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Emodin-induced hepatotoxicity was exacerbated by probenecid through inhibiting UGTs and MRP2

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ABSTRACT

Aggravating effect of probenecid (a traditional anti-gout agent) on emodin-induced hepatotoxicity was evaluated in this study. 33.3% rats died in combination group, while no death was observed in rats treated with emodin alone or probenecid alone, indicating that emodin-induced (150 mg/kg) hepatotoxicity was exacerbated by probenecid (100 mg/kg). In toxicokinetics-toxicodynamics (TK-TD) study, aspartate aminotransferase (AST) and systemic exposure (area under the serum concentration-time curve, *AUC*) of emodin and its glucuronide were significantly increased in rats after co-administrated with emodin and probenecid for 28 consecutive days. Results showed that the increased *AUC* (increased by 85.9%) of emodin was mainly caused by the decreased enzyme activity of UDP-glucuronosyltransferases (UGTs, decreased by 11.8%–58.1%). In addition, *AUC* of emodin glucuronide was increased 5-fold, which was attributed to the decrease of multidrug-resistant-protein 2 (MRP2) protein levels (decreased by 54.4%). Similarly, *in vitro* experiments proved that probenecid reduced the cell viability of emodin-treated HepG2 cells through inhibiting UGT1A9, UGT2B7 and MRP2. Our findings demonstrated that emodin-induced hepatoxicity was exacerbated by probenecid through inhibition of UGTs and MRP2 *in vivo* and *in vitro*, indicating that gout patients should avoid taking emodin-containing preparations in combination with probenecid for a long time.

1. Introduction

Emodin, an active anthraquinone derivative from *Rhubarb*, *Polygonum multiflorum*, and *Polygonum cuspidatum*, widely exists in > 800 kinds of Chinese medicine preparations (Dong et al., 2016). Emodin has been used as an active constituent of many herbal laxatives in the past (Qin et al., 2011; Zheng et al., 2014). In the recent years, it is often used for diminishing inflammation (Wang et al., 2008; Shrimali et al., 2013), treating gouty arthritis (Lin, 2012; Wang et al., 2014; Zhang, 2015; Liu and Zhang, 2017), and protecting kidney (Bi et al., 1982; Sheng et al., 1994; Khan et al., 2014) in clinical practice. Concomitant intake of herbal medicines and western medications is a usual practice in some chronic disease treatments and is widely accepted by the Chinese public. Common antiphlogistic preparations containing emodin (*e.g.*, Yi-Qing capsule, San-Huang tablet, and Da-Huang-Zhe-Chong pill) are frequently used by the public, especially the patients who are taking western medications. In addition, emodin-containing herbal medicines have also been wittingly used in combination with western medications to treat disease in some cases (Bhadauria, 2010; Zhang et al., 2013). Combinatorial prescription of probenecid and emodin-containing herbal medicines (*e.g.*, Tong-Feng-Shu tablet, Chuan-Hu-Tong-Feng mixture, and Tong-Feng-Shu capsule) are frequently issued by the doctor in China and usually consumed by the Chinese chronic gout patients for quite a long time. The rationale for the combination is that probenecid increases the elimination of urate (Robbins et al., 2012; Jones et al., 2017) while emodin could effectively treat gouty arthritis and protect kidney (Bi et al., 1982; Lin, 2012; Wang et al., 2014; Zhang, 2015; Zeng et al., 2016) since gout is associated with high incidence of renal impairment (Curiel and Guzman, 2012). Moreover, some herbal preparations containing emodin (such as San-Huang capsule and Rhubarb Oyster Decoction) are self-developed hospital preparation and not commercially available (Zhang, 2015; Liu and Zhang, 2017). In most cases, clinical data and toxicological studies of such combinatorial preparations are lacking.

Gout is a chronic disease and most medicine preparations for it need to be taken for a long time. Recently, cases of liver injury induced by

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herbs are increasingly being reported because most herbal medicine preparations need to be taken at high doses for a long period of time (Dong et al., 2014; Li et al., 2016; Tian et al., 2016). Some studies showed that short-term use of low doses of emodin might result in liver protection, whereas long-term use of high doses of emodin are more likely to cause liver toxicity (National Toxicology, 2001; Wang et al., 2011; Li et al., 2012; Islam et al., 2015; Dong et al., 2016). Moreover, some interactions between emodin-containing herbal medicines and therapeutic agents leading to adverse outcome are also reported (Lin et al., 2015; Wang et al., 2015). Thus, the precaution needs to be taken on possible interactions between emodin and probenecid to avoid severe damage.

Probenecid, one of the most useful anti-gout drugs, is used clinically in treatment of chronic gout diseases (Robbins et al., 2012; Jones et al., 2017). It could decrease uric acid and inhibit crystal formation in tissues of gout patients (Rice, 1959). Probenecid has been shown to be able to inhibit glucuronidation of drugs *via* UDP-glucuronosyltransferase (UGTs) (Sakai-Kato et al., 2004; Uchaipichat et al., 2004) and inhibit the biliary excretion of anionic compounds *via* multidrug-resistant-protein 2 (MRP2, also known as ABCC2) (Horikawa et al., 2002; Namkoong et al., 2007). Moreover, hydroxylation metabolites, glucuronides, and sulfonated metabolites of emodin are found by some researchers (Qin et al., 2016; Yu et al., 2017). While Yan et al. (Wu et al., 2014) and Liu et al.(Liu et al., 2010; Liu et al., 2011) have reported that glucuronidation is the major metabolic pathway for emodin, and emodin-3-O-β-D-glucuronide (emodin-3-G) is the main metabolite *in vivo* and *in vitro*. Furthermore, our recent study found that emodin is mainly glucuronidated by UGT1A9 and UGT2B7, and emodin-3-G is a substrate of MRP2 (Liu et al., 2012; Wu et al., 2018). Thus, it suggested that adverse reaction might occur when co-administration of emodin and probenecid for a long time.

The combined use of emodin and probenecid raises a concern of potential deleterious drug interaction *via* the modulation of the expression of drug-metabolized enzymes and transporters, resulting in unfavorable therapeutic outcomes. However, there is no direct information available regarding the interactions between emodin and probenecid. Accordingly, this study intends to explore the interactions between emodin and probenecid so as to guide the safety clinical use of the combination of these medicines, especially to the large Chinese population who regularly consume these formulations.

2. Materials and methods

2.1. Chemicals and reagents

Emodin (Emo), probenecid (Pro), genistein (Ges), mycophenolic acid (MPA), zidovudine (AZT), potassium phosphate, sucrose, alamethicin, and EDTA were purchased from Aladdin Industrial Corporation (China). Uridine diphosphate glucuronic acid (UDPGA), D-saccharic-1, 4-lactone monohydrate, tris base, magnesium chloride, and rabbit polyclonal antibody against UGT1A9 (SAB2102643) were purchased from Sigma-Aldrich (USA). All commercial reagents were of analytical grade and the purities were not < 98%. Acetonitrile, methanol, formic acid, and ammonium acetate were purchased from Merck Company (≥ 99%, HPLC grade, Germany). The assay kits for the measurements of alanine aminotransferase (ALT), aspartate aminotransferase (AST) creatinine (CRE), and blood urea nitrogen (BUN) were purchased from Nanjing Jiancheng Biological Engineering Institute (China). Protein assay kit was purchased from Bio-Rad Company (USA). Rabbit polyclonal antibody against UGT2B7 (ab126269), MRP2 (ab203397), GAPDH (ab22555), and HRP-conjugated goat anti-rabbit IgG (ab6721) were purchased from Abcam Company (USA). ECL western blotting substrate was purchased from Millipore (USA). HepG2 cell lines were purchased from American Tissue Culture Colection (ATCC, USA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO Company (USA). PrimeScript RT reagent kit was purchased from Takara Company (Japan). SYBR Green PCR Master Mix kit was purchased from Promega Company (USA).

2.2. Animals and experimental design

Sprague-Dawley (SPF) rats weighing between 180 and 220 g were obtained from laboratory animal center of Southern Medical University (Guangzhou, China). Animals were used in experiments after an acclimation period of one week. Animals were kept under conditioned environment (22–25 °C, 60–70% relative humidity and 12 h light/dark cycle) with free access to food and water. The use of animals were approved by Animal Ethics Committee of Southern Medical University and performed according to the guidelines of the Southern Medical University. Animal care and experimental procedures complied with the guidelines of the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). 48 male rats were randomly divided into four groups (n = 12): Control group (Con group; 0.5% carboxymethyl cellulose sodium, CMC−Na), Emodin alone group (Emo group; 150 mg/kg), probenecid alone group (Pro group; 100 mg/kg), and combination group (Pro + Emo group; 150 mg/kg probenecid and 100 mg/kg emodin). In this study, the dosages of emodin (150 mg/kg) was chosen according to the previous studies (Wu et al., 2018). The dosage is equivalent to 17.5 times of the suggested dosage for human in the 2015 edition of Chinese pharmacopoeia (8.6 mg/kg, converted to rat dose based on body surface area conversion). In addition, the non-toxic and therapeutic dosage of probenecid (100 mg/kg/d) was chosen according to the literature report (National Toxicology, 1991; Palylyk and Jamali, 1993; Carrillo-Mora et al., 2010). All the experimental rats received intragastric administration of drugs dissolved in 0.5% CMC-Na once a day for consecutive 4 weeks. The administered volume was controlled below 2 mL per day for each rat in this study. On the 1st and 28th day, blood samples were withdrawn at designed time points and stored at −80 °C for biochemical and toxicokinetic assays. On the 29th day, the rats were sacrificed. The fresh liver and kidney tissues were immediately removed. A small portion of the tissues were prepared for pathological examination and the rest were stored at liquid nitrogen for determining the gene expression, protein expression, and enzyme activity.

2.3. Biochemical analysis and histopathological observations

The blood samples were centrifuged at 4 °C, 5000 *g* for 8 min, the supernatant was obtained for biochemical detection. Biochemical parameters (ALT, AST, BUN, and CREA) were detected with a commercial kit by Microplate Absorbance Reader (Bio-Rad, USA).

Liver and kidney tissues for histopathologic examination were immediately fixed and preserved in 10% neutral buffered formalin. Tissues were sectioned to a thickness of 2–3 μm and stained with hematoxylin and eosin (H & E). Images of stained sections were observed under a light microscope (main images *vs* inset magnified images, 100× *vs* 200×, amplification; Nikon, Japan). The pathological changes of liver were graded: 0, normal; +1, mild degree (lobular disarray and cellular edema); +2 moderate degree (hepatic steatosis and inflammatory cell infiltration); and + 3, severe degree (hepatic fibrosis).

2.4. Toxicokinetics studies

Toxicokinetic experiments were performed on the 1st and 28th day. The rats of emodin alone group and combination group were treated with drug solution by oral administration. During experiments, 13% circulating blood from each rat within 24 h was collected, which was below the maximum blood sample volumes (15%) allowed for multiple sampling in rats (Diehl et al., 2001). Blood samples were collected from the orbital venous at 0, 0.25, 0.5, 1, 2, 4, 6, 10, and 24 h after dosing. Blood samples were centrifuged at 4 °C, 5000 *g* for 8 min and the