Antiviral Research 139 (2017) 69–78

Contents lists available at ScienceDirect

Antiviral Research

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

Activity of andrographolide against dengue virus

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ARTICLE INFO

Article history:
Received 27 July 2016
Received in revised form
29 November 2016
Accepted 21 December 2016
Available online 26 December 2016

Keywords:
Dengue virus
Andrographis paniculata
Andrographolide
Antiviral

ABSTRACT

Dengue is the most prevalent arthropod-transmitted viral illness of humans, with an estimated 100 million symptomatic infections occurring each year and more than 2.5 billion people living at risk of infection. There are no approved antiviral agents against dengue virus, and there is only limited introduction of a dengue vaccine in some countries. Andrographolide is derived from *Andrographis paniculata*, a medicinal plant traditionally used to treat a number of conditions including infections. The antiviral activity of andrographolide against dengue virus (DENV) serotype 2 was evaluated in two cell lines (HepG2 and HeLa) while the activity against DENV 4 was evaluated in one cell line (HepG2). Results showed that andrographolide had significant anti-DENV activity in both cell lines, reducing both the levels of cellular infection and virus output, with 50% effective concentrations (EC_{50}) for DENV 2 of 21.304 μM and 22.739 μM for HepG2 and HeLa respectively. Time of addition studies showed that the activity of andrographolide was confined to a post-infection stage. These results suggest that andrographolide has the potential for further development as an anti-viral agent for dengue virus infection.

1. Introduction

Dengue is the most prevalent arthropod-borne viral illness of humans, with an estimated 400 million infections occurring each year and more than 2.5 billion people living at risk of infection (Bhatt et al., 2013). Dengue virus (DENV) is a single-stranded RNA virus that belongs to the family *Flaviviridae*, genus *Flavivirus* and is transmitted to humans by *Aedes* mosquitoes, mainly *Aedes aegypti* and *Ae. albopictus*. There are four serologic types of DENV, DENV 1, 2, 3 and 4 (Calisher et al., 1989; Chambers et al., 1990). Human infection with DENV is asymptomatic in the majority of cases, but may result in a wide spectrum of clinical symptoms (Harris et al., 2000), ranging from classical dengue fever (DF) to more severe cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). A primary infection with any of the four DENVs results in a lifelong immunity to that DENV, but not against the other three heterologous DENVs. Sequential infections in the presence of heterologous anti-dengue antibodies generated from a primary infection often leads to a more severe secondary infection causing DHF or DSS (Burke et al., 1988; Guzman et al., 2002; Halstead and Simasthien, 1970; Recker et al., 2009; Thein et al., 1997). The more severe disease is believed to result from heterotypic non-neutralizing anti-dengue antibodies mediating infection of Fc receptor bearing cells in a process termed antibody dependent enhancement of infection or ADE (Halstead and O'Rourke, 1977a, b; Halstead et al., 1980). With the rapid expansion of dengue disease in most tropical and subtropical areas of the world, it is crucial to develop effective prevention and control measures, including vaccines and antiviral drugs against dengue disease. However, the development of a dengue vaccine is complicated by the process of ADE, and a commercial vaccine currently available in only a few countries is currently believed to be suboptimal (Rothman and Ennis, 2016). Currently treatment of DENV infection is limited to alleviation of symptoms, as there is no licensed anti-DENV therapeutic drug.

Andrographis paniculata (Burm. f.) Nees is a traditionally used medicinal plant in the family *Acanthaceae* that has been used for centuries in Asia to treat diseases such as gastro-intestinal tract disorders, upper respiratory infections, fever and herpes. In India it has considerable medical reputation, being part of 26 ayurvedic formulations as listed in the Indian pharmacopoeia (Mishra et al., 2007). In Thailand it was selected by the Ministry of Public Health as one of the medicinal plants to be included in "The National List of Essential Drugs A.D. 1999" (Pholphana et al., 2004).

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http://dx.doi.org/10.1016/j.antiviral.2016.12.014
0166-3542/

Extracts of the whole or part of the plant are widely used in China, India, Thailand and other Southeast Asian countries and the plant is also known as King of Bitters (English), Mahatikta (Sanskrit), Kiryato (Gujarati), Mahatita (Hindi), Kalmegh (Bengali) or Fah Talai Jone (Thai) (Li et al., 2007).

The main bioactive compound of *Andrographis paniculata* is believed to be the labdane diterpenoid andrographolide, and andrographolide has been reported to have activity against a number of viruses including HIV (Calabrese et al., 2000; Reddy et al., 2005), hepatitis B virus (Chen et al., 2014), herpes simplex virus (Seubsasana et al., 2011; Wiart et al., 2005), influenza virus (Chen et al., 2009; Yu et al., 2014), hepatitis C virus (Lee et al., 2014) and chikungunya virus (CHIKV) (Wintachai et al., 2015). In this study, we sought to evaluate the activity of andrographolide against DENV.

2. Materials and methods

2.1. Cells and virus

The human hepatoma cell line HepG2 (ATCC No. HB-8065) and the human cervical cancer cell line HeLa (ATCC No. CCL-2) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaitherburg, MD) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Gibco BRL, Gaitherburg, MD) at 37 °C with 5% CO_2. Dengue virus serotype 1 (DENV 1; strain 16007), serotype 2 (DENV 2; strain 16681), serotype 3 (DENV 3; strain 16562) and serotype 4 (DENV 4; strain 1036) were propagated in the *Ae. albopictus* derived cell line C6/36 (ATCC No. CRL-1660) as described previously (Sithisarn et al., 2003). Viruses were partially purified by centrifugation to remove cell debris and stored frozen at −80 °C. Virus titer was determined by standard plaque assay on LLC-MK2 cells (ATCC No. CCL-7) as described previously (Sithisarn et al., 2003).

2.2. Andrographolide

Andrographolide assessed as ≥ 98% pure by thin layer chromatography was purchased from Sigma-Aldrich (365645; Sigma-Aldrich, St. Louis, MO) was dissolved in 100% dimethyl sulfoxide (DMSO) to a final stock concentration of 100 mM and stored at −80 °C. Compound was diluted to various concentrations using complete DMEM. The final concentration of DMSO in media was less than 0.1%.

2.3. Virucidal assay

Stock DENV 2 was incubated directly with vehicle only or with medium only or with different concentrations (25, 50, 75, and 100 μM) of andrographolide in a final volume of 200 μl for 1 h at 37 °C, after which infectious virus titer was determined by standard plaque assay. Experiment was undertaken independently in triplicate with duplicate plaque assay.

2.4. Cell viability assay

HepG2 or HeLa cells were cultured in ninety-six well-tissue culture plates under standard conditions until the cells reached 90% confluence. The cell culture medium was removed and cells were incubated with various concentration of andrographolide (5, 10, 25, 50, 75, and 100 μM) diluted in complete medium with FBS prior to being cultured for 24 h under standard conditions before analysis using the Vybrant® MTT Cell proliferation assay kit (V13154; Invitrogen, Grand Island, NY) according to the manufacturers recommendations. Values were determined from 8 independent replicates. Negative controls (media only) and positive controls (5% DMSO) were included.

2.5. Andrographolide treatment of cells (DENV 2)

HepG2 or HeLa cells were cultured in six well culture plates and grown until the cells reached approximately 90% confluency. The cells were washed with PBS and then incubated with 0.1% DMSO (no treatment control) or with andrographolide at various concentrations (25, 50, 75, and 100 μM) for 1 h followed by washing twice with PBS before infection with DENV 2 at multiplicity of infection (MOI) of 20 or 1, respectively at 37 °C for 2 h in the absence of the compound. Cells were then washed three times with PBS and media supplemented with 10% FBS and containing appropriate concentration of each drug was added and cells were incubated under standard conditions for 24 h. For pre-treatment studies, cells at approximately 90% confluency were incubated with 100 μM andrographolide for 1 h, 30 min or 15 min prior to infection. HepG2 or HeLa cells were washed twice with PBS and infected with DENV 2 at MOI of 20 or 1, respectively under standard conditions. After 2 h infection cells were washed three times with PBS before the addition of complete media (without andrographolide) and incubated under standard conditions for 24 h. For post-infection treatment studies, cells were infected or mock infected as appropriate and 100 μM andrographolide was added in complete media at 0, 1, 2, 3, 4, 6, 9, and 12 h after infection. Cells were incubated under standard conditions until analyzed. For all experiments, supernatant and cells were harvested at appropriate time points, and all experiments were undertaken independently in triplicate with duplicate plaque assay. Control experiments were undertaken using 0.1% DMSO as vehicle.

2.6. Andrographolide treatment of cells (DENV 4)

HepG2 cells were seeded in six wells plates at a density obtain 90% confluency after 24 h. Cells were washed with PBS then mock infected or infected with DENV 4 (strain 1036) at MOI 1 and incubated at 37 °C for 2 h. Subsequently, cells were washed twice with PBS followed by culture with complete media supplemented with or without andrographolide at 100 μM or with 0.1% DMSO under standard condition. At the indicated times cells and supernatants were collected and all experiments were undertaken independently triplicate with duplicate plaque assay.

2.7. Flow cytometry

Mock or DENV infected cells (with or without compound treatment as appropriate) were harvested at appropriate time points and then incubated with 10% normal goat serum (NGS; Gibco BRL, Gaitherburg, MD) in PBS on ice for 30 min. The cells were washed twice with 1 ml of PBS followed by fixing with 200 μl of 4% paraformaldehyde in PBS-IFA at room temperature in the dark for 20 min. After washing twice with 1 ml of 1% BSA/PBS-IFA, the cells were permeabilized with 200 μl of 0.2% Triton X-100 in PBS-IFA for 10 min. Subsequently, the cells were washed twice with 1 ml of 1% BSA/PBS-IFA followed by overnight incubation with 50 μl of a pan specific mouse anti-dengue virus monoclonal antibody from hybridoma HB114 (Henchal et al., 1982) diluted 1:20 in 1% BSA/PBS-IFA with constant agitation at 4 °C. After washing 5 times with 1 ml of 1% BSA/PBS-IFA, the cells were incubated with 50 μl of a goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC; KPL, Guilford, UK) diluted 1:40 in 1% BSA/PBS-IFA for 1 h with constant agitation at room temperature in the dark. The cells were then washed 5 times with 1 ml of 1% BSA/PBS-IFA and resuspended in 200 μl of PBS-IFA. The fluorescence signal was analyzed by flow