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RESEARCH PAPER

5,7-Dimethoxyflavone and Multiple Flavonoids in Combination Alter the ABCG2-Mediated Tissue Distribution of Mitoxantrone in Mice

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ABSTRACT

Purpose The objective of our study was to investigate the effect of 5,7-DMF on the accumulation of mitoxantrone (MX) in BCRP-expressing normal cells and to investigate its impact on the PK and tissue distribution of MX in mice.

Methods The *in vitro* effect of 5,7-DMF on MX accumulation was examined in MDCK cells transfected with BCRP. The pharmacokinetic and tissue distribution of mitoxantrone, with and without co-administration of 5,7-DMF or multiple flavonoid combinations, were determined in mice.

Results In the presence of 2.5 μM or 25 μM of 5,7-DMF, the intracellular concentration of MX was significantly increased in MDCK/Bcrp1 and MDCK/BCRP cells, but not in MDCK/Mock cells. The AUC values of MX in several tissues were significantly increased when MX was co-administered with 5,7-DMF. The most substantial elevations of MX AUC in the presence of 5,7-DMF occurred in the liver (94.5%) and kidneys (61.9%), which is in apparent agreement with the relatively high levels of mouse Bcrp1 expression in these two tissues.

Conclusions Bcrp1-mediated DMF-MX interactions occur both *in vitro* and *in vivo*. 5,7-DMF represents a novel and very promising chemosensitizing agent for the BCRP-mediated MDR due to its low toxicity and potent BCRP inhibition.

KEY WORDS BCRP-based drug interaction · *in-vitro/in-vivo* correlation · mitoxantrone · tissue distribution · 5,7-dimethoxyflavone

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ABBREVIATIONS

ABC	ATP binding cassette
BCRP	breast cancer resistance protein
DMF	5,7-dimethoxyflavone
FTC	fumitremogin C
I.S	internal standard
MDCK	Madin-Darby canine kidney
MDR	multidrug resistance
MX	mitoxantrone
PK	pharmacokinetics

INTRODUCTION

Multi-drug resistance (MDR) has been implicated as a leading cause of the failure of cancer chemotherapy. The over-expression of ATP-binding cassette (ABC) transporters on the membrane of cancer cells, mainly P-glycoprotein (ABCB1, P-gp), MRP1 (ABCC1) and BCRP (ABCG2), is one of the best characterized mechanisms for this phenomenon (1). Compared with P-gp and MRP1, which were discovered several decades ago, BCRP is a relatively new efflux transporter discovered in 1998 (2). Many structurally distinct anticancer drugs, including mitoxantrone, topotecan, irinotecan, etoposide, and flavopiridol, have been reported to be substrates of BCRP (1). Diestra *et al.* (3) demonstrated that among 150 human solid tumors with 21 different tumor types, the expression of BCRP was detected in all tumor types examined. Besides its expression in various tumors, BCRP is also present in a number of normal tissues, including placenta, brain, intestine, liver, colon and kidney, and plays an important role in drug disposition (4,5).

To reverse transporter-mediated MDR, substantial research has been devoted to developing inhibitors/modulators of these efflux transporters (6,7). Many compounds have been identified as BCRP inhibitors *in vitro*, including elacridar (GF120918), fumitremogin C, Ko134, novobiocin, saquinavir, estrogen antagonists, and agonists (8,9). Our previous results revealed that many flavonoids are potent BCRP inhibitors: among a large number of flavonoids screened, twelve flavonoids exhibited potent BCRP inhibition with EC_{50} values <2.0 μM (10). Since flavonoids are widely present in the human diet and various herbal products, they represent very promising chemosensitizing agents due to their low toxicity. However, it should be noted that many flavonoids have poor bioavailability and undergo extensive metabolism (11,12). Therefore, for those flavonoids with unfavorable PK profiles, such as biochanin A (13), galangin and chrysin (14), the results of *in vitro* studies reporting potent BCRP inhibition may not result in *in vivo* interactions.

Recently, the Walle laboratory reported that methylated flavonoids have greatly improved intestinal absorption and metabolic stability compared with their demethylated analogs (15). For example, after oral administration (5 mg/kg each), 5,7-dimethoxyflavone (5,7-DMF) was clearly detected in plasma with a peak concentration of 2.5 μM, whereas chrysin, the demethylated analog of 5,7-DMF, was not detectable in plasma at any time point (16). Moreover, 5,7-DMF was also detected in tissues with concentrations in the liver exceeding those in the plasma by as much as seven-fold. Interestingly, both chrysin and 5,7-DMF are demonstrated to be potent BCRP inhibitors in our study, with EC_{50} values of 0.62 and 1.41 μM, respectively (10). Based on their different PK profiles, 5,7-DMF may represent a better inhibitor *in vivo*. 5,7-DMF is abundant in the leaves of *Piper caninum* (17) and *Leptospermum scoparium* (18,19). 5,7-DMF is also a major active constituent of *Kaempferia parviflora*, a perennial herb that has long been used traditionally in Thailand as a health-promoting herb and for the treatment of allergy, fungal infection and gastrointestinal disorders (20).

Since BCRP is expressed in both cancer cells and normal tissues, and 5,7-DMF demonstrates potent BCRP inhibition *in vitro* and has a favorable PK profile, our hypothesis is that 5,7-DMF not only can function as a chemosensitizing agent to reverse BCRP-mediated mitoxantrone resistance in cancer cells, but also is able to influence the transport of mitoxantrone (MX) in BCRP-expressing normal cells. Therefore, 5,7-DMF may impact the PK and tissue distribution of MX *in vivo*. In addition, there are several reports indicating that a total herb extract shows an enhanced activity when compared with equivalent doses of its individual constituents (21,22). We also hypothesized that multiple flavonoid combinations may have additive or synergistic effects on BCRP inhibition. In the present study, we first investigated the effect of 5,7-DMF on the accumulation of the BCRP substrate MX in BCRP-expressing normal cells. MDCK transfected with empty vector, human BCRP, or murine Bcrp1 were used in the current study. Second, to further evaluate whether BCRP inhibition occurs *in vivo*, a PK and tissue distribution study of MX, with and without co-administration of 5,7-DMF or a multiple flavonoid combination, was conducted in mice. Finally, since MX is not only a substrate of BCRP but also of P-gp, the effect of 5,7-DMF on the accumulation of mitoxantrone in P-gp over-expressing MCF7/ADR cells was also evaluated.

MATERIALS AND METHODS

Materials

5,7-dimethoxyflavone, 7,8-benzoflavone, 5,6,7-trimethoxyflavone and 8-methylflavone were purchased from Indofine (Hillsborough, NJ). Verapamil, mitoxantrone, sulfosalicylic acid, ascorbic acid, triethylamine, PEG200, PEG400, Tween 20, Cremophor, and hydroxypropyl-β-cyclodextrin (HPBCD) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640, phosphate-buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). Fumitremorgin (FTC) and ametantrone were kind gifts from National Cancer Institute (Bethesda, MD). All the other reagents or solvents used were commercially available and of reagent grade.

Cell Culture

The polarized Madin-Darby canine kidney cell line (MDCK-II) was used in the MX accumulation study. The MDCK-II and its subclones that were transduced with murine Bcrp1 or human BCRP cells were kindly provided by Dr. Alfred Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). Human breast cancer MCF-7 cells overexpressing with P-gp (MCF-7/ADR cells) or BCRP (MCF-7/MX100 cells) were also used in the mitoxantrone accumulation study. MCF-7/ADR and MCF7/MX100 cells were obtained from the National Cancer Institute. Cells were grown in a 5% CO_2 atmosphere at 37°C and cultured in 75 cm^2 flasks. Both MDCK cells and MCF-7 cells were cultured in culture media (DMEM and RPMI 1640, respectively) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Mitoxantrone Accumulation Studies

Effect of 5,7-DMF on the Accumulation of MX in BCRP-Expressing MDCK Cells or P-gp-Expressing MCF-7 Cells

The accumulation studies were carried out using flow cytometric analysis as previously reported (23). In brief, 1 ml of cell suspension (~10^6 cells) was pre-incubated with specific concentrations of 5,7-DMF, the vehicle (0.1% DMSO), or the positive control (10 μM FTC for MDCK cells and 100 μM verapamil for MCF-7/ADR cells) at 37°C for 15 min.

Effect of Excipients on the Accumulation of MX in BCRP-Expressing or BCRP-Negative MCF-7cells

Flavonoids usually have low solubility, and excipients are required when flavonoids are administered *in vivo*. To avoid the confounding factor of excipients themselves, the effects of several excipient candidates, namely Tween 20, Chremophor, PEG200, PEG400, ethanol, and HPBCD on BCRP were evaluated. Similarly as mentioned above, 1 ml of cell suspension (~10^6 cells) was pre-incubated with different vehicle candidates (0.1%), 0.1% DMSO (blank control), or the positive control (10 μM FTC) at 37°C for 15 min.

After 15 min of pre-incubation, 3 μM of mitoxantrone was then added into the cell suspension. After co-incubation for another 30 min, the accumulation was stopped by the addition of ice-cold PBS and centrifugation. The intracellular fluorescence of mitoxantrone was measured by a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a standard argon laser for 488-nm excitation and 670 nm bandpass filter. A total of 5000 cells, with the cell debris gated out, were analyzed for each sample.

Mitoxantrone PK and Tissue Distribution Study

Male ND4 Swiss Webster mice weighing 24–32 g were housed in a temperature-controlled environment with a 12-h light/12-h dark cycle and received a standard diet and water *ad libitum*. The study was approved by the Institutional Animal Care and Use Committee, University at Buffalo. Mice were randomized to three treatment groups. In all three groups, mitoxantrone at a dosage of 1 mg/kg was administered intravenously (i.v.) as a bolus injection under light anesthesia. In group 1, 5,7-DMF was administered i.p. (as a loading dose, 50 mg/kg) 30 min prior to and i.v. (25 mg/kg) 4–5 min prior to MX injection. In group 2, four flavonoids, namely 5,7-DMF, 7,8-benzoflavone (7,8-BF), 5,6,7-trimethoxyflavone (5,6,7-TMF) and 8-methylflavone (8-MF), were used as a combination and administered i.p first as a loading dose and then i.v. 4–5 min prior to MX injection. The total dose of flavonoids for the multiple flavonoid combination (group 2) was the same as for 5,7-DMF (group 1), and equal doses of each flavonoid were administered (*i.e.* 12.5 mg/kg of each flavonoid was administered i.p., followed by 6.25 mg/kg of each flavonoid i.v). In group 3, the vehicle was administered i.p. 30 min prior to and i.v. 4–5 min prior to the MX i.v injection (control group). The stock solution of 5,7-DMF or four flavonoids mixture was prepared in DMSO at a concentration of 50 mg/ml and was further diluted with a solution of 100% PEG400 when given i.p. or diluted with a solution of PEG400 and HPBCD (75%:25%, v/v) when given by i.v. bolus. MX was dissolved in saline at a concentration of 1.25 mg/ml. Both drug solutions were delivered as 5 μL/g body weight. At each time point (5, 15, and 30 min, and 1, 2, 4, 8, 12 and 24 hr), blood samples were obtained by aortic puncture and collected in tubes containing heparin as the anticoagulant, followed by a short centrifugation (5 min, 14000 rpm at 4°C) to separate plasma. To prevent oxidative degradation, 20 μl of 100 mg/ml ascorbic acid in 0.1 M citric buffer was added to each 200 μl of plasma sample. Immediately after blood collection, the mice were sacrificed by cervical dislocation, and heart, lung, liver, kidneys, spleen, intestine, muscle and brain were harvested. All samples were stored at −80°C until the time of HPLC analysis. Three mice were used for each time point in each group.

Mitoxantrone HPLC Analysis

The concentrations of MX in the plasma and tissue samples were determined using a validated HPLC method (24). Tissue samples were homogenized in 100 mg/ml ascorbic acid in 0.1 M citric buffer (pH 3.0). In brief, 5 μl or 10 μl of 5 μg/ml ametantrone (internal standard, I.S) was added to 100 μl of plasma sample or 200 μl tissue homogenates, respectively, to produce a final concentration of ametantrone of 250 ng/ml. An equal volume of sulfosalicylic acid and acetonitrile (25:75, v/v) was then added to precipitate proteins. After vigorous vortexing, the samples were centrifuged for 15 min at 14,000 rpm at 4°C. The supernatant (120 μl) was injected into the HPLC system. The HPLC analysis was carried out on a Nucleosil C18 (250 mm×4 mm, I.D.) column (Macherey-Nagel, Easton, WA). The isocratic mobile phase consisted of 19:81 (v/v) acetonitrile: sodium phosphate (pH 2.3 with 0.1% TEA), at a flow rate of 1.0 ml/min. The system consisted of a Waters 1525 pump, 717 plus autosampler, 2847 UV detector, and Waters Breeze workstation. Mitoxantrone and I.S were detected at 610 nm with a retention time of 6.4 and 4.2 min, respectively. The standard curve was linear over the concentration range of 5–1000 ng/ml in plasma and

12.5–2500 ng/ml in tissue homogenates. The lower limit of quantification of mitoxantrone was 5 ng/ml in plasma and 12.5 ng/ml in tissue homogenates.

Pharmacokinetic Analysis

The PK parameters of mitoxantrone in plasma and tissues were obtained by noncompartmental methods using WinNonlin (version 5.0, Pharsight Corporation, Palo Alto, CA). The area under the plasma and tissue concentration time curves (AUC) (0-infinity) was calculated using the linear trapezoidal method, with extrapolation to infinity. The systemic clearance (CL) was determined as Dose/AUC. Other basic pharmacokinetic parameters in plasma, such as the apparent volume of distribution at steady state (Vss) and the terminal half-life ($t_{1/2}$), were also determined.

Statistical Analysis

The differences between the mean values were analyzed for significance using a Student's *t*-test or one-way analysis of variance, followed by Dunnett's test. The *p*-values were considered statistically significant when they were less than 0.05.

RESULTS

Effects of 5,7-DMF on MX Accumulation in BCRP-Expressing MDCK Cells

The effects of 5,7-DMF on BCRP-mediated transport have only been investigated *in vitro* in cancer cells. Since BCRP is expressed not only in cancer cells but also a number of normal tissues, we investigated the effect of 5,7-DMF on MX accumulation in BCRP-expressing normal cells. MDCK cells transfected with empty vector (MDCK/Mock), murine Bcrp1 (MDCK/Bcrp1) or human BCRP (MDCK/BCRP) were used in the current study. FTC (10 μM), a potent and specific inhibitor of BCRP, was used as a positive control (5). As shown in Fig. 1, in the presence of 5,7-DMF at 2.5 μM or 25 μM concentrations, the intracellular concentration of MX was significantly increased in MDCK/Bcrp1 and MDCK/BCRP cells, whereas this phenomenon was not observed in MDCK/Mock cells. This indicated that 5,7-DMF has an inhibitory effect on BCRP/Bcrp1 in normal cells. In the presence of 2.5 μM of 5,7-DMF, the intracellular concentration of MX was increased by 41.8% in MDCK/Bcrp1 cells and 118% in MDCK/BCRP cells. The intracellular concentration of MX was increased by 181% in MDCK/Bcrp1 cells and 200% in MDCK/BCRP cells when MX was co-incubated with 25 μM of 5,7-DMF.

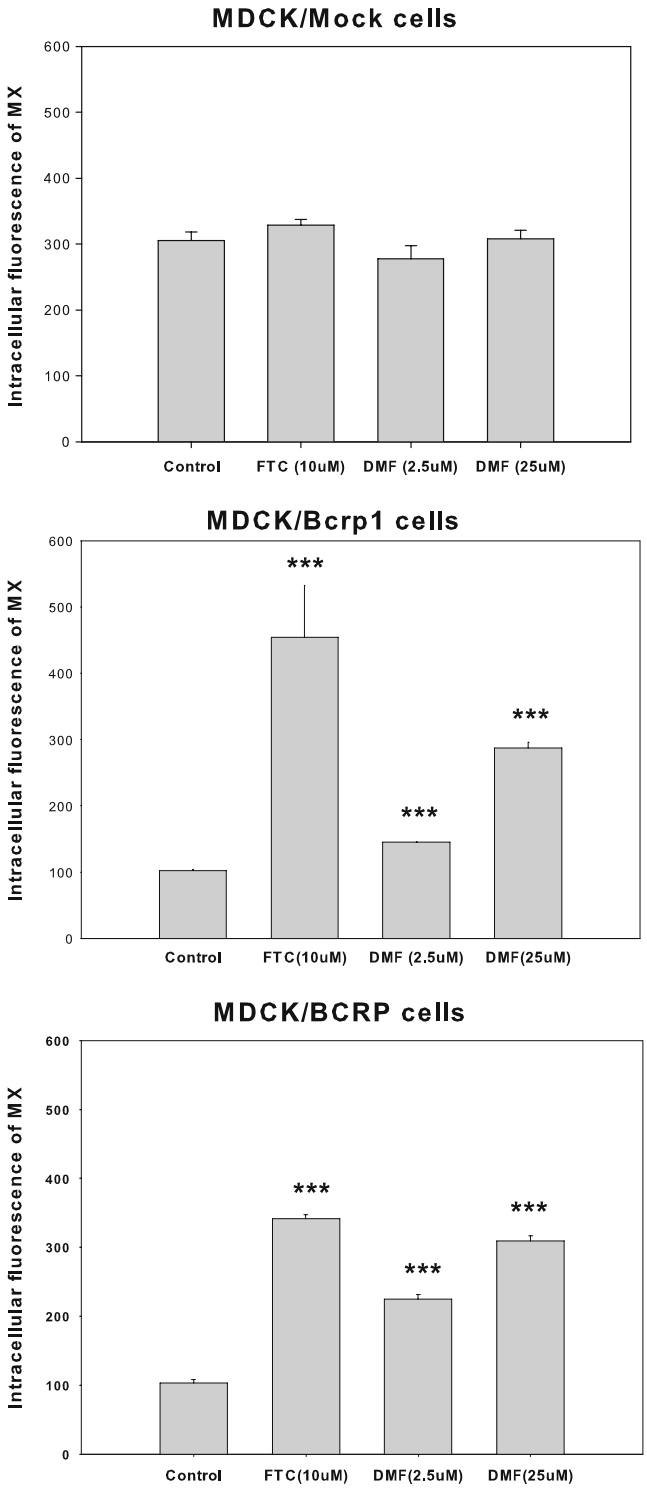

Fig. 1 Effects of 5,7-DMF on the accumulation of MX in MDCK cells transfected with empty vector (MDCK/Mock), murine Bcrp1 (MDCK/Bcrp1) or human BCRP (MDCK/BCRP). The intracellular fluorescence of MX after a 30 min incubation in MDCK cells in the presence of 0.1% DMSO (control), 10 μM FTC (positive control), 2.5 μM or 25 μM of 5,7-DMF was determined by flow cytometry, as described in Materials and Methods. Data are presented as mean ± SD, $n=3$. *, $p<0.05$; ***, $p<0.001$.

Effects of Excipients on BCRP

Due to the poor aqueous solubility of flavonoids, excipients are usually added to the formulation as solubilizing agents. Several excipients have been reported to exhibit an inhibitory effect on efflux transporters (25,26). Regarding the modulation effect of excipients on BCRP, only two excipients, namely Tween 20 and Pluronic P85, have been investigated, and both demonstrated BCRP inhibitory effects (27). To select the appropriate excipients for the four flavonoids, we screened several excipient candidates which are commonly used in drug PK studies, namely Cremophor, Tween 20, PEG200, PEG400, HPBCD and ethanol, and tested their effect on the accumulation of MX in both BCRP-negative and BCRP-overexpressing MCF-7 cells. As shown in Fig. 2, the intracellular concentration of MX was greatly increased in the presence of 0.1% of Cremophor and Tween 20 in MCF-7/MX100 cells, with no change in MCF-7/sensitive cells, suggesting their BCRP inhibitory effect. In contrast, no inhibitory effect on BCRP was found with 0.1% PEG200, PEG400, HPBCD and ethanol. Therefore, we further tested the solubility of flavonoids in the above four excipients, and the combination of PEG400 and HPBCD was selected as the vehicle for the flavonoids used in this study.

Effects of 5,7-DMF and the Multiple Flavonoid Combination on PK and Tissue Distribution of Mitoxantrone in Mice

To further evaluate whether the *in vitro* flavonoid-MX interactions via BCRP/Bcrp1 also occurred *in vivo* and to investigate the potentially additive or synergistic effect of multiple flavonoids on BCRP inhibition, the pharmacokinetic and tissue distributions of mitoxantrone, with and without co-administration of 5,7-DMF or multiple flavonoid combinations, were carried out in mice. In the multiple flavonoids treatment group, four flavonoids with low EC_{50} values, namely 5,7-DMF, 7,8-BF, 5,6,7-TMF and 8-MF, were included in the current study. 7,8-BF was selected, since it had the lowest EC50 value for BCRP inhibition among a large number of flavonoids that had been screened *in vitro* (10). 5,6,7-TMF and 8-MF were selected, since they exhibit potent BCRP inhibition *in vitro* and because methylated flavonoids have been reported to have greatly improved intestinal absorption and metabolic stability compared with their unmethylated analogs, indicating that methylated flavonoids have higher potential to have favorable PK profiles (15). The pharmacokinetic profiles of MX in the plasma of mice in the different treatment groups are illustrated in Fig. 3a. MX plasma levels declined in a biexponential manner, and MX plasma concentrations were not detectable at time points later than

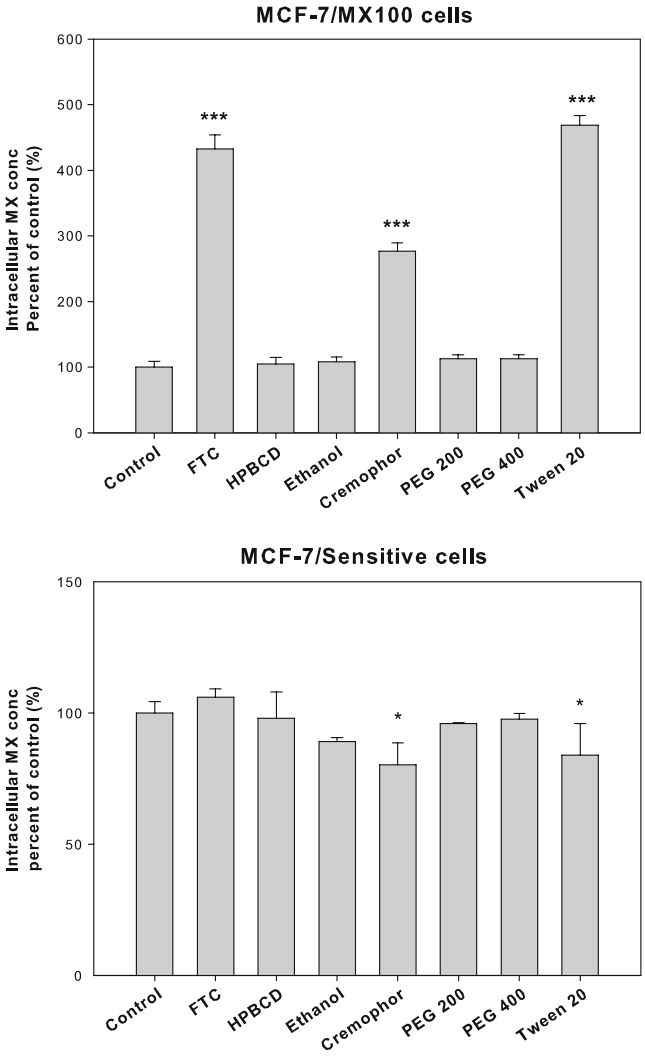

Fig. 2 Effects of excipients on the accumulation of MX in BCRP-expressing or BCRP-negative MCF-7 cells. The intracellular fluorescence of MX after a 30 min incubation in MCF-7 cells in the presence of 0.1% DMSO (control), 10 μM FTC (positive control), or 0.1% excipient candidates (Tween 20, Cremophor, PEG200, PEG400, ethanol, and HPBCD) was determined by flow cytometry, as described in Materials and Methods. Data are presented as mean ± SD, $n=3$. *, $p<0.05$; ***, $p<0.001$.

2 hours in all three groups. Compared with the vehicle-treated (control) group, MX plasma levels were higher in the flavonoid-treated groups at the last three time points (*i.e.* 30 min, 1 h and 2 h). The AUC of MX, however, was not significantly changed following the co-administration of flavonoid, with 0.098±0.028 μg*h/ml in the vehicle-treated group, 0.123±0.028 μg*h/ml in the 5,7-DMF-treated group, and 0.109±0.002 μg*h/ml in the multiple flavonoids-treated group. In addition to the AUC, other PK parameters of MX in plasma were also characterized, as shown in Table 1; none were significantly changed when MX was co-administered with flavonoids.

In a study conducted by Tanaka *et al.* (28), mouse Bcrp mRNA was detected in 17 different tissues, with highest

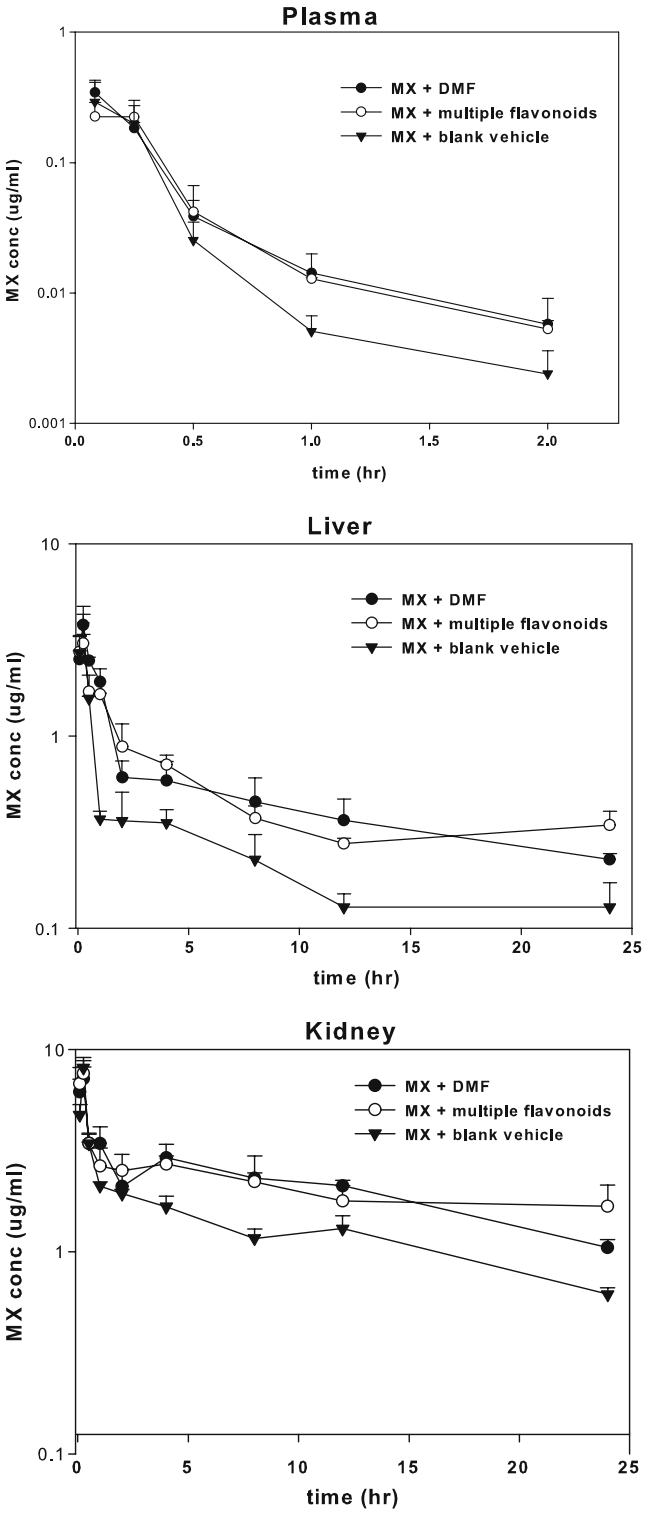

Fig. 3 The concentration-time profiles of MX in plasma, liver and kidney when MX was co-administered with 5,7-DMF, multiple flavonoid combination or vehicle. MX was administered at a dosage of 1 mg/kg in all three groups. The dose and dose regimen of flavonoids or vehicles were described in Materials and Methods. Plasma and tissue levels of mitoxantrone were determined by HPLC. Three mice were used at each time point in each treatment group. Data are presented as mean ± SD, $n = 3$.

Table 1 Plasma PK Parameters of MX in the Three Treatment Groups: Control, DMF and Multiple Flavonoids

	Control	DMF	Multiple flavonoids
AUCt(μg*h/ml)	0.098 ± 0.028	0.12 ± 0.028	0.11 ± 0.002
Cmax(μg/ml)	0.25 ± 0.051	0.35 ± 0.085	0.27 ± 0.024
$t_{1/2}$(h)	0.28 ± 0.03	0.47 ± 0.13	0.53 ± 0.17
V(L/kg)	113 ± 42.8	140 ± 58.6	167 ± 49.4
CL (L/h/kg)	268 ± 89.8	202 ± 37.5	220 ± 5.99

Data are presented as mean ± SD, $n = 3$. AUCt: $AUC_{0-2\ hr}$ for plasma

expression in kidney, followed by liver, ileum and testes. In our study, the concentrations and AUC values of MX in eight tissues with Bcrp1 mRNA expression, namely kidneys, liver, intestine, spleen, heart, lung, muscle and brain, were compared among different treatment groups. MX displayed extensive tissue distribution with high levels in kidneys, spleen, heart and lung, which is consistent with previous reports (29,30). In most tissues collected (except brain), the MX AUC values were one to two orders of magnitude greater than the plasma AUC. In contrast with the minor changes in MX concentrations in plasma, there were marked differences in MX tissue distribution in mice treated with 5,7-DMF or the multiple flavonoid combination. As shown in Table 2, in the presence of 5,7-DMF, the MX AUC values were significantly increased by 94.8% in liver, 61.9% in kidney and 18.4% in lung. With the co-administration of the multiple flavonoids, the AUC values for MX were significantly different in five different tissues, including heart (30.5%), liver (95.9%), kidney (63.3%), lung (30.7%), and muscle (33.8%). MX levels in brain were much lower than in other tissues, with no difference between flavonoid-treated or untreated groups. Although mean MX levels and AUC

Table 2 MX Tissue Exposure (AUCt) and Cmax in Tissues of Mice Treated with MX Alone, MX plus DMF, or MX Plus Multiple Flavonoids

	AUCt(μg*hr/ml)		
Tissues	control	DMF	Multiple flavonoids
Heart	19.1 ± 1.09	21.2 ± 0.72	24.9 ± 1.08*
Lung	38.8 ± 2.99	46.0 ± 1.72*	50.8 ± 1.35*
Liver	6.30 ± 0.59	12.3 ± 0.68*	12.3 ± 0.60*
Kidney	31.5 ± 0.35	51.1 ± 2.76*	51.5 ± 1.00*
Intestine	10.4 ± 2.17	14.2 ± 1.62	13.3 ± 1.91
Spleen	27.7 ± 2.85	32.8 ± 2.12	36.2 ± 4.41
Muscle	4.74 ± 0.15	5.45 ± 0.38	6.34 ± 0.85*
Brain	0.0249 ± 0.0032	0.0269 ± 0.0054	0.0275 ± 0.0041

Data are presented as mean ± SD, $n = 3$. *, $p < 0.05$. AUCt : $AUC_{0-1\ hr}$ for brain and $AUC_{0-24\ hr}$ for other tissues

values were higher in intestine and spleen in the flavonoid-treated groups compared with the vehicle-treated group, the differences were not statistically significant.

Effects of 5,7-DMF on MX Accumulation in P-gp-Expressing MCF-7 Cells

Since MX is not only a substrate of BCRP but also of P-gp, we investigated the effects of 5,7-DMF on MX accumulation in P-gp-expressing MCF-7 cells (MCF-7/ADR). Previous western blot results have shown that only P-gp, but not other efflux transporters, including BCRP and MRP1, was detected in MCF-7/ADR cells (31). Verapamil (100 μM), a well-documented P-gp inhibitor, was used as a positive control. Two different concentrations of 5,7-DMF, 10 μM and 50 μM, were selected in this study to represent the low and high concentration level of 5,7-DMF, respectively. As shown in Fig. 4, in the presence of 100 μM verapamil, the intracellular concentration of MX was greatly increased (3.2-fold) in MCF7/ADR cells, suggesting that MX is a P-gp substrate. The intracellular concentration of MX was significantly increased (1.62 fold) in the presence of 50 μM of 5,7-DMF in MCF-7/ADR cells, compared with that of the control group, indicating the inhibitory effect of 5,7-DMF on P-gp. Interestingly, although not reaching statistical difference, an opposite trend was observed when MX was co-incubated with a relatively low concentration of 5,7-DMF (10 μM): the intracellular accumulation of MX was decreased 32% compared with that of control group, suggesting a possible stimulatory effect of 5,7-DMF on P-gp at this concentration.

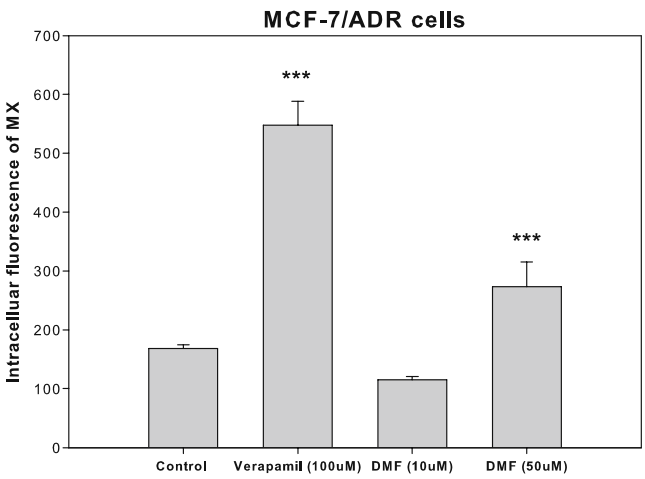

Fig. 4 Effects of 5,7-DMF on the accumulation of MX in P-gp-expressing MCF7/ADR cells. The intracellular fluorescence of MX after a 30 min incubation in MCF7/ADR cells in the presence of 0.1% DMSO (control), 100 μM verapamil (positive control), 10 μM or 50 μM of 5,7-DMF was determined by flow cytometry, as described in Materials and Methods. Data are presented as mean ± SD, $n = 3$. *, $p < 0.05$; ***, $p < 0.001$.

DISCUSSION

Previous studies have demonstrated that many flavonoids, including 5,7-DMF, can reverse BCRP-mediated MX resistance in BCRP-overexpressing breast cancer cells (10). Since BCRP is also expressed in normal tissues and is important for drug disposition, the aim of our study was to investigate the effect of 5,7-DMF on the MX accumulation in BCRP-expressing normal cells and to investigate its impact on the PK and tissue distribution of MX in mice. Moreover, continuing our interest in multiple flavonoid combinations, four flavonoids with potent BCRP inhibition, namely 5,7-DMF, 5,6,7-TMF, 7,8-BF and 8-MF, were selected, and their combination was also used as one treatment group in our MX PK and tissue distribution study.

In the MX accumulation study, 5,7-DMF significantly increased the intracellular concentration of mitoxantrone in BCRP-expressing normal cells. To further confirm our *in vitro* results, MX PK and tissue distribution studies, in the absence or presence of the single flavonoid 5,7-DMF or a multiple flavonoid combination, were conducted in mice. When MX was co-administered with 5,7-DMF or the multiple flavonoid combination, MX levels in plasma were higher at the last three time points than that in the vehicle-treated (control) group. BCRP is known to be located in the canalicular membrane of hepatocytes and the apical membrane of kidney proximal tubule cells and has the potential to increase biliary and urinary elimination of xenobiotics that are BCRP substrates (4). Since the elimination of MX is mainly through biliary and urinary excretion, the increase of MX plasma levels in the presence of flavonoids might be attributed to their inhibition of BCRP-mediated MX efflux, resulting in a reduction of MX biliary and/or urinary excretion. It should be noted that although MX plasma levels at the later times were increased in the presence of the tested flavonoids, no significant difference of AUC values and CL of MX was observed between the vehicle-treated group and flavonoid-treated groups. This is not surprising, because MX plasma levels decline very rapidly during the initial distribution phase, and the concentrations of MX at later times are low and therefore contribute little to the AUC of MX.

In contrast to the minor changes in plasma AUC values, MX AUC values in several tissues were significantly increased in both the DMF-treated and the multiple flavonoids-treated groups. In our study, the most substantial elevations of MX AUC in the presence of flavonoids occurred in the liver (>90%) and kidneys (>60%), and this observation is consistent with the high levels of mouse Bcrp1 expression in these two tissues (28). There was a significant increase of MX AUC observed in lung in both flavonoid-treated groups, albeit to a lesser extent than that

in liver and kidney. This might be due to the lower Bcrp1 expression level in murine lung. Although the expression level of murine Bcrp1 is relatively high in intestine, no significant increase of MX AUC in intestine was observed when MX was co-administered with 5,7-DMF or multiple flavonoids. However, MX was administered intravenously in these studies and would reach the intestine through biliary excretion. The intestinal concentrations of MX could be influenced by flavonoids by inhibition of biliary clearance and inhibition of intestinal cell efflux: these two processes have opposite effects on MX accumulation in intestinal cells, potentially resulting in no significant change in intestine MX concentration. It is interesting to note that, compared with 5,7-DMF, the multiple flavonoid combination had greater effects on MX tissue distribution. Besides liver, kidney and lung, the increase of MX AUC values in the presence of multiple flavonoids reached statistical difference in two more tissues—heart and muscle.

Since other transporters might also be involved in the DMF-MX interaction, we investigated the effects of 5,7-DMF on MX accumulation in P-gp-expressing MCF-7 cells, because MX is not only a substrate of BCRP but also P-gp. Our results revealed that 5,7-DMF only exhibited an inhibitory effect on P-gp at a high concentration (50 μM). Considering the high concentrations required for 5,7-DMF to exert P-gp inhibition, P-gp may only play a minor role in the *in vivo* DMF-MX interaction. In addition to P-gp, Patanasethanont *et al.* (32) examined the effect of flavone derivatives from *Kaempferia parviflora* on MRPs in A549 cells, a cell line expressing MRP1/2, but not P-gp. Their results demonstrated that 5,7-DMF significantly increased the cellular accumulation of doxorubicin at concentrations higher than 3 μM, indicating its inhibitory effect on MRPs. However, their results only indicate the overall inhibitory effect of 5,7-DMF on MRPs, and no specific information is available for MRP1 or MRP2. Moreover, MRP2 is not responsible for the transport of MX, and the role of MRP1 in MX transport remains controversial (33, 34). Taken together, the MRP-based DMF-MX interaction is unlikely to be important for MX. Besides modulation of transporters, another primary mechanism of drug-drug interactions is modulation of metabolizing enzymes. 5,7-DMF has been reported to directly inhibit the catalytic activity of CYP1A1, with an IC_{50} of 0.8 μM (35). However, CYP1A1 has not been reported to be involved in the metabolism of MX. Moreover, considering that MX only undergoes limited metabolism *in vivo* (30), enzyme inhibition may not contribute significantly to the DMF-MX interaction. Collectively, BCRP likely represents the main contributor to the *in vivo* DMF-MX interaction.

To understand the mechanism of flavonoids on Bcrp inhibition, we evaluated the role of Bcrp in the transport of several flavonoids, including 5,7-DMF and 8-MF, in Bcrp1-transfected MDCK/Bcrp1 cells. We used LC/MS/MS to measure the concentrations of investigated flavonoids. Our results showed that 5,7-DMF and 8-MF are not substrates of Bcrp (data not shown), suggesting that the inhibition may not be competitive in nature. However, the exact BCRP inhibitory mechanism requires further investigation.

Imai *et al.* (36) investigated the effect of flavonoids genistein, naringenin, and acacetin on the expression of BCRP after long-term incubation, and their western blot results showed that none of these flavonoids changed the expression level of BCRP compared to the control group. Whether flavonoids can induce BCRP expression has also been investigated in Caco-2 cells by Ebert *et al.* (37). Among six flavonoids they screened, flavone and quercetin demonstrated an induction effect on BCRP expression at the concentration of 25 μM (37). The effect of 5,7-DMF and the other flavonoids used in current study on BCRP expression is currently unknown.

To reverse transporter-mediated MDR, numerous studies have attempted to develop inhibitors/modulators of efflux transporters. However, except for P-gp inhibitors, the development of BCRP and MRP1 inhibitors are still limited to *in vitro* studies (6). Moreover, although many clinical trials have been conducted for P-gp inhibitors, most results have been disappointing, and the toxicity of these inhibitors themselves is one important factor that led to the failure of these studies. Compared with many P-gp inhibitors which demonstrate dose-limiting toxicity, 5,7-DMF, as a major constituent of several herbal products (17–19), represents an ideal compound for the BCRP-mediated MDR due to its low toxicity. Furthermore, in addition to potent BCRP inhibition, 5,7-DMF has also been reported to possess inhibitory effect on MRPs (32). Since the co-expression of BCRP with MRPs in tumors is not uncommon, another advantage of 5,7-DMF is that it might be used as a broad-spectrum modulator of MDR.

Our results may have important clinical implications for the oral administration of compounds with poor bioavailability due to BCRP. For example, the use of orally administered topotecan is often limited because of its poor and variable bioavailability (38). It has been reported that BCRP plays a key role in restricting the bioavailability of topotecan (39). Considering the strong BCRP inhibitory effect of 5,7-DMF observed both *in vitro* and *in vivo*, it is reasonable to speculate that the co-administration of 5,7-DMF might decrease the variability in topotecan absorption and improve its bioavailability and consequently its anticancer efficacy.

In conclusion, we observed strong inhibitory effects of 5,7-DMF on BCRP both *in vitro* and *in vivo*. The AUC values of MX in several tissues, especially those with high expression levels of BCRP, were significantly increased when MX was co-administered with 5,7-DMF. Compared

with 5,7-DMF, the multiple flavonoid combination had a greater effect on MX tissue distribution; this finding needs further investigation. Therefore, our results may have important clinical implications not only for tumors expressing BCRP but also for tissues with high expression levels of BCRP. While 5,7-DMF may represent a promising chemosensitizing agent for BCRP-mediated drug resistance by increasing tumor concentrations, the higher tissue concentrations of MX, observed in the present study, may result in possible toxic consequences.

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