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Ching-fang-pai-tu-san inhibits the release of influenza virus

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ABSTRACT

Ethnopharmacological relevance: Ching-fang-pai-tu-san (CFPTS) is a Chinese herbal decoction that is used as a cure for the common cold, fever, headache, and poor circulation. However, no previous studies have investigated the mode of action of CFPTS against influenza virus infections. To investigate the antiviral mechanism of CFPTS, we examined viral entry, transcription, translation, viral glycoprotein hemagglutinin (HA) transport, and budding of the influenza virus.

Materials and methods: The antiviral activity of nontoxic concentrations of CFPTS against influenza virus A/WSN/33 was examined by assaying (neutralization assay) its inhibition of the virus-induced cytopathic effects. The mode of CFPTS action was first examined with a time-of-addition assay of synchronized infections, followed by monitoring HA transport by immunofluorescence microscopy. Viral endocytosis was evaluated with attachment and penetration assays. The inhibition of viral replication was measured by quantitative real-time PCR, immunoblotting, and immunofluorescence microscopy. We also performed assays related to the inhibition of viral entry, such as neuraminidase activity and hemagglutinin activity assays.

Results: Based on the inhibition of the virus-induced cytopathic effect in Madin–Darby canine kidney cells, the EC_{50} of CFPTS was about 1.44 ± 0.22 mg/mL against influenza virus A/WSN/33. CFPTS displayed a broad spectrum of inhibitory activities against different strains of influenza A virus, as well as some enteroviruses. However, this extract proved less effective against clinical oseltamivir-resistant strains and influenza B viruses. CFPTS did not suppress viral RNA or protein synthesis. According to a time-of-addition assay, the antiviral mechanism of CFPTS may involve viral budding or intracellular viral glycoprotein transport. A plaque reduction assay showed that CFPTS reduced both the plaque size and plaque quantity. The intracellular transport of viral glycoprotein hemagglutinin was blocked by CFPTS by immunofluorescence microscopic analysis. Thus, it is possible that the antiviral mechanism of CFPTS might inhibit the assembly of progeny virions and/or their subsequent release.

Conclusions: Our results give scientific support to the use of CFPTS in the treatment of influenza virus infections. CFPTS has potential utility in the management of seasonal pandemics of influenza virus infections, like other clinically available drugs.

1. Introduction

Influenza virus is a respiratory pathogen that causes significant morbidity and mortality around the world. There have been four pandemic influenza outbreaks, in 1918 (Spanish flu, H1N1), 1957 (Asian flu, H2N2), 1968 (Hong Kong flu, H3N2), and 2009 (swine original influenza virus, SOIV, H1N1pdm) (Buchy et al., 2007; Guan et al., 2010; Michaelis et al., 2009; Yuen and Wong,

Abbreviations: AFM, atomic force microscopy; CC_{50}, 50% cytotoxic concentration; CFPTS, ching-fang-pai-tu-san; CPE, cytopathic effect; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate-buffered saline; EC_{50}, half maximal effective concentration; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde phosphate dehydrogenase; HA, hemagglutinin; HBSS, Hank's balanced salt solution; M1, matrix protein 1; M2, matrix protein 2; MDCK, Madin–Darby canine kidney; MEM, minimal essential medium; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MU-NANA, 2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; NA, neuraminidase; NEAA, nonessential amino acid; NP, nucleoprotein; PFA, paraformaldehyde; PBS, phosphate-buffered saline; pi, postinfection; P–v, peak-to-valley height; qRT–PCR, quantitative reverse transcription–PCR; Ra, average height; RD, rhabdomyosarcoma; Rrms, root-mean-square roughness; Rz, points mean roughness; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; vRNP, viral ribonucleoprotein.

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2005). In addition to humans, influenza virus can infect various other hosts, such as swine, cats, cattle, horses, and chickens (Suzuki, 2005). It is thought that genetic reassortment generated the above pandemic viruses (Guan et al., 2010). Recently, these various influenza viruses have been shown to undergo gene reassortment. For example, the SOIV genome has evolved into a composite of genes derived from human, swine, and avian influenza viruses (Guan et al., 2010; Shinde et al., 2009).

Influenza viruses belong to the family Orthomyxoviridae, contain negative-stranded RNA segments (Palese and Shaw, 2007), and are classified into A, B, and C types (Spackman, 2008). Influenza virus A can infect avian and mammalian species, whereas influenza virus B only infects humans (Palese and Shaw, 2007). Influenza virus C infects humans and swine, and can cause slight illness in children (Joosting et al., 1968; Kimura et al., 1997). Influenza virus A can be divided into different serotypes, based on two surface glycoproteins, neuraminidase (NA; 1–9 subtypes) and hemagglutinin (HA; 1–16 subtypes). The eight genomic segments encode 12 viral proteins, including the three transmembrane proteins of the viral envelope, HA, NA, and M2 (matrix protein 2). HA is the most abundant protein (about 80%), whereas NA accounts for about 17% of the envelope protein. HA binds exclusively to sialic acid and facilitates viral entry into the cell in the early stage of infection. NA promotes the release of virions by cleaving sialic acid (Palese and Shaw, 2007).

During the first stage of infection, HA binds to sialic acid receptors on the cell surface, with subsequent viral entry into the cell. NA also plays an important role in viral entry (Matrosovich et al., 2004; Su et al., 2009). The low-pH environment of the endosome activates the endosomal membrane to fuse with the virions, which then release viral ribonucleoproteins (vRNPs) into the cytosol. The vRNPs are transported into the nucleus and initiate viral transcription and replication. Viral mRNAs are exported to the cytosol and translated into viral proteins. The three envelope proteins (HA, NA, and M2) are transported to the endoplasmic reticulum (ER) and Golgi apparatus for maturation. Finally, these three proteins are conveyed to the cell surface. When new vRNPs arrive at the cell membrane, the progeny virions are assembled, followed by viral budding (Palese and Shaw, 2007).

Currently, there are two major classes of drugs used in the treatment of influenza: M2 ion-channel inhibitors and NA inhibitors. Adamantine derivatives of M2 inhibitors were the first drugs used to combat the influenza virus. Amantadine and rimantadine are representative of the adamantine derivative drugs and block viral replication after infection (Englund, 2002; Wang et al., 1993). However, drug-resistant strains have been found in both cell cultures and animals (Englund, 2002; Mast et al., 1991). Amantadine resistance has also been detected in human and avian H5N1 strains and SOIV (Hayden, 2006a, b; Mossad, 2009). NA inhibitors, including oseltamivir and zanamivir, are effective drugs against the influenza virus, but an increasing number of clinical strains have been confirmed as resistant to this class of inhibitors (Englund, 2002; Harrod et al., 2006; Mossad, 2009). Thus, increasing numbers of viruses have become resistant to current influenza drugs. Adamantine derivatives, for example, have proved ineffective in the treatment of all H3N2 strains and some SOIV strains (Garten et al., 2009; Mossad, 2009). These resistant viruses may cause outbreaks in the future, so the development of new anti-influenza drugs is vitally important. We screened traditional Chinese medicines that have been used for thousands of years to treat the symptoms of influenza, such as cough, rhinorrhea, and fever. We identified the antiviral activity of CFPTS by a cell-based neutralization qassay and we further determined its underlying antiviral mechanism.

2. Materials and methods

2.1. Cell culture and viruses

Madin–Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS; SAFC Biosciences, Kansas, USA), 2 mM L-glutamine (Gibco), 0.1 mM nonessential amino acid mixture (NEAA) (Gibco), 100 U/mL penicillin, and 0.1 μg/μL streptomycin (Sigma). Human lung carcinoma A549 cells were cultured in minimal essential medium (Invitrogen) containing 10% heat-inactivated FBS, 100 U/mL penicillin, and 0.1 μg/μL streptomycin. Human rhabdomyosarcoma (RD) cells were maintained in DMEM containing 10% heat-inactivated FBS, 100 U/mL penicillin, and 0.1 μg/μL streptomycin. Influenza viruses A/WSN/33 and PR8/34 were obtained from the American Type Culture Collection and propagated in MDCK cells. The sources and proliferation of the other influenza viruses, including H1N1pdm and the enteroviruses, have been described previously (Hsu et al., 2012).

2.2. Preparation of CFPTS extract

CFPTS contained 13 medicinal herbs and it was purchased from Sun Ten Pharmaceutical Co., Ltd (Cat. number G105H) (Taipei, Taiwan). CFPTS was provided as a powdered mixture that contained all of the ingredients and cornstarch. The plant species used, the plant parts used to produce the extracts and their relative quantitative composition, and the extraction procedure are shown in Table 1 for CFPTS. To prepare a CFPTS stock solution for use in the assays, CFPTS was weighed, dissolved in distilled water, and incubated at 37 °C in a water bath for 16 h. After sedimentation at 3000 × *g* for 10 min at 10 °C, the CFPTS supernatant was collected and subjected to a further round of centrifugation at 27,000 × *g* for 30 min. Finally, the aqueous extract of CFPTS was obtained by filtration through a 0.22 μm filter (Nalgene). The filtrate was treated as the stock and it was diluted directly in E0 (DMEM containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.1 mM NEAA mixture, and 2.5 μg/mL trypsin) without lyophilization. Original copies of the certificate of analysis (COA) were provided by the manufacturer (Fig. S5). The voucher specimen (CFPTS, lot number 060,941) used in the present study was deposited in the herbarium of Chang Gung University, Taoyuan, Taiwan. The HPLC analysis is also shown in Fig. S5. The HPLC chromatograms detected marker (indicator) constituents, i.e., glycyrrhizin and naringin, in *Glycyrrhiza uralensis* Fisch. and *Citrus aurantium* L., respectively. The extraction conditions and HPLC determination method have been described previously (Chang et al., 2002; Chang et al., 2011).

2.3. EC_{50} assay

A 96-well tissue culture plate was seeded with MDCK cells (2×10^4 cells/well) and incubated at 37 °C for 16–20 h under 5% CO_2. The medium was removed and the cells were washed once with Hank's balanced salt solution (HBSS). The cells were infected with influenza virus A/WSN/33 with MOI (multiplicity of infection) of 2×10^{-4} (equivalent to 9 $TCID_{50}$, the median tissue culture infective dose) and maintained in different concentrations of CFPTS in E0. After incubation at 37 °C under 5% CO_2 for 72 h, the medium was removed and the cells fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature, and then stained with 0.1% crystal violet for 20 min at room temperature. The density of the cells was measured with a microplate reader (VICTOR™ Multilabel Reader; Perkin Elmer, Shelton, CT). EC_{50} is defined as the concentration of CFPTS that inhibited the