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Magnesium lithospermate B and rosmarinic acid, two compounds present in *Salvia miltiorrhiza*, have potent antiviral activity against enterovirus 71 infections

Yi-Ching Chung [a], Feng-Chia Hsieh [b], Ying-Ju Lin [a], Tzong-Yuan Wu [c], Cheng-Wen Lin [d], Ching-Ting Lin [a], Nou-Ying Tang [a], Tzyy-Rong Jinn [a,*]

[a] School of Chinese Medicine, China Medical University, Taichung 40402, Taiwan, ROC
[b] Biopesticide Department, Agricultural Chemicals and Toxic Substances Research Institute, Taichung 41300, Taiwan, ROC
[c] Department of Science Technology, Chung Yuan Christian University, Chung Li 32023, Taiwan, ROC
[d] Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung 40402, Taiwan, ROC

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Salvianolic acid B (PubChem CID: 119177)
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ABSTRACT

The aim of this study was to identify the active ingredients responsible for the anti-EV71 activity produced by *Salvia miltiorrhiza* extracts. A pGS-EV71 IRES-based bicistronic reporter assay platform was used for rapid analysis of compounds that could specifically inhibit EV71 viral IRES-mediated translation. The analysis identified 2 caffeic acid derivatives, magnesium lithospermate B (MLB) and rosmarinic acid (RA), which suppressed EV71 IRES-mediated translation at concentrations of 30 μg/ml. We also found that MLB and RA inhibited EV71 infection when they were added to RD cells during the viral absorption stage. MLB had a low IC_{50} value of 0.09 mM and a high TI value of 10.52. In contrast, RA had an IC_{50} value of 0.50 mM with a TI value of 2.97. MLB and RA (100 μg/ml) also reduced EV71 viral particle production and significantly decreased VP1 protein production. We propose that these two derivatives inhibit EV71 viral entry into cells and viral IRES activity, thereby reducing viral particle production and viral RNA expression and blocking viral VP1 protein translation. This study provides useful information for the development of anti-EV71 assays and reagents by demonstrating a convenient EV71 IRES-based bicistronic assay platform to screen for anti-EV71 IRES activity, and also reports 2 compounds, MLB and RA, which are responsible for the anti-EV71 activity of *S. miltiorrhiza*.

1. Introduction

Enterovirus 71 (EV71) is a neurological pathogen mediating hand, foot, and mouth disease (HFMD). Infection by EV71 results in severe neurological complications and sudden death in infants and young children (Lum et al., 1998; Weng et al., 2010). Additionally, epidemiological studies indicate that EV71-induced HFMD is a global public health issue, especially in the Asia-Pacific region (Wong et al., 2010; Yip et al., 2013). However, there are no effective drugs and vaccines to treat and prevent EV71 infection (Huang and Shih, 2014; Wang and Liu, 2014; Yi et al., 2011). Thus, the search for agents with preventative and therapeutic effects against EV71 infections has garnered significant research attention.

Salvia miltiorrhiza radix, known as 'Danshen', is one of the most widely used traditional Chinese medicines (Zhou et al., 2005). The water-soluble extracts of *S. miltiorrhiza* have been reported to show anti-EV71 activity (Wu et al., 2007); however, its active ingredients are unknown. In this study, we investigated the caffeic acid derivatives of *S. miltiorrhiza*, because caffeic acid derivatives such as phenolic acids are the major water-soluble components present in *S. miltiorrhiza* extracts (Jiang et al., 2005; Li et al., 2009; Wang et al., 2007). Additionally, the caffeic acid derivatives of *S. miltiorrhiza* have been shown to possess structural diversity and a broad spectrum of biological activities (Jiang et al., 2005). Therefore, these derivatives merit further study and investigation. In this report, 5 *S. miltiorrhiza* caffeic acid derivatives (caffeic acid monomers and

* Corresponding author. Tel.: +886 4 22053366; fax: +886 4 22032295.
E-mail address: jinn@mail.cmu.edu.tw (T.-R. Jinn).

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oligomers), namely, sodium danshensu (SD; monomeric caffeic acid), rosmarinic acid (RA; dimeric caffeic acid), salvianolic acid A (Sal A; trimeric caffeic acid), salvianolic acid B (Sal B; tetrameric caffeic acid), and magnesium lithospermate B (MLB; tetrameric caffeic acid; Mg^{2+} salt of lithospermate B) were investigated.

The internal ribosome-entry site, known as the IRES element, plays an important role in EV71 viral protein translation via a cap-independent mechanism (Jackson, 1988; Martinez-Salas, 2008; Vagner et al., 2001); thus, the EV71 viral IRES was suggested as a potential target for anti-EV71 drugs (Gallego and Varani, 2002; Lin et al., 2013). For rapid and specific analyses of the candidate *S. miltiorrhiza* components responsible for inhibition of EV71 IRES activity, we used the pGS-EV71 bicistronic reporter assay platform with 2 reporter proteins, beta-galactosidase (β-gal) and secreted embryonic alkaline phosphatase (SEAP). This EV71 IRES-based bicistronic reporter assay platform, which was established by our laboratory in a previous study (Lee et al., 2005; Wu et al., 2007), provided a convenient assay to evaluate candidate components for inhibition of EV71 viral IRES-mediated translation and/or eukaryotic promoter-mediated translation. The findings reported herein elucidate the potential biological functions of caffeic acid derivatives from *S. miltiorrhiza*, particularly its anti-EV71 IRES activity.

2. Materials and methods

2.1. Reagents

Magnesium lithospermate B (MLB; PubChem CID: 6918234; Purity ≧98%) was purified from the dried roots of *S. miltiorrhiza* (Tzen et al., 2007). Sodium danshensu (SD; PubChem CID: 23711819; Purity ≧98%), Rosmarinic acid (RA; PubChem CID: 528179; Purity ≧96%), Salvianolic acid A (Sal A; PubChem CID: 5281793; Purity ≧95%), Salvianolic acid B (Sal B; PubChem CID: 119177; Purity ≧95%), and Amantadine (PubChem CID: 64150; Purity ≧99%) were purchased from Sigma-Aldrich (St. Louis. MO, USA). These components were dissolved in PBS at a concentration of 10 mg/ml and stored at 4 °C.

2.2. Cell culture and virus

COS-1 cell line, a fibroblast-like cell line derived from monkey kidney tissue, was purchased from Bioresource Collection and Research Center, Hsinchu, Taiwan. Human rhabdomyosarcoma (RD) cell line (ATCC accession no. CCL-136) and enterovirus 71 (EV71) (GeneBank accession number HM807310) were kindly provided by the clinical virology laboratory of China Medical University Hospital in Taichung, Taiwan. COS-1 cells and RD cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml) and maintained in 37 °C incubator with 5% CO_2.

2.3. Cytotoxicity assay

COS-1 cells (1.5×10^5 cells/well) or RD (1×10^5 cells/ml) were seeded into a 24-well plate and incubated for overnight, and then treated with different candidate components. After 24 h of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5 Diphny-l-tetrazolium bromide (MTT reagent; Sigma, St. Louis, MO) was added at 100 μl/well and incubated for 1 h, and then the DMSO (100 μl/well) was added to dissolve the crystals. The absorbance at 540 nm was measured by an ELISA reader (Molecular Devices, LLC, at Biocompare.com.). The viability of the untreated cells was taken as 100%. Viability (% of control) = 100 × (absorbance of treated sample)/(absorbance of control). Triplicate wells were used for each treatment.

2.4. Plasmids and transfection

Plasmid pGS-EV71 consists of an IRES element derived from enterovirus 71 (EV71), which is flanked by the β-galactosidase (β-gal) and secreted alkaline phosphatase (SEAP) reporter genes (Lee et al., 2005). The pGS-EV71 plasmid was transferred to COS-1 cells using the Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). COS-1 cells were plated onto 24-well plate at a density of 1×10^5 cells/well with approximate 75–80% confluence. The cells were repeatedly washed with serum-free media to remove all traces of sera prior to transfection. One microgram of plasmid was diluted in 200 μl of serum-free DMEM medium, and then 1 μl of the Lipofectamine reagent was added. The DNA–Lipofectamine mix was incubated for 20 min. Afterwards, the DNA–Lipofectamine complex was added to the COS-1 cells at a final volume of 0.5 ml of serum-free medium. The pGS-EV71 plasmid-transfected cells were incubated at 37 °C in 5% CO_2. After 5 h, the transfection media were replaced by media containing 10% FCS with various concentrations of the candidate components for 24 h. The treated cell supernatants and cell pellets were collected for the examination of the SEAP and β-gal activity, respectively. The determination of SEAP and β-gal activity is described below.

2.5. Measurement of SEAP and β-gal activity

The treated cell supernatants were collected for SEAP activity using BD Great EscApe SEAP detection kits (Clontech, Mountain View, CA). On the other side, the treated cell pellets were collected for the β-gal activity assay; the cell pellets were lysed in 300 μl of culture cell lysis reagent for 10 min. After centrifugation at 200*g* for 30 min, the lysate supernatant was measured using Luminescent β-galactosidase Detection Kit II (BD Biosciences, Franklin Lakes, NJ, USA). The chemiluminescent intensities reflecting relative β-gal activity and SEAP activity were detected with a chemical luminescence counter (Mithras LB 940; Berthold Technologies). To control for variation in transcription, SEAP activity was normalized to the β-gal activity in the cell lysates. The β-gal and SEAP activities of the untreated-transfected cells were considered as 100%.

2.6. Anti-EV71 infection assays

To determining the effects of MLB and RA on anti-EV71 infection, RD (1×10^5 cells/ml) were seeded into 24-well plates and incubated overnight (Lu et al., 2011; Zhu et al., 2011). The cells were infected with EV71 at an MOI of 3 and simultaneously treated with different concentrations of MLB or RA for 1 h. After virus absorption, the virus–medium mixture was removed and the cells were overlaid with 1 ml of culture medium containing 10% FCS (with the same concentration of MLB or RA as was initially added) until 24 h after infection. The EV71 induced-cytopathic effect (CPE) was observed by a microscope and quantitatively measured by MTT assay. The concentration that reduced the virus-induced CPE by 50% was taken as the IC_{50}, and RD cells infected by EV71 virus only were used as a positive control (taken as 100% CPE). To further validate the ability of MLB and RA to inhibit EV71 progeny virus yield, a plaque reduction assay was also carried out. RD cells (1×10^6 cells/well) were seeded into a six-well plate and incubated overnight. The cells were washed twice with PBS and then treated with different concentrations of MLB or RA while simultaneously being infected with EV71 at an MOI of 3. After adsorption for 1 h at 37 °C, the cells were washed twice and overlaid with 1 ml of plaque medium (containing the same concentration of MLB or RA as was initially added, 1.2% carboxymethylcellulose, and 2% FCS). After 48 h of incubation, the cells were fixed with 10% formaldehyde and stained with 1% crystal violet, and the plaques were observed. The plaque