Antiviral Research 151 (2018) 27–38

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

A novel flavanone derivative inhibits dengue virus fusion and infectivity

Pimsiri Srivarangkul[a,1], Wanchalerm Yuttithamnon[a,1], Aphinya Suroengrit[b], Saran Pankaew[a], Kowit Hengphasatporn[c], Thanyada Rungrotmongkol[c,d], Preecha Phuwapriasirisan[e], Kiat Ruxrungtham[f], Siwaporn Boonyasuppayakorn[f,g,*]

[a] Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[b] Graduate Program, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330, Thailand
[c] Bioinformatics and Computational Biology Program, Graduated School, Chulalongkorn University, Bangkok, 10330, Thailand
[d] Structural and Computational Biology Research Group, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[e] Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[f] Chula Vaccine Research Center (Chula VRC), Faculty of Medicine, Chulalongkorn University, Bangkok, 10330, Thailand
[g] Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330, Thailand

ARTICLE INFO

Keywords:
Flavanone
Dengue
Envelope
Drug discovery
Flavivirus

ABSTRACT

Dengue infection is a global burden affecting millions of world population. Previous studies indicated that flavanones were potential dengue virus inhibitors. We discovered that a novel flavanone derivative, 5-hydroxy-7-methoxy-6-methylflavanone (FN5Y), inhibited DENV2 pH-dependent fusion in cell-based system with strong binding efficiency to DENV envelope protein at K (P83, L107, K128, L198), K' (T48, E49, A50, L198, Q200, L277), X' (Y138, V354, I357), and Y' (V97, R99, N103, K246) by molecular dynamic simulation. FN5Y inhibited DENV2 infectivity with EC_{50}s (and selectivity index) of 15.99 ± 5.38 (> 6.25), and 12.31 ± 1.64 (2.23) μM in LLC/MK2 and Vero cell lines, respectively, and inhibited DENV4 at 11.70 ± 6.04 (> 8.55) μM. CC50s in LLC/MK2, HEK-293, and HepG2 cell lines at 72 h were higher than 100 μM. Time-of-addition study revealed that the maximal efficacy was achieved at early after infection corresponded with pH-dependent fusion. Inactivating the viral particle, interfering with cellular receptors, inhibiting viral protease, or the virus replication complex were not major targets of this compound. FN5Y could become a potent anti-flaviviral drug and can be structurally modified for higher potency using simulation to DENV envelope as a molecular target.

1. Introduction

Dengue virus causes a global burden with 390 million infections per year (Bhatt et al., 2013). This viral hemorrhagic fever was first emerged in South East Asia in 1970s and has become a regional public health burdens (Ooi and Gubler, 2009). Moreover, a rapid increase by 30-fold to all tropical regions within the recent decade was statistically shown (WHO, 2012). Dengue virus is a member of the family *Flaviviridae* consisting of 4 serotypes (DENV1-4). The genome is a single stranded positive sense RNA with a guanosine cap but no poly-A tail (Lindenbach et al., 2007). The transmission occurred by infected *Aedes aegypti* and *Aedes albopictus* mosquitoes injecting the virus to human while taking blood meal. The virus infected dendritic cells and was carried into circulatory system causing systemic infection. Severe dengue is a pathological condition with plasma leakage, internal bleeding, or multiple organ failure, hypovolemic shock, and death. Those serious pathological manifestations are caused by secondary heterotypic infection that the immune system misdirectedly responds to the previously infected serotype (WHO, 2009). Cellular and humoral immune-mediated responses create excessive cytokine production, endothelial damage, and coagulopathy which leads to vascular leakage, hemorrhage and shock.

A vaccine (Dengvaxia, CYD-TDV) is currently available but the efficacy against DENV1 and 2 were still limited (Vannice et al., 2017). This vaccine is recommended only in endemic population at the age group of 9–45 years old (WHO, 2016). Besides preventive vaccines, therapeutic drugs were extensively developed in order to alleviate the clinical severity of infected individuals. Cumulative evidences suggested the level of viral load was associated with progression to severe dengue (Libraty et al., 2002; Pozo-Aguilar et al., 2014; Wang et al., 2003); therefore, a small molecule that inhibits viral replication should reduce the viral load, and eventually prevent the disease progression

* Corresponding author. Department of Microbiology, Faculty of Medicine, Chulalongkorn University,1873 Rama 4 Road, Pathumwan, Bangkok, 10330, Thailand.
E-mail address: Siwaporn.b@chula.ac.th (S. Boonyasuppayakorn).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.antiviral.2018.01.010
Received 13 June 2017; Received in revised form 10 October 2017; Accepted 17 January 2018

(reviewed by Lim et al. (2013)). Dengue drug discovery was extensively studied in recent decades since it has acquired technological advancements of target identification, screening, and validation (Lim et al., 2013).

Flavonoid derivatives are potential inhibitors to flaviviral replications (Frabasile et al., 2017) (Allard et al., 2011; Du et al., 2016; Johari et al., 2012; Moghaddam et al., 2014; Sanchez et al., 2000; Zandi et al., 2011; Zhang et al., 2012). Previous reports suggested potential molecular targets of the compounds were at flaviviral protease (de Sousa et al., 2015) (Senthilvel et al., 2013), RdRP (Coulerie et al., 2012, 2013) and envelope (Ismail and Jusoh, 2016). Moreover, our preliminary results showed that flavanone derivatives were potentially inhibited DENV2 infectivity analyzed by plaque assay (Boonyasuppayakorn et al., 2016). In this study, we verified the previous findings and characterized a flavanone derivative, 5-hydroxy-7-methoxy-6-methylflavanone, as a dengue virus fusion inhibitor blocking DENV envelope protein to perform dimer to trimer conformational change.

2. Materials and methods

2.1. Cells and viruses

LLC/MK2 (ATCC® CCL-7), and C6/36 (ATCC® CRL-1660) cell lines were maintained in minimal essential medium (Gibco®, Langley, USA) supplemented with 10% fetal bovine serum (Gibco®, Langley, USA), 100 I.U./ml penicillin (Bio Basic Canada®, Ontario, Canada), and 100 µg/ml streptomycin (Bio Basic Canada®, Ontario, Canada), and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma Aldrich®, St. Louis, USA) at 37 °C under 5% CO_2 for LLC/MK2 and 28 °C for C6/36 cell line. Vero (ATCC® CCL-81) was maintained in Medium 199 (Gibco®, Langley, USA) supplemented with 10% fetal bovine serum, 100 I.U./ml penicillin, and 100 µg/ml streptomycin, and 10 mM HEPES at 37 °C under 5% CO_2. HEK-293 (ATCC® CRL-1573) and HepG2 (ATCC® HB-8065) were maintained in DMEM (Gibco®, Langley, USA) supplemented with 10% fetal bovine serum, 100 I.U./ml penicillin, and 100 µg/ml streptomycin, and 10 mM HEPES at 37 °C under 5% CO_2.

Reference strains of DENV2 (New Guinea C strain, NGC), and DENV4 (c0036) were propagated in Vero cell line with minimal essential medium supplemented with 1% fetal bovine serum, 100 I.U./ml penicillin, and 100 µg/ml streptomycin at 37 °C under 5% CO_2.

2.2. Isolation of natural flavonoids

Natural flavonoids in this experiment were isolated and purified from the leaves of rose apple, *Syzygium samarangense*, collected from Nakhonratchasima, Thailand, in October 2011. The dried leaves (214 g) were extracted (2 × 2L) at 80 °C for 3 h. the aqueous extract was partitioned with EtOAc to afford the organic extract. The EtOAc extract was fractionated with silica gel flash column chromatography eluted with CH_2Cl_2, MeOH-CH_2Cl_2 (5:95, 15:85 and 3:7) MeOH-CH_2Cl_2 and MeOH, thus yielding 5 combined fractions. Fraction 1 was triturated with hot hexane to afford hexane soluble fraction and insoluble solid. The hexane soluble fraction was further purified using silica gel (3:2 CH_2Cl_2-hexane) to obtain 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (F5Y), 5-hydroxy-7-methoxy-6-methylflavone (FN5Y), pinostrobin (F7Y) and aurentiacin (F8Y). The hexane insoluble solid was crystallized in 9:1 hexane-CH_2Cl_2 to obtain demthoxymatteucinol (F9Y). Fraction 2 was purified by Sephadex LH20 (5:4:1 CH_2Cl_2-hexane-MeOH) followed by silica gel column chromatography (1:1 CH_2Cl_2-hexane) to yield pinocembrin (F12Y). The purity and identity of the isolated flavonoids were verified by NMR.

2.3. Cytotoxicity test

LLC/MK2, Vero, HEK-293, or HepG2 cells were seeded at 10^4 cells per well of 96-well plate and incubated overnight. Compounds were prepared to 6–10 different concentrations in filter-sterilized dimethylsulfoxide (Merck®, Darmstadt, Germany) before addition to the cells. Plates were incubated for 48, 72, or 120 h as indicated in the experiment. MTS reagent (Promega®, Madison, USA) was added to cells according to manufacturer's protocol and incubated for 4 h before analysis by spectrophotometry at A_{450nm}. Each compound was tested in triplicate. Cytotoxic concentrations (CC_{50}) were calculated using nonlinear regression analysis and the results were reported as means and standard deviation of three independent experiments.

2.4. Effective concentration (EC_{50}) test

LLC/MK2 at 5 × 10^4 cells per well were seeded into 24-well plate in growth medium and incubated overnight at 37 °C under 5% CO_2. Cells were infected with DENV2 at the multiplicity of infection (MOI) of 0.1 for 1 h with gentle rocking every 15 min. Cells were washed with PBS and incubated with MEM supplemented with 1% fetal bovine serum, 100 I.U./ml penicillin, and 100 µg/ml streptomycin. The compound was added to the virus-infected cells during and after infection. Cells were incubated for 72 h, unless otherwise indicated, at 37 °C under 5% CO_2. Supernatants were collected and the viral infectivity were analyzed by 96-well plaque titration (Boonyasuppayakorn et al., 2016). Data were plotted and the EC_{50} values were calculated by nonlinear regression analysis. Each concentration was tested in duplicate and the results were reported as means and standard deviation of three independent experiments. Selectivity index was calculated from the ratio of CC_{50} and EC_{50}.

2.5. Time of drug addition study

LLC/MK2 cells were seeded into 24-well plates and incubated as previously described. Cells were then infected with DENV2 at the M.O.I. of 0.1 for 1 h with gentle rocking every 15 min. FN5Y at 25 µM was added at the following conditions; (i) 1 h prior to DENV infection; (ii) during DENV infection; or (iii) after DENV infection at 1, 2, 4, 8, 12, 24, 48, and 72 h. Supernatants and cells were collected at 72 h post infection to determine the viral titers by plaque titration and RT-qPCR, respectively. RT-qPCR was performed by total intracellular RNAs extraction using TRIzol reagent (Invitrogen™ Carlsbad, USA) and purification using Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, USA). The RT-qPCR was performed with a Step-One Plus Real-Time PCR System (Applied Biosystem, Foster City, CA, USA) with 1 × Power SYBRGreen PCR Master Mix (Applied Biosystem, Foster City, CA, USA), 50 µM each of DN-F and DN-R primers (Shu et al., 2003) under the following condition. Each reaction mixture at 20 µl contains 5 µl of sample RNA, 50 nM of each primer, and 1 µg of total RNA. The reactions were then cycled at 48 °C for 30 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 20s (denaturation), 55 °C for 30s (annealing), 72 °C for 30s (extension). Each sample was analyzed in triplicated and results were confirmed by three independent experiments.

2.6. Pre-incubation study

LLC/MK2 cells were seeded in 24-well plates and incubated as previously described. FN5Y at 25 µM was added to DENV2 for 1 or 2 h before adsorption (pre-incubation). Cells were then infected with the FN5Y-treated DENV2 (M.O.I. of 1) at 4 °C for 1 h with continuous rocking, followed by washing three times with cold PBS. Cells were incubated at 37 °C, under 5% CO_2 for 2 days before supernatant collection. The level of virus production was quantified by plaque titration. DMSO-treated samples were used as a no-inhibition control. Results were confirmed by three independent experiments.