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Coupling of UDP-glucuronosyltransferases and multidrug resistance-associated proteins is responsible for the intestinal disposition and poor bioavailability of emodin

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ABSTRACT

Emodin is a poorly bioavailable but promising plant-derived anticancer drug candidate. The low oral bioavailability of emodin is due to its extensive glucuronidation in the intestine and liver. Caco-2 cell culture model was used to investigate the interplay between UDP-glucuronosyltransferases (UGTs) and efflux transporters in the intestinal disposition of emodin. Bidirectional transport assays of emodin at different concentrations were performed in the Caco-2 monolayers with or without multidrug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP) efflux transporter chemical inhibitors. The bidirectional permeability of emodin and its glucuronide in the Caco-2 monolayers was determined. Emodin was rapidly metabolized to emodin glucuronide in Caco-2 cells. LTC4, a potent inhibitor of MRP2, decreased the efflux of emodin glucuronide and also substantially increased the intracellular glucuronide level in the basolateral-to-apical (B–A) direction. MK-571, chemical inhibitor of MRP2, MRP3, and MRP4, significantly reduced the efflux of glucuronide in the apical-to-basolateral (A–B) and B–A directions in a dose-dependent manner. However, dipyridamole, a BCRP chemical inhibitor demonstrated no effect on formation and efflux of emodin glucuronide in Caco-2 cells. In conclusion, UGT is a main metabolic pathway for emodin in the intestine, and the MRP family is composed of major efflux transporters responsible for the excretion of emodin glucuronide in the intestine. The coupling of UGTs and MRP efflux transporters causes the extensive metabolism, excretion, and low bioavailability of emodin.

Introduction

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), a medicinal extract present in many herbs, such as rhubarb (*Rheum officinale* B.), aloe (*Aloe barbadensis* M.), senna (*Cassia angustifolia*), and thunberg (*Polygonum multiflorum*), has been widely used as a traditional medicine in many countries, especially in Eastern Asia. Emodin has been known to have numerous pharmacological effects, including laxative, anti-allergic (Lim et al., 2007; Liu et al., 2005), anti-inflammatory, anti-cancer, and anti-diabetic activities (Feng et al., 2010; Garg et al., 2005; Huang et al., 2007). In traditional Chinese medicines, emodin was commonly used for the treatment of pain-related diseases (Sui et al., 2010). Moreover, the laxative property of emodin has been its primary mechanism of action for promoting weight loss (Matsuda et al., 2008).

Nevertheless, noticeable potential side effects of emodin have been reported. Genotoxic and mutagenic effects of emodin in vivo and in vitro have been reported in several studies (Krivobok et al., 1992; Masuda and Ueno, 1984; Masuda et al., 1985; Morita et al., 1988). The toxicity of emodin has been reported to be caused by the generation of reactive oxygen species, which led to lipid peroxidation, DNA oxidation, and protein damage (Huang et al., 1992). The National Toxicology Program (NTP) found that the exposure of rats to emodin increased the risk of renal tubule hyaline droplets and the preponderance of renal tubule pigmentation in both female and male rats (NTP, 2000). Furthermore, numerous studies have recently shown that emodin might have genotoxic (Liang et al., 2010), nephrotoxic (Wang et al., 2008), and hepatotoxic effects (Westendorf et al., 1990). The intestinal absorption and metabolism of xenobiotics, such as emodin, are the keys to understanding their toxicity and effectiveness. Therefore,

Abbreviations: AIC, Akaike's information criterion; UGT, UDP-glucuronosyltransferase; UDPGA, uridine diphosphoglucuronic acid; LTC4, leukotriene C4; NTP, National Toxicology Program; SD, Sprague–Dawley; DMEM, Dulbecco's modified Eagle's medium; AP, apical; BL, basolateral; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; HBSS, Hank's balanced salt solution; CL, intrinsic clearance; TEER, transepithelial electrical resistance; UPLC, ultra-pressure liquid chromatography.

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the absorption and metabolism of emodin should be studied carefully for its safe medical use.

Our previous pharmacokinetic study found that the absolute oral bioavailability of total emodin was extremely low (5% in male rats) in Sprague–Dawley (SD) rats (Liu et al., 2011). This poor oral bioavailability was thought to be the result of extensive phase II metabolism predominantly intestinal and hepatic glucuronidation in male and female rats (Liu et al., 2011). Efflux transporters expressed on the apical (AP) membrane of the intestine can transport hydrophobic compounds, such as glucuronide conjugates, from the cells to the intestinal cavity. Our previous studies found that breast cancer resistance protein (BCRP) plays a dominant role in genistein glucuronide excretion from the intestine (Jiang et al., 2011; Zhu et al., 2010). Multidrug resistance-associated proteins (MRPs) and organic anion transporters are involved in the intestinal efflux of apigenin glucuronide (Hu et al., 2003). Emodin glucuronide was also found in the perfusate of our previous in situ perfusion experiment (Liu et al., 2010). However, no information is available for the possible involvement of efflux transporters in the excretion of emodin glucuronide in both in vivo and in vitro settings. Although we have made some progress on the pharmacokinetic and metabolic characteristics of emodin, few studies on the mechanisms responsible for the absorption and excretion of emodin and its metabolites in the intestine have been reported. The interplay between efflux transporters, including multidrug resistance-associated protein 2 (MRP2), BCRP, and drug-metabolizing enzymes, such as UDP-glucuronosyltransferases (UGTs), has been deemed to play a major role in determining the absorption and disposition of polyphenols, such as flavonoids (Chen et al., 2005; Jia et al., 2004; Liu and Hu, 2002). Also, our previous investigation preliminarily implied that the interplay between UGTs and efflux transporters possibly contributes to the low bioavailability of emodin (Liu et al., 2010, 2011). Therefore, to proceed with the mechanistic studies involving emodin disposition and metabolism in the intestine, we used Caco-2 TC7 cells, one of the cloned Caco-2 variants, for increased homogeneity and cell population stability. Using the Caco-2 cell model, the "gold standard" of intestinal drug absorption (Hubatsch et al., 2007), it was demonstrated as a perfect interplay of conjugating enzymes with efflux transporters in the elimination of drugs (Nies et al., 2008). This model has also been used by many investigators to study human intestinal disposition (Hu et al., 1999; Pontier et al., 2001). Teng et al. (2007) found that emodin was glucuronidated and sulfated in the Caco-2 cell model. However, no efflux transporters were observed in their study.

Moreover, metabolic enzymes, along with efflux transporters, play an important role in drug detoxification and effectiveness. Therefore, this study aims to determine the contribution of efflux transporters on intestinal absorption and to delineate the disposition mechanism of the emodin conjugate in Caco-2 cells.

Materials and methods

Chemicals and reagents. Caco-2 TC7 cells were provided by Dr. Ming Hu (Department of Pharmaceutical Sciences College of Pharmacy University of Houston, USA). Emodin (≥98%, HPLC grade; confirmed by LC/MS) was purchased from Chengdu Mansite Pharmaceutical Company (China). Glucose, $NaHCO_3$, Hank's balanced salt solution (HBSS; powdered form), uridine diphosphoglucuronic acid (UDPGA), alamethicin, D-saccharic-1,4-lactone monohydrate, magnesium chloride, leukotriene C4 (LTC4), and MK-571 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dipyridamole was purchased from Zelang Medical Technology Company (Nanjing, China). HPLC-grade acetonitrile and water were used in the experiments. All other chemicals used were of analytical grade.

Cell culture. Caco-2 TC7cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 1% nonessential amino acids, 1% L-glutamine (Sigma-Aldrich), and 1% antibiotics (penicillin/streptomycin). The cells were cultured in an atmosphere of 5% CO_2 and 90% relative humidity at 37 °C. The cells were seeded on 3 μm porous 6 well plate polycarbonate cell culture inserts (Catalog number: 137435, Nalge Nunc International, Naperville, IL, USA), with a seeding density of 100,000 cells/cm^2 (4.2 cm^2 per monolayer). The culture medium was routinely replaced by fresh medium every other day. The cells were passaged upon reaching approximately 90% confluence using 2 ml to 3 ml of trypsin-EDTA (Sigma-Aldrich). Only the cells that grew for 19 to 22 days were used for the Caco-2 permeability studies (Chen et al., 2003; Yang et al., 2010).

Caco-2 permeability studies. The transport experiments were conducted as described previously (Liu and Hu, 2002). Briefly, prior to each experiment, the media were removed and the Caco-2 monolayers were washed twice with 37 °C blank transport buffer (HBSS), 500 ml HBSS containing 4.9 g HBSS power, 2.2 mM $NaHCO_3$, 12.5 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), and 9.71 mM D-glucose (pH 7.4). The transepithelial electrical resistance values were measured, and those with the values less than 420 Ω/cm^2 were discarded. Various concentrations (ranging from 2.4 μM to 13 μM) of emodin were loaded onto the AP or basolateral (BL) sides of the Caco-2 cell monolayer. Samples (500 μl) were collected from both sides of each transwell at predetermined time points (0, 30, 60, 90, and 120 min). The total volume of each chamber was 2.5 ml. The same volume of the solution on the AP or BL side was replaced after each sampling, and 100 μl of the internal standard solution (10 μM testosterone in acetonitrile) was immediately added to 200 μl of each sample to stabilize them until further analysis. All the samples were centrifuged for 30 min at 13,000 rpm, and the supernatant was measured by ultra-pressure liquid chromatography (UPLC) to analyze the concentrations of emodin and its metabolites at both sides.

Inhibition experiment. The inhibition experiments were carried out as described above for the Caco-2 permeability studies with minor modifications. Dipyridamole (10 μM) was used as a selective BCRP efflux transporter chemical inhibitor (Zhang et al., 2005). MK571 (5, 10, and 20 μM) was used as a non-selective MRP efflux transporter family chemical inhibitor and LTC4 (0.1 μM) was used as a highly selective MRP2 efflux transporter chemical inhibitor (Loe et al., 1996; Luders et al., 2009). Chemical inhibitor used in the assay was always added to the apical side 1 h before the transport study during the pre-incubation period. When used, the chemical inhibitor concentration was maintained in the apical side solution throughout the emodin bidirectional transport study. Emodin (13 μM) was added to either the AP or BL side of the Caco-2 cell monolayer.

Preparation of Caco-2 cell lysates. Mature Caco-2 cell monolayers in the culture flasks were washed twice with 10 ml HBSS at 37 °C. All the cells were scraped from the bottom of the flask and pooled in 6 ml of 50 mM potassium phosphate buffer (pH 7.4). The cell suspensions were ultrasonicated in an ice bath for 10 min. The cell lysates were centrifuged at 1000 rpm for 5 min, and the concentration of each cell lysate was determined using a commercial protein assay kit (Bio-Rad, Hercules, CA, USA).

Glucuronidation of emodin in Caco-2 cell lysate. The incubation procedures for measuring UGT activities from the cell lysates were the same as those published previously (Chen et al., 2005; Hu et al., 2003; Jeong et al., 2004). Briefly, the cell lysate (with a final concentration of approximately 0.2 mg protein/ml) was mixed with magnesium chloride (0.88 mM), saccharolactone (4.4 mM), alamethicin (0.022 mg/ml), different concentrations of emodin in a 50 mM potassium phosphate buffer (pH 7.4), and UDPGA (3.5 mM). The mixture (with a final volume of 200 μl) was incubated at 37 °C for a predetermined period. The reaction was stopped by the addition of 100 μl 94% acetonitrile/6% glacial acetic acid containing 50 μM testosterone as the internal standard.