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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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**A B S T R A C T**

**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 \(\mu\)g/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 Kitchington, 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 Kitchington, 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 famiclovir. 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*...
tanguticum (R. tanguticum), Rheum palmatum, Rheum officinale, etc. The pharmaceutically relevant compounds in rhubarb are sennosides, anthraquinones, stilbenes, glucose gallates, naphthothenes, 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% CO2. 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 strain 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 (TCID50/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. 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 H2O 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 × 104 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) in a humidified atmosphere containing 5% CO2. 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% CO2 for 8 h and 12 h, respectively. The cells were then washed with PBS for twice and challenged with 100 TCID50/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 titer of drug therapy group/viral titer of viral control group. All data presented are results of experiments performed in triplicate.

2.3.4. Virucidal assay

Viral suspensions containing 100 TCID50/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% CO2 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% CO2 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 TCID50/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.
The antiviral effect of emodin and ACV on the replication of HSV-1 and HSV-2.

| Compound | Dosage (µg/ml) | HSV-1 | HSV-2 |
|----------|----------------|-------|-------|
| Emodin   |                |       |       |
| 5        | 5.4 ± 0.24 (1.03) | 6.0 ± 0.27 (1.00) |
| 15       | 5.4 ± 0.25 (1.11) | 4.4 ± 0.21 (1.50) |
| 25       | 4.1 ± 0.18 (1.46) | 3.1 ± 0.17 (1.94) |
| 50       | 2.9 ± 0.21 (2.07) | 1.7 ± 0.24 (3.53) |
| 100      | 1.9 ± 0.21 (3.21) | 1.1 ± 0.14 (5.35) |
| 200      | 1.1 ± 0.09 (5.55) | 0.6 ± 0.11 (10.0) |
| 300      | 0.7 ± 0.11 (8.71) | 0.2 ± 0.14 (30.0) |
| Acyclovir|                |       |       |
| 5        | 5.7 ± 0.25 (1.05) | 5.8 ± 0.21 (1.03) |
| 15       | 5.6 ± 0.23 (1.07) | 5.6 ± 0.24 (1.07) |
| 25       | 4.5 ± 0.20 (1.20) | 3.6 ± 0.21 (1.67) |
| 50       | 2.5 ± 0.22 (2.40) | 2.1 ± 0.23 (2.86) |
| Control  | 6.1 ± 0.31       | 6.0 ± 0.34       |

* Viral titer represent geometric mean ± SD of three independent experiments.

2.4. In vivo experiments

BALB/c mice were infected intracerebrally with 20 µl viral suspension containing 100 LD<sub>50</sub>/0.1 ml of HSV-1 or -2. The mice infected with virus were randomly divided into 6 groups; emodin at a dose of 3.3, 6.7, 11.3 g/kg/day, and acyclovir at a dose of 0.1 g/kg/day were orally administered three times daily to the mice (at 8 h intervals) for 7 days beginning 24 h post-virus exposure. The placebo controls received 0.9% sodium chloride; the virus controls (at 8 h intervals) for 7 days beginning 24 h post-virus exposure. The virus medium was removed after 2 h incubation and replaced with overlay medium containing 2% agarose. The agarose overlay was virus medium removed after 2 h incubation and replaced with overlay medium containing 2% agarose. The agarose overlay was removed after incubation for 72 h. The cells were fixed by 10% formalin and stained with 0.5% crystal violet. Plaques were counted.

2.5. Statistical analysis

The data were analyzed by SPSS 13.0 software. The data of in vitro experiments was analyzed using Student’s t-test, and that of in vivo experiments was analyzed using Wilcoxon test for survival rates, analysis of variance (ANOVA) for mean time to death (MTD) and Kaplan–Meier method for survival analysis.

3. Results

3.1. In vitro experiment

3.1.1. Cytotoxicities of emodin on HEp-2 cells

The cytotoxicities of emodin for HEp-2 cells were evaluated. Subconfluent monolayers treated with emodin at concentrations ranging from 5 to 300 µg/ml did not show any visible changes in cell morphology or cell density. Cells treated with emodin at concentrations of 5, 15, 25, and 50 µg/ml respectively. No virucidal effect of emodin was observed. The mixed suspension could still infect cells and propagates. The direct inhibitory effect of emodin was not able to block viral adsorption to HEp-2 cells.

3.1.2. Drug treatment before infection

Cells were treated with emodin and subsequently infected with HSV-1 or HSV-2 as described in Section 2. Emodin at concentrations of 100, 200, and 300 µg/ml did not show any inhibitory activity against HSV-1 or HSV-2. The titers were between 5.8 ± 0.24–6.1 ± 0.18, and the antiviral indexes were assigned.

3.1.3. Virucidal activity

To investigate the direct inactivating effect of emodin, viruses were treated for 6 h, 12 h, and 24 h with concentrations of emodin ranging from 5 to 300 µg/ml, respectively. No virucidal effect of emodin was observed. The mixed suspension could still infect cells and propagates. The mixture treated with 0.1–1.02 g/ml did not show any visible changes in cell morphology.

3.1.4. Drug treatment after infection

Subconfluent cells were infected with virus and then incubated with the drugs as described in Section 2. As shown in Table 1, the effective dose of emodin on HSV-1 started from 50 µg/ml, and 25 µg/ml on HSV-2. The virus titers at these dosages decreased 2-fold compared with the virus control group (p<0.01). The antiviral effect enhanced correspondingly with the increase of the dosage. The antiviral index of emodin on HSV-2 was 3.4 times as that on HSV-1 when the concentration reached 300 µg/ml. The antiviral activity of emodin against HSV-2 was stronger than that against HSV-1 at the same concentrations according to the antiviral index. The antiviral effects of emodin and ACV are equivalent at concentrations of 5, 15, 25, and 50 µg/ml regarding to the antiviral index (p>0.05).

3.2. In vivo experiment

3.2.1. Characteristics of HSV infection on mice

The signs of viral infection were observed in the mice at Day 4 after inoculation, such as weight-loss, tendencies to huddle, ruffled fur, paralysis of limbs, spasm and diminished vitality. The clinical manifestations showed no difference between the mice infected with HSV-1 and HSV-2. However, HSV-2 infected mice showed 1
6.7 g/kg/day emodin group showed favorable survival rates in both HSV subtype infected mice and longer MTD in HSV-1 infected mice (4.8 ± 0.3 days) compared to ACV treatment (19.0 ± 1.5) group (p < 0.01).

3.2.2. Elimination of viral antigens in the HSV infected mice

The viral antigens in the organs of the infected mice (brain, heart, liver, and ganglion) were detected by indirect IF assay. Viral antigens were positive in all of the organs at the early stage of infection (within 1 week after infection). The viral antigens disappeared over different lengths of time corresponding to various dosages of the treatment medicines (Table 2).

3.2.3. Changes of virus titers in HSV infected mice

The changes of the virus titers in brain, heart, liver and ganglion of the HSV-1 and HSV-2 infected mice from different groups are shown in Fig. 2. The administration of emodin (6.7 g/kg/day and 11.3 g/kg/day groups) significantly reduced the virus titers of mice organ homogenates compared to 0.9% NaCl control group (p < 0.05), and the time needed for virus elimination was dose-dependent. The virus titers in brains, hearts and livers of the emodin groups began to decrease at day 5 after treatment (day 6 after infection). The reduction of virus titers in the ganglion was observed a 2-day delay compared to other organs. The 6.7 g/kg/day and 11.3 g/kg/day emodin group showed faster virus elimination than 3.3 g/kg/day group (p < 0.05). These results suggest that emodin is effective for treatment of HSV infection.

4. Discussion

In the current study, we demonstrated the antiviral activity of emodin, extracted from R. tanguticum, against HSV-1 and HSV-2 both in vitro and in vivo. In vitro, emodin could not prevent HEp-2 cells from HSV infection in the pre-treatment assay, nor could it show virucidal effect against HSV-1 and HSV-2. However, it presented antiviral activities after HSV infection. Our results revealed that the antiviral activity of emodin was more potent against HSV-2 than HSV-1. However, the decrease of CPE and viral titers in culture media was dose-dependent in both HSV types. These were equivalent to the antiviral activities of ACV against both HSV types. Our results imply that emodin may inhibit HSV biological synthesis rather than directly inactivating the viruses or blocking their absorption to the susceptible cells in vitro.

We employed an HSV-infected BALB/c mice mouse model which can be used to screen and evaluate the anti-HSV agents. The emodin-treated mice showed better survival rate, prolonged MTD, decreased viral titers and faster elimination of virus in tissues compared to the virus control group. The most effective dose was 6.7 g/kg/day which was equivalent to ACV at a dose of 0.1 g/kg/day. Mice died in 6.7 g/kg/day emodin groups after 20 days due to the remnant virus existing in the organs (brain and ganglion, other than heart and liver).

Hsiang and Ho (2008) found that emodin specifically inhibited HSV-1 UL 12 activity, which is a viral protein involved in viral DNA processing and capsid egression, and then exerted its antiviral activity by accumulating nucleocapsids in the nucleus and reducing the HSV-1 yields. This can explain why emodin decreased HSV replication in our experiment. Several other studies demonstrated that emodin inhibited casein kinase 2 (CK2), which involves in the phosphorylation of many viral proteins that are essential to their life cycle (Yim et al., 1999; Battistutta et al., 2000). The potent antiviral capacity of emodin could also be based on its ability to disrupt the lipid bilayer, resulting in the effective inactivation of envelope virus (Alves et al., 2004).

HSV-2 infection is currently the most common cause for genital ulcerative disease, which is one of the risk factors for human
### Table 2
The effect of emodin on viral antigen changes in mice organs.

| Virus type | Compound and dosage (g/kg/day) | The time of the viral antigen disappearing (days after treatment) |
|------------|--------------------------------|---------------------------------------------------------------|
|            |                                | Brain | Heart | Liver | Ganglion |
| HSV-1      | Emodin group                   | NO    | NO    | NO    | NO       |
|            | 3.3                             |       |       |       |          |
|            | 6.7                             | 16    | 16    | 10    | 20       |
|            | 11.3                            | 12    | 16    | 10    | 20       |
|            | ACV group (0.1)                 | 12    | 16    | 10    | 20       |
|            | 0.9% NaCl                       | NO    | NO    | NO    | NO       |
| HSV-2      | Emodin group                   | NO    | NO    | NO    | NO       |
|            | 3.3                             |       |       |       |          |
|            | 6.7                             | 12    | 12    | 8     | 16       |
|            | 11.3                            | 12    | 16    | 8     | 20       |
|            | ACV group (0.1)                 | 12    | 10    | 10    | 16       |
|            | 0.9% NaCl                       | NO    | NO    | NO    | NO       |

**Note:** NO means “not disappeared”.

immunodeficiency virus (HIV) acquisition (Celum et al., 2004; Anuradha et al., 2008). Studies have shown that HIV-infected patients have a significant seroprevalence of HSV-2 (Chen et al., 2000; Mbopi-Keou et al., 2000; Mostad et al., 2000; Anuradha et al., 2008), and the latter alters the host immunity (Sheth et al., 2008) and facilitates HIV transmission and acquisition (Celum, 2004). Anti-HSV therapy should therefore be evaluated as a strategy for HIV prevention (Celum et al., 2005) and may benefit HIV-infected...
patients as well (Abu-Raddad et al., 2008; Mayaud et al., 2008). In our study, emodin showed potent anti-HSV-2 activity both in vitro and in vivo. It may serve as a promising agent in combating HSV-2 and simultaneously prevent the high risk population from acquiring HIV.

In summary, our results indicated that emodin was an effective antiviral agent against both HSV-1 and HSV-2 infection. It was observed to be more potent in inhibiting HSV-2 than HSV-1. Other studies have also demonstrated antiviral activities of emodin against some enveloped viruses, such as hepatitis B virus, human cytomegalovirus, and severe acute respiratory syndrome-corona virus; and non-enveloped viruses, such as poliovirus (Barnard et al., 1992; Cohen et al., 1996; Semple et al., 2001; Shuangsoo et al., 2006; Ho et al., 2007; Hsiang and Ho, 2008). Considering these findings, emodin may potentially play a significant role in anti-HSV management with broad spectrum of antiviral activities.

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