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Enhanced absorption and inhibited metabolism of emodin by 2, 3, 5, 4'-tetrahydroxystilbene-2-*O*-*β*-D-glucopyranoside: Possible mechanisms for Polygoni Multiflori Radix-induced liver injury

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[ABSTRACT] Polygoni Multiflori Radix (PMR) has been commonly used as a tonic in China for centuries. However, PMR-associated hepatotoxicity is becoming a safety issue. In our previous *in vivo* study, an interaction between stilbenes and anthraquinones has been discovered and a hypothesis is proposed that the interaction between stilbene glucoside-enriching fraction and emodin may contribute to the side effects of PMR. To further support our previous *in vivo* results in rats, the present *in vitro* study was designed to evaluate the effects of 2, 3, 5, 4'-tetrahydroxystilbene-2-*O*-*β*-D-glucopyranoside (TSG) on the cellular absorption and human liver microsome metabolism of emodin. The obtained results indicated that the absorption of emodin in Caco-2 cells was enhanced and the metabolism of emodin in human liver microsomes was inhibited after TSG treatment. The effects of the transport inhibitors on the cellular emodin accumulation were also examined. Western blot assay suggested that the depressed metabolism of emodin could be attributed to the down-regulation of UDP-glucuronosyltransferases (UGTs) 1A8, 1A10, and 2B7. These findings definitively demonstrated the existence of interaction between TSG and emodin, which provide a basis for a better understanding of the underlying mechanism for PMR-induced liver injury.

[KEY WORDS] Polygoni Multiflori Radix; Hepatotoxicity; Emodin; 2, 3, 5, 4'-Tetrahydroxystilbene-2-*O*-*β*-D-glucopyranoside; Component interaction

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Introduction

Polygoni Multiflori Radix (PMR), also known as Heshouwu in Chinese, originated from the dried root of *Polygonum multiflorum* Thunb. (Family Polygonaceae), has been widely used in many prescriptions for the treatment of various diseases associated with aging in Asian countries for centuries [1]. Modern pharmacological researches have revealed that PMR exhibits many biological activities such as immuno-enhancement, anti-hyperlipidaemia, neuroprotective, anti-cancer, and anti-inflammatory effects [2]. Nevertheless, PMR-induced liver injury in clinic has been constantly reported in recent years [2-3]. A retrospective study on herb-induced liver injury (HILI) shows that PMR is ranked first among all Chinese herbal medicines listed [4]. Supervisions and managements of the clinical application of PMR are strengthened in Canada, British, and Australia [3]. In China, a series of pharmacovigilance reports about usage of PMR-containing preparations have been announced by drug administration agency [5].

Although many investigations on the toxic ingredients of PMR have been conducted, there is still no clear consensus [6-12]. In general, anthraquinones were considered to be hepatotoxic components in PMR [6]. Our previous cytotoxicity tests have demonstrated a concentration- and time-dependent toxic effect of emodin on L02 cells [8]. Lv *et al*. have compared the cytotoxicities of different extracts of PMR in L02 cells, and found that the ethanolic extract is more toxic than the water extract due to its higher emodin levels [6]. However, several *in vivo* studies have revealed that free anthraquinones have very poor bioavailability. Anthraquinones such as emodin and

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physcion are rapidly and largely converted into glucuronide conjugates in intestine undergoing phase II metabolism, resulting in trace or even undetectable levels of anthraquinones in plasma of rats [13-16]. Thus, it is debatable whether anthraquinones are fully responsible for the toxic effects of PMR.

Recently, we have proposed a hypothesis that the interaction between stilbenes and anthraquinones might contribute to the PMR-induced liver injury, and an interaction between these two types of components has been discovered by our research team [17]. The exploratory study shows that the pharmacokinetics of emodin is significantly influenced, and the *in vivo* exposure of emodin is steadily increased after the treatment of stilbene glucoside fraction. Thus, to verify the *in vivo* results, the present study was designed to evaluate interaction between two major components co-occurring in PMR, 2, 3, 5, 4'-tetrahydroxystilbene-2-*O*-*β*-D-glucopyranoside (TSG, the predominant stilbene in PMR) and emodin (the representative anthraquinone compound in PMR). In the experimental study, a high-performance liquid chromatography-mass spectrometry (HPLC-MS) method was established and employed for qualitative and quantitative determination of emodin in incubation systems of Caco-2 cells and human liver microsomes by TSG treatment. Gene expression analysis in Caco-2 cells was performed for investigating the regulation of UDP-glucuronosyltransferases (UGTs) expression with a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) method.

Materials and methods

Chemicals and reagents

TSG, emodin, aloe-emodin, and phlorizin were purchased from Chengdu Must Bio-technology Co. (Chengdu, China). MK-571, dulbecco's modified eagle medium (DMEM), and Hank's balanced salt solution (HBSS) were from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Sijiqing (Hangzhou, China). Bicinchoninic acid (BCA) assay kit was from Beyotime (Shanghai, China). Uridine 5'-diphosphate glucuronic acid (UDPGA), alamethicin, and saccharolactone were from Sigma (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris) was from Aladdin (Shanghai, China). Human liver microsomes were purchased from BD Gentest (Franklin Lakes, NJ, USA). Acetonitrile of HPLC grade was purchased from TEDIA Company (Fairfield, OH, USA). Formic acid (HPLC grade) was from ROE (Neward, New Castle, DE, USA). Deionized water was prepared using a Milli-Q purification system produced by Millipore (Milford, MA, USA).

Caco-2 cell culture

Caco-2 cells were obtained from Cell Bank, Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U·mL^{-1} of penicillin, 100 μg·mL^{-1} of streptomycin and maintained in a humidified atmosphere of 95% O_2−5% CO_2 at 37 ºC. The pH of the medium was maintained at 7.4. At the beginning of the experiment, when plated cells had reached about 85% confluence, the growth medium was removed, the cells were washed with PBS, and fresh medium was used as base for treatment with the drugs.

Cellular uptake in Caco-2 cells

Cellular uptake was assayed as described previously [18], with minor modifications. Caco-2 cells were treated in 6-well culture plates. The content of TSG was usually higher than emodin in PMR, so we set the concentrations of TSG no less than those of emodin. The cells were exposed to mixtures of emodin (50 μmol·L^{-1}) and TSG at different proportions in 1 : 1 and 1 : 10, or treated with emodin only (control), and incubated for 5, 15, 30, 60, 90, and 120 min. Additionally, SGLT1 inhibitor phlorizin (50 μmol·L^{-1}) and MRP2 inhibitor MK-571 (50 μmol·L^{-1}) were respectively subjected to the Caco-2 cells incubation system to investigate the absorption of emodin. At the end of the incubation, the cells were washed three times with HBSS before repeated freezing-thawing, and then the tested samples were centrifuged at 13 000 r·min^{-1} for 15 min at 4 ºC after vortexing for 2 min. The supernatant (200 μL) was extracted twice with 1 mL of ethyl acetate followed by the addition of aloe-emodin as internal standard (IS). The resultant organic layer was dried under nitrogen and thendissolved in 100 μL of methanol for HPLC-MS analysis. Another 200 μL of supernatant was taken to determine the protein concentration with BCA assay kit, and the emodin levels were expressed as μg·mL^{-1} per mg protein.

Incubation with human liver microsomes

The assays were conducted as described previously [14], with slight modifications. Emodin (10 μmol·L^{-1}) and TSG (10 and 100 μmol·L^{-1}) in methanol were added to human liver microsomes (0.25 μg protein/mL) suspended in Tris-HCl buffer (pH 7.4) containing alamethicin (1.25 μg·mL^{-1}), $MgCl_2$ (10 mmol·L^{-1}), and saccharolactone (5 mmol·L^{-1}) in a final volume of 200 μL. The mixture was incubated at 37 ºC for 5 min before adding UDPGA (1 mmol·L^{-1}) to initiate the reaction. After 15 min incubation, the reaction was quenched with 200 μL of ice acetonitrile (containing 10 μmol·L^{-1} of aloe-emodin) at −20 ºC. The mixture was centrifuged at 13 000 r·min^{-1} for 15 min at 4ºC. The supernatant (20 μL) was injected to HPLC-MS for analysis.

Semi-quantitative RT-PCR analysis

The Caco-2 cells were treated with TSG (25, 50, and 100 μmol·L^{-1}) or vehicle only (DMSO) for 24 h. Total RNA was isolated from Caco-2 cells using Trizol reagent (Invitrogen, USA) in accordance with the manufacturer's protocol. A two-step RT-PCR was conducted by reverse transcribing 1 μg of total RNA to cDNA using Reverse Transcription Reagents (TaKaRa, Japan). The DNAs of UGT 1A1, UGT 1A3, UGT 1A8, UGT 1A9, UGT 1A10, UGT 2B4 and UGT 2B7 were prepared by RT-PCR. cDNA was denatured at 95 ºC for 5 min and subsequently submitted to various amplification cycles composed of 94 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 20 s, for 38 cycles. The final extension step was performed at 72 ºC