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Potential modulation on P-glycoprotein and CYP3A by soymilk and miso: *In vivo* and *ex-vivo* studies

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ABSTRACT

P-glycoprotein (P-gp) and CYP3A4 both play very important roles in drug bioavailability, resistance and interactions. Our *in vitro* studies indicated that P-gp function was activated by many isoflavones. This study investigated the *in vivo* effects of soymilk and miso, isoflavone-rich soy foods, on P-gp and CYP3A by tracing the pharmacokinetics of cyclosporine (CSP), a probe drug of P-gp. Rats were orally administered CSP with and without soymilk or miso. A specific monoclonal fluorescence polarisation immunoassay was used to determine the blood concentration of CSP. The results showed that soymilk and miso significantly decreased the C_{max} of CSP by 64.5% and 78.3%, and reduced the AUC_{0-540} by 64.9% and 78.3%, respectively. Mechanism studies revealed that the activities of P-gp and CYP3A4 were induced by soymilk and miso. In conclusion, ingestion of soymilk and miso significantly activated the functions of P-gp and CYP3A.

1. Introduction

Soy foods, a major source of isoflavones, are a very common diet in Asian countries. Many epidemiological studies have demonstrated that consumption of soy products could lower the incidences of cardiovascular diseases, osteoporosis and estrogenic-related cancers (Magee & Rowland, 2012; Peterson, Dwyer, Jacques, & McCullough, 2012; Romagnolo & Selmin, 2012; Xiao, 2008). Soymilk and miso, a fermented soybean product originated from Japan, are traditional soy foods consumed by Asian people. They contain several isoflavones, including daidzin, genistin, daidzein and genistein (chemical structures shown in Fig. 1), which have shown a variety of beneficial pharmacological activities, such as chemoprevention (Romagnolo & Selmin, 2012; Zhao & Mu, 2011), estrogenicity (Choi et al., 2008; Vitale, Piazza, Melilli, Drago, & Salomone, 2012) and anti-inflammation (Blay et al., 2010; Khan et al., 2012).

P-glycoprotein (P-gp), one of the ATP-binding cassette (ABC) drug transporters, is expressed on the apical membrane of several tissues, including the intestines, liver, kidney and brains; it mediates the inside-out efflux of a number of different endogenous compounds and xenobiotics (König, Müller, & Fromm, 2013). P-gp has a broad spectrum of substrates including lipophilic compounds and cations, such as cardiovascular drugs, immunosupressants, HIV protease inhibitors and anticancer agents, etc. (Li et al., 2013). Recent studies have reported that flavonoids could modulate the functions of P-gp and cytochrome P450 enzymes (CYP450), which play important roles not only in drug bioavailability, but also in drug resistance and drug interactions (Alvarez et al., 2011; Lee, Cook, Reyner, & Smith, 2010; Moon, Wang, & Morris, 2006; Yu et al., 2011; Yi, 2013). Among isoflavones, daidzein and genistein have been reported to inhibit P-gp function (Limtrakul, Khantamat, & Pintha, 2005), however, an opposite effect of inductive modulation was demonstrated in a different model (Okura, Ibe, Umegaki, Shinozuka, & Yamada, 2010). Besides, there are *in vitro* studies reporting that CYP3A4 was modulated by daidzein and genistein (Li, Ross-Viola, Shay, Moore, & Ricketts, 2009; Sergent et al., 2009).

Cyclosporine (CSP), an immunosuppressant agent, is a substrate of P-gp and CYP3A4. Clinically, the blood level of CSP should be monitored due to its narrow therapeutic window. A supra-therapeutic CSP level in the blood would cause adverse effects, including neurotoxicity, hepatotoxicity and nephrotoxicity. Conversely, a sub-therapeutic level in the blood would cause allograft rejection in transplant patients (Burke et al., 1994). Therefore, we suspected that when soymilk or miso was concomitantly ingested with CSP, the level of CSP in the blood might be affected. In order to clarify the virtual modulation effect of soymilk and miso on P-gp and CYP3A4 *in vivo*, CSP pharmacokinetics were investigated in rats,

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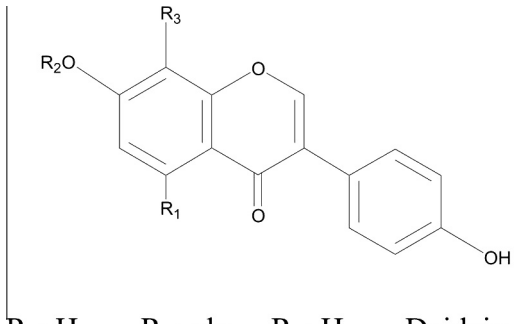

R_1: H	R_2: glu	R_3: H	Daidzin
R_1: H	R_2: H	R_3: H	Daidzein
R_1: OH	R_2: glu	R_3: H	Genistin
R_1: OH	R_2: H	R_3: H	Genistein

Fig. 1. Chemical structures of daidzin, daidzein, genistin and genistein.

with and without co-administration of soymilk or miso. Furthermore, the underlying mechanisms were investigated using *in vitro* and *ex-vivo* models.

2. Materials and methods

2.1. Chemicals and reagents

Genistin, genistein, rhodamine 123, sodium dodecyl sulphate (SDS), dimethyl sulfoxide (DMSO), 3-(4′,5′-dimethylthiazol-2′-yl)-2, 5-diphenyltetrazolium bromide (MTT), Triton X-100, verapamil, β-glucuronidase (type B-1 from bovine liver), sulfatase (type H-1 from Helix pomatia, containing 14,400 units/g of sulfatase and 574,000 units/g of β-glucuronidase) and ethyl paraben were purchased from Sigma (St. Louis, MO, USA). Daidzin and daidzein were obtained from Fluka Chemie GmbH (Buchs, Switzerland). L(+)-Ascorbic acid and ortho-phosphoric acid were purchased from RdH Laborchemikalien GmbH & Co. KG (Seelze, Germany). Dulbecco's Modified Eagle Medium (DMEM), Hank's Buffered Salt Solution (HBSS), nonessential amino acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and trypsin/EDTA were purchased from Invitrogen (Grand Island, NY, USA). Cyclosporine (Neoral®, 100 mg/ml) was kindly provided by Novartis (Taiwan) Co. Ltd. Acetonitrile (LC grade) was obtained from J.T. Baker, Inc. (Phillipsburg, NJ, USA). Methanol and ethyl acetate (LC grade) were supplied by Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). TDx kit was supplied by Abbott Laboratories (Abbott Park, IL, USA). Milli-Q plus water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Preparation and characterisation of soymilk powder and miso

Soybean and miso were purchased from a supermarket in Taichung, Taiwan. Soybean (250 g) was weighed and soaked with 500 ml of water for 8 h, ground by a blender with 1 l of water and then filtered with gauze. The filtrates were heated on a gas stove until boiling. After cooling to room temperature, sufficient water was added to make to 1 l. The filtrates was lyophilized to afford soymilk powders (83 g) and frozen at −20 °C for later use.

Soymilk powders (1.0 g) and miso (1.0 g) were extracted twice with 10 ml of 60% acetonitrile by ultrasonic shaking for 30 min and filtered. Sufficient 60% acetonitrile was added to the combined filtrates to make to 20 ml. After dilution with methanol and centrifugation, the supernatant (180 μl) was added to 20 μl of 5,7-dimethoxycoumarin solution (200 μg/ml in methanol as internal standard) and 20 μl was subjected to HPLC analysis (Hou, Chi, Tsai, & Chao, 2011). The instrumentation included a pump (LC-10AT, Shimadzu, Japan), a diode array detector (SPD-M10AVP, Shimadzu, Japan) and an automatic injector (SiL-10A, Shimadzu, Japan). A LiChrospher® 100 RP-18e column (4 × 250 mm, 5 μm) was equipped and the mobile phase consisted of acetonitrile (A) and 0.1% phosphoric acid (B). A gradient elution was programmed as follows: A/B: 11/89 (0 min), 18/82 (10 min), 50/50 (20 min) and 11/89 (35 min). The detection wavelength was set at 250 nm and the flow rate was 1.0 ml/min.

2.3. Cell line and culture conditions

LS 180, the human colon adenocarcinoma cell line, was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (Biological Industries Ltd., Kibbutz Beit Haemek, Israel), 0.1 mM nonessential amino acid, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 292 μg/ml of glutamine. Cells were grown at 37 °C in a humidified incubator containing 5% CO_2. The medium was changed every other day and cells were subcultured when 80–90% confluency was reached.

2.4. Cell viability assay

The effects of test drugs, verapamil and DMSO on the viability of LS 180 cells was evaluated by an MTT assay (Mosmann, 1983). Cells were seeded into a 96-well plate. After overnight incubation, the tested agents were added into the wells and incubated for 72 h, then 15 μl of MTT (5 mg/ml) was added into each well and incubated for an additional 4 h. During this period, the MTT was reduced to formazan crystals in live cells. Acid–SDS (10%) solution was added to dissolve the purple crystal at the end of incubation and the optical density was detected at 570 nm by a microplate reader (BioTex, Highland Park, Winooski, VT, USA).

2.5. Effects of daidzin, daidzein, genistin and genistein on P-gp activity

A transport assay of rhodamine 123 was performed to observe the effects of daidzin, daidzein, genistin and genistein on the activity of P-gp as described previously (Hou, Lin, & Chao, 2012; Yang, Tsai, Hou, & Chao, 2012). Briefly, LS 180 cells (1×10^5) were seeded in a 96-well plate. After overnight incubation, the old medium was replaced with rhodamine 123 solution and incubated for 1 h. Then, each isoflavone, verapamil (as a positive control of the P-gp inhibitor) and DMSO were added to corresponding wells and incubated at 37 °C. After 4 h incubation, the cells were washed and 100 μl of 0.1% triton X-100 was added to lyse the cells and the fluorescence was measured with excitation wavelengths at 485 nm and emission at 528 nm. The relative intracellular accumulation of rhodamine 123 was calculated by comparing with that of control and corrected with protein contents.

2.6. Drug administration and blood collection

Sprague–Dawley rats weighing between 300– 400 g were fasted for 12 h before the experiment and food was withheld for another 3 h. Water was supplied *ad libitum*. The CSP oral solution was prepared by diluting Neoral® with deionised water to afford a