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Alpha-mangostin inhibits both dengue virus production and cytokine/chemokine expression

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ABSTRACT

Since severe dengue virus (DENV) infection in humans associates with both high viral load and massive cytokine production – referred to as "cytokine storm", an ideal drug for treatment of DENV infection should efficiently inhibit both virus production and cytokine expression. In searching for such an ideal drug, we discovered that α-mangostin (α-MG), a major bioactive compound purified from the pericarp of the mangosteen fruit (*Garcinia mangostana* Linn), which has been used in traditional medicine for several conditions including trauma, diarrhea, wound infection, pain, fever, and convulsion, inhibits both DENV production in cultured hepatocellular carcinoma HepG2 and Huh-7 cells, and cytokine/chemokine expression in HepG2 cells. α-MG could also efficiently inhibit all four serotypes of DENV. Treatment of DENV-infected cells with α-MG (20 μM) significantly reduced the infection rates of four DENV serotypes by 47–55%. α-MG completely inhibited production of DENV-1 and DENV-3, and markedly reduced production of DENV-2 and DENV-4 by 100 folds. Furthermore, it could markedly reduce cytokine (IL-6 and TNF-α) and chemokine (RANTES, MIP-1β, and IP-10) transcription. These actions of α-MG are more potent than those of antiviral agent (ribavirin) and anti-inflammatory drug (dexamethasone). Thus, α-MG is potential to be further developed as therapeutic agent for DENV infection.

1. Introduction

Dengue virus (DENV) infection is the most widespread mosquito-borne viral disease caused by any one of the four DENV (DENV-1-4) serotypes. Up to 50–100 million infections with 20,000 deaths in over 100 endemic countries are estimated annually and about half of world population is now at risk of infection (WHO, 2012). A wide spectrum of illness caused by DENV infection ranges from asymptomatic condition, mild form as dengue fever (DF), to severe forms as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The severe forms are characterized by vascular leakage, hemo-concentration, thrombocytopenia, coagulopathies, pleural effusion, hypovolumic shock, and organ failure (Simmons et al., 2012). Recently, the first dengue vaccine, Dengvaxia®, has been approved but its efficacy is limited (Khetarpal and Khanna, 2016). Till now, there is no effective antiviral drug available for DENV infection. Treatment is only supportive care based on clinical symptoms. Thus, effective drugs for DENV infection are urgently required.

It has been shown that severe DENV infection correlated with high viral load and abnormal immune responses of the patients (Tricou et al., 2011; Vaughn et al., 2000; Takhampunya et al., 2009). Several risk factors may explain the development of DHF/DSS, one of which is antibody dependent enhancement (ADE) in secondary infection with heterotypic DENV. Non-neutralizing antibodies enhance viral entry into host cells leading to increased viral load and immune activation associated with disease severity (Balsitis et al., 2010). Furthermore, during secondary infection, viral antigens on infected cells trigger activation of serotype-cross-reactive memory T-cells, resulting in massive pro-inflammatory cytokine production that may ultimately lead to plasma leakage in the severe disease (Rothman, 2011). Several cytokines/chemokines markedly increase in the patients with DHF/DSS and play a role in the pathogenesis of the severe infection, such as IL-1β, IL-6, IL-10, TNF-α, IFN-γ, IP-10, RANTES, and MIP-1β (Bozza et al., 2008; Nguyen et al., 2004; Rathakrishnan et al., 2012). The correlation between the severity of infection and high viral titer associated with excessive cytokine production – the so called "cytokine storm", leading to

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our hypothesis that concurrent inhibition of DENV and cytokine production should be more effective for treatment of DENV infection. Therefore, the use of single drug with combined effects on antiviral and anti-inflammatory is a promising therapeutic strategy for treatment of DENV infection.

Recently, several antiviral and anti-inflammatory agents such as the alkaloidal fraction from *Uncaria tomentosa* (Willd.) DC. (Reis et al., 2008), propolis compound (PropoelixTM) (Soroy et al., 2014), and dexamethasone (Reis et al., 2007) have been tested and proposed as a potential therapeutic drug for DENV infection. Our group showed the effect of anti-inflammatory compound, compound A (CpdA), against DENV infection. CpdA reduced DENV-induced cytokine secretion and DENV production in the infected HepG2 cells (Suttitheptumrong et al., 2013). Interestingly, another study from our group demonstrated that combination of antiviral agent (ribavirin) and anti-inflammatory compound (CpdA) offers a greater efficiency in reduction of DENV and cytokine/chemokine production comparing to individual treatment (Rattanaburee et al., 2015), supporting our hypothesis on the concurrent inhibition of DENV and cytokine production for more effective treatment of DENV infection.

To search for a new promising therapeutic agent against DENV infection, we select the natural compound exhibiting both antiviral and anti-inflammatory activities to evaluate its properties for inhibiting DENV infection and the massive cytokine production. α-Mangostin (α-MG) is a major bioactive compound purified from the pericarp of the mangosteen fruit (*Garcinia mangostana* Linn.), which possesses a wide range of biological activities including anti-inflammatory, anti-allergic, antiviral, antibacterial, antifungal, anti-parasitic, antioxidant, and anticancer properties (Ibrahima et al., 2016). The mangosteen has been used as a traditional medicine for the treatment of trauma, diarrhea, wound infection, pain, fever, and convulsion (Cui et al., 2010). A number of studies demonstrated the antiviral property of α-MG. The ethanol extract from *G. mangostana* Linn. showed potent inhibitory activity against HIV-1 protease which interfered the virus replication cycle (Chen et al., 1996). α-MG could reduce rotavirus infectivity (Shaneyfelt et al., 2006) and suppress hepatitis C virus (HCV) replication (Choi et al., 2014). Furthermore, the anti-inflammatory and anti-allergic effects of α-MG were demonstrated in several models. It significantly inhibited nitric oxide (NO) and PGE_2 production from lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and inhibited mice carrageenan-induced paw edema in acute inflammation model (Chen et al., 2008). α-MG also reduced LPS-induced inflammatory cytokines, TNF-α and IL-4, in U937 cells (Liu et al., 2012) and allergic mediators IL-6, prostaglandin D2 (PGD2), and leukotriene C4 (LTC4) in bone marrow derive mast cell (Chae et al., 2012). It has also been studied in allergic diseases and demonstrated for its potent in alleviating allergic inflammatory responses (Gopalakrishnan et al., 1980; Jang et al., 2012). Nevertheless, α-MG has never been tested for its effects against DENV infection. Based on aforementioned information, we hypothesize that α-MG possesses combined therapeutic potential, for both antiviral and anti-inflammatory effects, against DENV infection. We therefore tested this hypothesis by investigating the effects of α-MG on DENV-infected cells and viral production in DENV-infected HepG2 and Huh-7 cells, in addition to cytokine/chemokine expression in DENV-infected HepG2 cells. Our results demonstrated that α-MG efficiently inhibits both DENV and cytokine/chemokine production in DENV-infected cells.

2. Materials and methods

2.1. DENV propagation

DENV-1 strain Hawaii, DENV-2 strain 16681, DENV-3 strain H87, and DENV-4 strain H241 were propagated in *Aedes albopictus* C6/36 cell line. The amount of each DENV serotype in the culture supernatant was titrated by focus-forming units (FFU) assay as previously described (Rattanaburee et al., 2015). In experiments with UV-inactivated DENV (UV-DENV), the viruses from the same virus stock (used for infection) were inactivated by exposure to UV irradiation for 3 h and then confirmed their inactivation by FFU assay.

2.2. Cell cultures

African green monkey kidney (Vero) cells were grown in Minimum Essential Medium (MEM) (Gibco, USA) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and 1.2% (v/v) antibiotics at 37 °C, 5% CO_2 for DENV titration by FFU assay.

Human hepatocellular carcinoma (HepG2) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1% (v/v) non-essential amino acids (NEAA), 1 mM sodium pyruvate and 1.2% (v/v) antibiotics at 37 °C, 5% CO_2 for determining antiviral and anti-inflammatory activities of α-MG in DENV infection.

Human hepatocellular carcinoma (Huh-7) cells were grown in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1% (v/v) NEAA and 1.2% (v/v) antibiotics at 37 °C, 5% CO_2 for determining antiviral activity of α-MG in DENV infection.

2.3. DENV infection and compound treatment

To determine antiviral activity of α-MG in DENV infection, HepG2 and Huh-7 cells were cultured in complete DMEM medium for 24 h and infected with DENV-2 at MOI 5 and 0.5, respectively. After absorption at 37 °C for 2 h, unbound DENV was discarded and the cells were replenished with the medium containing either α-MG (Wako Pure Chemical Industries, Japan) or ribavirin (RV) (Sigma, USA) or dexamethasone (DEX) (Sigma, USA) or ethanol solvent (EtOH) and incubated for 24, 48, and 72 h. Cisplatin (Cis), a chemotherapy drug which has cytotoxic properties, was used as the control to display cell death in cell viability assay. The cells without DENV infection were used as mock control. The treated cells were detected for DENV-infected cells by flow cytometry. Culture supernatants were collected for determination of DENV production by FFU assay.

To determine antiviral and anti-inflammatory activities of α-MG in DENV infection, HepG2 cells were cultured in complete DMEM medium for 24 h and infected with DENV-1, DENV-2, DENV-3 and DENV-4 at MOI 20, 5, 40 and 5, respectively, to control the percentage of infected cells at over 60%. After absorption at 37 °C for 2 h, unbound DENV was discarded and the cells were replenished with the medium containing either α-MG or RV or DEX and incubated for 24 h. The HepG2 cells without DENV infection were used as mock control. Culture supernatants were collected for determination of DENV production by FFU assay. The treated cells were detected for DENV-infected cells by flow cytometry and immunofluorescence assay, and determined for cytokine/chemokine transcription by real-time RT-PCR.

2.4. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

To determine cytokine/chemokine transcription, total RNA was extracted from mock and DENV-infected cells, with or without treatment as indicated, by using Trizol™ reagent (Invitrogen, New Zealand). Cytokine/chemokine mRNA was quantified by real-time RT-PCR technique using specific primers (Table 1). Total RNA was reverse-transcribed into cDNA using Superscript®III reverse transcriptase (Invitrogen, New Zealand). Amplification of cDNA by real-time PCR was carried out in a reaction mixture of the LightCycler® 480 SYBR Green I Master reagent (Roche, Germany). The relative mRNA expression was normalized against β-actin mRNA level by using a comparative Ct method.