Differential Effects of Chrysin on Nitrofurantoin Pharmacokinetics Mediated by Intestinal Breast Cancer Resistance Protein in Rats and Mice

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ABSTRACT

Purpose. The activities of breast cancer resistance protein (Bcrp/ABCG2) as well as P-glycoprotein (P-gp) and drug-metabolizing enzymes can be inhibited by several flavonoids or drugs in rats. However, the species, sex and regional differences of effects of flavonoids on Bcrp/ABCG2 in rats and mice remain unclear, although Bcrp, like P-gp, is also important in controlling drug absorption and disposition. **Methods**. We used chrysin as a model flavonoid because it possesses anti-inflammatory and antioxidative properties and is used as a dietary supplement. We examined the pharmacokinetics of nitrofurantoin, a specific Bcrp substrate, after oral or intravenous administration in rats and mice treated with chrysin. Bcrp mRNA levels were measured in liver, kidney, duodenum, jejunum and ileum in rats and mice. **Results**. Oral chrysin increased plasma concentrations of nitrofurantoin in rats but not mice. Intraperitoneal injection of chrysin into rats or mice had little effect on the elimination of nitrofurantoin. The $AUC_{0\text{-}t}$ in female mice was 1.5-2.0 folds higher than in male mice after oral and intravenous administration of nitrofurantoin. Absorption of nitrofurantoin from apical to basal sides was significantly increased by chrysin in both duodenum and jejunum as well as in ileum in rat small intestine. **Conclusions.** Chrysin-nitrofurantoin interactions it takes place in the small intestine and occur in rats, but not in mice, possibly due to the higher levels of Bcrp in the small intestine in rats as compared with mice.

INTRODUCTION

Drug efflux transporters such as P-glycoprotein (P-gp; ABCB1), breast cancer resistance protein (Bcrp; ABCG2) and multidrug resistance-associated proteins 1 and 2 (ABCC1 and ABCC2) are membrane-embedded proteins that limit intracellular concentrations of substrates by pumping them out of the cell (1). These transporters are involved in the phenomenon of multidrug resistance encountered during cancer therapy and can affect the disposition of many clinically used drugs, because of their wide tissue distributions and broad substrate specificities (2).

Bcrp has one ATP-binding cassette and six putative transmembrane domains and is, therefore, referred to as a half-ABC transporter, which most likely functions as a homodimer (3). Bcrp was initially shown to confer resistance to mitoxantrone, doxorubicin, daunorubicin and topotecan (4, 5). In normal human tissues, BCRP mRNA is most highly expressed in placental tissue. Furthermore, the substrate specificity of Bcrp shows considerable, but variable, overlap with that of P-gp (6, 7) suggesting that Bcrp plays a similar role to P-gp in the pharmacokinetics of substrate drugs.

Effective and safe modulators of Bcrp, such as elacridar (GF120918), gefitinib and flavonoids, have recently been discovered by many investigators (8-14).

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It is well known that flavonoids can inhibit the activities of P-gp and/or drug-metabolizing enzymes (15, 16), but the effects of flavonoids on Bcrp remain unclear. Any differences in their effects between rats and mice also remain unknown, and it is important to determine these in order to identify an appropriate animal model for testing the inhibitory effects of such compounds on human BCRP.

In this study, we investigated the species, sex and regional different effects of flavonoids on Bcrp in rats and mice. We chose chrysin as a model flavonoid due to its potent anti-inflammatory and antioxidative properties, and because it is used as a dietary supplement (17, 18). Wang et al. demonstrated that chrysin (5,7-dihydroxyflavone) affected the pharmacokinetics of nitrofurantoin, a specific Bcrp substrate, in female rats (19). Chrysin is a natural flavonoid which is contained in many plant extracts, honey, and propolis (20, 21). Nitrofurantoin(1-[(5-nitro-2-furanyl)methylene]amino-2,4-imidazolidione) is a nitrofuran-derivative which is widely used as an antibacterial agent in human and veterinary medicine.

METHODS

Materials

Chrysin, nitrofurantoin and glycofurol were obtained from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide and furazolidone were purchased from Kishida Chemical (Osaka, Japan) and Wako (Kyoto, Japan), respectively. All other chemicals used were of the highest purity available.

Animals

Eight-week old male Wistar rats (210-250 g) and 5-week-old female and male ddY mice (15-20 g) were purchased from Japan SLC Co. (Shizuoka, Japan). They were housed in a temperature-controlled room with free access to standard laboratory chow and water. The experiments were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University School of Pharmaceutical Science.

Pharmacokinetic studies

We performed pharmacokinetic studies as described previously (19, 22, 23), with some modifications. To clarify the effects of chrysin on intestinal Bcrp and on hepatic or renal Bcrp, we investigated the effects of chrysin on the levels of orally or intravenously administered nitrofurantoin. In rats, jugular vein cannulation was performed following intraperitoneal injection of 50 mg/kg pentobarbital. Rats were fasted overnight but had free access to tap water. To examine the effect of chrysin on orally administered nitrofurantoin, 100 mg/kg chrysin dissolved in glycofurol, or glycofurol alone (as control) was administered orally to rats by gavage. Chrysin was administered as a solution at 10 μL/g body weight. Five minutes later, the animals were administered 10 mg/kg nitrofurantoin orally (3 mg/ml dissolved in 50:50% (v/v) ethanol:polyethylene glycol 400. Blood samples (150 μL) were taken from the jugular vein cannula at 0 (10 min before injection), 5, 15, 30, 60, 120 and 180 min. Jugular cannula was rinsed with heparinized saline solution between sampling and the blood sample volume compensated with saline. To study the effects on intravenously administered nitrofurantoin, 50 mg/kg chrysin (dissolved in dimethyl sulfoxide at a concentration of 20 mg/ml) or vehicle (dimethyl sulfoxide) was first given to rats via intraperitoneal injection. Five minutes later, a dose of 2.5 mg/kg nitrofurantoin [0.5 mg/ml dissolved in 10% (v/v) ethanol, 40% (v/v); polyethylene glycol 400, and 50% phosphate-buffered saline] was injected into the tail vein. Blood samples were sequentially collected at 0, 5, 10, 20, 30, 60 and 120 min after nitrofurantoin administration.

In mice, animals were fasted overnight with free access to tap water. For drug interaction studies in the small intestine, the animals were orally administered 100 or 500 mg/kg chrysin (dissolved in glycofurol at a concentration of 10 mg/ml or 50 mg/ml, respectively) or glycofurol alone (as control). Five minutes later, the animals were administered 10 mg/kg nitrofurantoin (3 mg/ml dissolved in 50% (v/v) ethanol and 50% (v/v) polyethylene glycol 400) by gavage into the stomach. They were anesthetized with euther and

blood samples were collected from the vena cava under ether anesthesia at 15, 30, 60, 120 and 180 min after nitrofurantoin administration. Subsequently, they were euthanized by over anesthesia. For intravenous administration of nitrofurantoin, 50 mg/kg chrysin (dissolved in dimethyl sulfoxide at a concentration of 20 mg/ml) or vehicle (dimethyl sulfoxide) was first given to rats via intraperitoneal injection. Five minutes later, a dose of 2.5 mg/kg nitrofurantoin (0.5 mg/ml dissolved in 10% (v/v) ethanol, 40% (v/v) polyethylene glycol 400, and 50% phosphate-buffered saline) was injected into the tail vein. Blood samples were collected at 5, 15, 30 and 60 min after nitrofurantoin administration.

All heparinized blood samples were centrifuged immediately at 3,000 *g* for 15 min, and plasma was collected and stored at –20° C until analuzed using HPLC.

Everted sac studies

Small intestines were removed from 18-h-fasted rats and mice after induction of anesthesia by inhalation of ether, and euthanasia by cervical dislocation. In rats, the part of duodenum and jejunum consisted of 10 cm removed from 2–3 cm below the ligament of Treitz, while the part of ileum consisted of 10 cm removed from 5 cm above the cecum. In mice, the duodenum and jejunum part was dissected at 1–15 cm from the stomach and the part of ileum from 8 cm above the cecum. Each intestinal segment was ligated at one end and then everted. The open end of the everted sacs was ligated after the insertion of a polyethylene tube. The length of the prepared everted sacs was 5 cm. Four hundred μL of Krebs-Ringer buffer (KRB), pH 6.4 was added to the serosal side of the everted sacs through a cannula. The everted sacs were incubated in KRB for 10 min at 37° C; transferred into KRB containing 10 μM nitrofurantoin tubes and incubated at 37° C. Aliquots (200 μL) of the mucosal solution were collected at 0 and 30 min after the addition of nitrofurantoin. The fluid volume was checked to confirm the integrity of everted sacs after each experiment. All samples were stored at –20° C until HPLC analysis.

HPLC analysis

The concentrations of nitrofurantoin in the plasma and in the samples from the everted sac study were analyzed according to the HPLC method of Merino et al. (22), with minor modifications. In brief, a 50 μL aliquot of sample was spiked with 10 μL of 12.5 μg/ml furazolidone solution as an internal standard, and then 50 μL of cold methanol (–20°C) was added. The mixture was vortexed vigorously for 60 s and incubated at –20°C for 15 min. The organic and water phases were separated by centrifugation at 12,000 *g* for 10 min at 4 °C, and 30 μL of the supernatant was injected into the HPLC system (Agilent SERIES 1100, Agilent Technologies, Palo Alto, CA, USA). The HPLC analysis was performed using a CAPCELL PAK MGII (C_{18} column, 150 × 4.6 mm, 5 μm particle size) (SHISEIDO, Tokyo, Japan). The mobile phase was composed of 25 mM potassium phosphate buffer (pH 3)/acetonitrile (90:10). The mobile phase was delivered isocratically at a flow rate of 1.0 ml/min. UV absorbance was measured at 366 nm. Nitrofurantoin and the internal standard eluted out at approximately 3.8 and 4.3 min, respectively, with no interference peaks. The concentrations of nitrofurantoin were determined from a standard curve prepared using the same procedures. The calibration curve was linear over the range of 0.1–20 μg/ml with coefficient of variation not exceeding 5%.

Pharmacokinetic Analysis

The area under the plasma concentration-time curve (AUC_{0-t}) was calculated using the trapezoidal method. The AUC extrapolated to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + Ct/Ke$, where Ct is the last measurable concentration and the elimination rate constant (β) was obtained from the least square fitted terminal log-linear portion of the plasma-concentration time profile. The terminal half-life ($t_{1/2}$) was calculated as ln2/β, and β was determined from the slope of the terminal regression line. The systemic clearance (CL) and oral clearance (CL/F) were calculated as the i.v. and p.o. dose divided by AUC, respectively. The bioavailability (F) was determined by

(AUCp.o./Dosep.o.)/(AUCi.v./Dosei.v.). All pharmacokinetic parameters of nitrofurantoin were estimated by noncompartmental analysis using WinNonlin version 4.1 (Pharsight, Mountain View, CA).

Sample preparation for the determination of Bcrp mRNA levels

The liver, kidney, duodenum, jejunum and ileum were removed from rats and mice after induction of anesthesia by inhalation of ether, and euthanasia by cervical dislocation. Each part of the small intestine of rats or mice that had been treated repeatedly with oral chrysin for 4 or 10 days (100 mg/kg) was also removed. In rats, the excised small intestine was flushed with 10 ml of ice-cold saline and was divided into six 5 cm segments, from the stomach. The first, third and fifth segments were used as samples of duodenum, jejunum and ileum, respectively. In mice, the excised small intestine was flushed with 5 ml of ice-cold saline. The duodenum (1–8 cm from the stomach), jejunum (10–18 cm from the stomach), and ileum (8 cm above the cecum) were dissected. After flash freezing with liquid nitrogen, each sample was preserved at –80° C until analyzed.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from approximately 100 mg of mouse or rat liver, kidney, duodenum, jejunum and ileum using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Following RNase-free DNase I treatment (TaKaRa, Shiga, Japan), approximately 500 ng of total RNA, as evaluated by UV absorption at 260 nm, was reverse-transcribed to complementary DNA (cDNA) using a PrimeScript-RT reagent Kit (TaKaRa), according to the manufacturer's instructions. The reactions were incubated for 15 min at 37° C and for 5 s at 85°C. The reverse-transcribed cDNA provided the template for real-time PCR. Amplification was performed in 50 μl of reaction mixture containing 2×SYBR Premix Ex Taq (TaKaRa), 0.2 μM of the primer sets for 28S rRNA (mice) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (rats) as endogenous references. Amplification and detection were performed using an ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA). The PCR reactions were incubated at 95° C for 10 s, and amplified by 40 three-step cycles at 95° C for 5 s, 55° C for 20 s and 72° C for 31 s. The amount of 28S rRNA or GAPDH in each sample was also measured for normalization. For the PCR amplifications, we used the following oligonucleotide sequences designed by Primer Express 2.0 (Applied Biosystems): Bcrp: 5'-TGCCAGGCGCTCATTTAAAA-3' and 5'-CCAGCGGCATCATATTTCAGA-3', 28S rRNA: 5'-CGGCTCTTCCTATCATTGTG-3' and 5'-CCTGTCTCACGACGGTCTAA-3' and GAPDH: 5'-CAACGACCCCTTCATTGA-3' and 5'-CAGTGATGGCATGGACTG-3'.

Data were analyzed using ABI Prism 7000 SDS Software (Applied Biosystems), particularly the comparative method for multiplex analysis. The relative quantification of the amounts of target messages in the tested tissue samples was accomplished by measuring Cycle thresholds (*Ct*).

To determine the quantity of the target gene-specific transcripts present in kidney, duodenum, jejunum, and ileum relative to those in liver, their respective *Ct* values were first normalized by subtracting the *Ct* value obtained from the 28S rRNA control (ΔCt = *Ct*, target – *Ct*, control). The concentration of gene-specific mRNA in kidney, duodenum, jejunum or ileum relative to liver was then calculated by subtracting the normalized *Ct* values obtained for liver from those obtained from kidney, duodenum, jejunum or ileum ($\Delta\Delta Ct$ = ΔCt, liver – ΔCt, kidney, duodenum, jejunum or ileum) and the relative concentration was determined ($2^{-\Delta\Delta Ct}$).

Statistical analysis

Significant differences between mean plasma concentrations or gene expression levels were estimated using unpaired Student's *t*-test.

RESULTS

To assess the effects of chrysin on Bcrp activities, the pharmacokinetics of orally and intravenously administered nitrofurantoin were determined in rats and mice, treated with or without chrysin. We also investigated the effects of repeated oral administration of chrysin for 10 days on Bcrp mRNA levels in rats and mice.

Figure 1 depicts the plasma concentrations of orally or intravenously administered nitrofurantoin in rats treated with or without chrysin. Significantly higher plasma concentrations of orally administered nitrofurantoin were observed at 15, 30 and 60 min in chrysin-treated rats, compared with controls. However, similar nitrofurantoin plasma concentration profiles were observed in chrysin-treated and control rats treated after intravenous administration of nitrofurantoin. Table 1 shows the estimated pharmacokinetic parameters for rats. $AUC_{0-\infty}$ for plasma nitrofurantoin and the maximum concentration (C_{max}) of nitrofurantoin were significantly (about 2.5-fold) increased by coadministration of chrysin. Figures 2 and 3 depict the time courses of the plasma concentrations of nitrofurantoin after oral and intravenous administration, respectively, to female and male mice treated with or without chrysin. Neither of the chrysin doses (100 and 500 mg/kg) had any significant effect on the plasma concentrations of nitrofurantoin in mice. Nor did the intravenous nitrofurantoin doses were influenced by chrysin in either sex. These results indicate inhibitory effect of chrysin on Bcrp mediated efflux of nitrofurantoin after oral administration to rats but not to mice. After oral and intravenous administration of nitrofurantoin, the $AUC_{0-\infty}$ values for nitrofurantoin exhibited sex differences in both control and treated mice. The AUC_{0-t} in female mice was 1.5-2.0 fold higher than in male mice (data not shown).

Bcrp expression in the small intestine shows regional differences (Tanaka et al. 2005). We used everted intestinal sacs to determine any regional differences throughout the small intestine in terms of the effects of chrysin on Bcrp inhibition (Figure 4). In rats, absorption of nitrofurantoin from apical to basal sides was significantly increased by chrysin in both duodenum and jejunum as well as in ileum in small intestine. However, chrysin had little effect on the amounts of nitrofurantoin absorbed in mice (data not shown). We examined the relationship between the effects of chrysin on the pharmacokinetics of nitrofurantoin and Bcrp mRNA expression in rats and mice. Figure 5 shows the Bcrp mRNA levels in liver, kidney, duodenum, jejunum and ileum of rats and female or male mice. In rats, the Bcrp mRNA levels in kidney, duodenum and jejunum were significantly higher than those in liver. In contrast, only the Bcrp mRNA levels in kidney were significantly higher than those in liver in mice. Bcrp mRNA expression levels were relatively low in the small intestine in mice. No sex differences in Bcrp mRNA levels in the examined tissues were observed in mice.

Dietary supplements and herbal preparations containing flavonoids are extensively used chronically. We, therefore, investigated the alterations in Bcrp mRNA expression after repeated oral administration of chrysin to female and male rats and mice for 4 or 10 days (Figure 6). Bcrp mRNA levels in any part of the small intestine was not statistically significant. After 4 days of chrysin administration, the Bcrp mRNA levels also exhibited little change in any part of the small intestine (data not shown).

DISCUSSION

The present study demonstrates that chrysin-nitrofurantoin interactions, mediated by Bcrp, differes between rats and mice. We also demonstrated that treatment with chrysin for 10 days had little effect on intestinal Bcrp mRNA levels. Pharmacokinetic studies of orally administered nitrofurantoin in rats showed that the $AUC_{0-\infty}$ values for nitrofurantoin were dramatically increased by oral chrysin, whilst intravenously administered nitrofurantoin was unaffected. This suggests that chrysin affected the nitrofurantoin pharmacokinetics at the intestinal level. Nitrofurantoin is well absorbed through the small intestine without metabolism (24, 25).

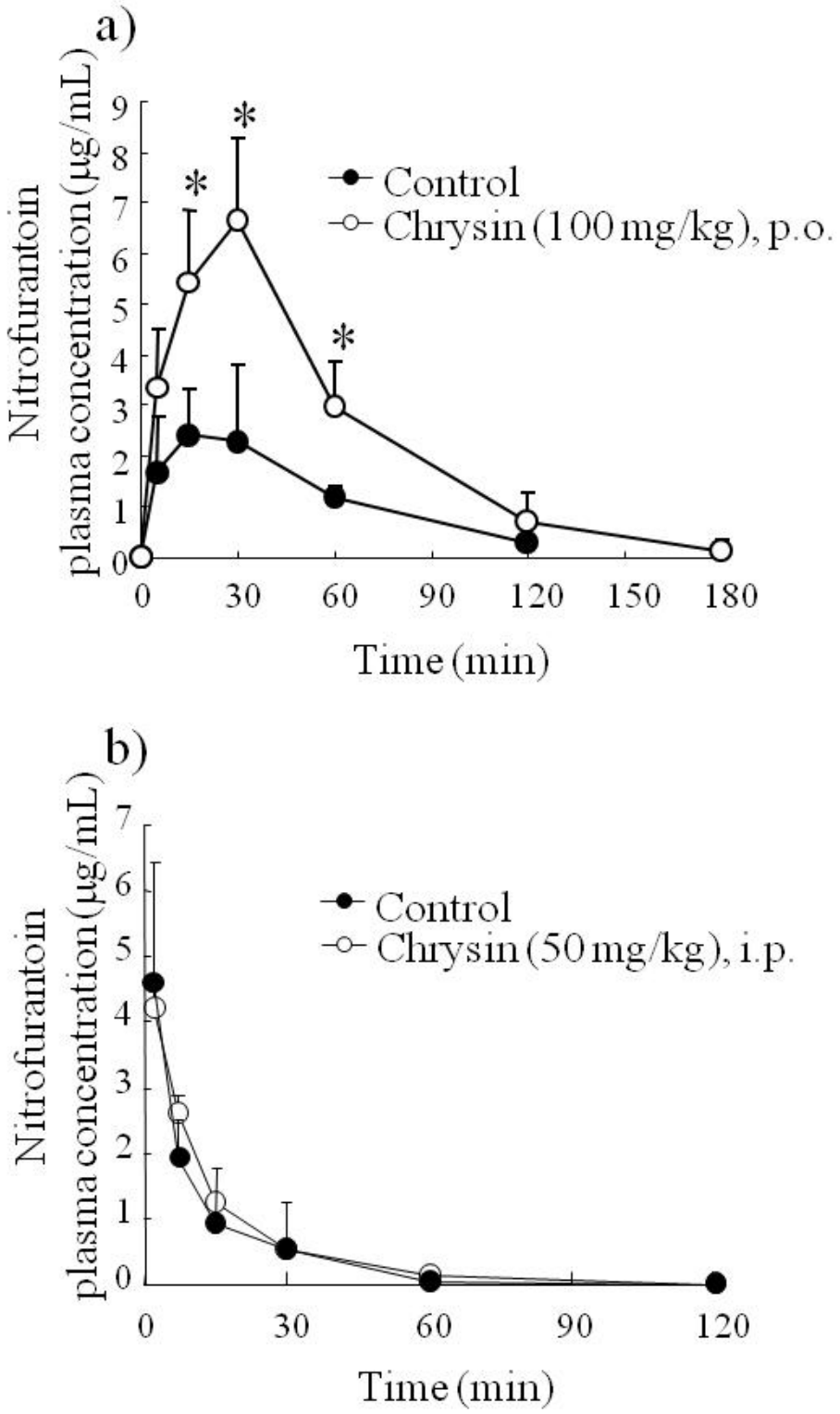

Figure 1. Plasma concentration versus time profile of nitrofurantoin after a) oral or b) intravenous administration in rats treated with either control vehicle or chrysin. Plasma levels of nitrofurantoin were determined by HPLC. Data are expressed as mean ± SD (n = 4). *, $p < 0.05$ compared with the control group.

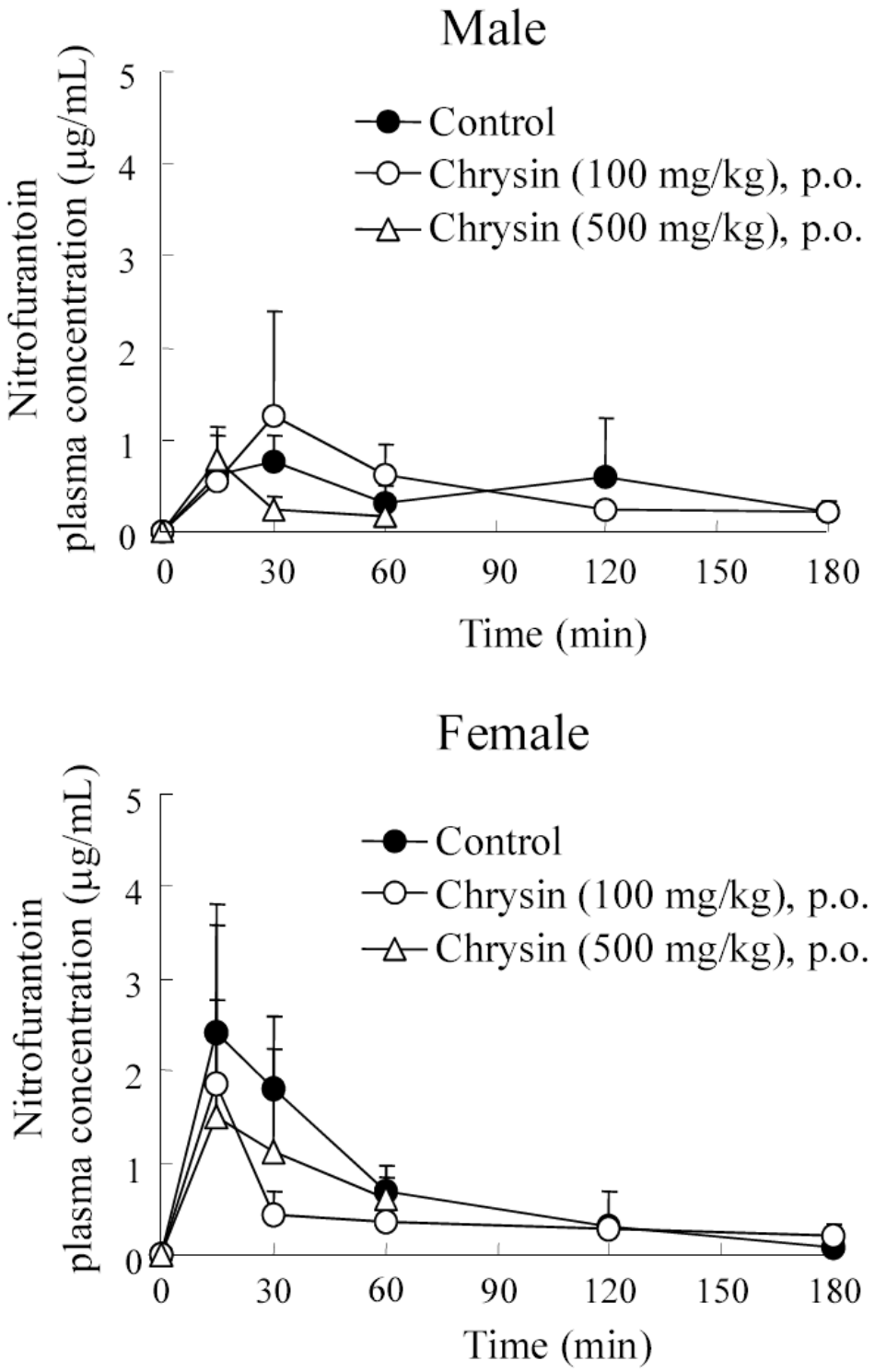

Figure 2. Plasma concentration versus time profile of nitrofurantoin after oral administration in male or female mice treated with either control vehicle or chrysin. Plasma levels of nitrofurantoin were determined by HPLC. Data are expressed as mean ± SD (n = 4).

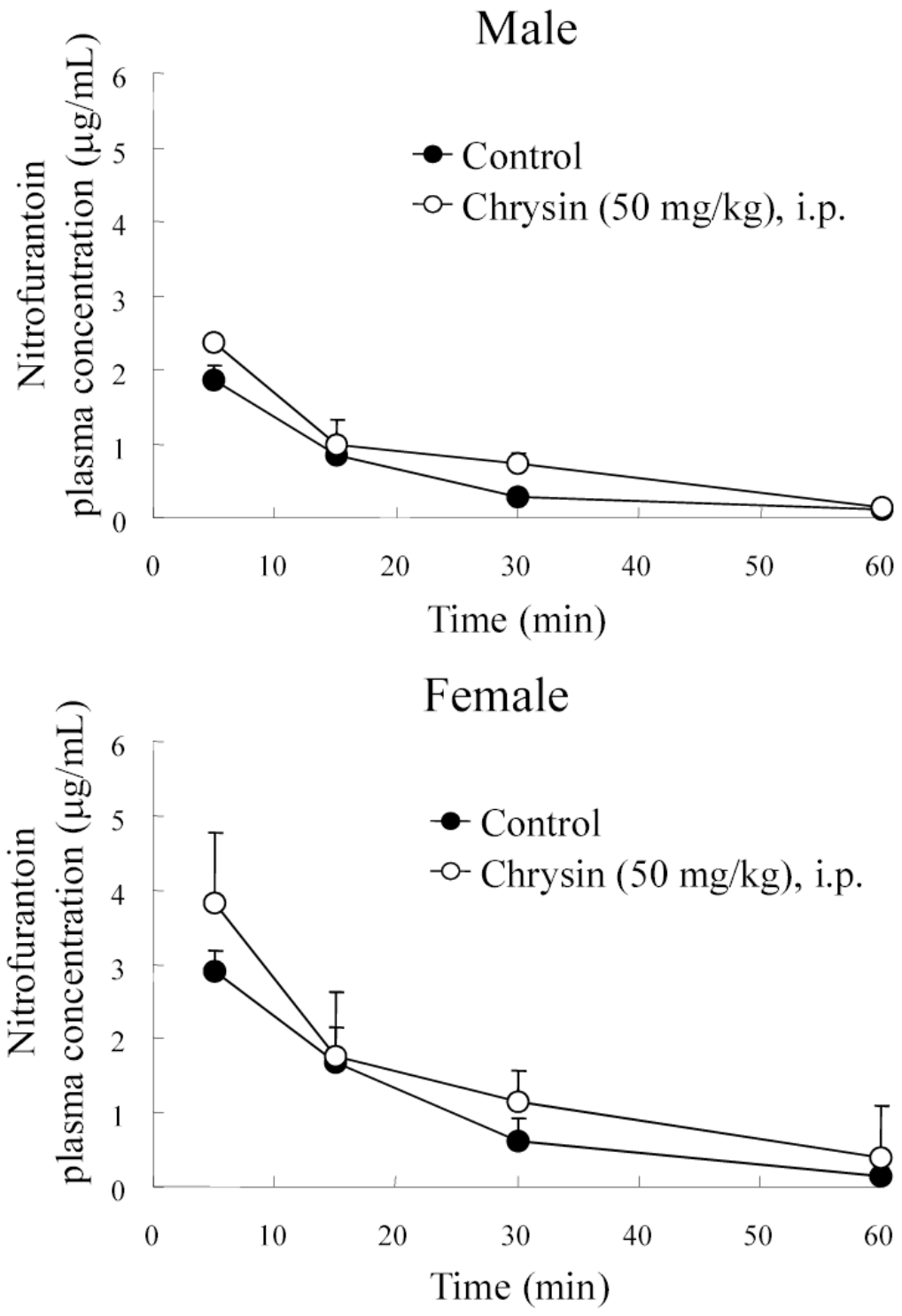

Figure 3. Plasma concentration versus time profile of nitrofurantoin after intravenous administration in male or female mice treated with control vehicle or chrysin. Plasma levels of nitrofurantoin were determined by HPLC. Data are expressed as mean ± SD (n = 3-6).

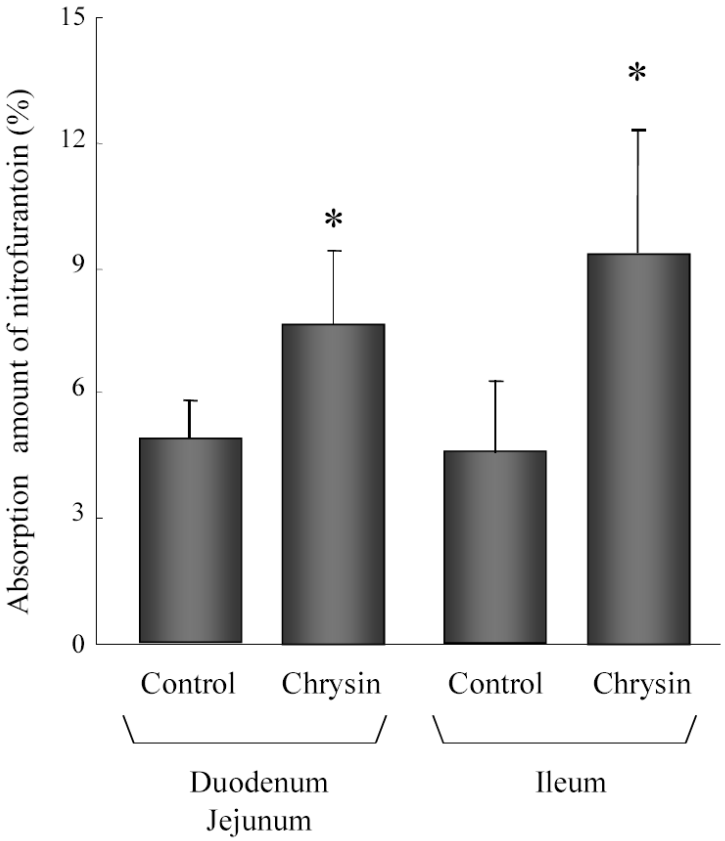

Figure 4. Absorption of nitrofurantoin in everted intestinal sacs from rat upper and lower intestine. The data are expressed as mean ± S.D. (n = 7-8). *, $p < 0.05$ compared with the control group.

The values of bioavailability of nitrofurantoin calculated from $AUC_{0\text{-}T}$ are about 50% in both control rats and mice. Therefore, we considered that the effect of chrysin on nitrofurantoin pharmacokinetics was induced by alteration of Bcrp activity. Wang et al. demonstrated that the flavonoid chrysin significantly inhibited nitrofurantoin transport mediated by human BCRP and rat Bcrp1 *in vitro*. In this study, we demonstrated that Bcrp inhibition was observed in rats but not in mice. No obvious effects of chrysin on plasma concentration of nitrofurantoin were observed in mice even after administration of 5 times the dose of chrysin used in rats (500 mg/kg). It is possible that test compounds have similar effects on rat and human BCRP, but not on mouse Bcrp. To clarify the reasons for the inter-species differences in the inhibitory effects of chrysin on Bcrp, we examined the Bcrp mRNA expression in both rats and mice. Little information is available on the relationship between Bcrp expression and the inhibitory effects of flavonoids on Bcrp activities. It has been reported that chrysin inhibited intestinal Bcrp in female rats (19). However, it is unclear whether or not similar effects of chrysin on Bcrp were also observed in male rats, and in female and male mice. The relative Bcrp mRNA levels in rat small intestines were higher than in those of mice, indicating that high intestinal Bcrp mRNA expression in rats could be related to the inhibitory effects of chrysin. Levels of Bcrp protein were not measured in this study, but it has been reported that the disposition of Bcrp mRNA was similar to that of Bcrp protein (26).

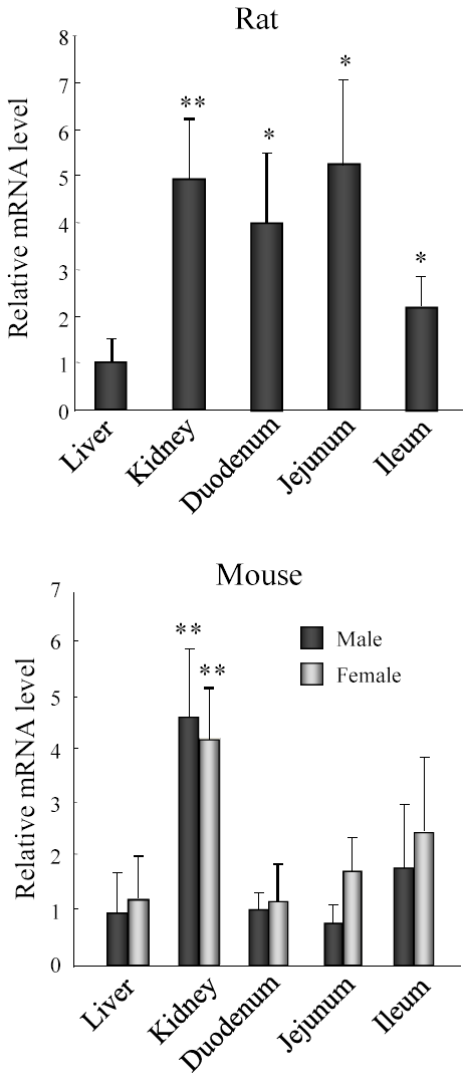

Figure 5. Relative Bcrp mRNA levels in liver, kidney, duodenum, jejunum and ileum of rats or male or female mice. The mRNA levels were determined by real-time RT PCR. The data are expressed as mean ± S.D. (n = 3-4). *, $p < 0.05$; **, $p < 0.01$ compared with the control group.

Therefore, Bcrp protein levels, as well as mRNA levels, could differ between rats and mice. These results are also consistent with previous reports on the tissue distribution and sex differences of Bcrp expression in rats and mice (27, 28). Tanaka et al. demonstrated that rat Bcrp mRNA levels were high in intestine and male kidney, and intermediate in testes, and that mouse Bcrp expression was highest in kidney, followed by liver, ileum, and testes. The tissue distribution of Bcrp shows extensive overlap with that of P-gp (5, 29, 30). We suggest that the species differences in tissue distribution of Bcrp could determine the inhibitory effects of chrysin on intestinal Bcrp in rats and mice.

Chrysin is used orally as a dietary supplement in humans. The recommended dose of chrysin supplements is 500–2000 mg/day (31). High concentrations of chrysin could therefore be found in the small intestine after the intake of supplements, which could result in drug interactions via Bcrp.

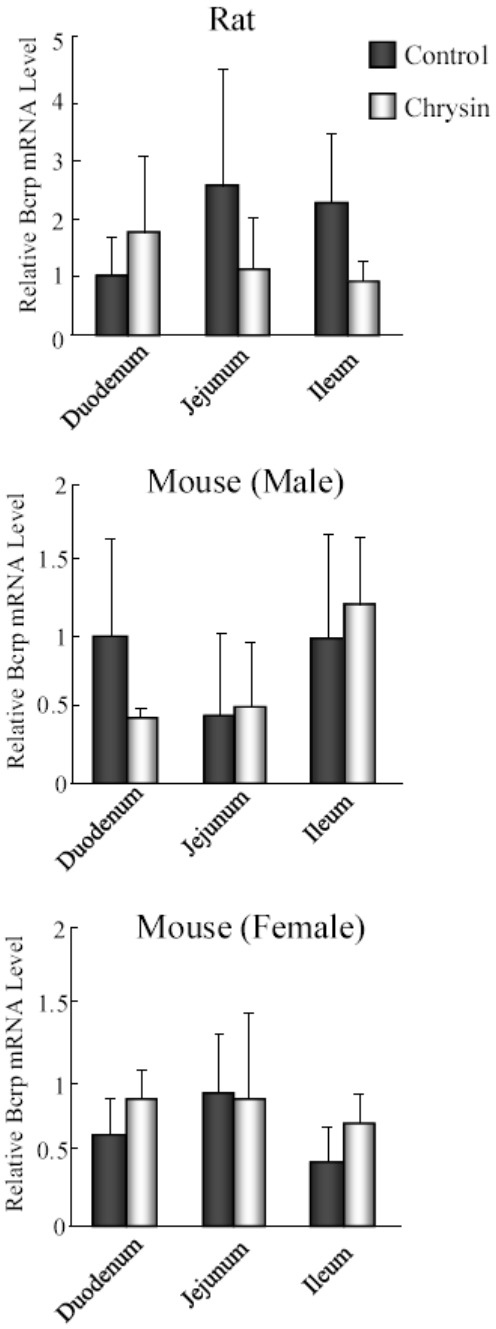

Figure 6. Relative Bcrp mRNA levels in duodenum, jejunum and ileum of rats or male or female mice treated with or without chrysin for 10 days. The mRNA levels were determined by real-time RT PCR. The data are expressed as mean ± SD (n = 3-4). Mark significant differences.

The intestinal expression of BCRP in humans has been shown to be greater than that of P-gp (32) and the effects of BCRP on oral drug bioavailability could be comparable to those of P-gp. In general, flavonoids have been shown to alter the activities and/or expression levels of phase-I (33, 34) as well as phase-II enzymes (35, 36) in the intestine, thereby affecting the plasma levels of xenobiotics (37, 38). P-gp expression was significantly increased by flavonoids, including chrysin (39). It is therefore possible that chrysin could alter the expression of some factors, including Bcrp, that

affect the pharmacokinetics of drugs. The expression of P-gp and Bcrp are regulated by nuclear receptors such as the pregnane X receptor and the constitutive androstane receptor (40, 41). We examined the effects of chrysin on Bcrp after treatment for 4 or 10 days, because dietary supplements and herbal preparations containing flavonoids are generally used over long periods. After 10 days of treatment with oral chrysin, little change in intestinal Bcrp mRNA levels were observed, suggesting that the pharmacokinetics of Bcrp substrates in rats and mice was not affected by chrysin treatment.

CONCLUSION

Inter-species differences in the inhibitory effects of chrysin on Bcrp exist between rats and mice. Little sex differences of chrysin on Bcrp were observed in mice. These species differences need to be taken into account when investigating drug interactions mediated via Bcrp.

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