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Mechanism by which ma-xing-shi-gan-tang inhibits the entry of influenza virus

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ABSTRACT

Ethnopharmacological relevance: Ma-xing-shi-gan-tang (MXSGT, aka *maxing shigan powder*), a Chinese herbal decoction, has been used for the treatment of the common cold, fever, and influenza virus infections. However, the underlying mechanisms of its activity against the influenza virus are not fully understood. In this study, we examined the antiviral effects of MXSGT in influenza-virus-infected MDCK cells and their underlying mechanisms, including the damage of the viral surface ultrastructure and the consequent inhibition of viral entry.

Materials and methods: The antiviral activity of nontoxic concentrations of MXSGT against influenza virus A/WSN/33 was examined by assaying (neutralization assay) its inhibition of the virus-induced cytopathic effects. The mode of MXSGT action was first examined with a time-of-addition assay of synchronized infections, followed by viral attachment and penetration assays. Viral endocytosis was evaluated with attachment and penetration assays. We also performed assays related to the inhibition of viral entry, such as neuraminidase activity, hemagglutinin activity, and phosphoinositide-3-kinase (PI3K)/AKT phosphorylation assays. The inhibition of viral replication was demonstrated by quantitative real-time PCR, immunoblotting, and immunofluorescence microscopy. The surface ultrastructure of the MXSGT-treated virus was revealed by atomic force microscopy.

Results: MXSGT exhibited an EC_{50} of 0.83 ± 0.41 mg/ml against influenza virus A/WSN/33 (H1N1), with broad-spectrum inhibitory activity against different strains of human influenza A viruses, including clinical oseltamivir-resistant isolates and an H1N1pdm strain. The synthesis of both viral RNA and protein was profoundly inhibited when the cells were treated with MXSGT. The time-of-addition assay demonstrated that MXSGT blocks the virus entry phase. This was confirmed with attachment and penetration assays, in which MXSGT showed similar inhibitory potencies (IC_{50} of 0.58 ± 0.07 and 0.47 ± 0.08 mg/ml). High-resolution images and quantitative measurements made with atomic force microscopy confirmed that the viral surface structure was disrupted by MXSGT. We also established that viral entry, regulated by the PI3K/AKT signaling pathway, was abolished by MXSGT.

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

Abbreviations: AFM, atomic force microscopy; CC_{50}, 50% cytotoxic concentration; CPE, cytopathic effect; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate-buffered saline; EC_{50}, half maximal effective concentration; FBS, fetal bovine serum; H, hemagglutinin; HBSS, Hank's balanced salt solution; HI, hemagglutination inhibition; KKT, ko-ken-tang; M1, matrix protein 1; 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; MXSGT, ma-xing-shi-gan-tang; NA, neuraminidase; NEAA, nonessential amino acid; NP, nucleoprotein; pi, postinfection; P-v, peak-to-valley height; PI3K, phosphoinositide-3-kinase; qRT–PCR, quantitative reverse transcription-PCR; Ra, average height; RBC, red blood cell; RD, rhabdomyosarcoma; Rrms, root-mean-square roughness; Rz, points mean roughness; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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1. Introduction

Influenza viruses cause respiratory illness and pandemic outbreaks worldwide. From the 1918 Spanish influenza virus (H1N1) pandemic, the 1957 Asian influenza virus (H2N2) pandemic, and the 1968 Hong Kong influenza virus (H3N2) pandemic to the 2009 swine-origin pandemic virus (H1N1pdm) pandemic, influenza has resulted in millions of deaths (Guan et al., 2010; Michaelis et al., 2009). The avian influenza virus (H5N1) and H1N1pdm are highly contagious in both humans and domestic animals (Michaelis et al., 2009; Sambhara and Poland, 2010), and antigenic shifts allow pandemic influenza viruses to adapt to different environments (Guan et al., 2010).

The influenza viruses belong to the *Orthomyxoviridae* family of negative RNA strains and have eight genomic segments. There are three types of influenza virus: A, B, and C. The influenza viruses A include different serotypes, defined according to their viral surface proteins neuraminidase (NA, N1 to N9 subtypes) and hemagglutinin (H, H1 to H16 subtypes) (Spackman, 2008). The eight viral genomic segments encode 12 proteins. The outside of the viral envelope contains three transmembrane proteins: H, NA, and M2 (Das et al., 2010). The H and NA proteins are abundant on the viral surface, constituting more than 95% of its proteins (Nayak et al., 2009). H binds specifically to sialic acid and has epitopes susceptible to neutralizing antibodies (Nayak et al., 2009; Nicholls et al., 2008). NA facilities the release of progeny virus by sialic acid cleavage at the viral budding step (Air and Laver, 1989). However, NA has recently been shown to facilitate viral entry (Matrosovich et al., 2004; Su et al., 2009).

After infection, the viral RNA is released into the cytosol, where its transcription and translation begin (Das et al., 2010). Many host cellular factors are involved in viral replication and antiviral signaling (Konig et al., 2010; Ludwig et al., 2003, 2006; Stertz and Shaw, 2011; Watanabe et al., 2010). The phosphoinositide-3-kinase (PI3K)/AKT signaling pathway is induced during the viral replication cycle. The influenza virus activates AKT phosphorylation at serine 473 during the viral entry and transcription steps (Ehrhardt et al., 2006). Inhibition of PI3K activity in the early stage of infection leads to a reduction in viral progeny production (Ehrhardt et al., 2007). Therefore, viral protein synthesis is reduced by the suppression of AKT phosphorylation (Shin et al., 2007; Wu et al., 2011).

There are two major classes of anti-influenza drugs currently available: M2 inhibitors and NA inhibitors. Adamantane derivatives belong to the M2 inhibitors and representative drugs include amantadine and rimantadine (Englund, 2002; Wang et al., 1993). However, when 7000 influenza viruses A were analyzed from 1994 to 2005, the number of clinical M2-resistant infections had increased markedly. In 1994–1995, 0.4% of viruses were resistant, but this had reached 12.3% in 2004–2005 (Bright et al., 2005). Two currently available NA inhibitors, oseltamivir and zanamivir, are effective in the treatment of influenza virus infections (Englund, 2002). However, oseltamivir-resistant viruses have become more prevalent and have quickly spread worldwide (Hurt et al., 2009; Yang et al., 2010). Therefore, the development of new antiviral drugs is desperately required.

The search for influenza-inhibiting drugs is particularly important with the emergence of new pandemic strains and the high rate of emergent influenza strains resistant to several existing influenza antiviral drugs (Dawood et al., 2009; Layne et al., 2009; Webster and Govorkova, 2006). In this study, we screened a collection of folk medicines that have been prescribed to treat fever, inflammation, and seasonal influenza in the search for influenza-inhibiting drugs. We identified an anti-influenza activity in ma-xing-shi-gan-tang (MXSGT, also known as ma-xing-gan-shih-tang) and determined the potential mode of action of MXSGT in antagonizing viral infections. A time-of-addition assay of different treatment protocols during one infectious cycle of viral replication was used to determine the step in which the inhibition by MXSGT was exerted. Viral uptake assays, in which viral attachment and penetration were monitored, demonstrated that MXSGT affects viral entry. Atomic force microscopy (AFM) was used to demonstrate that MXSGT disrupts the viral ultrastructure and hence reduces viral uptake.

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; JRH Biosciences), 2 mM L-glutamine (Gibco), 0.1 mM nonessential amino acid (NEAA) mixture (Gibco), 100 U/ml penicillin, and 0.1 μg/μl streptomycin (Sigma). Human rhabdomyosarcoma (RD) cells were cultured in DMEM containing 10% heat-inactivated FBS and penicillin/streptomycin. Human lung carcinoma A549 cells were cultured in minimal essential medium (MEM; Invitrogen) containing 10% heat-inactivated FBS and penicillin/streptomycin. Influenza viruses A/WSN/33 and PR8/34 were obtained from the American Type Culture Collection and were propagated in MDCK cells. The sources and proliferation of the other influenza viruses, including H1N1pdm, and enteroviruses have been described previously (Hsu et al., 2012).

2.2. Preparation of MXSGT extract

MXSGT, which is composed of *Ephedrae herba* (ma huang), *Armeniacae amarum semen* (ku-xing-ren), *Glycyrrhizae radix preparata* (gan-cao; licorice), and *Gypsum fibrosum* (shi-gao; calcium sulfate), was purchased from Sun Ten Pharmaceutical Co., Ltd (Taipei, Taiwan). Every 7.5 g of MXSGT consisted of 6.5 g of dry crude extract (from 8 g of *Ephedrae herba*, 6 g of *Armeniacae amarum semen*, 4 g of *Glycyrrhizae radix preparata*, and 16 g of *Gypsum fibrosum*) and 1 g of cornstarch. The specimen number (lot number 152042) of the MXSGT used in the present study was recorded and will be stored for 10 years. The HPLC profile of the MXSGT used in this study indicates that the chemical components contain amygdalin, ephedrine, and pseudoephedrine. The HPLC analysis and certificate of analysis are in Fig. S4. To prepare an MXSGT solution for assay, the MXSGT was first 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 MXSGT supernatant was collected and subjected to one more round of centrifugation at 27,000*g* for 30 min. Finally, the aqueous extract of MXSGT was obtained by filtration through a 0.22 μm filter (Nalgene).

2.3. EC_{50} assay

MDCK cells (2×10^4 cell/well) were seeded in a 96-well tissue culture plate and incubated at 37 °C for 16–20 h under 5% CO_2. The medium was removed and the cells washed once with Dulbecco's phosphate-buffered saline (DPBS). The cells were infected with virus (9 $TCID_{50}$, MOI = 2×10^{-4} for influenza virus A/WSN/33) in the presence of various concentrations of MXSGT 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). After incubation at 37 °C under 5% CO_2 for 72 h, the medium was removed and the cells fixed with 4% paraformaldehyde for 1 h at room temperature, followed by staining with 0.1%