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Gan-Lu-Siao-Du-yin, a prescription of traditional Chinese medicine, inhibited enterovirus 71 replication, translation, and virus-induced cell apoptosis

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ABSTRACT

Ethnopharmacological relevance: Gan-Lu-Siao-Du-yin (GLSDY) is a prescription of traditional Chinese medicine. GLSDY contains 11 ingredients and is commonly used for endemic diseases. Enterovirus 71 (EV71) is an endemic disease that can cause meningoencephalitis with mortality and neurologic sequelae without any effective management. It is unknown whether GLSDY is effective against EV71 infection.
Aim of the study: To test the hypothesis that GLSDY can protect cell from EV71-induced injury.
Materials and methods: Effects of a hot water extract of GLSDY on EV71 were tested in human foreskin fibroblast cells (CCFS-1/KMC) and human rhabdomyosarcoma cells (RD cells) by plaque reduction assay and flow cytometry respectively. Inhibition of viral replication was further examined by reverse quantitative RT-PCR (qRT-PCR). Its effect on viral protein translation and virus-induced apoptosis were examined by western blot.
Results: GLSDY was dose-dependently effective against EV71 infection ($p < 0.0001$) in both CCFS-1/KMC cells and RD cells. GLSDY was highly effective when supplemented after viral inoculation ($P < 0.0001$) with an IC_{50} of 8.7 μg/mL. GLSDY inhibited viral RNA replication ($P < 0.0001$), formation of viral structural proteins (VP0, VP1, VP2 and VP3) and non-structural proteins (protease 2B and 3AB). Furthermore, 300 μg/mL GLSDY is effective to inhibit virus-induced apoptosis possibly through direct inhibition of caspase-8 and indirectly by inhibition of Bax.
Conclusions: GLSDY is cheap and readily available to manage EV71 infection by inhibiting viral replication, viral protein formations, and EV71-induced apoptosis.

1. Introduction

Enteroviruses are small, non-enveloped, single-stranded, positive-sense RNA viruses of *Picornaviridae*. Enterovirus is one of the most common viruses causing symptomatic diseases, including CNS infections (Cohen, 2015). Among enteroviruses, enterovirus 71 (EV71) is a common agent that causes several large outbreaks of hand-foot-mouth disease (HFMD), pulmonary edema, and neurological diseases in the Asia-Pacific region (Hu et al., 2015; Kim, 2010; Luo et al., 2015; Solomon et al., 2010). EV71 has also caused small epidemics in United Kingdom (Bible et al., 2008), Greece (Siafakas et al., 2011) and United States (Fowlkes et al., 2008). EV71 infection can cause severe aseptic meningitis and brain-stem encephalitis. Brain-stem encephalitis, particularly affecting the medulla, associates with cardiopulmonary dysfunction that may cause death (Biswas, 2012; Khanh et al., 2012). EV71-

Abbreviations: ATCC, the American Type Culture Collection; CC_{50}, 50% Cytotoxic Concentration; CCFS-1/KMC, Human Foreskin Fibroblast Cell Line; EV71, Enterovirus 71; FCS, Fetal Calf Serum; GLSDY, Gan-Lu-Siao-Du-Yin; IC_{50}, Minimal Concentration Required to Inhibit 50% Cytopathic Effect; DMEM, Dulbecco's Modified Eagle's Medium; hr, Hour; M.O.I, Multiplicity of Infection; PBS, Phosphate-Buffered Saline; pfu, Plaque Forming Unit; RD cells, Human Rhabdomyosarcoma Cell; TCM, Traditional Chinese Medicine

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infected children who survived from both neurological and cardiopulmonary complications may display signs of neurologic sequelae (Chang et al., 2007). Therefore, EV71 infection is a major public health issue worldwide that needs to be effectively controlled. Unfortunately, no therapy has been approved to manage this miserable disease (Kok, 2015). EV71 is far more easily transmitted in the developing countries with poor public hygiene by the fecal–oral route (Brian et al., 2013; Thoa le et al., 2013). Economical factors can be a determinant to counteract the disease during outbreaks. A cheap, readily available, effective therapeutic modality is urgently needed.

In temperate climates, EV71 infection is commonly endemic in the summer and fall (Cohen, 2015). Several herbal prescriptions of traditional Chinese medicine (TCM) have been used to manage endemic infections in ancient China. Gan-Lu-Siao-Du-yin (GLSDY), also named Gan-Lu-Siao-Du-dan or Pu-Ji-Siao-Du-dan in its form of tablet, is a famous prescription of TCM that helps Chinese to counteract endemic diseases in the summer. GLSDY was first mentioned in Wen-Re-Jing-Wei, which was published in 1852 A.D. during the Qing Dynasty of China. Wen-Re-Jing-Wei is a TCM book providing medical prescriptions for clinicians to initiate symptoms-based management of various diseases. In this book, GLSDY has been claimed to effectively manage endemic diseases with fever, fatigue, and sore throat with ulcers. These symptoms are commonly found in HFMD (Cohen, 2015). GLSDY contains 11 ingredients (Table 1) and has been empirically used to manage enteroviral infection (Chen, 2009). Each ingredient of GLSDY has also been used to manage HFMD to ameliorate the symptoms and to shorten the course in combination with other herbs according to their traditional use (Zhang et al., 2010). Besides, among these ingredients, *Pogostemon cablin* (Kiyohara et al., 2012), *Artemisia capillaris* (Zhao et al., 2014) and active compounds from *Forsythia suspensa* (Li et al., 2014) have been proved to have antiviral activities against various DNA and RNA viruses. However, GLSDY does not have any proven antiviral activity against enterovirus. Therefore, the authors hypothesized that GLSDY has anti-EV71 to be beneficial to manage EV71 infection. We used both human foreskin fibroblast cell line (CCFS-1/KMC) and human rhabdomyosarcoma cell line (RD cells) to validate this hypothesis.

2. Materials and methods

2.1. Preparation of a hot water extract of GLSDY

All air-dried medicinal plants of GLSDY (Table 1) were collected from herbal shops in South Taiwan. A voucher specimen was prepared and deposited at Kaohsiung Medical University (KMU) Herbarium. Their authenticities were examined by at least two experts through morphological and anatomical identification. A hot water extract of GLSDY was prepared (Yen et al., 2014). Briefly, 100 g of air-dried ingredients of GLSDY were decocted for one hr with 1000 mL of distilled water each, and repeatedly for three times. The decoctions were collected, mixed, filtered by gauze, concentrated under reduced pressure, and lyophilized to dry. The w/w yield of GLSDY was 9.9%. The extracts were dissolved in Dulbecco's Modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 2% or 10% fetal calf serum (FCS) to the final concentrations of 10 μg/mL, 30 μg/mL, 100 μg/mL, and 300 μg/mL for bioactivity assay and up to 3000 μg/mL for cytotoxicity tests prior to the experiments.

2.2. Chromatographic conditions and test samples and standard reference preparation

Fingerprint of a hot water extract of GLSDY was examined on a Hitachi chromatograph system (HITACHI Co. Inc., Japan), equipped with a quaternary pump (L-7100), an autosampler (L-7200) and a UV–vis detector (L-7420). The chromatographic data were recorded and processed with the D-7000 Muti-HSM Manager software. A Mighty SIL RP-18 GP column (5 μm, 4.6 × 250 mm; Kanto Chemical Co. Inc., Tokyo, Japan) was used at room temperature. The mobile phase was composed of (A) aqueous phosphoric acid (0.1%, v/v) and (B) acetonitrile, using a gradient elution of 100–90% (A) at 0–2 min, 90–85% (A) at 2–10 min, 85–80% (A) at 10–22 min, 80–75% (A) at 22–36 min, 75–70% (A) at 36–45 min, 70–65% (A) at 45–52 min, 65–58% (A) at 52–60 min, 58–30% (A) at 60–70 min. The sample injection volume was 20 μL and the flow rate was 1.0 mL/min. The detection wavelength was set at 272 nm. Then, 200 mg dried powder of a hot water extract of GLSDY (HWE-GLSDY) was dissolved in 10 mL methanol and was extracted under sonication for 20 minutes. Studies have demonstrated chlorogenic acid in *Mentha haplocalyx* (Dong et al., 2015), *Forsythia suspensa* (Cui et al., 2010), and *Artemisia capillaries* (Tan et al., 2008; Yang et al., 2014b). Forsythin can be found in *Forsythia suspensa* (Cui et al., 1992). Baicalin and wogonoside were the main constituents of *Scutellaria baicalensis* (Islam et al., 2012; Zhou et al., 2009). Therefore, for quality control, we used four standard references to evaluate the quality and to establish the HPLC-UV chromatographic fingerprint of HWE-GLSDY. All the test samples were filtered through a 0.45 μm membrane filter before chromatographic analysis. The standard HPLC-UV fingerprint reference of HWE-GLSDY was recorded and compared to those of four standard references.

2.3. Human cell lines, virus, chemicals, and reagents

Human foreskin fibroblast cell line (CCFS-1/KMC) (Chiang et al., 1992) and rhabdomyosarcoma cell line (RD cell, ATCC CCL-136) were maintained in DMEM supplemented with 10% FCS and antimicrobials, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Reagents and medium for cell culture were

Table 1
Composition of Gan-Lu-Siao-Du-Yin (GLSDY).

Chinese medicine plant	Family	Weight (gm)	Used part	Voucher specimens[a]
Soapstone [$Mg_3 \cdot (Si_4O_{10}) \cdot (OH)_2$]		6.0		Talcum-1
Artemisia capillaris Thunb.	Compositae	4.4	Seedling	COM/ACP-7
Scutellaria baicalensis Georgi	Labiatae	4.0	root	LAB/SBC-9
Acorus gramineus Soland.	Araceae	2.4	Rhizome	ARA/AGM-2
Fritillaria cirrhosa D. Don	Liliaceae	2	Bulb	LIL/FCR-1
Clematis armandii Franch.	Ranunculaceae	2	Rattan and stem	RAN/CAM-2
Pogostemon cablin (Blanco) Benth.	Labiatae	1.6	plant shoot	LAB/PCL-1
Belamcanda chinensis (L.) DC.	Iridaceae	1.6	Rhizome	IRI/BCN-3
Forsythia suspensa (Thunb.) Vahl	Oleaceae	1.6	Fruits	OLE/FSP-2
Mentha haplocalyx Briq.	Labiatae	1.6	Stem and leaf plot	LAB/MHL-6
Amomum kravanh Pierre ex Gagnep.	Zingiberaceae	1.6	Fruits	ZIN/AKV-2

[a] Voucher specimens were deposited at Herbarium of Materia Medica Laboratory, School of Pharmacy, Kaohsiung Medical University.