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Luteolin restricts dengue virus replication through inhibition of the proprotein convertase furin

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ABSTRACT

In many countries afflicted with dengue fever, traditional medicines are widely used as panaceas for illness, and here we describe the systematic evaluation of a widely known natural product, luteolin, originating from the "heat clearing" class of herbs. We show that luteolin inhibits the replication of all four serotypes of dengue virus, but the selectivity of the inhibition was weak. In addition, ADE-mediated dengue virus infection of human cell lines and primary PBMCs was inhibited. In a time-of-drug-addition study, luteolin was found to reduce infectious virus particle formation, but not viral RNA synthesis, in Huh-7 cells. During the virus life cycle, the host protease furin cleaves the pr moiety from prM protein of immature virus particles in the *trans*-Golgi network to produce mature virions. Analysis of virus particles from luteolin-treated cells revealed that prM was not cleaved efficiently. Biochemical interrogation of human furin showed that luteolin inhibited the enzyme activity in an uncompetitive manner, with Ki value of 58.6 μM, suggesting that treatment may restrict the virion maturation process. Luteolin also exhibited *in vivo* antiviral activity in mice infected with DENV, causing reduced viremia. Given the mode of action of luteolin and its widespread source, it is possible that it can be tested in combination with other dengue virus inhibitors.

1. Introduction

Dengue fever, a mosquito-borne viral disease, is caused by dengue virus (DENV), transmitted by female mosquitoes, mostly *A. aegypti*., and mainly found in tropical and sub-tropical regions, urban and semi-urban areas. Dengue has become a global burden with approximately 500,000 people with severe dengue symptoms needing hospital care annually and with a 2.5% mortality rate (WHO, 2015; Messina et al., 2014; Bhatt et al., 2013).

DENV enter cells by binding to cellular receptors such as heparin sulphate and unknown co-receptors through receptor-mediated endocytosis. The low pH in the endosomes promotes fusion of viral envelope with the host endosomal membrane releasing the nucleocapsid into the cytoplasm (Kuhn et al., 2002). Following a poorly understood uncoating mechanism the viral 5' capped RNA genome of ~11k is translated into a precursor polyprotein which is co- and post-translationally processed by host signalises as well as the virus encoded serine protease into the three structural and seven non structural proteins (NS) in the order of C (Core) – prM (pre-Membrane) – E (Envelope) – NS1 – NS2A – NS2B – NS3 – NS4A – NS4B – NS5 (Chambers et al., 1997). Immature progeny virion enveloped within prM-E heterodimers is assembled and traffic to the Golgi where the prM protein is cleaved by the host proprotein convertase furin within the *trans*-Golgi network to produce infectious mature virus particles that are released by exocytosis from the cell and ready to infect other cells (Screaton et al., 2015). Efforts to discover antiviral drugs targeting the various steps in entry, replication and release in the virus life cycle have been reviewed extensively e.g. (Lim et al., 2013). Despite many advances there is no licensed drug for dengue fever at present. A

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tetravalent vaccine is available in a handful of countries for protection from dengue infection, however its efficacy against dengue virus is not ideal (Hadinegoro et al., 2015; Halstead and Russell, 2016).

Traditional Chinese medicines (TCM) continue to be widely used in eastern societies as treatments for symptoms such as fever, pain, bloating, rashes, and their benefits are also being recognized by the growing numbers of TCM users in westernized societies seeking natural remedies. In an attempt to identify new antiviral substances active against dengue virus, we tested hundreds of crude extracts from TCM plants classified as antipyretic herbs, detoxifying herbs, blood cooling herbs or involuntary perspiration inducing herbs (Fig. S1A) (Li et al., 2015). We isolated 13 candidate compounds that were tested for antiviral activity in DENV infected cells or AG129 mice. We found that the widely occurring flavonoid, luteolin, that was isolated as one of the main components (0.15 mg/g), from the herb *Viola yedoensis Makino*, inhibited DENV replication. Our studies demonstrated that luteolin has the ability to obstruct the later stages of DENV viral lifecycle in infected cells by inhibiting, albeit weakly, the host proprotein convertase furin. Consistent with our finding, previous studies have shown that luteolin exhibits inhibitory effects on Epstein-Barr Virus, Japanese encephalitis virus, HIV-1, Hepatitis B virus, Hepatitis C virus, enterovirus 71, coxsackievirus A16, and chikungunya virus (Wu et al., 2016; Fan et al., 2016; Mehla et al., 2011; Bai et al., 2016; Rehman et al., 2016; Xu et al., 2014; Murali et al., 2015). Nevertheless, there have been no studies on anti-DENV activity of luteolin *in vitro* or *in vivo*. This finding opens the possibility that TCMs may be a source for potential drugs against dengue fever.

2. Material and methods

2.1. Cells, virus and antibodies

Huh-7 (hepatocellular carcinoma cells, ATCC), Vero (African green monkey kidney cells, ATCC) and HEK-293T (human embryonic kidney 293 cells, ATCC) cells were cultured in DMEM medium containing 10% FBS, 1% penicillin/streptomycin (P/S) at 37 °C in 5% CO_2. BHK-21 (baby hamster kidney fibroblast cells, ATCC) cells were cultured in RPMI 1640 medium containing 10% FBS, 1% P/S, at 37 °C in 5% CO_2. A549 (adenocarcinomic human alveolar basal epithelial cells, ATCC) cells were cultured in F12/K medium containing 10% FBS, 1% P/S, at 37 °C in 5% CO_2. C6/36, an *Aedesalbopictus* cell line (ATCC), was maintained in RPMI 1640 medium containing 25 mM HEPES, 10% FBS and 1% P/S, at 28 °C in the absence of CO_2. Hybridoma cell lines (ATCC) for 4G2 and 3H5 were grown in PFHM-II (Gibco) medium with 1% P/S at 37 °C in 5% CO_2.

DENV-1 (EDEN-1, GenBank accession EU081230), DENV-2 (EDEN-2, GenBank accession EU081177), DENV-3 (EDEN-3, GenBank accession EU081190) and DENV-4 (EDEN-4, GenBank accession GQ398256) were obtained from the Early Dengue infection and outcome (EDEN) study in Singapore (Low et al., 2006). All virus strains were grown in C6/36 cells and the supernatants were stored at −80 °C. Virus titer was determined by plaque assay on BHK-21 cells.

DENV-specific mouse monoclonal antibodies, 4G2 and 3H5 against E protein, were prepared from hybridoma cell lines as described previously (Watanabe et al., 2012).

2H2 against prM MAbs was a generous gift from Prof. Shee Mei Lok, Duke-NUS Medical School.

2.2. Extracts preparation

Two-gram of each herb (KANGMEI Pharmaceutical Co. Ltd) was smashed and extracted with 20 ml distilled water by heating reflux extraction for 30 min or extracted with 20 ml 70% ethanol by ultrasonic extraction for 30 min. The extracts were condensed under vacuum to obtain the residue and diluted with 20 ml of distilled water for antiviral activity assay.

2.3. Therapeutic compounds

Compounds in plant extracts (Purity > 95%) were purified and isolated by macro-reticular resin column (Cangzhou Bon Adsorber Technology Co. Ltd), RP-C18 column (Merck), sephadex LH-20 (Sigma) and reversed-phase preparative HPLC column (Phenomenex, 10 × 250 mm, 5 μm) as described previously (Peng et al., 2016).

NITD008 used as a positive control for DENV infection (Yin et al., 2009) was a generous gift from Novartis Institute for Tropical Disease, Singapore.

2.4. Cell viability assay

For measurement of compound cytotoxicity, Huh-7, A549, HEK-293T, BHK-21 and Vero cells, were seeded at 2×10^4 cells per well with 10% FBS medium in 96-well white flat-bottom plate. Cells were incubated with various concentrations of compound for an additional 48 h. Cell viability was measured using CellTiterGlo® Luminescent cell viability Assay (Promega) kit according to the manufacturer's instructions. Luminescence was measured on microplate reader (Tecan Infinite 200 PRO) with a 100 m/s integration time. For crude extracts, cell viability was measured using Cell Counting Kit-8 (Dojindo) according to the manufacturer's instructions. Cell viability curve presented as percentage of luminescence derived from treated samples to that of the untreated control.

2.5. Virus infection

Huh-7 cells were seeded in a 24-well plate at 1×10^5 cells per well. Eight concentrations (50, 25, 12.5, 6.25, 3.125, 1, 0.1, 0.01 μM) were used to generate an inhibition curve. Cells were infected with DENV strains at a multiplicity of infection (MOI) equal to 0.3 as indicated in the Results section in the presence of compounds for 1 h. Virus/drug inoculums were removed and fresh medium containing the indicated concentrations of compounds were added. Cells were incubated for additional 48 h at 37 °C and the supernatants were collected. Virus titers in the supernatants were determined by plaque assay using BHK-21 cells. EC_{50} were determined using GraphPad Prism software.

For antibody-dependent enhanced (ADE) infection, peripheral blood mononuclear cells (PBMCs) were isolated from human blood using Ficoll-Paque (GE Healthcare) extraction method according to manufacturer's instructions. DENV-1 (MOI of 10) and humanized 4G2 (0.05 μg) were mixed and incubated on ice for 1 h to allow the formation of immune complexes. PBMCs (1×10^6) were infected with the immune complexes for 2.5 h at 37 °C with shaking. Cells were then washed once with PBS before resuspending in RPMI medium containing indicated concentrations of the compounds for a further incubation of 48 h. After 48 h, supernatants were harvested, subjected to virus titer determination by plaque assay and IL6 ELISA assay using Ready-Set-Go! ELISA kit (eBioscience) according to manufacturer's instructions.

2.6. Time of drug addition assay

To determine the mode of action of luteolin, 1×10^5 Huh-7 cells were seeded into a 24-well plate and infected with DENV-2 at MOI 1 for 1 h as described above. Infected cells were treated with 10 μM