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The effect of emodin, an anthraquinone derivative extracted from the roots of *Rheum tanguticum*, against herpes simplex virus *in vitro* and *in vivo*

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ABSTRACT

Aim of the study: Herpes simplex viruses (HSV-1 and -2) are important pathogens for humans and the discovery of novel anti-HSV drugs with low toxicity deserves great efforts. Rhubarb is one of the oldest and best-known traditional Chinese medicines. We initiated this study to test if emodin is the active ingredients from *Rheum tanguticum* (*R. tanguticum*, one of the Chinese Rhubarb) against HSV infection and to investigate its antiviral activity on HSV infection in tissue culture cells and in a mouse model.

Materials and methods: Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) was extracted and purified from *R. tanguticum* (cultivated at high mountainous area in Qinghai) and the purity was determined by high performance liquid chromatography. The antiviral experiments of emodin against HSV infection were performed *in vitro* and *in vivo*. *In vivo*, the HSV-infected mice were orally administered with emodin beginning at 24 h post-HSV exposures with dosages of 3.3 g/kg/day, 6.7 g/kg/day, and 11.3 g/kg/day, respectively, for 7 days.

Results: Emodin was found to inhibit the replication of HSV-1 and HSV-2 in cell culture at the concentration of 50 μg/ml with antiviral index of 2.07 and 3.53, respectively. The emodin treatment increased the survival rate of HSV-infected mice, prolonged survival time and showed higher efficacy of HSV elimination from brain, heart, liver and ganglion, compared to the viral controls. In addition, the antiviral activity of emodin was found to be equivalent to that of acyclovir *in vivo*.

Conclusions: Our results indicate that emodin has the anti-HSV activity *in vitro* and *in vivo* and is thus a promising agent in the clinical therapy of HSV infection.

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1. Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are common human pathogens of the family of Herpesviridae, and cause infections worldwide with an estimated 60–95% of human adults infected at least by one of them (Brady and Bernstein, 2004). Either type can establish lesions at any site of the human body with indistinguishable diseases (Patel et al., 2007). HSV is transmitted through direct contact of the infected secretions and presents the common biological features of herpesviruses, including latency and reactivation (Chakrabarty et al., 2004; Kleymann, 2005; Greco et al., 2007; Ramachandran and Kinchington, 2007). Clinical manifestations of HSV infection vary from asymptomatic infection to mucocutaneous lesions, life-threatening encephalitis, and fatal dissemination, depending on the portal of viral entry, host immune competence, and primary or secondary nature of the disease (Leflore et al., 2000; Nadelman and Newcomer, 2000; Brady and Bernstein, 2004)

To date, HSV infections are incurable and may persist during lifetime of the host, resulting in a series of psychosocial problems. Significant efforts have been made to develop vaccines and agents against HSV infections. However, no effective vaccine is currently available (Rajcani and Durmanova, 2006; Us, 2006; Ramachandran and Kinchington, 2007). Acyclovir (ACV) remained the reference treatment for more than thirty years after its discovery (Greco et al., 2007). Drug-resistant strains emerged due to the extensive clinical use of ACV and its analogues, such as valacyclovir, penciclovir and famciclovir. Although there are some novel treatments available, the uncertainty of their efficacies and the high costs limited their clinical use (Brady and Bernstein, 2004; Chakrabarty et al., 2004; Kleymann, 2005; Greco et al., 2007; Kovalchuk et al., 2007). Therefore, it remains a continuous need to discover antiviral agents against HSV with lower resistant rate, toxicity and cost.

Rhubarb is a group of plants that belong to the genus *Rheum* in the family *Polygonaceae* and is one of the oldest and best-known traditional Chinese medicines. Chinese Rhubarb includes *Rheum*

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tanguticum (R. tanguticum), Rheum palmatum, Rheum officinale, etc. The pharmaceutically relevant compounds in rhubarb are sennosides, anthraquinones, stilbenes, glucose gallates, naphthalenes, and catechins (Ye et al., 2007). Hsiang et al. (2001) observed that the extract of rhubarb prevented the process of HSV attachment and penetration. Wang et al. (2003) reported that the extract of rhubarb showed antiviral activity against HSV that was comparable to acyclovir *in vivo*. A number of anthraquinones and anthrones isolated from plants and lichens have been shown to exhibit virucidal activity and non-virucidal antiviral activity against enveloped viruses, including HSV (Meruelo et al., 1988; Schinazi et al., 1990; Tang et al., 1990; Andersen et al., 1991; Sydiskis et al., 1991; Barnard et al., 1992; Cohen et al., 1996; Hsiang et al., 2001; Semple et al., 2001). The anthraquinones in rhubarb are thus potentially the effective component against HSV. The anthraquinone derivatives in rhubarb include physcion, emodin, rhein, aloe-emodin, chrysophanol and their glucosides (Ye et al., 2007). Among these, emodin is the most abundant one (Huang et al., 2007). We initiated this study to test if emodin is the active ingredients from *R. tanguticum* against HSV infection and to investigate its antiviral activity HSV infection in tissue culture cells and in a mouse model.

2. Materials and methods

2.1. Cell culture, viruses, and animals

HEp-2 (human laryngeal carcinoma) cells were routinely grown in RPMI-1640 medium (HyClone) supplemented with 10% heat-inactivated fetal calf serum, 0.1% L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2. The test medium used for the cytotoxic assay and antiviral assays contained 2% of the appropriate serum. The overlay medium for the plaque assay consisted of RPMI-1640 medium plus 2% fetal calf serum, 2% agarose, and antibiotics as described above.

HSV-1 and HSV-2 used in this study were HSV-1F stain and HSV-2 333 stain. The viruses were propagated in HEp-2 cells. The virus titer was estimated from cytopathic effect (CPE) induced by viral infection and expressed as 50% tissue culture infectious doses/ml ($TCID_{50}$/ml) by Reed–Muench method.

In vivo experiments were carried out with specific-pathogen-free BALB/c mice, 5–7 weeks old, obtained from Animal Center of Wuhan University. The virus titer for inoculation was expressed as median lethal dose (LD50/0.1 mL) and the titers of isolated virus were expressed as PFU/ml determined by plaque assay. All the animal research was conducted in accordance with the internationally accepted principles and guidelines for Care and Use of Laboratory Animals of Wuhan University.

2.2. Preparation and purification of emodin

R. tanguticum were cultivated and collected from at high mountainous area in Qinghai, China. The extract was produced and purified by Department of Plant Chemistry, Hubei College of Traditional Chinese Medicine, Hubei, China. Fifty grams of the dried powdered roots of *R. tanguticum* was extracted by 8-fold of 85% ethanol under reflux three times (1.5 h each time). Concrete was obtained from the ethanol solution and dissolved in 500 mL H_2O overnight. The solution was mixed with 500 mL 80% ethanol: acetone (1:1, v/v, pH = 4.0) and followed by ultrasonication for 30 min. The final product was obtained after filtering and drying. The main component is emodin (3-methyl-1,6,8-trihydroxyanthraquinone). The quantity of emodin was observed and determined by high performance liquid chromatography (HPLC). The content of emodin monomer is 84% and has a little impurities (Hou et al., 2003). Acyclovir (ACV, 9-(2-hydroxyethoxymethyl)guanine) was synthesized by Keyi Pharmaceutical Co. Ltd., Hubei, China.

2.3. In vitro experiments

2.3.1. Cytotoxicity assay

HEp-2 cells were seeded at 3.5×10^4 cells per well in 96-well plates and grown at subconfluence. After removal of the growth medium, cells were incubated with various concentrations of emodin (5, 10, 100, 200, 300, 500, 1000, and 1500 μg/ml, dissolved in 200 μl test medium) for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2. The cytotoxicity of emodin was evaluated on the basis of the morphological changes of the cells under microscope.

2.3.2. Indirect immunofluorescence (IF) assay

The infected cells were mounted on 10-well slides, air-dried and fixed with ice-cold methanol at room temperature for 10 min. The slides were blocked with 5% BSA in PBS for 1 h at 37 °C followed by incubation with rabbit serum reactive against HSV for 1 h at 37 °C. The slides were then washed with PBS for three times and incubated with fluorescein isothiocyanate (FITC)-conjugated goat immunoglobulin against rabbit IgG (Santa Cruz, 1:6000) for 1 h at 37 °C. Results were observed under fluorescence microscope.

2.3.3. Drug treatment before virus infection

HEp-2 cells were preincubated with test mediums containing different concentrations of emodin (100, 200, 300 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2 for 8 h and 12 h, respectively. The cells were then washed with PBS for twice and challenged with 100 $TCID_{50}$/ml of HSV-1 and HSV-2, respectively. After 1 h incubation for virus adsorption, the cells were rinsed twice with PBS and further incubated with test medium for about 72 h until typical CPE was visible. The inhibition of virus-induced CPE was scored by observation under microscopy and the virus titration was measured by indirect IF assay. Four untreated virus controls and four uninfected cell controls were included in all assays. The antiviral index was calculated using the following formula: antiviral index = viral $\text{titers}_{drug\ therapy\ group}$/viral $\text{titers}_{viral\ control\ group}$. All data presented are results of experiments performed in triplicate.

2.3.4. Virucidal assay

Viral suspensions containing 100 $TCID_{50}$/ml of virus were incubated with an equal volume of medium containing different concentrations of emodin (5, 10, 20, 50, 100, 200, 300 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2 for 6 h, 12 h, and 24 h, respectively. One hundred microliters of the mixed suspensions were then added to subconfluent monolayers of HEp-2 cells. After 1 h incubation for virus adsorption, the mixed suspensions were removed; the cell monolayers were rinsed carefully with PBS and maintained in test medium at 37 °C in a humidified atmosphere containing 5% CO_2 for 72 h. The virucidal effect was determined using indirect IF assay following the procedures described above.

2.3.5. Drug treatment after virus infection

The experiment was carried out as stated above with the following difference: monolayers of subconfluent HEp-2 cells were challenged with 100 $TCID_{50}$/ml HSV-1 and -2 for 1 h. The cells were washed with PBS and overlaid with 200 μl test medium containing different concentrations of emodin (5, 15, 25, 50, 100, 200, 300 μg/ml). The antiviral effect was determined using indirect IF assay following the procedures described above.

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