The antiviral activity of lichen metabolites against dengue virus

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โรคไข้เลือดออกเป็นหนึ่งในโรคติดเชื้อที่มียุงเป็นพาหะที่มีการระบาดอยู่ทั่วโลก ปัจจุบันยังไม่มีวิธีการรักษาที่จำเพาะ สารกลุ่มเดพไซด์และเดพซิโดนเป็นหนึ่งในสารเมทาบอไลต์ทุติยภูมิที่พบมากที่สุดในไลเคนซึ่งผลิตออกมาเพื่อการอยู่รอดในสภาวะแวดล้อมสุดขั้ว สารกลุ่มนี้เคยมีรายงานประสิทธิภาพในการยับยั้งไวรัสตับอักเสบซีซึ่งอยู่ในวงศ์ *Flaviviridae* การศึกษานี้ทดสอบประสิทธิภาพของสารกลุ่มเดพไซด์และเดพซิโดนต่อการยับยั้งไวรัสเดงกี่ซีโรไทป์ 2 และความเป็นพิษต่อเซลล์ พบสารเดพซิโดนหนึ่งชนิดคือ VK-0014 และสารเดพไซด์สองชนิดคือ TT-031 และ TT-032 ที่มีประสิทธิภาพยับยั้งไวรัสเดงกี่ซีโรไทป์ 2 โดยมีค่าความเข้มข้นที่ยับยั้งไวรัสที่ 50 เปอร์เซนต์อยู่ที่ 17.42 ± 3.21, 2.43 ± 0.19 และ 0.91 ± 0.15 ไมโครโมลาร์ ตามลำดับ และมีค่าความเข้มข้นที่เป็นพิษต่อเซลล์ที่ 50 เปอร์เซ็นต์อยู่ที่ 155.83 ± 7.77, 50.13 ± 7.45 และ 12.10 ± 0.38 ไมโครโมลาร์ ตามลำดับ สาร TT-031 ซึ่งมีประสิทธิภาพดีที่สุดถูกนำไปศึกษาประสิทธิภาพเพิ่มเติมในไวรัสเดงกี่ซีโรไทป์ 1-4 และไวรัสซิกา พบว่ามีประสิทธิภาพใกล้เคียงกัน การศึกษากลไกระดับโมเลกุลพบว่าเป้าหมายของสารอาจเป็นองค์ประกอบในเซลล์มากกว่าโปรตีนของไวรัสแต่ยังไม่ชัดเจน การศึกษานี้พบว่าสารกลุ่มเดพไซด์และเดพซิโดนจากไลเคนมีประสิทธิภาพในการยับยั้งไวรัสเดงกี่เป็นครั้งแรก และอาจเป็นสารที่มีประสิทธิภาพสำหรับพัฒนาต่อไปเพื่อเป็นยาต้านไวรัสเดงกี่ได้

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....
ปีการศึกษา 2563 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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Naphat Loeanurit : The antiviral activity of lichen metabolites against dengue virus. Advisor: Assoc. Prof. SIWAPORN BOONYASUPPAYAKORN, M.D., Ph.D Co-advisor: Asst. Prof. WARINTHORN CHAVASIRI, Ph.D.

Dengue fever is one of the mosquito-borne infectious diseases globally. Currently, no specific treatment was established. Depsidones and depsides are one of the most common lichen secondary metabolites produced for the organism's survival in extreme environments. These compounds have been reported as antivirals against the other virus in *Flaviviridae* family, hepatitis C virus. In this study, depsidones and depsides were explored for anti-dengue efficacy and cellular toxicity. One depsidone, VK-0014, and two depsides, TT-031 and TT-032, exhibited an anti-DENV2 with effective concentration (EC_{50}) of 17.42 ± 3.21, 2.43 ± 0.19, and 0.91 ± 0.15 μM, respectively. Cytotoxicity concentration (CC_{50}) in Vero cells were 155.83 ± 7.77, 50.13 ± 7.45 and 12.10 ± 0.38 μM, respectively. The most effective compound, TT-031, was further study against DENV1-4 and ZIKV and found similar efficacies. The molecular mechanism studies revealed that the possible targets were cellular components rather than viral protein but the molecular target remains unclear. This study reports lichen depsidones and depsides an anti-DENV and ZIKV leads for the first time and could serve as effective candidates for further development as anti-DENV drugs.

Field of Study:	Medical Microbiology	Student's Signature .....
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LIST OF ABBREVIATIONS

ATCC	=	American Type Culture Collection
CC_{50}	=	50% Cytotoxicity concentration
CHIKV	=	Chikungunya virus
DDW	=	Double deionized water
DENV	=	Dengue virus
DMSO	=	Dimethyl sulfoxide
DW	=	Deionized water
EC_{50}	=	50% Effective concentration
EDTA	=	Ethylenediaminetetraacetic acid
EV-A71	=	Enterovirus A71
FBS	=	Fetal bovine serum
HEPES	=	4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid
hpi	=	Hours post infection
MES	=	2-(N-Morpholino) ethanesulfonic acid
mg	=	Milligram
mL	=	Milliliter
mM	=	Millimolar
M.O.I.	=	Multiplicity of infection
MTS	=	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NGS	=	Next generation sequencing
NS	=	Non-structural protein
PBS	=	Phosphate buffer saline
PFU	=	Plaque forming unit
RNA	=	Ribonucleic acid
rpm	=	Revolutions per minute
RT-qPCR	=	Reverse transcription-quantitative polymerase chain reaction

TOA	=	Time-of-addition
ZIKV	=	Zika virus

CHAPTER 1

INTRODUCTION

Dengue hemorrhagic fever is caused by dengue virus (DENV) infection with a mosquito (*Aedes aegypti* and *Aedes albopictus*) as a carrier. Estimate cases of dengue infection are 390 million per year, of which 96 million clinically manifest the illness (1). In Thailand, the incidence of dengue fever in 2015-2019 showed a 0.13% mortality rate (2). For the year 2020, the number of cases from January to October reached 65000 cases with 46 deaths (2). Moreover, dengue diseases can be prevented by vaccination and vector control. The vaccine efficacy against DENV1-4 was 58.4, 47.1, 73.6, and 83.2% with age limitation to 9-45 years old (3, 4). Another method is vector control by preventing mosquito bites and eliminating breeding sites. The infection could cause asymptomatic or severe clinical manifestations such as multiple organ failure, hypovolemic shock, and death. In severe cases, the etiology involves the secondary heterotypic infection, or the second infection with the subtype differed by the previous infection(5). The disease progresses in three stages; febrile, critical, and recovery. The clinical severity during the febrile stage such as high fever, abdominal pain, myalgia, nausea, and vomiting (6) determines a poor prognosis in the critical phase. Moreover, previous reports suggested the correlation between the viremic titer and the clinical severity (7). Therefore, administering the anti-dengue drugs in a febrile stage would potentially benefit the treatment. Currently, hundreds of anti-dengue leads were reported with potential targets locating at various stages in the virus life cycle. only a few were chosen for further clinical development because of inadequate efficiency towards all serotypes, improper pharmacokinetics properties, or adverse reaction in an animal study (8). Nine drugs have been studied in clinical trials such as chloroquine, prednisolone, balapiravir, and celgosivir. However, none of them have been clinically proven to relieve the symptoms or reduce the viral load significantly (9). The antiviral drug is still important for development to help in reduced viral load in patients to limit the progression to severe dengue.

Natural products have been one of the rich sources of novel antimicrobial drugs (10). Lichen, a symbiont of fungi and algae or cyanobacteria, is considered one of the interesting sources

because it produces a wide variety of secondary metabolites. The metabolites facilitate its survival in extreme environment including temperature, salinity, pathogens, UV irradiation, as well as protecting the organism from herbivores (11). Depsides and depsidones are major components with several reports on biological activities such as antioxidant, anti-cancer, antibacterial, immunostimulatory, and antiviral activities (12-19). To date, there is no report of depside and depsidone as an anti-dengue but there was one compound, atranorin, inhibited hepatitis c virus (HCV) at entry stage. (14). In this study, we aimed to explore the potentials of depside and depsidone as new anti-dengue leads. The compounds were extracted, purified, and identified for the first time (20-22). We determine the structure-activity relationship of depside and depsidone against dengue virus and broad-spectrum activities against other flaviviruses. We also search for potential molecular targets and evaluate the possibility of these compounds for further development as anti-dengue drugs.

Objectives

1. To screen naturally derived depsides and depsidones against dengue virus serotype 2
2. To examine the broad-spectrum activity of selected compounds.
3. To search for the possible target of the selected compound

CHAPTER 2

LITERATURE REVIEWS

1. Dengue virus

1.1 Dengue viral structures and replication cycle

Dengue virus (DENV) belongs to the family *Flaviviridae*, the genus *Flavivirus* and comprised four serotypes (DENV1-4). DENV is a positive-sense single-strand RNA in the size of about 11 kb with a single open reading frame which translated to a single polyprotein including three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (23) (**Figure 1**). The replication cycle starts from the attachment of viral envelope proteins and host receptors before entering the cell via receptor-mediated endocytosis. The viral membrane is then fused with the host endosomal membrane to release the viral genome. The positive-sense RNA genome is also an mRNA which will directly translate to a single polyprotein using host ribosomal machinery. The polyprotein is subsequently cleaved to eleven proteins by host-derived and viral proteases. The viral replication complex, including viral NS2B-3-4A-4B-5 and host factors, is assembled at the endoplasmic reticulum (ER) surface for the negative-sense RNA synthesis. The negative-sense RNA becomes an antigenome template for the synthesis of the positive-sense RNA genome (23). The proportion of negative to positive-sense RNA is one-tenth in quantity (24). The viral genome and proteins are assembled at the ER membrane to form a virion and travel through the trans-Golgi network via a cellular transport system. The prM protein at the virion surface is cleaved by a host protease called furin, thus creating a ME rearrangement or a maturation. The mature virion is released by exocytosis (25) (**Figure 2**).

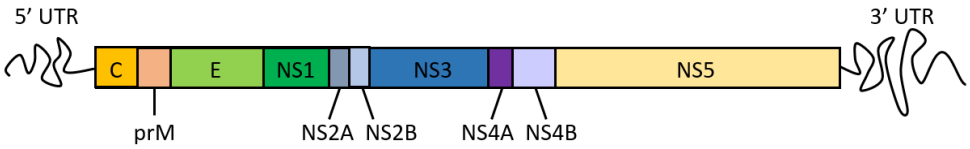


Figure 1 *Schematic of the DENV genome organization*

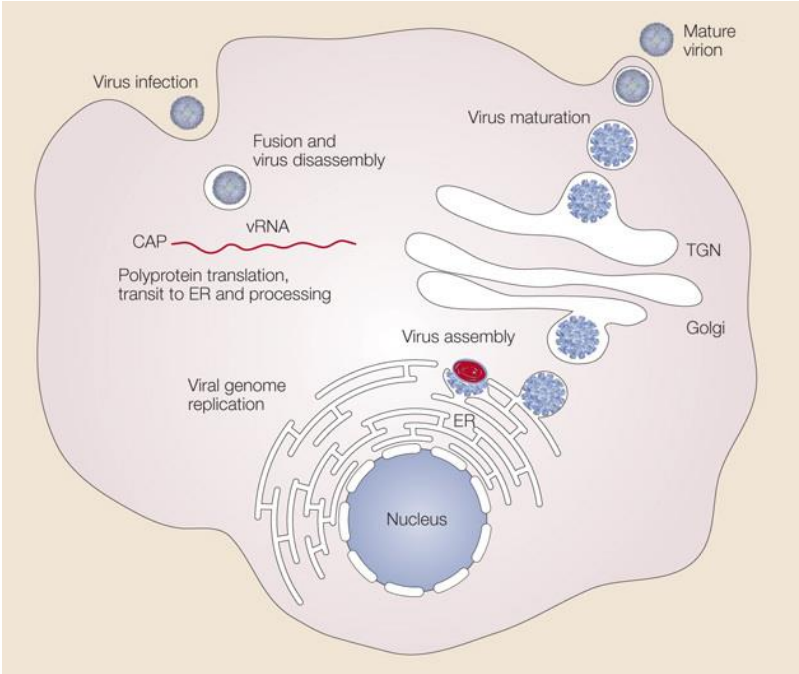


Figure 2 *Schematic of DENV replication cycle (Retrieved from Mukhopadhyay et al. (25))*

1.2 Anti-dengue drugs

Currently, there is no commercialized drug to treat dengue infection despite tremendous efforts. Many reports were on anti-dengue agents which inhibit viral components or host target that support viral replication (**Table 1**). Nine drugs and compounds have been studied in clinical trials (**Table 2**). However, any of these has no statistical significance in reducing the viremia. (23, 26, 27)

Table 1 *Anti-dengue agents reported on DENV inhibition*

Target	Compound	Mechanism of action	Reference
E-protein	NITD-448,	Bind to β-OG pocket of E-protein and prevent membrane fusion	Poh et al. (2009) (28)
	DN59 peptide	Bind to E-trimer to block stem binding	Hrobowski et al. (2005) (29)
Capsid	ST-148	Bind to the capsid protein	Byrd et al. (2013) (30)
NS2B/NS3 protease	BP2109	Interfere interaction between NS2B and NS3	Yang et al. (2011) (31)
NS3 helicase	ST-610	Inhibit helicase function	Byrd et al. (2013) (32)
NS4B	NITD-618	Hinder NS3/NS4B complex formation	Xie et al. (2011) (33)
NS5 MTase	Compound 10	Bind to NS5 MTase	Lim et al. (2011) (34)
NS5 RdRp	NITD-008	Function as nucleoside analog	Yin et al. (2009) (35)
	NITD-107	Bind to RNA binding site of the polymerase	Noble et al. (2013) (36)
Host protein	NITD-982	Inhibit host enzyme required for pyrimidine synthesis	Wang Et al. (2011) (33)

Table 2 *Anti-dengue inhibitors in clinical trials*

Drug	Mechanism of action	Study site	Clinical results	Reference
Balapiravir	NS5 nucleoside inhibitor	OUCRU, Ho Chi Minh City, Vietnam	No change in virological and immunological endpoints	Nguyen et al. (2012)(37), Reviewed by (27)
Celgosivir	ER-associated α-glucosidase inhibitor	SGH/Duke-NUS, Singapore	No reduction in viral load or fever	Low et al. (2014) (38), Reviewed by (27)
Chloroquine	Lysosomal fusion inhibitor	OUCRU, Ho Chi Minh City, Vietnam	No change in viremia or NS1 antigenemia	Tricou et al. (2010) (39), Reviewed by (27)
Lovastatin	Improving endothelial function and stabilizing lipid membranes	OUCRU, Ho Chi Minh City, Vietnam	No evidence of the beneficial effect on clinical progress or DENV viremia	Whitehorn et al. (2015) (40), Reviewed by (27)
Prednisolone	Anti-inflammatory activity	OUCRU, Ho Chi Minh City, Vietnam	No change in hematological virological or clinical endpoints	Tam et al. (2012) (41), Reviewed by (27)
Ivermectin	Anti-parasitic agent, helicase inhibitor	Mahidol University/Siriraj Hospital, Thailand	NS1 antigenemia and fever reduction (preliminary)	Avirutnan et al. (2016) (42), Reviewed by (23)

Ribavirin	Nucleoside analog	Guangzhou 8th People's Hospital	Pending	Reviewed by (23)
UV4B	ER-associated α-glucosidase inhibitor	Emergent BioSolutions, Maryland USA	Pending	Reviewed by (43)
Ketotifen	Anti-histamine and mast cell stabilizer	SGH/Duke-NUS, Singapore	Pending	Reviewed by (23)

2. Natural products as anti-dengue agents

Natural products are large sources of active compounds against fungal, microbial, and viral infection. Up to 40 percent of modern drugs are derived or chemically-inspired from natural products (44) due to versatility and biologically-relevance. Many compounds from natural sources have been reported on anti-dengue activity (Reviewed by (45)). Some of the natural compounds and derivatives with anti-DENV were showed in **Table 3**.

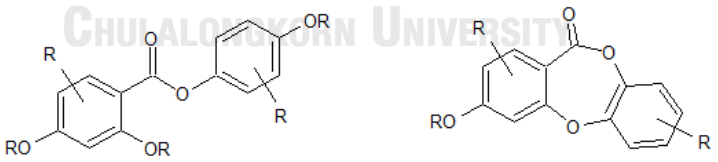
Table 3 Natural compounds and derivatives previously reported as anti-DENV agents

Compound group	Example	Mechanism of action	Reference
Polysaccharides	Fucoidan	Interact with DENV2 envelope glycoprotein	Hidari et al. (2008) (46)
	DL-galactan	Interfere the binding of DENV2 envelope glycoprotein to cell receptor	Pujol et al. (2002) (47)
Flavonoids	Chartaceone	NS5 RdRp inhibitor	Allard et al. (2011) (48)
	5-hydroxy-7-methoxy-6-methylflavanone	Bind to E protein, prevent envelope fusion	Srivarangkul et al. (2018) (49)
	4-hydroxypanduratin A, panduratin A	NS2B-NS3 protease inhibitor	Kiat et al. (2006) (50)

Alkaloids	Castanospermine	α-glucosidase inhibitor, disrupt E and prM folding	Whitby et ail. (2005) (51)
	Anisomycin	Inhibit DENV replication	Quintana et al. (2020) (52)
Phenolics	Cardol triene	Bind to β-OG pocket of E protein, prevent envelope fusion	Kanyaboon et al. (2018) (53)
	Methyl gallate	NS2B-NS3 protease inhibitor	Rahman et al. (2006) (54)

2.1 Lichen metabolites

Lichen is a symbiotic organism consist of fungi (mycobiont) and algae or cyanobacteria (photobiont). Lichen is a source of many unique metabolites in the amount of 0.1 to 10 % of the dry weight with a wide range of biological activities (11, 55). Aromatic polyketides such as depside and depsidone are the most common chemicals (**Fig. 3**), with previous reports of various biological activities listed in **Table 4**. (11). A previous study showed that atranorin, one of the depsides, and its two derivatives, were actively inhibiting hepatitis C virus, another member of the family *Flaviviridae*. The mechanism of drug action was suggested to both early and late stages but the actual molecular target(s) is still elusive and requires further investigations (14).



Depside derivatives Depsidone derivatives

Figure 3 *Core structures of depside and depsidone*

Table 4 *Biological activities of some depsides and depsidones with known function*

Compound		Biological activities	Function	Reference
Depside	Atranorin	Antiproliferative	G0/G1 cell cycle block and induced apoptosis	Kosanić et al. (2014) (13)
		Antioxidant	Reduced H_2O_2 and inhibit Lipid peroxidation	Marante et al. (2003) (15)
	Barbatic acid	Pro-apoptotic	Induce caspase-3 activity, accumulation of cells in Go/G1 phase	Reddy et al. (2019) (16)
	Sekikaic acid	Anti-RSV, immunostimulation	Induce IFN-γ and IL-2 production by T-cells	Odimegwu et al. (2018) (19)
	Diffractaic acid	Induce immunostimulation	Stimulated an increase of NO release in macrophage cells	Santos et al. (2004) (17)
Depsidone	Fumarprotocetraric acid			
		Antioxidant	Had free radical scavenging activity	Kosanić et al. (2014) (13)
	Salazinic acid	Antioxidant	Had free radical scavenging activity	Manojlović et al. (2012) (18)

3. DENV infection and regulation of apoptosis

Apoptosis is a programmed cell death essential for removing damaged or infected cells required for regulation of homeostasis (56). Flavivirus induced cellular apoptosis during infection through caspase activation such as caspase-3, -7, -8, and -9 (57-60) and induced late activation of caspase-1 and IL-1 production for pyroptosis to facilitate viral replication and prevent cell

immune response against infection (61). Moreover, the introduction of a caspase inhibitor resulted in viral inhibition (60, 62). The previous report showed that barbatic acid, one of the depside, selectively inhibits cancer cell lines via caspase-3 activation lead to cell apoptosis (**Fig. 4**)(16). Inhibition of caspase activity might serve as an alternative way for viral inhibition via help maintain cellular function. In contrast, competitive activation or alteration in the caspase pathway may also serve as an alternative pathway in controlling viral replication by inducing apoptosis in infected cells.

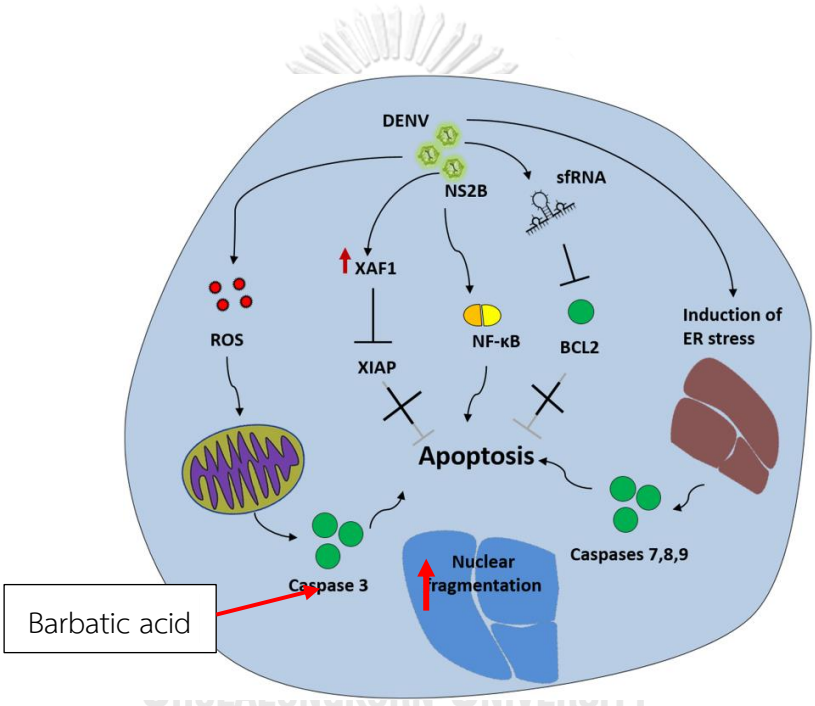


Figure 4 *Cellular apoptotic pathway induced by DENV infection (retrieved from Castillo et al. (61)) and the reported activity of barbatic acid in caspase-3 activation shown in red arrows*

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The development of antiviral agents by targeting apoptosis or caspase has been studied, as shown in **Table 5.** A study in chronic hepatitis C patients found that 50% of patients did not respond to medication. Caspase level in the patient serum who responded to the drug was higher than those who did not. Caspase activity was also related to the HCV viral load (63), indicating that caspase activity was necessary to eliminate the virus.

Table 5 *Some antiviral agents targeting the apoptotic pathway*

Agent	Virus	Mechanism of action	Reference
DRACO (dsRNA Activated Caspase Oligomerizer)	DENV, Influenza, and others	induce apoptosis of infected cells by detection of viral dsRNA	Rider TH et al. (2011)(64)
Aurintricarboxylic acid	Zika virus	inhibits viral production and protect against cell death caused by a viral infection	Park JG et al. (2019)(65)
zVAD.fmk, zIETD.fmk	Rhinovirus	inhibit viral 2A proteinase, caspase-8 inhibitor	DesZcZ L et al. (2004)(66)
Deptropine	Hepatitis E virus	induce NF-ΚB and caspase	Qu C et al. (2019)(67)

CHAPTER 3

METHODS

1. Cells

1.1 Vero cells

Vero cells (African green monkey kidney cells) (ATCC®CCL-81) were maintained in M199 medium (Gibco, Langley, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Massachusetts, USA), 100 I.U./mL penicillin (Bio Basic Canada, Ontario, Canada) and 100 µg/mL streptomycin (Bio Basic Canada, Ontario, Canada), 10mM HEPES (4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid) (Sigma Aldrich, St. Louis, USA) at 37°C humidified air with 5% CO_2.

1.2 LLC/MK2 cells

LLC/MK2 cells (Rhesus monkey kidney cells) (ATCC®CCL-7) were maintained in minimal essential medium (MEM) (Gibco, Langley, USA) supplemented with 10% FBS, 100 I.U./mL penicillin and 100 µg/mL streptomycin and 10mM HEPES at 37°C humidified air with 5% CO_2.

1.3 C6/36 cells

C6/36 cells (Asian tiger mosquito larva cells) (ATCC®CRL-1660) were maintained in MEM supplemented with 10% FBS, 100 I.U./mL penicillin and 100 µg/mL streptomycin and 10mM HEPES at 28°C.

1.4 BHK-21 cells

BHK-21 cells (Baby hamster kidney cells) (ATCC®CCL-10) were maintained in MEM supplemented with 5% FBS, 100 I.U./mL penicillin, and 100 µg/mL streptomycin and 10mM HEPES at 37°C humidified air with 5% CO_2.

1.5 THP-1 cells

THP-1 (Human monocytic leukemia cells) (ATCC®TIB-202) were maintained in RPMI-1640 medium (Gibco, Langley, USA) supplemented with 10% FBS, 100 I.U./mL penicillin and 100 µg/mL streptomycin and 10mM HEPES at 37°C humidified air with 5% CO_2.

1.6 HepG2, Huh7, HEK-293, and RD cells

HepG2 (Human hepatocellular carcinoma cells) (ATCC®HB-8065), Huh7 (Huamn hepatoma cells) (JCRB0403), HEK-293 (Human embryonic kidney cells) (ATCC®CRL-1573), and RD (Human rhabdomyosarcoma cells) (ATCC®CRL-1573™) cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, Langley, USA) supplemented with 10% FBS, 100 I.U./mL penicillin and 100 µg/mL streptomycin and 10mM HEPES at 37°C humidified air with 5% CO_2.

Vero, LLC/MK2, and C6/36 were courtesy of Prof. Kiat Ruxrungtham, M.D., and Assist. Prof. Chutitorn Ketloy, Ph.D. THP-1 and HepG2 were courtesy of Prof. Tanapat Palaga, Ph.D. Huh7 was courtesy of Assoc. Prof. Justin Chu, Ph.D. HEK-293 was received from Assoc. Prof. Parvapan Bhattarakosol, Ph.D. RD was purchased from ATCC (ATCC, Maryland, USA).

2. Viruses

2.1 Virus stock

Reference strains of DENV1 (16007), DENV2 (New Guinea C, NGC), DENV3 (16562) and CHIKV (25) were courtesy of Prof. Padet Siriyasatien, M.D., Ph.D., and DENV4 (c0036) was received from Prof. Kiat Ruxrungtham, M.D., and Assist. Prof. Chutitron Ketloy, Ph.D. ZIKV (SV0127/14) was received from Armed Forces Research Institute of Medical Sciences (AFRIMS), and Department of Disease Control, Ministry of Public Health, Thailand. EV-A71 (BRCA) was received from the Department of Medical Sciences, Ministry of Public Health, Thailand.

2.2 Viral propagation

DENV, ZIKV, and CHIKV were propagated in C6/36 cells. The monolayer cells in T-25 culture flask were infected with a stock virus at room temperature with gentle rocking for 1 hour. The maintenance medium was added and incubated at 28°C for three days. The supernatant was collected and repeated infect to monolayer C6/36 cells for the next two passages to yield a high viral titer. Supernatants were centrifuged to removed cell debris at 1500 rpm, 4°C for 5 minutes and FBS was added to the final 20-25% of total volume. The virus was stored in aliquoted at -70°C. The viral titer was quantified by plaque titration assay in 24-well plate.

EV-A71 was propagated in RD cells. The monolayer cells in T-25 culture flask were infected with a stock virus at 37°C with gentle rocking every 15 minutes for 1 hour. The maintenance medium was added and incubated at 37°C until a cytopathic effect was observed. The culture flask was frozen at -80°C for 30 minutes then thawed at 37°C in a water bath. The freezing and thawing steps were repeated for 3 times. The supernatant was collected and centrifuged to remove cell debris at 2000 rpm, 4°C for 10 minutes and FBS was added to 20-25% of the total volume to preserve the titer. The virus was aliquotted and stored at -70°C. The viral titer was quantified by plaque titration assay in 24-well plate using RD cells.

3. Compound collection

The powdered depsides and depsidones were kindly provided from Assist. Prof. Warinthorn Chavasiri, Ph.D., from the Department of Chemistry, Faculty of Science, Chulalongkorn University. VK-0014 – VK-0020 and VK-0025 were extracted from lichen *Usnea baileyi* (21). VK-0021 – VK-0023 were extracted from lichen *Parmotrema dilatatum* (22). VK-0024 was extracted from lichen *Parmotrema tsavoense* (20).

TT-029 – TT-034 were extracted from lichen *Usnea aciculifera*. Briefly, air-dried ground lichen was maceration with hexane, dichloromethane, ethyl acetate, acetone, and methanol, respectively. The solution of each solvent was filtered and evaporated under reduced pressure to yield an extract. The dichloromethane extract was separated using silica gel column and silica gel chromatography to give fractions of isolated compounds.

All compounds were stored as a solid at room temperature. The stock solution of compounds was prepared by diluted in DMSO (PanReac AppliChem, Hesse, Germany) to a final concentration of 50 mM and stored in aliquoted at -20°C until use.

4. Primary screening for DENV inhibition

The efficacy of DENV inhibition was primarily studied in Vero. Vero cells were seeded for $5x10^4$ cells per well in a 24-well plate and incubate at 37°C under 5% CO_2 overnight. Cells were infected with DENV2 NGC at a multiplicity of infection (M.O.I.) of 0.1, and compounds were added at a final concentration of 10 μM then incubate for 1 hour with gentle rocking every 15

minutes. Supernatants were removed, maintenance medium, and compounds were added then incubated at 37°C under 5% CO_2 for 3 days. Supernatants were used for estimating viral titers by plaque titration assay. Compounds that show over 90% inhibition were used for further investigation.

5. Plaque titration assay

DENV, CHIKV, and EV-A71 titers were quantified by 96-well plaque titration assay (68). Briefly, culture supernatants containing virus were 10-fold serially diluted in maintenance medium at 50 µL in 96-well plate. For DENV and CHIKV, LLC/MK2 cells were added for $1x10^4$ cells per well. For EV-A71, RD cells were seeded for $2.5x10^4$ cells per well. The viral-cell mixture was incubated at 37°C under 5% CO_2 for 3 hours. Overlay medium was added and culture was maintained for five to ten days until plaque became visually observed under microscopy for DENV and CHIKV and two days for EV-A71. Cells were stained and fixed using 1% crystal violet and 10% formaldehyde for one hour then wash out. Viral titers were calculated in a plaque-forming unit (PFU) with formula.

Viral titers (PFU/mL) = No. of plaques x dilution factor x 20

DENV2 16681 and ZIKV titer were quantified in 24-well plate. LLC/MK2 cells were seeded for $5x10^4$ cells per well in 24-well plate and incubate at 37°C under 5% CO_2 overnight. the medium was removed, virus was 10-fold serially diluted in maintenance medium then added to cells for 100 µL and incubated for 1 hour with gentle rocking every 15 minutes. Supernatants were removed and an overlay medium was added. Cultures were maintained for seven to ten days until plaque became visually observed under microscopy. Cells were stained and fixed using 1% crystal violet and 10% formaldehyde for one hour then wash out. Viral titers were calculated in plaque-forming unit (PFU) with formula.

Viral titers (PFU/mL) = No. of plaques x dilution factor x 10

6. Efficacy study

The efficacies of represented compound(s) against DENV2 were evaluated. Compounds were diluted into 8-10 concentrations and added to DENV2 NGC-infected Vero cells (M.O.I. of 0.1), 1% DMSO was used as a mock treatment. Cultures were incubated for 1 hour with gentle rocking every 15 minutes. Supernatants were removed, maintenance medium and compounds were added then incubated at 37°C under 5% CO_2 for 3 days. Supernatants were used for estimating viral titers by plaque assay. The efficacies were calculated in efficacy concentrations (EC_{50}) from nonlinear regression analysis.

7. Cytotoxicity study

The toxicity of representing compound to cells was measured using MTS assay. Vero cells were seeded for $1x10^4$ cells per well in 96-well plate overnight. Compounds were diluted into 8-10 concentrations and DMSO at 1% was used as a mock control. Cultures were maintained for 2 days. Cytotoxicity was measured using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega, Wisconsin, USA) according to the manufacturer's instruction and analyzed by spectrophotometry at A_{450nm}. The toxicity was calculated in cytotoxic concentration (CC_{50}) from nonlinear regression analysis.

8. Examine broad-spectrum activities of compound

The represent compound(s) were tested for efficacies (EC_{50}) in all serotypes of DENV, ZIKV, CHIKV, and EV-A71 as previously described.

An efficacy study in EV-A71 was performed using RD cells. Briefly, RD cells were seeded for $1x10^5$ cells per well in 24-well plate and incubate at 37°C under 5% CO_2 overnight. Cells were infected with EV-A71 at a multiplicity of infection (M.O.I.) of 0.1. Compound were added in various concentrations and 1% DMSO was used as a mock treatment incubate for 1 hour with gentle rocking every 15 min. Supernatants were removed, maintenance medium and compound were added then incubated at 37°C under 5% CO_2 for 1 day. Supernatants were used for estimating viral titers by plaque titration assay in 96-well plate as previously described using RD cells at $3 x10^4$ cells per well. Plates were incubated for 2 days then stained and fixed using 1% crystal violet and 10% formaldehyde. Viral titers were calculated in a plaque-forming unit (PFU) with formula above.

9. RNA extraction and Quantitative RT-PCR (RT-qPCR)

Viral RNA in infected cells was extracted by NucleoZOL™ reagent (MACHEREY-NAGEL, Dueren, Germany) and Direct-zol™ RNA MiniPrep Kit (Zymo Research, California, USA) according to the manufacturer's instruction. Total RNA was quantified by Nanodrop (Eppendorf Bio Photometer D30, New York, USA) and stored at -70°C until use. The viral genome in the experiment was determined by quantitative RT-PCR. The RT-qPCR was performed using *Power* SYBR® Green RNA-to-CT™ *1-Step* kit (Applied Biosystems™, California, USA) with a Step-OnePlus Real-Time PCR System ABI 7500 (Applied Biosystems™, California, USA).

10. Examine the target of the compound

10.1 Time-of-addition assay

The possible targets of the compound were preliminarily investigated by time-of-addition (TOA) assay. Briefly, Vero cells were seeded for $5x10^4$ cells per well in 24-well plate and incubate at 37°C under 5% CO_2 overnight. Cells were infected with DENV2 NGC (M.O.I. of 0.1) and compounds were added at a final concentration of 10 μM at different time points (0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60 and 72 hours post-infection). Supernatants were collected and estimate viral titers by plaque titration assay. The impairment in the viral stage was confirmed with the functional assay.

11. Functional inhibition assay

11.1 Viral entry inhibition assay

The anti-attachment assay was used to verify the activity of the compound at the entry step. DENV2 NGC (M.O.I. of 1) was inoculated to Vero cells or previously incubated with compounds before added to cells for 1 hour then incubated cells and viruses for 1 hour at 4°C. Supernatants were removed and replaced with a maintenance medium contained the compound for 2 days. Supernatants were collected and determined viral titers by plaque titration assay.

11.2 Viral fusion inhibition assay

To verify the activity of the compound in impairment the fusion and post-assembly step (53, 69). C6/36 cells were seeded for $2x10^5$ cells in 24-well plate and incubated at 28°C for one day (53). Cells were infected with DENV2 NGC (M.O.I. of 1) along with the compounds TT-031.

DMSO at 1% and 4G2 antibody (ATCC, Virginia, USA) were used as no inhibition and positive inhibition controls, respectively. Cultures were incubated for 2 days. Acidic condition (pH 5-6) was induced by adding 0.5 M MES (2-(N-Morpholino) ethanesulfonic acid) (Bio Basic Canada, Ontario, Canada) and further incubated for 1-2 days until fused cells were observed under the microscope. Cell fusion was observed under Nikon ECLIPSE TS100 Inverted Routine Microscope (Nikon, New York, USA).

11.3 Replicon inhibition assay

BHK-21 cells were seeded at $5x10^4$ cells per well in a 24-well plate and incubate at 37°C under 5% CO_2 overnight. A subgenomic DENV2 16681 replicon expressing GFP was transfected into the cells using jetprime® according to the manufacturer's protocol (Polypus transfection®). The compound TT-031 or DMSO control was added to the transfected cells at 6 h after transfection. Cells were incubated for 48 h before visualization under fluorescence microscopy. Cells were lysed and the RNAs were extracted using RNeasy mini kit. The viral and actin RNAs were quantified using RT-qPCR as previously described (70).

12. Generation of compound resisted mutation

Huh7 cells were infected with DENV2 16681 (M.O.I. of 1) for 1 h at 37°C under 5% CO_2. After infection, cells were washed with PBS. The compound at designated concentrations or 1% DMSO was added to the virus-infected cells and maintained for 2-3 days or until the 50% cytopathic effect was observed. Supernatants were collected for subsequent infection in Huh7 cells and analyzed by plaque titration assay. The process was repeated for 10-15 rounds. The viral samples from drug-treated and DMSO-treated groups were collected for RNA extraction before proceeding to NGS sample preparation. The RNA library was prepared for a next generation sequencing (NGS) using SeqCap EZ HyperCap (Roche, California, USA) and NEBNext® Magnesium RNA Fragmentation Module (New England Biolabs, Massachusetts, USA) according to the manufacturer's instruction. The sequences were sent for analysis (Genewiz, Guangzhou, China).

13. Caspase-3 activity test

Caspase activation was determined at the transcription level and activity level. For Caspase-3 activity, Vero cells were seeded at $3x10^5$ cells per well in a 6-well plate. HepG2 and HEK-293 were seeded at $5x10^5$ cells per well and incubated at 37°C under 5% CO_2 overnight. Cells were washed and maintenance media were added. The compound TT-032, a known inhibitor (cardanol mixture), and 1% DMSO were added to the cells and incubated for 48-72 hours. Cells were collected and caspase-3 activities were quantified using Caspase-3 Colorimetric Assay Kit (Biovision, California, USA). Briefly, cells were harvested and counted to $2x10^6$ cells and lysis using cell lysis buffer. Samples were centrifuged and collected supernatant then quantified protein concentration by Bradford assay (Bio-Rad, California, USA). The sample protein at 200 µg was mixed with 2X reaction buffer containing DTT. DEVD-*p*NA substrate was added and incubated at 37° for 2 hours. Samples were read by spectrometry at A_{405nm} in a microplate reader.

For transcription level, Vero cells were seeded at $5x10^4$ cells per well in a 24-well plate and incubated at 37°C under 5% CO_2 overnight. Cells were infected with DENV2 (M.O.I. of 0.1) for 1 hour at 37°C under 5% CO_2. The compound or DMSO at 1% was added to the infected cells and maintained for 72 hours. Cells were lysed and mRNAs were extracted and converted to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA). Caspase-3 mRNA levels were determined by qPCR compared to β-actin as internal controls. Primer pairs used for qPCR were used as followed; caspase-3 forward primer 5' TGCATACTCCACAGCACCTGGTTA 3', caspase-3 reverse primer 5' CATGGCACAA AGCGA CTGGATGAA 3', β-actin forward primer 5' GGCATCCTCACCCTGAAGTA 3' and β-actin reverse primer 5' GGGGTGTTGAAGGTCTCAAA 3'.

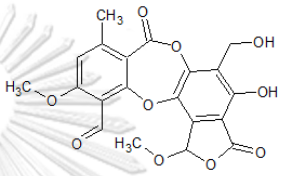
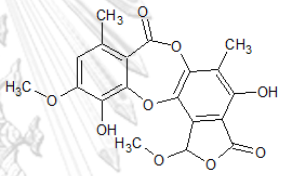
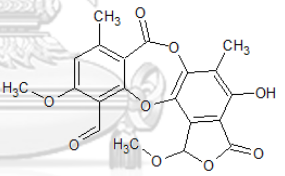
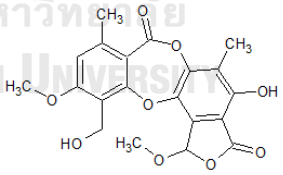
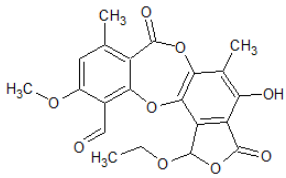
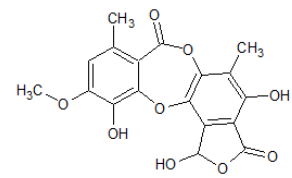
CHAPTER 4

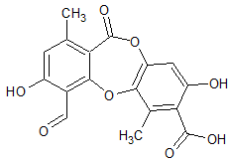
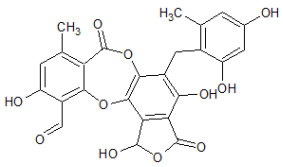
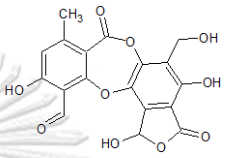
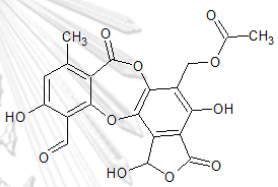
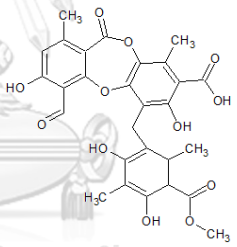
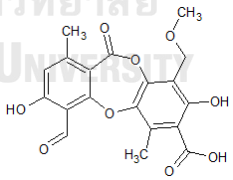
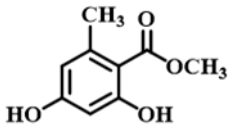
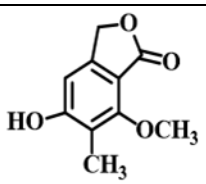
RESULTS

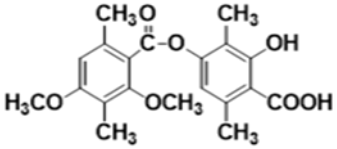
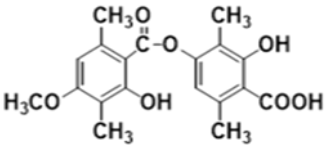
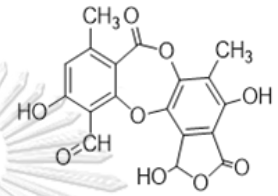
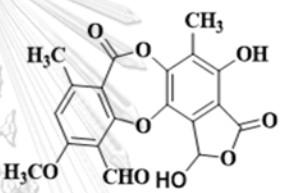
1. Compound purification results

The chemical structures of all compounds were elucidated by 1D, 2D NMR and HRESIMS data. The structures of compounds were shown in **Table 6**.

Table 6 *List of depsidones and depsides extracted from lichens*

No.	Code	Common Name	Structure	Formula	MW
1	VK-0014	8′-O-methylconstictic acid		$C_{20}H_{16}O_{10}$	416.07
2	VK-0015	8′-O-methylmenegazziaic acid		$C_{19}H_{16}O_9$	388.08
3	VK-0016	Methylstictic acid		$C_{20}H_{16}O_9$	400.08
4	VK-0017	8′-O-methylcryptostictic acid		$C_{20}H_{18}O_9$	402.0951
5	VK-0018	8′-O-ethylstictic acid		$C_{21}H_{18}O_9$	414.0951
6	VK-0019	Menegazziaic acid		$C_{18}H_{14}O_9$	374.06

7	VK-0020	Subvirensic acid		$C_{17}H_{12}O_8$	344.275
8	VK-0021	Parmosidone F		$C_{25}H_{18}O_{11}$	494.0849
9	VK-0022	Salazinic acid		$C_{18}H_{12}O_{10}$	388.043
10	VK-0023	Galbinic Acid		$C_{20}H_{14}O_{11}$	430.0536
11	VK-0024	Parmosidone C		$C_{28}H_{26}O_{12}$	554.504
12	VK-0025	9′-O-methylprotocetraric acid		$C_{19}H_{16}O_9$	388.08
13	TT-029	Methyl orselinate		$C_9H_{10}O_4$	182
14	TT-030	7-hydroxy-5-methoxy-6-methylphthalide		$C_{10}H_{10}O_4$	194

15	TT-031	Diffractaic acid		$C_{20}H_{22}O_7$	374
16	TT-032	Barbatic acid		$C_{19}H_{20}O_7$	360
17	TT-033	Norstictic acid		$C_{18}H_{12}O_9$	372
18	TT-034	Stictic acid		$C_{19}H_{14}O_9$	386

2. Initial screening of compounds against DENV2

A previous study suggested that a depside, atranorin, extracted from lichen *Stereocaulon evolutum* inhibited the hepatitis C virus at the entry stage (14). Therefore, other depsides and a chemically related core such as depsidones, could also inhibit DENV2. We initially screen depsides and depsidones against DENV2 NGC in Vero cells with a compound concentration of 10 μM. The DENV2 NGC at the M.O.I. of 0.1 and compounds were added to Vero cells and incubated for 72 hours. Supernatants were analyzed by plaque titration assay as a percentage of a no inhibition control or the DMSO-treated cells. Our data suggested that one depsidone (VK-0024) and two depsides (TT-031 and TT-032) at a concentration of 10 μM inhibited 96.48±2.90, 99.98±0.04, and 99.99±0.02% of DENV2 titer. The viability of Vero cells was counterscreened by the addition of compound at the same concentration to the cells and incubated for 48 hours before quantification by MTS assay. None of the compounds show any cytotoxic effects to the cells (**Table 7**). The three compounds (VK-0024, TT-031, and TT-032) with the highest

selectivity index were selected for further efficacy (EC_{50}) and cytotoxicity (CC_{50}) analysis (**Fig. 5**).

***Table** 7 Primary screening of depsides and depsidones against DENV2 NGC and cytotoxicity in Vero cells.*

compound	Primary screening at 10 μM (in Vero)	
	% DENV2 inhibition (plaque)	% Cell viability
VK-0014	Not inhibited	112.63±17.52
VK-0015	Not inhibited	122.40±4.58
VK-0016	Not inhibited	118.00±4.69
VK-0017	Not inhibited	108.59±5.75
VK-0018	Not inhibited	97.88±5.73
VK-0019	Not inhibited	92.00±11.41
VK-0020	Not inhibited	114.15±15.82
VK-0021	Not inhibited	120.97±12.71
VK-0022	Not inhibited	120.10±6.87
VK-0023	80.50±4.85	112.03±3.55
VK-0024	96.48±2.90	123.58±17.10
VK-0025	Not inhibited	102.53±3.32
TT-029	Not inhibited	106.62±8.0
TT-030	Not inhibited	119.68±11.66
TT-031	99.98±0.04	114.72±5.88
TT-032	99.99±0.02	113.31±7.29
TT-033	78.00±7.55	111.18±5.63
TT-034	Not inhibited	108.02±4.34

Data represent the mean ± standard deviation of a triplicate result of a single experiment.

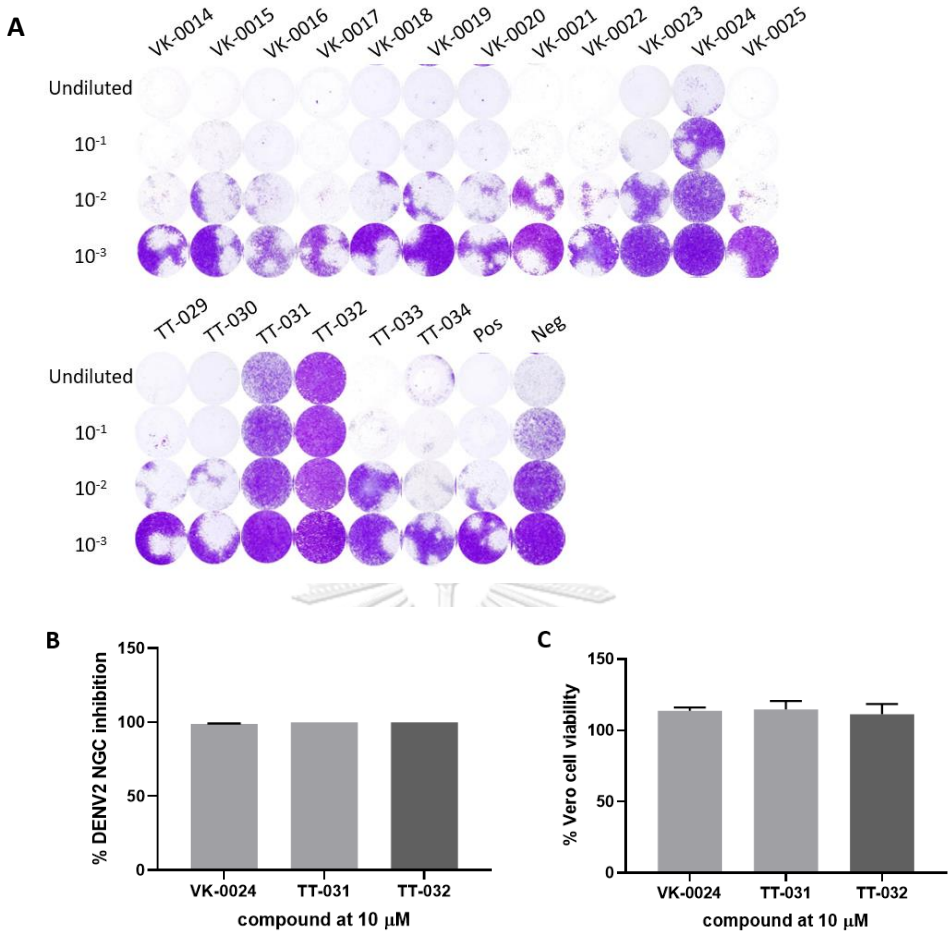


Figure 5 *Primary screening results of compounds against DENV2 (NGC) infected Vero cells.* **A)** Plaque formation of compounds in primary screening against DENV2 inhibition. **B)** Three compounds (VK-0024, TT-031 and TT-032) exhibited over ninety percent inhibition at concentration of 10 µM against DENV2 (M.O.I. of 0.1) in Vero cells. **C)** Compounds showed non-toxicity to cells at the same concentration. Cytotoxicity was measured by MTS assay. Data represent the mean ± standard deviation of a triplicate result of a single experiment.

3. Efficacy and cytotoxicity of selected compounds

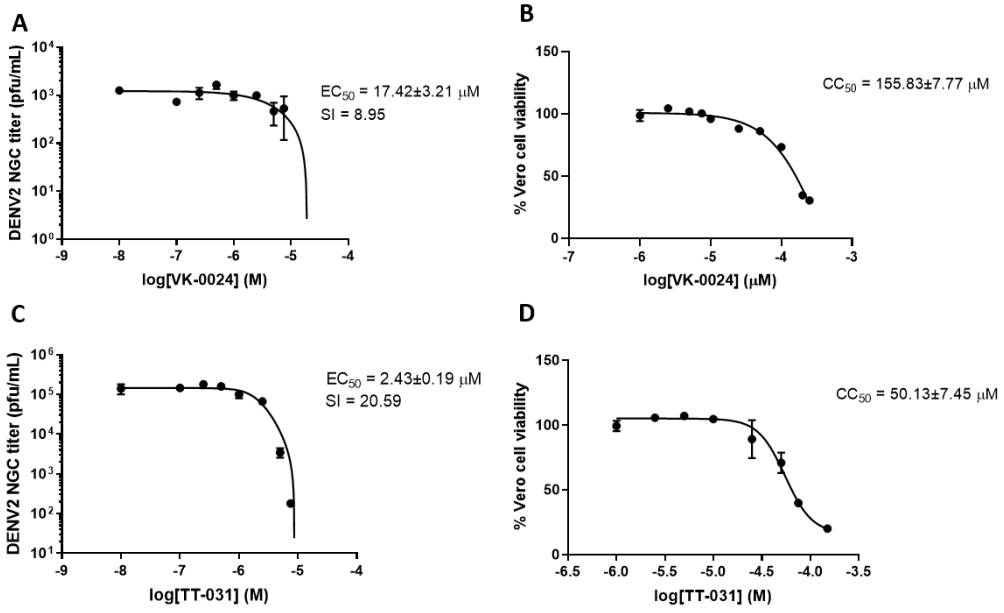
Three selected compounds (VK-0024, TT-031, TT-032) were tested for the efficacy (EC_{50}) against DENV2 NGC. Compounds at various concentrations were added to DENV2-infected Vero cells (M.O.I. of 0.1) and supernatants were collected for viral inhibition by plaque titration assay as previously described. TT-032 showed the most effective EC_{50} value, followed by TT-031, and VK-024 at 0.91±0.26, 2.43±0.19, 17.42±3.21µM, respectively (**Fig. 6A, C and E**). In contrast, the

compound with the highest cytoprotective effect was VK-0024, followed by TT-031, and TT-032 with the cytotoxicity value (CC_{50}) in Vero cells at 155.83±7.77, 50.13±7.45, 12.10±038 μM, respectively (**Fig. 6B, D and F**). Selectivity index was calculated from the ratio of CC_{50} and EC_{50}. Out of the three compounds, TT-031 showed the highest selectivity index of 20.59. Therefore, TT-031 was selected as a representative compound of depsides and depsidones for further molecular target identification and inhibition against other viruses.

The efficacy of TT-031 was confirmed using human hepatoma cell line infected with DENV2 16681. Results showed similar efficacy at the EC_{50} of 3.89±0.07 μM, while the Huh7 cells was less toxic than Vero cells (CC_{50} > 100 μM) (Data courtesy of Assist. Prof. Siwaporn Boonyasuppayakorn, M.D., Ph.D., performed at National University of Singapore) (**Fig. 6G and Fig. 7E**).

Table 8 *efficacy and cytotoxicity of selected compounds in Vero cells*

compound	EC_{50} (μM)	CC_{50} (μM)	SI
VK-0024	17.42±3.21	155.83±7.77	8.95
TT-031	2.43±0.19	50.13±7.45	20.59
TT-032	0.91±0.15	12.10±0.38	13.33



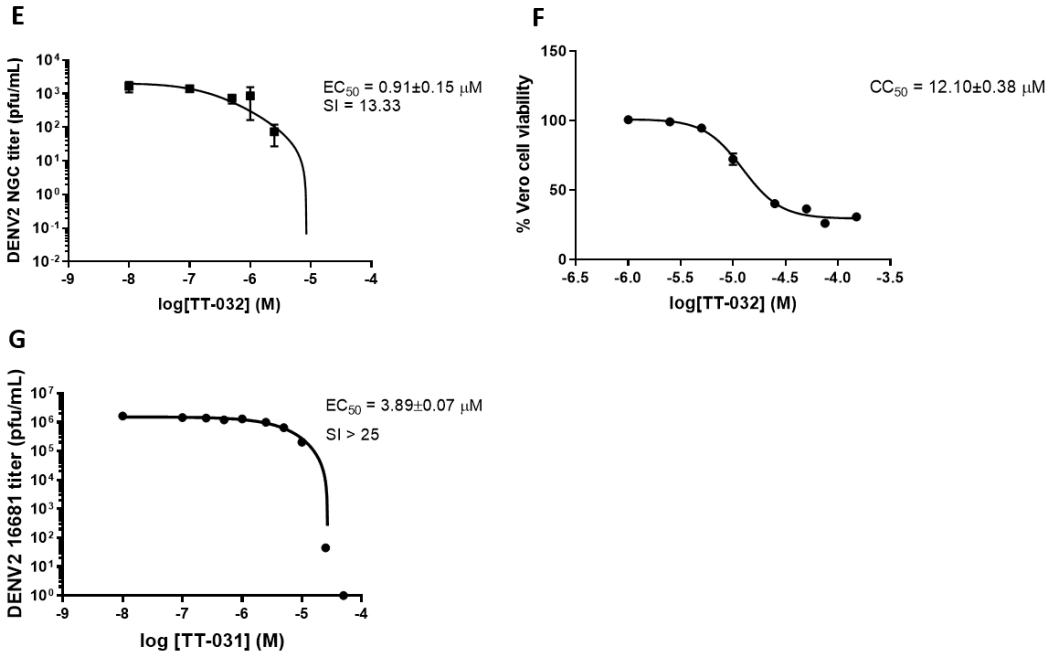


Figure 6 *Dose-response curves of VK-0024, TT-031 and TT-032 efficacies against DENV2 NGC and cytotoxicity of compounds in Vero cells.*

TT-031 efficacy against DENV2 16681 was performed in Huh7 cells. Each graph represents one of three independent experiments.

4. Broad-spectrum activity of TT-031

The representative compound, TT-031, was tested for cytotoxicity in other human-derived cell lines (THP-1, HepG2, RD, HEK-293, and Huh7). The cytotoxicity varied among the cells but the toxicity was most and least obvious in HepG2 and Huh7 cells, respectively (**Table 9** and **Fig.7**).

Table 9 *Cytotoxicity of TT-031 in various cell lines*

Cell lines	**CC_{50} (μM)**
HepG2	39.32±6.11
THP-1	49.60±1.06
HEK-293	70.44±0.33
RD	64.34±5.04
Huh7[a,b]	>100

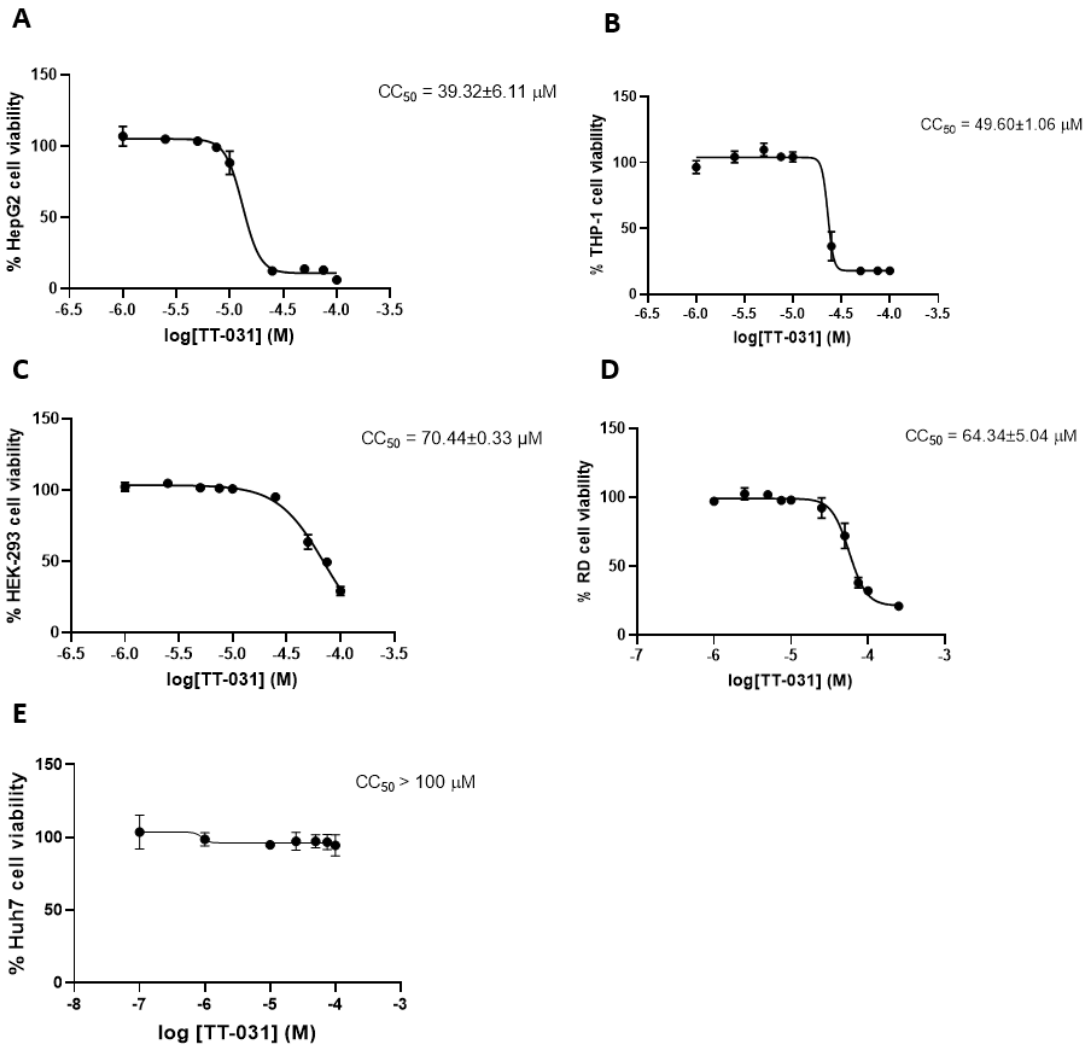


***Figure** 7 Dose-response curves of TT-031 cytotoxicity in various cells.*

Each graph represents one of three independent experiments. Data represent means ± stand error of the means of three independent experiments. a = cytotoxicity in Huh7 cells was performed by alarmaBlue cytotoxicity assay (71). b = Data courtesy of Assist. Prof. Siwaporn Boonyasuppayakorn, M.D., Ph.D.

The broad-spectrum efficacy of TT-031 was examined against four serotypes of DENV (DENV1-4) (M.O.I. of 0.1), ZIKV (M.O.I. of 0.01), CHIKV (M.O.I. of 0.1) and EV-A71 inhibition (M.O.I. of 0.1). Results showed that TT-031 was similarly effective in DENV1-4, ZIKV, and CHIKV and less effectively inhibited EV-A71 (**Table 10 and Fig. 8**). From these results, the activity of TT-031 seems to be conserved in flaviviruses.

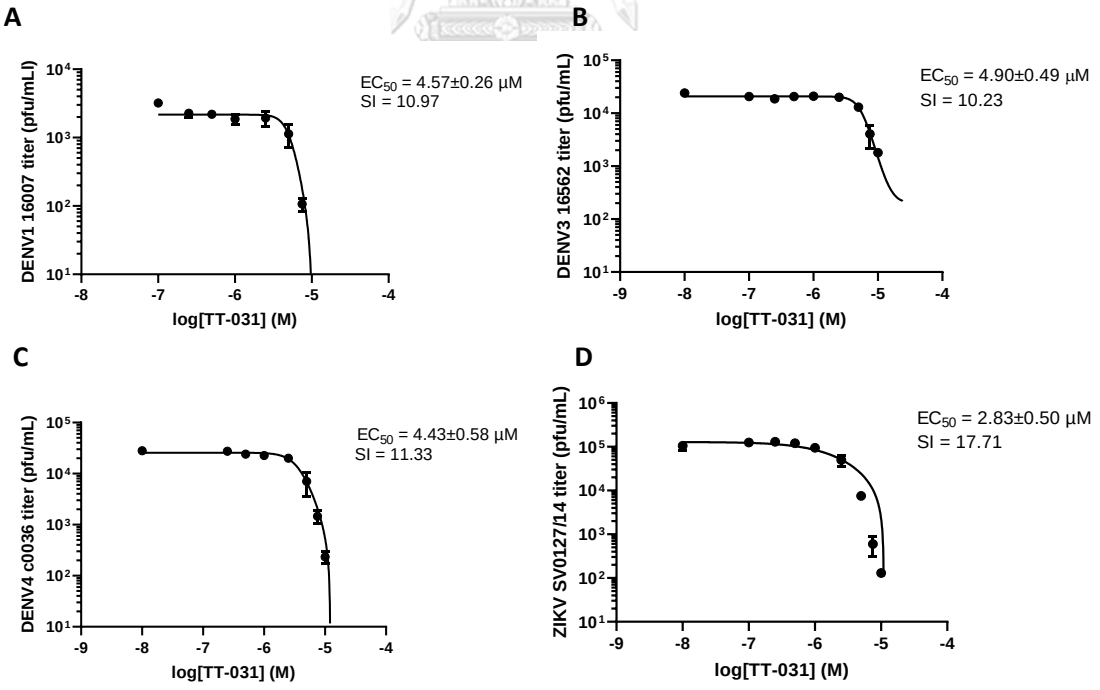
Table 10 *Efficacy of TT-031 against four serotypes of DENV and other viruses*

Virus	EC_{50} (μM)	SI
DENV1 (16007)	4.57±0.26	10.97
DENV2 (NGC)	2.43±0.15	20.59
DENV2 (16681)[a]	3.89±0.07	>25
DENV3 (16562)	4.90±0.49	10.23
DENV4 (c0036)	4.43±0.58	11.33
ZIKV (SV0127/14)	2.83±0.50	17.71
CHIKV (25)	6.21±0.69	8.07
EV-A71 (BRCA)**	19.48±2.86	3.30

*Data represented means and standard error of the means of three independent experiments.

** Data represented means and standard error of the means of two independent experiments.

a = Data courtesy of Assist. Prof. Siwaporn Boonyasuppayakorn, M.D., Ph.D.



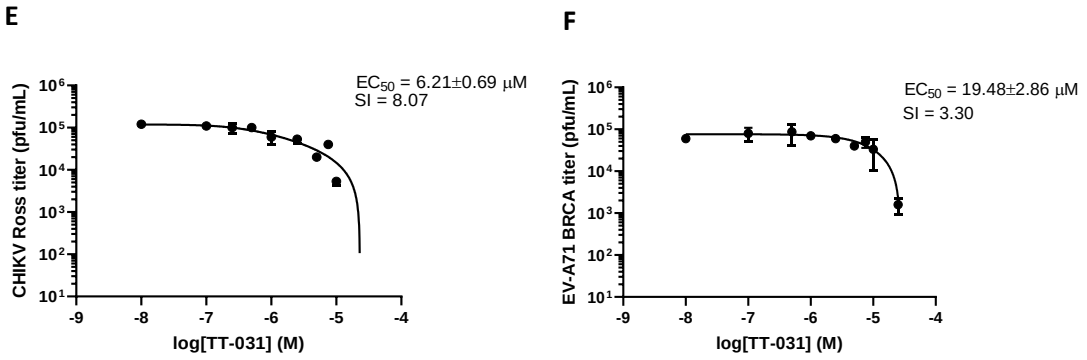


Figure 8 *Dose-response curves of TT-031 efficacy against DENV1-4, ZIKV, CHIKV and EV-A71.*

Data represent mean ± stand error of means of three independent experiments. Each graph represents one of three independent experiments.

5. Mechanism of action study

5.1 Time-of-addition and removal assays

The possible target of TT-031 was initial screening using time-of-addition (TOA) assay. The objective is to determine whether the target resides in early or late time points. Vero cells were infected with DENV2 NGC (M.O.I. of 0.1) and the compound was added to infected cells at various times (0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, and 72 hours post-infection). DMSO was used as a no inhibition control. Culture supernatants were collected and determined viral inhibition by plaque titration assay. The result showed that DENV titer decreased significantly 4-log from 2 to 10 hours post-infection (hpi), reduced to 3-log from 12 to 60 hpi, and 2-log at 72 hpi (**Fig. 9**). This result indicated that TT-031 inhibits viral production at post entry, preferentially at the early stage of DENV infection such as fusion or translation stage.

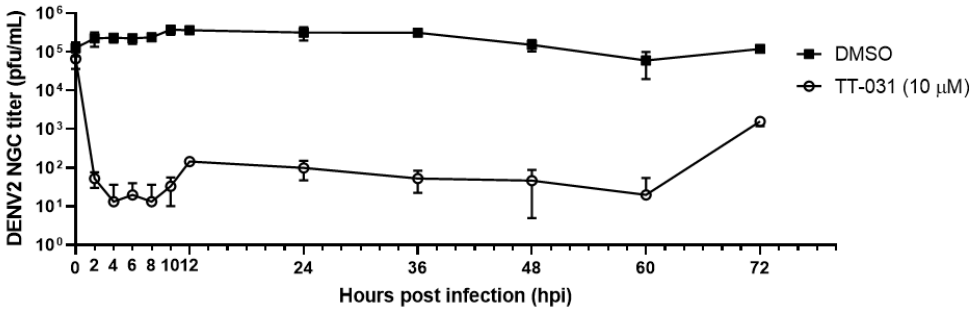


Figure 9 *Mechanism of action of TT-031 using time-of-addition assay.*

The compound was added to DENV2-infected Vero cells at various times post infection and measured DENV titer by plaque titration assay. The DMSO-treated sample was used as no inhibition control. Graph represents one of three independent experiments.

5.2 Anti-attachment assay

The anti-attachment assay was used to confirm that the compound inhibits DENV infection after the entry step. The compound was incubated with DENV2 NGC at 4°C before, during, or after infecting cells (M.O.I. of 1) and incubated for 48 hours. DMSO was used as no inhibition control. Culture supernatants were collected to determined viral inhibition by plaque titration assay. The result showed that TT-031 did not interfere with the neutralization of DENV2 at pre- or co-incubation but inhibited 99.94±0.08% DENV2 virion progeny at post attachment step (**Fig. 10**). Data represent the triplicate results from one of three independent experiments.

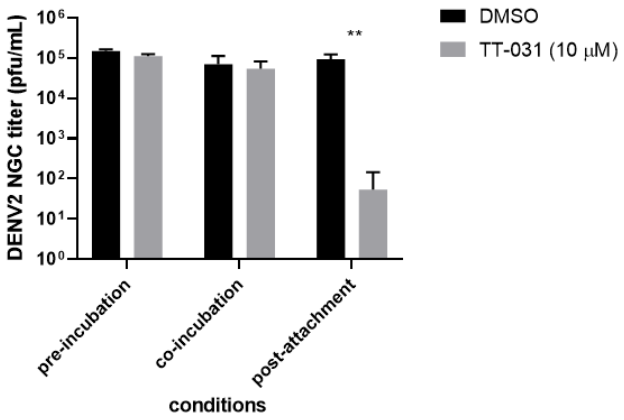


Figure 10 *Inhibition of DENV2 NGC-infected Vero cells treated with TT-031 at pre-, co-, or post-infection condition.*

The graph represents one of three independent experiments. ** *P*-value < 0.01

5.3 Fusion inhibition assay

Next, fusion inhibition assay was tested for inhibition of the conformational change of DENV envelope protein and initiate phospholipid membrane fusion Syncytial formation under acidic condition (pH 5-6) (72). Briefly, TT-031 (10 μM) was added to DENV2 NGC infected C6/36 cells (M.O.I. of 1) and incubated for 2 days. DMSO and 4G2 preincubated with DENV2 before the

C6/36 infection were used as no inhibition and positive inhibition control. Cell fusion or syncytial formation was induced by adding 0.5 M MES to induced acidic condition (pH5-6) and incubated for 24-48 hours. If the compound inhibited the DENV envelope conformational change, syncytial formation would not be observed under a microscope. However, TT-031 did not prevent cell-to-cell fusion (**Fig. 11**), therefore, it was unlikely that a DENV envelope protein would be a molecular target of TT-031.

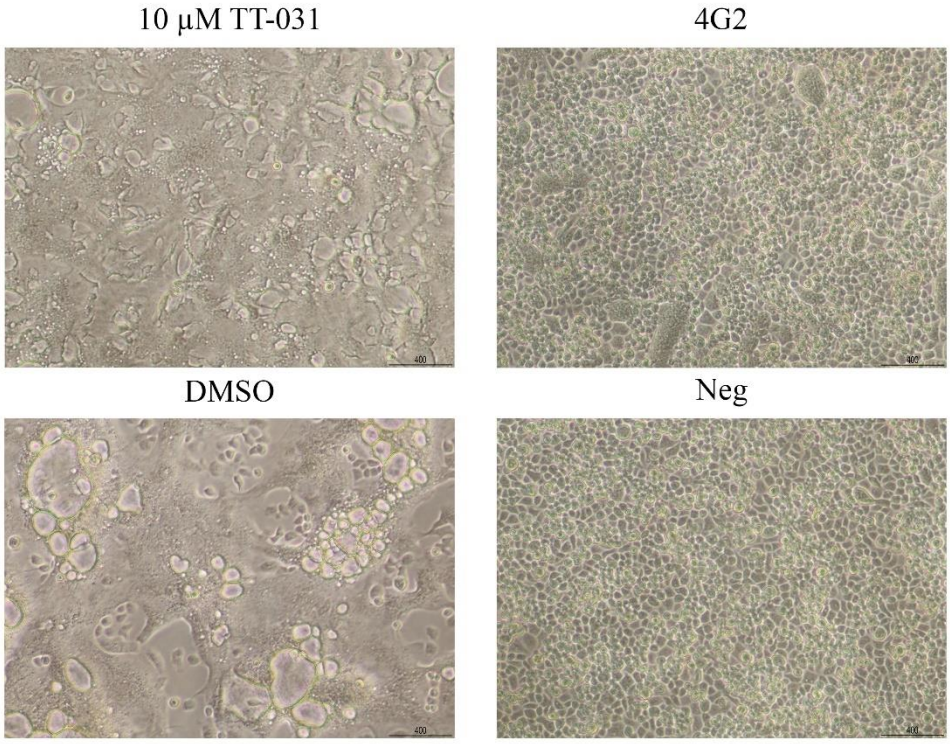


Figure 11 *Inhibition of DENV2-infected C6/36 cell fusion. 4G2 and DMSO were used as positive and no inhibition control.*

The pictures were taken by Nikon ECLIPSE TS100 at 100x magnification.

5.4 Replicon inhibition

DENV2 replicon expressing GFP reporter was transfected into BHK-21 cells and the compound TT-031 at 1, 10, 25, and 50 μM was added at 6 hours after transfection. Ribavirin was used as a positive inhibitor. Cells were incubated for 48-72 hours, and the replicon inhibition was determined by GFP visualization and RT-qPCR. Results showed that the DENV replicon was not

inhibited in the presence of the compound TT-031 (**Appendix A Fig. 14A**). Instead, the enhancement was observed in 1 and 25 μM samples (**Appendix A Fig. 14B**). Noted that the replicon was not inhibited by ribavirin which supposes to be a known inhibitor. Therefore, the assay optimization is still required for accurate interpretation (Data courtesy of Assoc. Prof. Siwaporn Boonyasuppayakorn, M.D., Ph.D., performed in Assoc. Prof. Justin Chu laboratory at the National University of Singapore).

5.5 Generation of mutation

DENV2 16681 was continuously subpassaged under the selective pressure of a sublethal dose of TT-031. The escape mutation would reveal the viral genome's interacting position to the compound, thus implying a possible target. In this experiment, TT-031 at the increasing doses (1, 2.5, 5, 25 μM) was added to several passages of DENV2 16681-infected Huh7 cells, and DMSO was used as control (**Appendix A Fig. 15A**). Supernatants at the 15th passage were extracted and prepared for whole-genome sequencing. The sequence was compared with the DMSO-treated virus to the 15th subpassage at the same condition. DENV2 16681 strain (Accession no.: KU725663.1) was used as a template for a multiple sequence alignment analysis by Si-Xian Ho of Assoc.Prof. Justin Chu laboratory (National University of Singapore). A single nucleotide substitution (G5178A) was discovered (**Appendix A Fig. 15B**). This position belongs to a C-terminal domain of NS3 between ATP-binding and Mg2+ binding sites of ATPase/RTPase viral enzyme. Molecular docking and simulation results from Kowit Hengprasatporn, Ph.D. showed that the prediction site belongs to the active site of helicase (Site 3, with the binding energy of -7.6 kcal/mol), not the mutation site (NS3 helicase domain I) (**Appendix A Fig. 16**). Therefore, the molecular docking suggested that DENV NS3 helicase was not targeted by TT-031.

5.6 Caspase-3 activity

Caspase activity was determined to verify the effect of the compound to control viral propagation by induced or inhibited apoptosis in DENV-infected cells. Vero cells were incubated with TT-032 (barbatic acid) (16, 73, 74), and DMSO was used as uninduced control. Caspase-3 activity was quantified using a colorimetric assay kit by detection of *p*NA light emission cleaved from DEVD-*p*NA substrate by caspase-3. The absorbances were weak or not different from even

blank compound previously reports to induced cell apoptosis via inducing of caspase-3 activity (**Table 11**).

Table 11 *Activity of caspase-3 by detection of pNA chromophore at 405 nm in compound-treated Vero cells.*

Cell type	Condition	A_{405}
-	Blank	0.048
Vero	DMSO	0.049
	TT-032 5 μM	0.055

Blank was used as background reading using lysis buffer instead of sample protein. DMSO-treated cells were used as uninduced control.

As no caspase-3 activity was found, cell type was changed to human cell lines, HepG2, and HEK-293 cells. The result was the same as the previous result, with no activity of caspase-3 was detected (**Table 12**). From these results, we assumed the negative results might cause by the sensitivity of reaction mixtures or substrate in the kit. The experiment was returned to check the expression of caspase-3 at the transcription level by RT-qPCR method.

Table 12 *Activity of caspase-3 by detection of pNA chromophore at 405 nm in compound-treated HepG2 and HEK-293cells.*

Cell type	Condition	A_{405}
-	Blank	0.047
HepG2	DMSO	0.061
	TT-032 5 μM	0.061
HEK-293	DMSO	0.058
	TT-032 5 μM	0.055

Blank was used as background reading using lysis buffer instead of sample protein. DMSO-treated cells were used as uninduced control.

Caspase-3 expression was examined at mRNA expression level by RT-qPCR. An experiment condition was the same as the efficacy study. Compound-treated DENV2 infected

cells condition did not change in mRNA level significantly compared to nontreated infected cells (**Fig. 12**). We expected that 72-hour incubation was too long for differentiated mRNA level detection. We changed the time point to 24 hours and increased M.O.I. to 1. Relative mRNA expression also not different in both TT-031 and TT-032-treated conditions (**Fig. 13**). The results concluded that TT-031 did not control DENV propagation via changing caspase-3 activity.

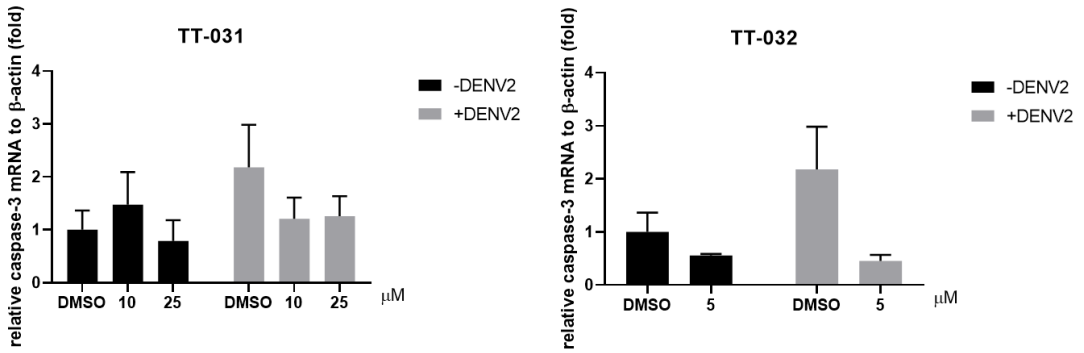


Figure 12 *Relative caspase-3 mRNA expression level in DENV2 infected Vero cells or mock infection at 72 hours (M.O.I of 0.1).*

Data represents mean ± standard deviation.

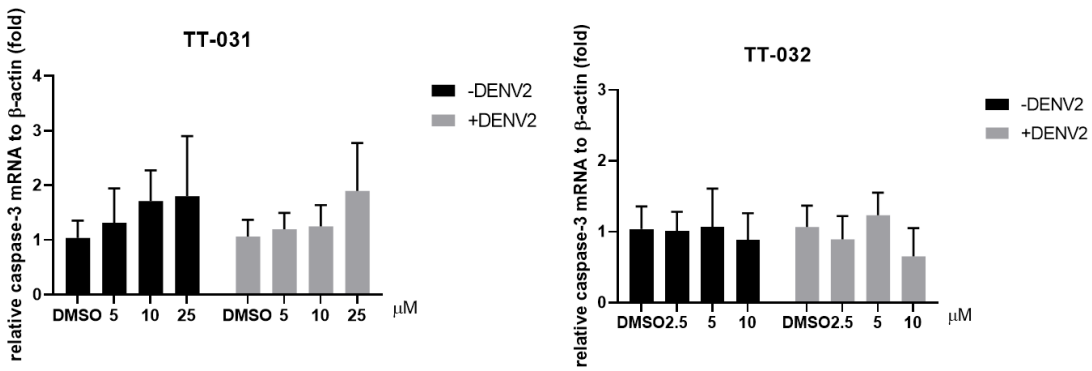


Figure 13 *Relative caspase-3 mRNA expression level in DENV2 infected Vero cells or mock infection at 24 hours (M.O.I of 1).*

Data represent the mean ± standard deviation.

CHAPTER 5

DISCUSSION

Depsidones and depsides are secondary metabolites from lichens. Previous reports revealed several activities such as anti-cancer, antibacterial, antioxidant, anti-parasitic, and antiviral activities (13-19, 55). We screened anti-dengue activity of depsidones and depsides extracted from lichens *Usnea baileyi, Usnea aciculifera and Parmotrema dilatatum* and founded three compounds with activity against DENV2 over 90 percent at 10 μM including VK-0024 (EC_{50} = 17.42±3.21 μM), TT-031 (EC_{50} = 2.43±0.19 μM) and TT-032 (EC_{50} = 0.91±0.15 μM). TT-031 and TT-032 had promising and similar EC_{50} value, this may be due to similar structures of these compounds

Out of the three compounds, VK-0024 was the least cytotoxic in Vero cells (CC_{50} = 155.83±7.77 μM) while being the least effective against DENV2 NGC. This depsidone compound was also non-toxic against MCF-7 (human breast cancer) cells with IC_{50} over 100 μM (20). Interestingly, VK-0024 is the only depsidone showing >90% DENV2 inhibition in primary screening. However, with the EC_{50} result, VK-024 was unlikely the promising lead for further investigation. Therefore, the depsidone core is unlikely as an active candidate for further analysis.

The two depsides, TT-032, and TT-031, contain one different moiety (-OH and $–OCH_3$), contributing to various properties such as solubility, toxicity, and DENV2 inhibition. TT-032 was toxic to many cancer cells but not toxic to PBMC (75). Our results showed that TT-032 was toxic to Vero cells (CC_{50} = 12.10±038 μM), corresponding to previous reports of TT-032 as pro-apoptosis (16). The structure could be modified to have less toxicity and improved solubility to provide better efficacy.

The selectivity index revealed TT-031 was the highest among the three compounds (SI = 20.59); therefore, it was further investigated for cytotoxicity, molecular target identification, and broad-spectrum antiviral activity. Our results found that TT-031 exhibited various cellular toxicities in THP-1, HepG2, Huh7, RD, and HEK-293 from 39.32 to >100 μM or 14,705.68 to >37,400 μg/l. Similarly, TT-031 was the least toxic at 70.44 μM in HEK-293 due to its anti-cancer activity (76, 77). TT-031 was challenged via an oral route into mice, and the lethal dose (LD_{50}) was 962 mg/kg

(78). The lethal dose was at least 65 times higher than those of the CC_{50}s. It is possible that the compound TT-031 was poorly absorbed or quickly metabolized and excreted.

The TT-031 was broadly inhibited DENV1-4 and ZIKV with similar EC_{50} values to DENV (EC_{50} ranges of 2.43-4.90 μM). The compound was slightly less effective against an enveloped alphavirus (CHIKV EC_{50} at 6.21±0.69 μM) and was attenuated with a non-enveloped enterovirus (EV-A71 EC_{50} at 19.48±2.86 μM). EV-A71 replication cycle differs from those enveloped +ssRNA viruses mainly in pH-independent fusion, IRES-dependent translation, initiation factors of viral replication, and packaging steps (79). Moreover, DENV, ZIKV, and CHIKV are mosquito-borne viruses; therefore, the target could be the viral or host components shared among arboviruses.

A time-course experiment (TOA) revealed that TT-031 inhibited DENV production early after the entry stage. The anti-attachment assay confirmed the inhibition of DENV2 after attachment. However, TT-031 did not inhibit C6/36 pH-induced membrane fusion implying that TT-031 did not interfere with the DENV envelope protein conformational change triggering the membrane fusion. Moreover, the replicon inhibition assay still required further optimization, such as cell seeding density or replicon concentration used in transfection; therefore, the results could not be used for interpretation.

We generated a compound-driven DENV2 16681 mutant by the repetitive passage of the virus under the selective pressure of the compound TT-031 at sublethal doses. The mutation site could imply the viral-compound interaction, and the virus escaped to become resistant to the compound TT-031. The silent mutation was found at the nucleotide G5178A located at ATPase/RTPase domain of NS3 C-terminus. To confirm the contribution of this TT-031-induced mutation towards the NS3 functionality, site-direct mutagenesis could be used as a tool. The mutated virus should be resistant to the compound TT-031. Another possible solution is to generate the alkyne-tagged compound to pull down the molecular target and further analysis using mass spectrophotometry.

As G5178A substitution was a silent mutation, the target molecules of TT-031 could possibly be the cellular proteins. Virtual screening of TT-031 in ZINC database (ZINC1687273)

(80) revealed the interaction of the compound with cellular protein such as FAD-linked sulfhydryl oxidase ALR, thiopurine S-methyltransferase, Potassium voltage-gated channel subfamily B member 1, Glucose-6-phosphate exchanger SLC37A4, and L-lactate dehydrogenase B chain. However, none of these proteins directly correlated with DENV protein, but the TT-031 activity could interfere with the cellular defense mechanism against viral infection.

Computational docking and drug-likeness analysis revealed TT-031 as one of the cyclooxygenase-2 (COX-2) inhibitor candidates (81). COX-2 overexpression was founded in many viral infections, including HCV, EV71, cytomegalovirus (CMV), hepatitis B virus (HBV), DENV, and ZIKV (82-86). COX-2 inhibitors or non-steroidal anti-inflammation drugs (NSAIDs) treatment 39attenuated viral inhibition (87-90). Moreover, TT-031 has been reported as a 5-lipoxygenase (5-LOX) inhibitor (91). 5-LOX expression was founded in DENV-infected human neutrophils (92). Dual inhibition of 5-LOX/COX-2 serves as anti-inflammation (93). Targeting COX-2 and/or 5-LOX may serve as DENV therapeutic agents by reducing inflammation as inflammation involved in DENV pathogenesis.

In conclusion, we reported for the first time that lichen depsides, diffractaic acid (TT-031), and barbatic acid (TT-032) had antiviral activity against DENV. The most effective compound, diffractaic acid, exhibited significant inhibition against all four serotypes of DENV and ZIKV. The molecular mechanism studies revealed the possible targets of host components involved in viral propagation and required further studies to find an exact target molecule. The compound modification also required reduced toxicity and improved the activity for the higher potency of the anti-dengue lead.

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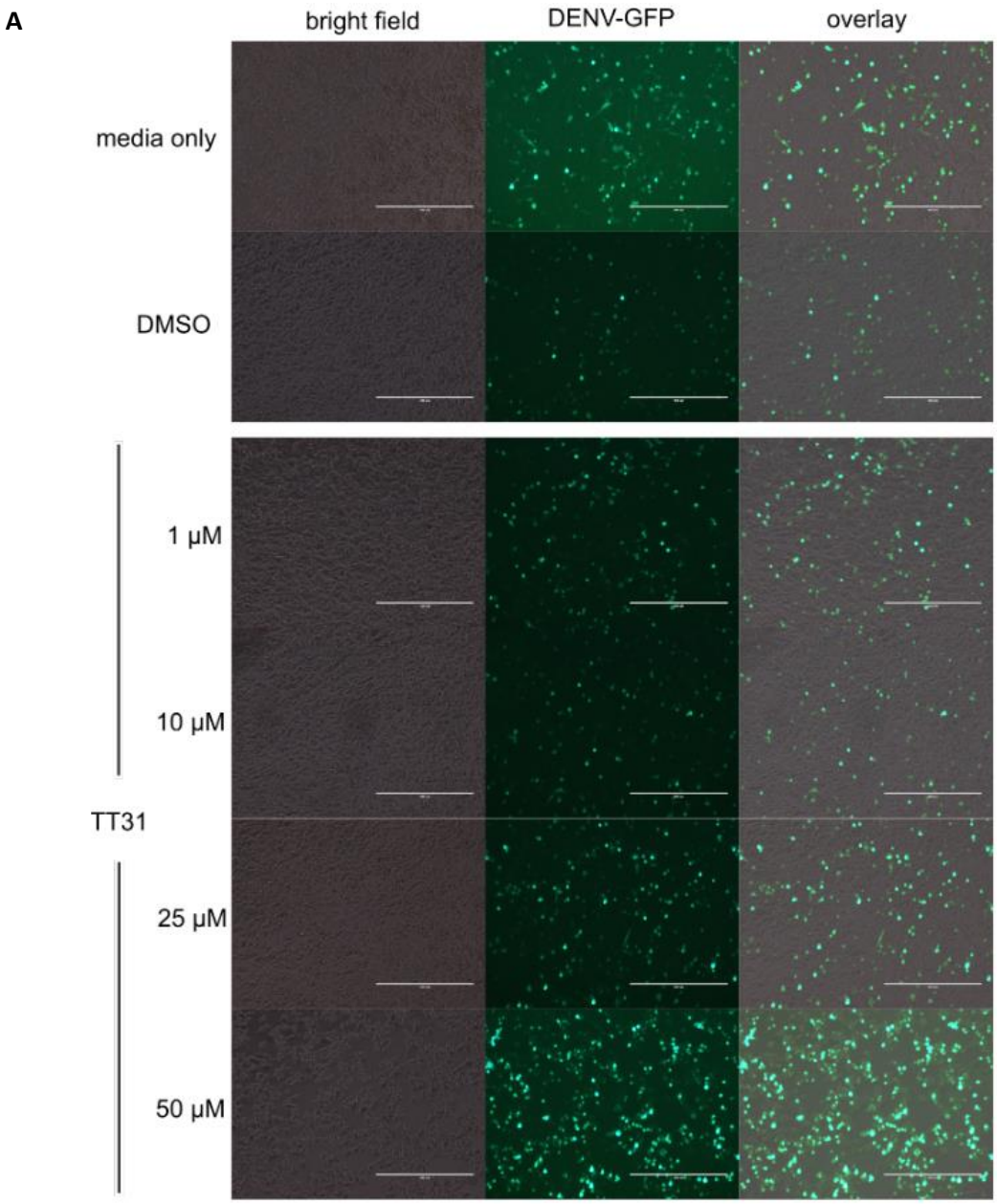
APPENDIX A

SUPPLEMENTARY FIGURES



B

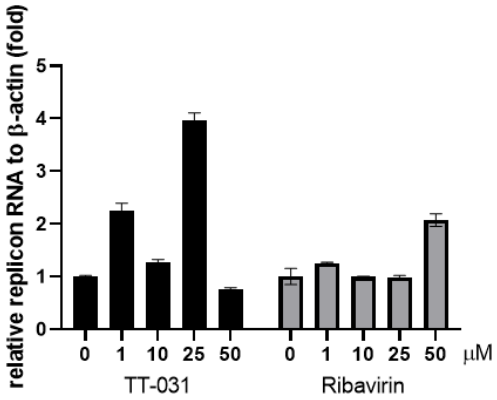


Figure 14 *DENV replicon inhibition of TT-031.*

A) TT-031 was treated in various concentrations and DMSO was used as no inhibition control. DENV replicon replication was observed by fluorescence microscope to detected GFP signal. **B**) Relative replicon RNA expression level in BHK-21/DENV2 replicon treated with TT-031 and ribavirin. Data courtesy of Assoc. Prof. Siwaporn Boonyasuppayakorn, M.D., Ph.D.

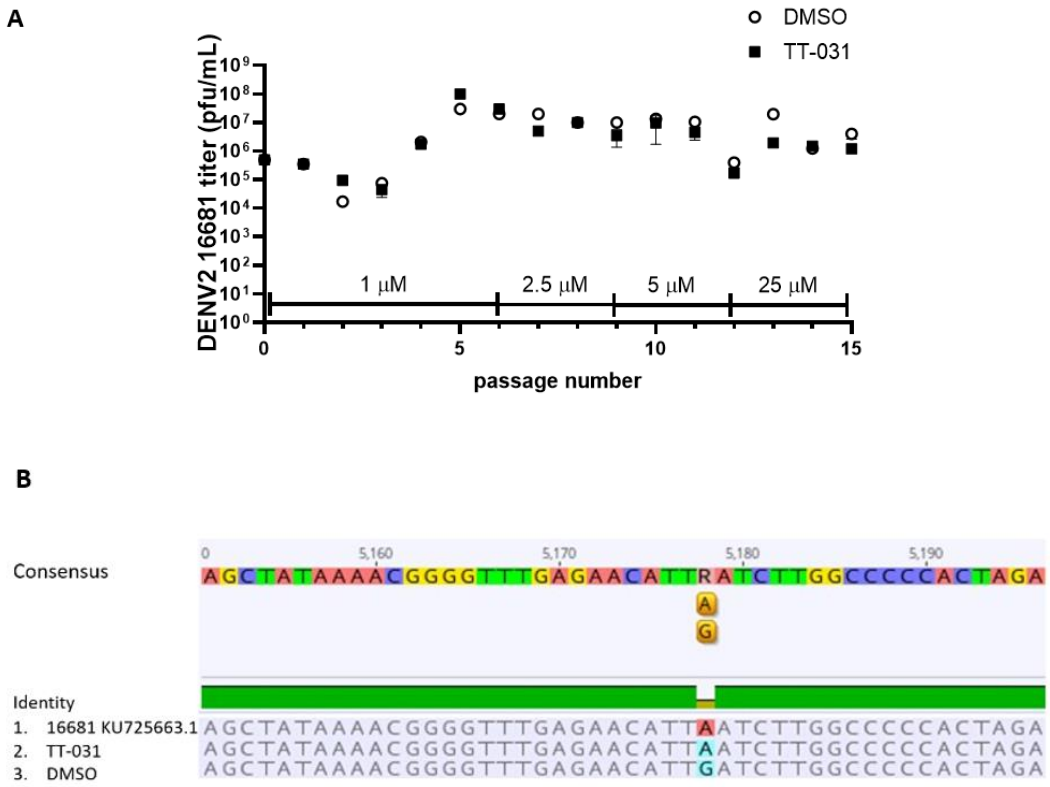


Figure 15 *Generation of TT-031-resisted DENV2.*

A) DENV2 16681-infected Huh-7 cells compared to DMSO as control. **B**) A single nucleotide substitution (G5178A) was discovered from TT-031 treatment compared with DMSO wildtype. Data courtesy of Assoc. Prof. Siwaporn Boonyasuppayakorn, M.D., Ph.D., Si-Xian Ho, and Assoc. Prof. Justin Chu, Ph.D. (National University of Singapore).

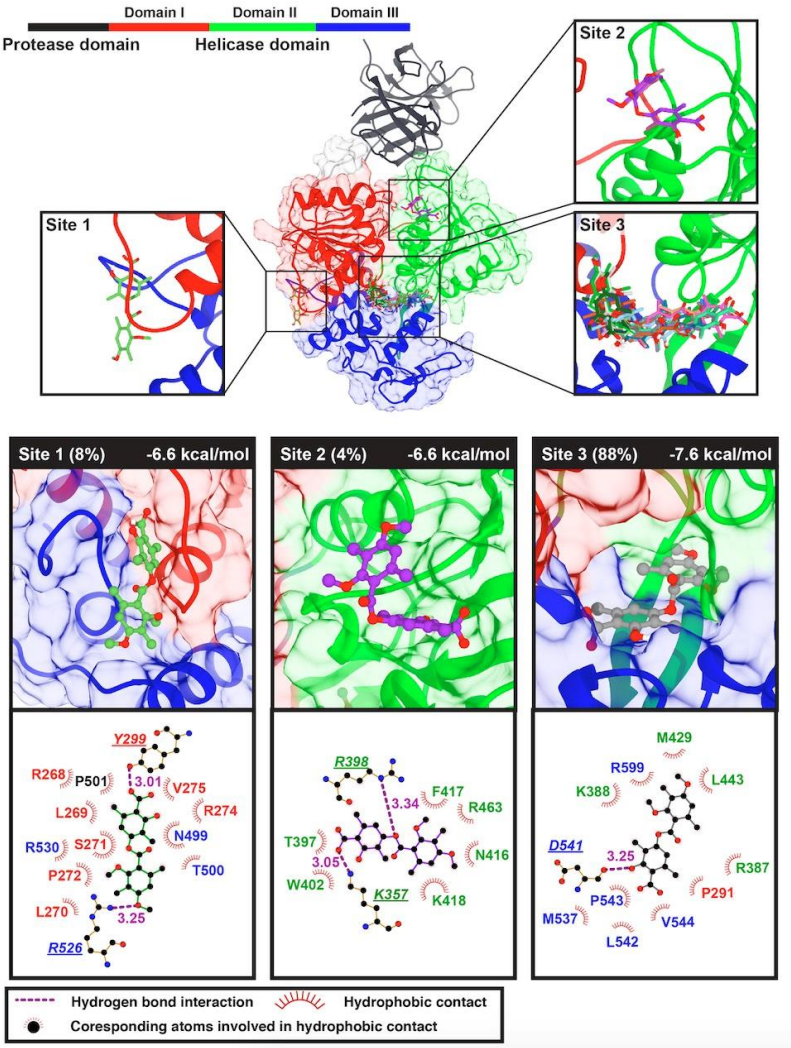


Figure 16 *Schematic of the DENV NS3 helicase and TT-031 interaction.*

The helicase domain of DENV NS3 protein was docked with TT-031. Data courtesy of Kowit Hengprasatporn, Ph.D

APPENDIX B

REAGENTS, MATERIALS, and INSTRUMENTS

Reagents

Absolute ethanol	(Merck, Darmstadt, Germany)
Crystal violet	(Merck, Darmstadt, Germany)
Dimethyl sulfoxide	(PanReac AppliChem, Hesse, Germany)
Direct-zol™ RNA MiniPrep Kit	(Zymo Research, California, USA)
Disodium hydrogen phosphate	(Sigma Aldrich, St. Louis, USA)
DMEM	(Gibco, Langley, USA)
EDTA	(Bio Basic Canada, Ontario, Canada)
Fetal Bovine serum	(Hyclone, Massachusetts, USA)
Formaldehyde	(QReC, New Zealand)
Geneticin (G418)	(Bio Basic Canada, Ontario, Canada)
Gum tragacanth	(Sigma Aldrich, St. Louis, USA)
HEPES	(Sigma Aldrich, St. Louis, USA)
Isopropanol	(Merck, Darmstadt, Germany)
M199	(Gibco, Langley, USA)
MEM	(Gibco, Langley, USA)
MES (2-(N-Morpholino) ethanesulfonic acid)	(Bio Basic Canada, Ontario, Canada)
Caspase-3 Colorimetric Assay Kit	(Biovision, California, USA)

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit

	(Promega, Wisconsin, USA)
NucleoZOL™ reagent	(MACHEREY-NAGEL, Dueren, Germany)
Penicillin G, sodium salt	(Bio Basic Canada, Ontario, Canada)
Potassium chloride	(Merck, Darmstadt, Germany)
Potassium dihydrogen phosphate	(Bio Basic Canada, Ontario, Canada)
Power SYBR® Green RNA-to-CT™ *1-Step* kit	(Applied Biosystems, California, USA)
RevertAid First Strand cDNA Synthesis Kit	(Thermo Scientific, Massachusetts, USA)
Ribavirin	(TCI, Tokyo, Japan)
RPMI-1640	(Gibco, Langley, USA)
Sodium bicarbonate	(Sigma Aldrich, St. Louis, USA)
Sodium chloride	(EMSURE, Darmstadt, Germany)
Streptomycin sulfate	(Bio Basic Canada, Ontario, Canada)
Trypsin	(Bio Basic Canada, Ontario, Canada)

Materials

Centrifuge tubes (15 and 50 mL)	(JET BIOFIL, Guangzhou, China)
Microcentrifuge tube (1.5 mL)	(JET BIOFIL, Guangzhou, China)
Flat 24- and 96-well plate	(Corning, New York, USA)
Tissue culture flask (T25 and T75)	(NUNC, Roskilde, Denmark)

Instruments

Autoclave (model-SX-700)	(Tomy, Tokyo, Japan)
Biophotometer (D30)	(Eppendorf, Connecticut, USA)

Centrifuge (Biofuge Stratos)	(Thermo Scientific, Massachusetts, USA)
CO_2 incubator	(Thermo Scientific, Massachusetts, USA)
Incubator	(Memmert, Schwabach, Germany)
Invert microscope (Eclipse TS100)	(Nikon, New York, USA)
Microcentrifuge (model: Forc 1418)	(Edison, New Jersey, USA)
Microplate reader (model: VICTOR™X3)	(PerkinElmer, Massachusetts, USA)
Mixer-vortex	(Science industrial, Pennsylvania, USA)
Step-OnePlus Real-time PCR System (ABI 7500)	(Applied Biosystems, California, USA)
Water bath	(Julabo, Baden-Württemberg, Germany)

APPENDIX C

CULTURE MEDIA and REAGENTS PREPARATION

Culture media

Growth medium for Vero cells

• 2X M199 with L-glutamine	50	mL
• Fetal bovine serum	10	mL
• Pen/Strep mixture	1	mL
• 1M HEPES	500	µL
• 10% $NaHCO_3$	2	mL
• Sterilized DDW	36.5	mL

Growth medium for LLC/MK2 cells

• 2X MEM with L-glutamine	50	mL
• Fetal bovine serum	10	mL
• Pen/Strep mixture	1	mL
• 1M HEPES	500	µL
• 10% $NaHCO_3$	2	mL
• Sterilized DDW	36.5	mL

Growth medium for C6/36 cells

• 2X MEM with L-glutamine	50	mL
• Fetal bovine serum	10	mL
• Pen/Strep mixture	1	mL
• 1M HEPES	700	µL
• 10% $NaHCO_3$	500	µL
• MEM non-essential amino acids	1	mL
• Sterilized DDW	36.5	mL

Growth medium for HepG2, HEK-293 and RD cells

• 2X DMEM	50	mL
• Fetal bovine serum	10	mL
• Pen/Strep mixture	1	mL
• 1M HEPES	500	µL
• 10% $NaHCO_3$	2	mL
Sterilized DDW	36.5	mL

Growth medium for THP-1 cells

• 2X RPMI 1640	50	mL
• Fetal bovine serum	10	mL
• Pen/Strep mixture	1	mL
• 1M HEPES	500	µL
• 10% $NaHCO_3$	2	mL
Sterilized DDW	36.5	mL

Maintenance medium for Vero cells

• 2X M199 with L-glutamine	50	mL
• Fetal bovine serum	1	mL
• Pen/Strep mixture	1	mL
• 1M HEPES	500	µL
• 10% $NaHCO_3$	2	mL
Sterilized DDW	45.5	mL

Maintenance medium for LLC/MK2 cells

• 2X MEM with L-glutamine	50	mL
• Fetal bovine serum	1	mL
• Pen/Strep mixture	1	mL

- 1M HEPES 500 µL
- 10% $NaHCO_3$ 2 mL

  Sterilized DDW 45.5 mL

Maintenance medium for C6/36 cells

- 2X MEM with L-glutamine 50 mL
- Fetal bovine serum 1 mL
- Pen/Strep mixture 1 mL
- 1M HEPES 700 µL
- 10% $NaHCO_3$ 500 µL
- MEM non-essential amino acids 1 mL

  Sterilized DDW 45.5 mL

0.8% Plaque overlay medium for LLC/MK2 cells

- 2X MEM with L-glutamine 50 mL
- 1.6% Gum tragacanth 50 mL
- Fetal bovine serum 1 mL
- Pen/Strep mixture 1 mL
- 1M HEPES 500 µL
- 10% $NaHCO_3$ 4 mL

1.2% Plaque overlay medium for RD cells

- 2X DMEM 50 mL
- 2.4% gum tragacanth 50 mL
- Fetal bovine serum 1 mL
- Pen/Strep mixture 1 mL
- 1M HEPES 500 µL
- 10% $NaHCO_3$ 4 mL

2X M199

- M199 with L-glutamine 19 g
- Sterilized DDW 1000 mL

  Sterilized by filtration and stored at 4°C

2X MEM

- MEM with L-glutamine 19.2 g
- Sterilized DDW 1000 mL

  Sterilized by filtration and stored at 4°C

2X DMEM

- DMEM (high glucose) with L-glutamine 27 g
- Sterilized DDW 1000 mL

  Sterilized by filtration and stored at 4°C

2X RPMI 1640

- RPMI medium 1640 with L-glutamine 10.4 g
- Sterilized DDW 500 mL

  Sterilized by filtration and stored at 4°C

1.6% Gum tragacanth

- Gum tragacanth 1.6 g
- Sterilized DDW 100 mL

  Sterilized by autoclaved and stored at 4°C

Reagents

Penicillin (10000 I.U./mL)/Streptomycin (10 mg/mL) mixture

- Penicillin G sodium salt 0.6 g
- Streptomycin sulfate 1 g
- Sterilized DDW 100 mL

  Sterilized by filtration and stored in aliquoted at -20°C

1M HEPES (4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid)

- HEPES monohydrate 11.915 g
- Sterilized DDW 50 mL

  Sterilized by autoclaved and stored at 4°C

10% $NaHCO_3$

- $NaHCO_3$ 10 g
- DDW 100 mL

  Sterilized by autoclaved and stored at 4°C

10X Phosphate buffer saline (PBS)

- NaCl 40 g
- KCl 1 g
- Na_2HPO_4 7.2 g
- KH_2PO_4 1.2 g
- Sterilized DDW 500 mL

  Sterilized by autoclaved and stored at room temperature

1X PBS

- 10X PBS 40 mL
- Sterilized DDW 360 mL

5% Trypsin

- Trypsin 5 g
- Sterilized DDW 100 mL

  Sterilized by filtration and stored in aliquoted at -20°C

1% EDTA

- EDTA 1 g
- Sterilized DDW 100 mL

  Sterilized by filtration and stored at 4°C

0.25% Trypsin-EDTA

- 5% Trypsin 2 mL
- 1% EDTA 800 µL
- 1X PBS 37.2 mL

  Stored at 4°C

0.05% Trypsin-EDTA

- 0.25% Trypsin 4 mL
- 1% EDTA 320 µL
- 1X PBS 15.68 mL

  Stored at 4°C

Crystal violet staining dye

- Crystal violet 1 g
- Isopropanol 5 mL
- 37% Formaldehyde 27 mL
- DW 68 mL

  Stored at room temperature

0.5M MES (2-(N-Morpholino) ethanesulfonic acid)

- MES hydrate 0.98 g
- Sterilized DDW 10 mL

Sterilized by filtration and stored at room temperature

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