIBCO, USA)
Ribavirin	(TargetMol, USA)
Sodium bicarbonate	(Sigma aldrich, USA)
Sodium chloride	(EMSURE, Germany)
Sodium phosphate	(Merk, Germany)
Streptomycin	(Bio Basic Inc., Canada)
TRIZOL reagent	(Ambion, USA)
Trypsin	(Bio Basic Inc., Canada)

Materials

Centrifuge tube	(JET BIOFIL, China)
Flat 24, 96 well-plate	(SPL LIFE SCIENCE, Korea)
Microcentrifuge tube	(JET BIOFIL, China)
Tissue culture flask (T25 and T75)	(NUNC, Denmark)

Instruments

Autoclave (model-SX-700)	(Tomy, Japan)
Biophotometer (D30)	(Eppendorf, USA)
Centrifuge (Biofuge Stratos)	(SORVALLR, Germany)
CO_2 incubator	(Thermo Forma, USA)
Incubator	(Memmert, Germany)
Inverted microscope (Eclipse TS100)	(Nikon, USA)
Microcentrifuge (model: Forc 1418)	(Edison, USA)
Microplate reader (model: VICTOR™ X3)	(PerkinElmer, USA)
Mixer-vortex	(Science industrial, USA)
Step-OnePlus Real-Time PCR System	(Applied Biosystems, USA)
Water bath	(Julabo, Germany)

APPENDIX B

REAGENTS PREPARATION

Reagents and media for cell culture

2X MEM

MEM with L-glutamine	19.2	g
Sterilized DDW	1000	ml

Sterilized by filtration and stored at 4 ^{o}C

2X M199

M199 with L-glutamine	19	g
Sterilized DDW	1000	ml

Sterilized by filtration and stored at 4 ^{o}C

2X DMEM

DMEM	13.5	g
Sterilized DDW	500	ml

Sterilized by filtration and stored at 4 ^{o}C

2X RPMI 1640

RPMI 1640	10.4	g
Sterilized DDW	500	ml

Sterilized by filtration and stored at 4 ^{o}C

10% MEM (Growth media for LLC/MK2 cells)

2X MEM with L-glutamine	50	ml
Fetal bovine serum	10	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml
Sterilized DDW	36.5	ml

Stored at 4 ^{o}C

10% MEM (Growth media for BHK- DENV2 replicon cells)

2X MEM with L-glutamine	50	ml
Fetal bovine serum	10	ml
10 mM HEPES	500	µl
Geneticin (G418) (50 mg/ml)	600	µl
10% Na_2HCO_3	2	ml
Sterilized DDW	36.5	ml
Stored at 4 ºC		

10% MEM (Growth media for C6/36 cells)

2X MEM with L-glutamine	50	ml
Fetal bovine serum	10	ml
10 mM HEPES	700	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	500	µl
Tryptone	1	ml
Sterilized DDW	36.5	ml
Stored at 4 ºC		

10% M199 (Growth media for Vero cells)

2X M199 with L-glutamine	50	ml
Fetal bovine serum	10	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml
Sterilized DDW	36.5	ml
Stored at 4 ºC		

10% DMEM (Growth media for HepG2 cells)

2X DMEM	50	ml
Fetal bovine serum	10	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml

Sterilized DDW	36.5	ml
Stored at 4 ºC		

10% RPMI (Growth media for THP-1 cells)

2X RPMI	50	ml
Fetal bovine serum	10	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml
Glutamax	2	ml
Pyruvate	1	ml
Sterilized DDW	33.5	ml
Stored at 4 ºC		

1% MEM (Maintenance medium for LLC/MK2 cells and BHK-DENV2 replicon cells)

2X MEM with L-glutamine	50	ml
Fetal bovine serum	1	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml
Sterilized DDW	45.5	ml
Stored at 4 ºC		

1% MEM (Maintenance medium for C6/36 cells)

2X MEM with L-glutamine	50	ml
Fetal bovine serum	1	ml
10 mM HEPES	700	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	500	µl
Sterilized DDW	45.5	ml
Stored at 4 ºC		

1% M199 (Maintenance medium for Vero cells)

2X M199 with L-glutamine	50	ml
Fetal bovine serum	1	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml
Sterilized DDW	45.5	ml

Stored at 4 ^oC

1% DMEM (Growth media for HepG2 cells)

2X DMEM	50	ml
Fetal bovine serum	1	ml
10 mM HEPES	500	µl
Penicillin (100 I.U./ml) and Streptomycin (1000 µg/ml) antibiotic	1	ml
10% Na_2HCO_3	2	ml
Sterilized DDW	36.5	ml

Stored at 4 ^oC

5% Trypsin

Trypsin	5	g
Sterilized DDW	100	ml

Sterilized by filtration and stored at -20 ^oC

0.25% Trypsin-EDTA

5% Trypsin	2	ml
1% EDTA	800	µl
1X PBS	37.2	ml

Stored at -20 ^oC

0.05% Trypsin-EDTA

0.25% Trypsin	4	ml
1% EDTA	320	µl
1X PBS	15.68	ml

Stored at 4 ºC

1% EDTA

EDTA	1	g
Sterilized DDW	100	ml

Sterilized by autoclaved and stored at 4 ºC

10X PBS pH 7.4

NaCl	40	g
KCl	1	g
Na_2HPO_4	5.75	g
KH_2HPO_4	1	g
Sterilized DDW	500	ml

Sterilized by autoclaved and stored at room temperature

1X PBS

10X PBS	20	ml
Sterilized DDW	180	ml

Stored at room temperature

10 mM HEPES

HEPES	11.915	g
Sterilized DDW	50	ml

Sterilized by autoclaved and stored at 4 ºC

10% Na_2HCO_3

Na_2HCO_3	5	g
Sterilized DDW	50	ml

Sterilized by autoclaved and stored at 4 ºC

0.5 M MES (N-morpholino ethanesulfonic acid)

MES	0.98	g
DDW	10	ml

Sterilized by filtration and stored at room temperature

Plaque overlay medium

1.6% Gum tragacanth

Gum tragacanth	1.6	g
Sterilized DDW	100	ml

Sterilized by autoclaved and stored at 4 ºC

0.8% Overlay medium

2X MEM with L-glutamine	50	ml
1.6% Gum tragacanth	50	ml
Fetal bovine serum	1	ml
1M HEPES	500	µl
Penicillin (100 I.U./ml) and Streptomycin (100 µg/ml) antibiotic	1	ml
10% $NaHCO_3$	4	ml

Stored at 4 ºC

1% Crystal violet staining dye

Crystal violet	1	g
5% Isopropanol	5	ml
10% Formaldehyde	25	ml
Sterilized DDW	70	ml

Stored at room temperature

APPENDIX C

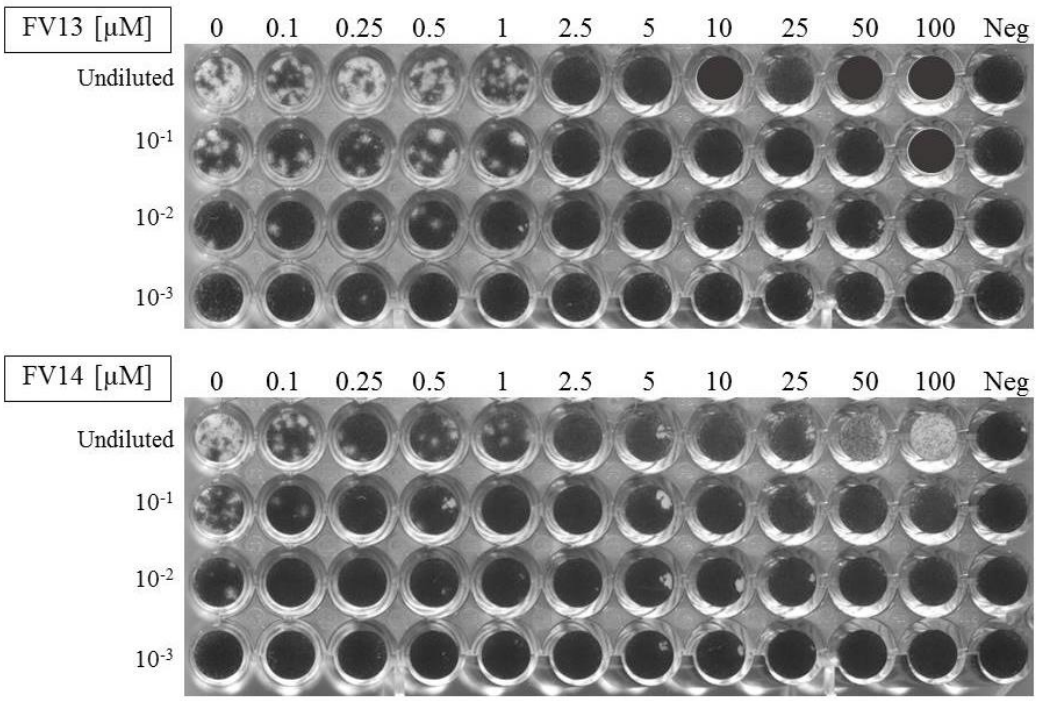
PRIMERS SEQUENCES

Primers	Sequences
DN-F	5’-CAA TAT GCT GAA ACG CGA GAG AAA-3’
DN-R	5’-CCC CAT CTA TTC AGA ATC CCT GCT-3’
D2-F NS1	5’-CTG CGA CTC AAA ACT CAT GTC AG-3’
D2-R NS1	5’-GGC TTT CTC TAT CTT CCA TGT GTC-3’

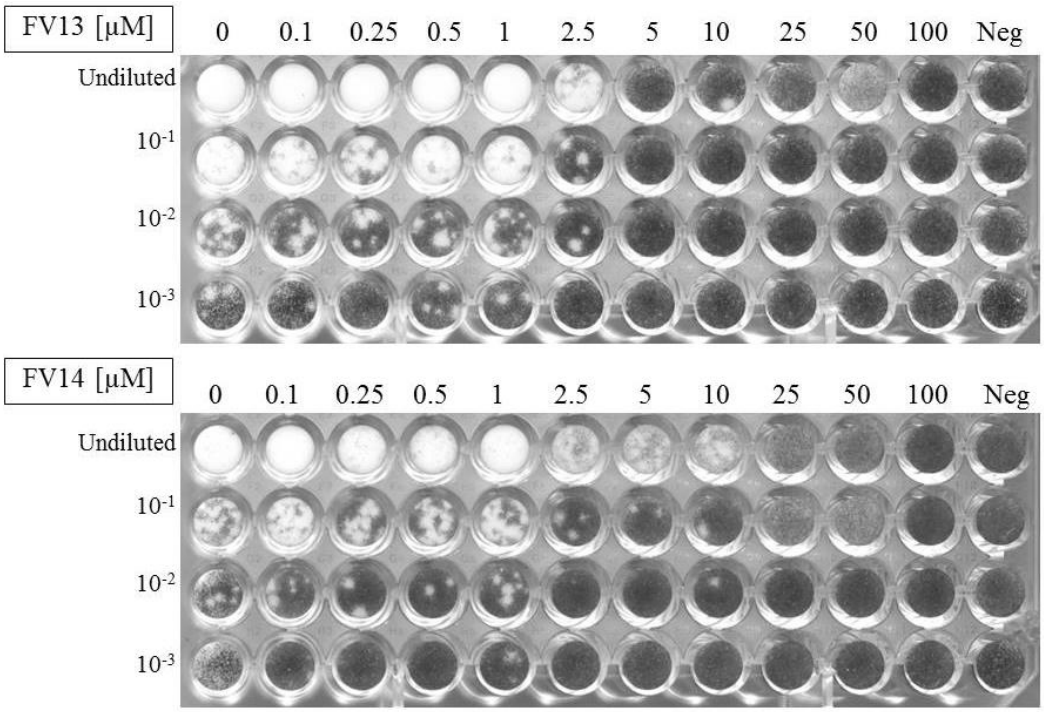
APPENDIX D

EC_{50} PLAQUE FORMATION OF DENV1-4 AND ZIKV

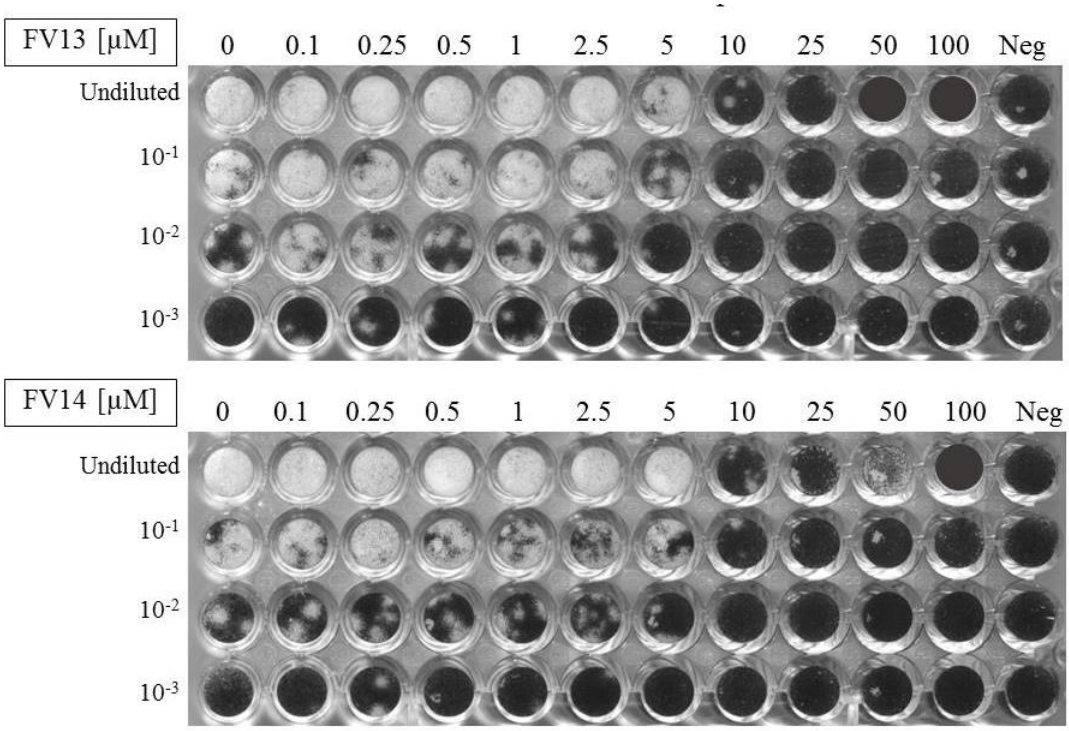
Serially diluted DENV1 supernatants in LLC/MK2 cells of representative plate



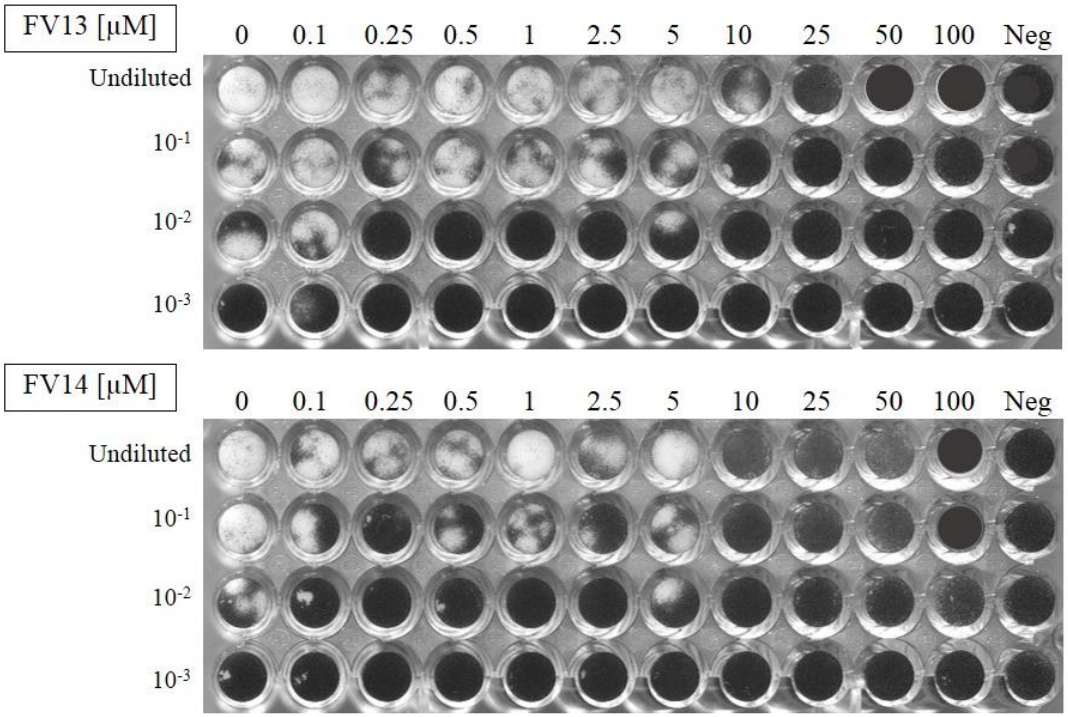
Serially diluted DENV2 supernatants in LLC/MK2 cells of representative plate



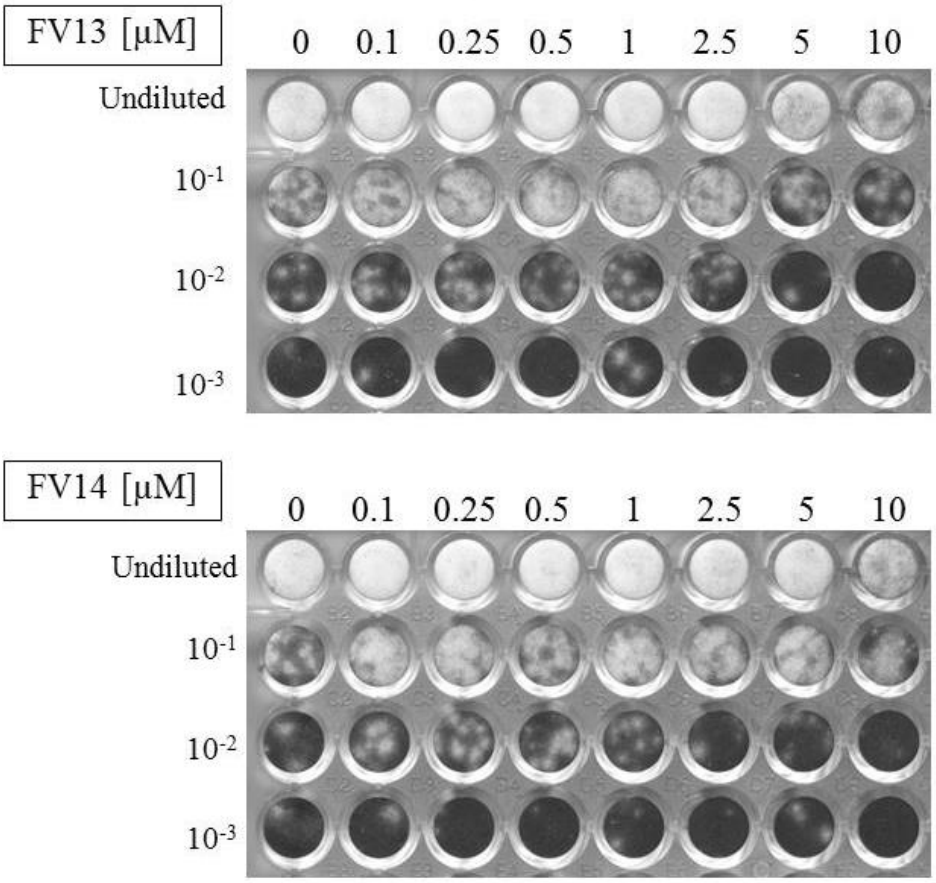
Serially diluted DENV3 supernatants in LLC/MK2 cells of representative plate



Serially diluted DENV4 supernatants in LLC/MK2 cells of representative plate



Serially diluted DENV2 supernatants in HepG2 cells of representative plate



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