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An acute herb-drug interaction of Magnoliae Officinalis Cortex with methotrexate via inhibiting multidrug resistance-associated protein 2

An acute herb−drug interaction of Magnoliae Officinalis Cortex with methotrexate *via* inhibiting multidrug resistance-associated protein 2

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Abstract

Magnoliae Officinalis Cortex (MOC), an herbal drug, contains polyphenolic lignans mainly magnolol (MN) and honokiol (HK). Methotrexate (MTX), a critical drug for cancers and autoimmune deseases, is a substrate of multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP). This study investigated the effect of coadministration of MOC on the pharmacokinetics of MTX and relevant mechanisms. Sprague−Dawley rats were orally administered MTX alone and with single dose (2.0 and 4.0 g/kg) and repeated seven doses of MOC (2.0 g/kg thrice daily for 2 days, the 7th dose given at 0.5 h before MTX). The serum concentrations of MTX were determined by a fluorescence polarization immunoassay. The results showed that a single dose of MOC at 2.0 g/kg significantly increased the AUC_{0-t} and MRT of MTX by 352% and 308%, and a single dose at 4.0 g/kg significantly enhanced the AUC_{0-t} and MRT by 362% and 291%, respectively. Likewise, repeated seven doses of MOC at 2.0 g/kg significantly increased the AUC_{0-t} and MRT of MTX by 461% and 334%, respectively. Mechanism studies indicated that the function of MRP2 was significantly inhibited by MN, HK and the serum metabolites of MOC (MOCM), whereas BCRP was not inhibited by MOCM. In conclusion, coadministration of MOC markedly enhanced the systemic exposure and mean residence time of MTX through inhibiting the MRP2-mediated excretion of MTX.

Keywords: Herb-drug interaction, Magnoliae Officinalis Cortex, Methotrexate, MRP2, Pharmacokinetics

1. Introduction

Magnoliae Officinalis Cortex (MOC), the dried bark of *Magnolia officinalis* Rehder & E.H. Wilson), is a widely prescribed herbal medicine in Asia countries for thousand years. According to the 4th Taiwan Herbal Pharmacopeia, the indications of MOC are for drying dampness and resolving phlegm, directing qi downward and eliminating fullness. The usual daily dose of MOC is 3.0−11.5 g. Nowadays, MOC is often used for the treatments of a variety of disorders, such as digestive disturbance, mental diseases and allergic diseases [1−3]. The major constituents of MOC are magnolol (MN) and honokiol (HK), which are polyphenolic lignans exhibiting various beneficial activities, such as anti-inflammation [4], anti-spasmodic [5], anti-anxiety [6,7] and anti-cancer effects [8]. Therefore, MOC and its components are contained in many nutraceuticals available in recent markets [9,10].

Based on previous pharmacokinetic studies, the conjugated metabolites of MN and HK are abundant in the bloodstream after oral administrations of MN, HK or MOC [10−12]. In recent decades, the conjugated metabolites existing as anions in the blood were known as substrates of multidrug

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resistance-associated proteins (MRPs) or breast cancer resistance protein (BCRP) [13–15]. Besides, our recent study verified that MN and HK were substrates and inhibitors of BCRP [16]. Because MRP2 and BCRP are efflux drug transporters expressed at the apical membranes of multiple tissues, including intestine, liver and kidney tubules [17], the inhibitions of MRP2 and BCRP might result in decreased excretion of their substrate drugs.

Methotrexate (MTX), a critical drug existing as anions in blood, is also a substrate of MRPs and BCRP like the conjugated metabolites [18–20]. MTX is clinically prescribed for the treatments of cancers [21,22] and autoimmune diseases, such as rheumatic arthritis and psoriasis [23–25], but with narrow therapeutic index. Nephrotoxicity and hepatoxicity were the common side effects of MTX [24,26,27], therefore, the serum concentration of MTX should be monitored for reducing the risks of adverse events. Moreover, patients treated with MTX often suffered from serious drug interactions, such as MTX-NSAIDs interactions, which were highly attributed to the inhibition of MRPs and BCRP [28,29]. Kidney is the major route for MTX elimination [30], thus any modulation on the function of renal efflux transporters like MRPs and BCRP would alter the elimination of MTX and its efficacy or toxicity. Based on the above rationale, we hypothesized that the conjugated metabolites of MN and HK might compete with MTX for the MRP2- or BCRP-mediated efflux transports and decrease the renal excretion of MTX. Therefore, this study aimed to investigate the effect of coadministration of MOC on the pharmacokinetics of MTX and relevant mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

MOC was obtained from a Chinese drugstore (Taichung, Taiwan) and identified through visual inspection and microscopic examination by Dr. Yu-Chi Hou. A voucher specimen (CMU-P-1905-13) was deposited in China Medical University. MN and HK were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MTX (25 mg/mL) was supplied by Wyeth Pharma Gmbh (Wolfratshausen, Germany). Mitoxantrone (MXR) was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Butylparaben was obtained from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), Hank's Buffered Salt Solution (HBSS) and 5-chloromethylfluorescein diacetate (CMFDA) were purchased from Invitrogen (Grand Island, NY, USA). Acetonitrile and methyl alcohol (MeOH) were supplied by Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). Milli-Q plus water (Millipore, Bedford, MA, USA) was used for all processes.

2.2. Preparation and characterization of MOC

The preparation of MOC was modified from our previous study [31]. Briefly, crude drugs of MOC (100 g) were immersed with 4000 mL of water. The other procedures were the same as previous described. Finally, the filtrate was concentrated to make 200 mL to afford a concentration of 0.5 g/mL of MOC decoction. Then, 500 μL of MOC was mixed with 500 μL of butyl paraben solution (40 μg/mL in MeOH as internal standard). After vortexed and centrifuged, 20 μL of the supernatant was subjected to HPLC analysis. The HPLC apparatus included one pump (LC-10AT; Shimadzu, Japan), an UV detector (SPD-10A; Shimadzu, Japan), an autosampler (Series 200, PerkinElmer, USA), and a RP-C18 column (4.6 × 250 mm, 5 μm; Alltech Associates Inc., USA). The mobile phase was acetonitrile/0.1% phosphoric acid (60:40) and run isocratically. The detection wavelength was set at 290 nm and the flow rate was 1.0 mL/min.

2.3. Animals and drug administration

Male Sprague–Dawley rats (350–450 g) were purchased from BioLASCO Taiwan Co., Ltd. (Yi-Lan, Taiwan) and housed in a 12-h light–dark cycle, constant temperature environment at the Animal Center of China Medical University (Taichung, Taiwan) prior to study. The study was conducted in accordance with the recommendations by "The Guidebook for the Care and Use of Laboratory Animals" published by the Chinese Society for the Laboratory Animal Science, Taiwan, ROC. This protocol (CMUIACUC-2019-127-2) was approved by the Institutional Animal Care and Use Committee (IACUC), China Medical University (Taichung, Taiwan).

Rats were randomly divided into four groups and a parallel study was conducted. Before experiment, rats were fasted overnight, but drinking water was allowed *ad libitum.* Food was supplied 3 h after drug administration. The 1st group (n = 6) was orally given MTX (5.0 mg/kg) alone with an equal volume of water as MOC. The 2nd groups (n = 6) and 3rd groups (n = 6) were concomitantly administered with 2.0 g/kg and 4.0 g/kg of MOC at 0.5 h before MTX (5.0 mg/kg), respectively. The dose selection was based on the daily dosage of MOC in the 4th Taiwan Herbal Pharmacopeia [32] and the USFDA 2005 guideline for "Conversion of Animal Doses to

Human Equivalent Doses" [33]. The 4th group (n = 5) was orally administered 2.0 g/kg of MOC thrice daily for two days and the 7th dose was given at 0.5 h before MTX.

2.4. Blood collection and the determination of MTX in serum

The blood samples (0.5 mL) were collected *via* the tail vein at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36 and 48 h. Then, the serum concentration of MTX was measured by a fluorescence polarization immunoassay (Abbott, Abbott Park, IL, USA). All the procedures, calibration curve and validation were conducted according to the manufacturer's instructions.

2.5. Cell line, culture conditions and MTT assay for cell viability

Madin–Darby canine kidney type II wild-type (WT) cells and two transfected cells with overexpression of MRP2 (MDCKII-MRP2) and BCRP (MDCKII-BCRP) were all kindly obtained from Professor Dr. Piet Borst (Netherlands Cancer Institute, Amsterdam, Netherlands) [34]. Cells were grown in DMEM medium supplemented with 10% of fetal bovine serum (Biological Industries Inc., Kibbutz, Beit Haemek, Israel), 1% of penicillin-streptomycin-glutamine (Invitrogen, Grand Island, NY, USA) at 37 °C in a humidified incubator containing 5% CO_2 [31,34]. Furthermore, the effects of tested agents on the viability of MDCKII-WT, MDCKII-MRP2 and MDCKII-BCRP cells were evaluated by MTT assay [35] with minor modification [31].

2.6. Preparation of the serum metabolites of MOC (MOCM)

Three rats were orally administered two doses of MOC (2.0 g/kg) with an interval of 30 min, then the blood was collected at 30 min after the second dose. In parallel, the blood was collected from another three rats untreated with MOC to prepare the serum as blank control. Other procedures were the same as our previous study [31].

2.7. Effects of MOC, MN, HK and MOCM on MRP2 activity

The effects of MOC, MN, HK and MOCM on the function of MRP2 were evaluated by using MDCKII-WT and MDCKII-MRP2 cells [31,36], respectively, and CMFDA (1 μM) was used as a fluorescent probe for evaluating the activity of MRP2. MK 571 (Enzo Life Sciences, Inc, Farmingdale, NY, USA) was used as a positive control of MRP2. Briefly, MDCKII-WT cells (1×10^5 cells/well) were incubated with the tested agents at 37 °C for 30 min. The fluorescence was set at 485/528 nm (excitation/emission). The relative intracellular accumulation of glutathione-methylfluorescein (GSMF, a metabolite of CMFDA), a fluorescent substrate of MRP2, was calculated by comparing with that of control. Besides, before experiments, MOCM sample was re-dissolved in 1 mL of water to afford a solution with 10-fold serum concentration and filtered with 0.2-μm pore size filter (Sartorius Stedim Biotech Gmbh, Gottingen, Germany). Furthermore, 10-fold serum concentration of MOCM was diluted with HBSS to afford a series solution of 1-fold and 1/2-fold serum concentration. Then, MDCKII-MRP2 cell suspension (5×10^5 cells/tube) was co-incubated with test agents and CMFDA at 37 °C for 30 min. The intracellular accumulation of GSMF was monitored by a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a standard argon laser. All data of MOCM were obtained after correction with the correspondent concentration of blank serum control. The transport studies were performed in triplicates.

2.8. Effects of MOC and MOCM on BCRP activity

The effect of MOC and MOCM on the function of BCRP was evaluated by using MDCKII-BCRP cells [16,31]. MXR (5 μM) was used as a probe for evaluating the activity of BCRP and Ko143 (Enzo Life Sciences, Inc, Farmingdale, NY, USA) was used as a positive control of BCRP. Briefly, MDCKII-BCRP cells (1×10^5 cells/well) were incubated with the tested agents at 37 °C for 2 h. After the cells were collected and lysed, the fluorescence of MXR was monitored at 607/684 nm (excitation/emission). On other hand, MDCKII-BCRP cell suspension (5×10^5 cells/tube) was pre-incubated with tested agents at 37 °C for 15 min. MXR was co-incubated for 30 min. The intracellular accumulation of MXR was determined by a FACScan flow cytometer equipped with a standard HeNe laser. All data of MOCM were obtained after correction with the correspondent concentration of blank serum control. The transport studies were performed in triplicates.

2.9. Data analysis

Pharmacokinetic parameters were calculated by using noncompartment model with the aid of Phoenix WinNonlin (version 8.1, Pharsight Corp., NC, USA). The differences of pharmacokinetic

parameters of rats among four treatments were analyzed by one-way ANOVA with Scheffe test, and unpaired Student's *t*-test was used for cell studies, taking $P < 0.05$ as significant level.

3. Results

3.1. Characterization of MOC

The HPLC chromatogram of MOC is shown in Fig. 1. The peaks of MN and HK were satisfactorily resolved within 18 min by an isocratic elution. The calibration curves (2.0–250.0 μg/mL) of MN and HK both showed good linearities with a correlation coefficient of 0.999. The coefficients of variation of intraday and interday analysis were less than 9% and the relative errors were below 16%. The recoveries were 94–108% and 94–100% for MN and HK, respectively. The quantitation results showed that MOC (0.5 g/mL) contained 0.9 and 0.5 μmol/mL of MN and HK, respectively. Accordingly, a dose of 2.0 g/kg of MOC contained 3.6 and 2.0 μmol/kg of MN and HK, and a dose of 4.0 g/kg of MOC contained 7.2 and 4.0 μmol/kg of MN and HK, respectively.

3.2. Effect of coadministration of MOC on MTX pharmacokinetics in rats

The serum MTX profiles after oral dosing of MTX alone and coadministrations with single dose (2.0 g/kg and 4.0 g/kg) and the 7th dose (2.0 g/kg) of MOC are shown in Fig. 2(A). The pharmacokinetic parameters of MTX after four treatments are listed in Table 1. After coadministration with single dose of 2.0 g/kg of MOC, the AUC_{0-t} and MRT of MTX was significantly increased by 352% and 308%, respectively. While 4.0 g/kg of MOC was coadministered, the AUC_{0-t} and MRT of MTX was significantly increased by 362% and 291%, respectively. After coadministration of the 7th dose of MOC at 2.0 g/kg, the AUC_{0-t} and MRT of MTX was significantly increased by 461% and 334%, respectively.

3.3. Cell viability assay

MTT assay indicated that 150 μM of MN and HK in MDCKII-WT cells, 100 μM of MN and HK in MDCKII-BCRP cells and 1-fold serum concentration of MOCM in both MDCKII-MRP2 and MDCKII-BCRP cells all exhibited no toxic effects on the cell viability (data not shown).

3.4. Effects of MOC, MN, HK and MOCM on MRP2 activity

The effects of MOC, MN and HK on the intracellular accumulation of GSMF in MDCKII-WT cells are shown in Fig. 3(A) and (B). The results revealed that MOC at 0.2, 1 and 5 mg/mL did not influence the intracellular accumulation of GSMF. As a positive control of MRP2 inhibitor, MK571 significantly increased the accumulation of GSMF by 203%. MN

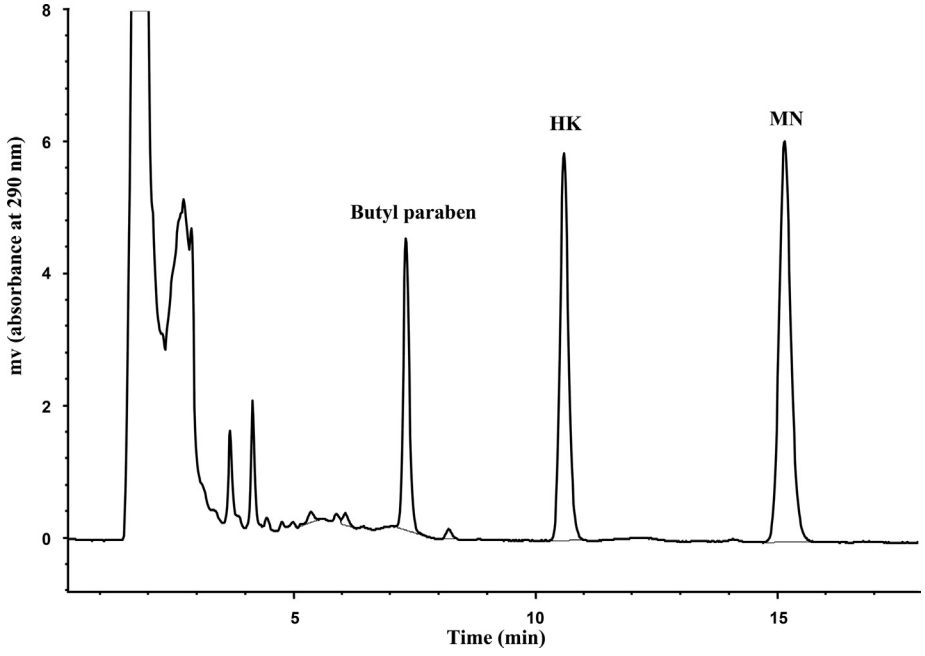

Fig. 1. HPLC chromatogram of MN, HK and butyl paraben (internal standard) in MOC.

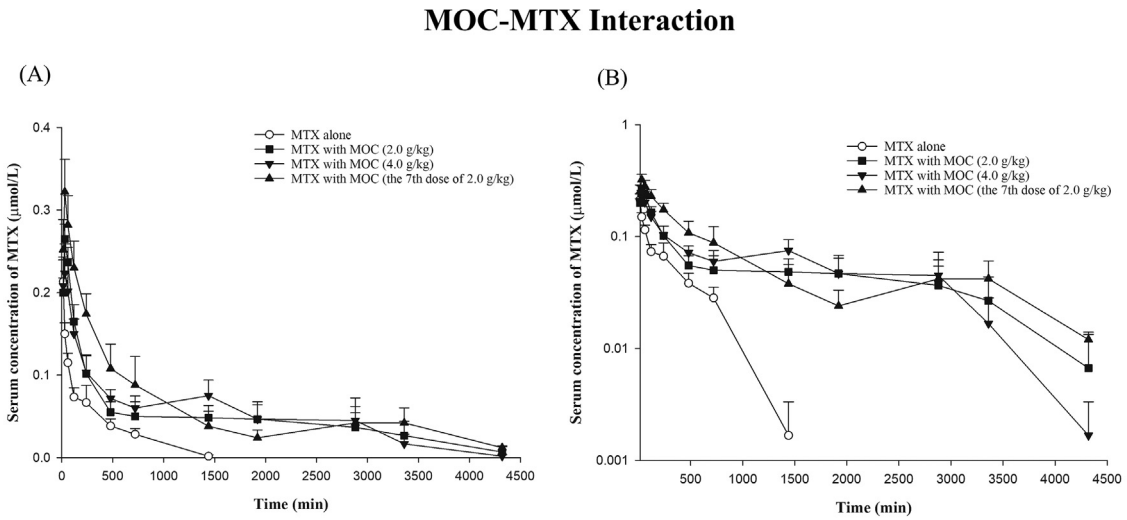

Fig. 2. (A): Mean (± S.E.) serum concentration–time profiles of MTX after oral doing of MTX alone (5.0 mg/kg) (○, n = 6) and coadministration with single dose of 2.0 g/kg (■, n = 6), 4.0 g/kg (▼, n = 6) and the 7th dose of 2.0 g/kg (▲, n = 5) of MOC and (B): the semi-log diagram of (A).

at 50 μM significantly increased the accumulation of GSMF by 137%; HK at 25 and 50 μM significantly increased the accumulation of GSMF by 34% and 75%, respectively. As a positive control of MRP2 inhibitor, MK 571 increased the accumulation of GSMF by 331%.

The effect of MOCM on the accumulation of GSMF in MDCKII-MRP2 cells is shown in Fig. 3(C). The results indicated that at 1-fold serum concentration, MOCM significantly increased the accumulation of GSMF by 17%. As a positive control of MRP2 inhibitor, MK 571 increased the accumulation of GSMF by 72%.

3.5. Effects of MOC and MOCM on BCRP activity

The effects of MOC and MOCM on the intracellular accumulation of MXR in MDCKII-BCRP cells are shown in Fig. 4. The results revealed that MOC at 0.2, 1 and 5 mg/mL significantly decreased the accumulation of MXR by 6%, 13% and 29%, respectively. As a positive control of BCRP inhibitor, Ko143 significantly increased by 55%.

On other hand, MOCM did not influence the intracellular accumulation of MXR. As a positive control of BCRP inhibitor, Ko143 significantly increased the accumulation of MXR by 60%.

4. Discussion

The determination of MN and HK concentrations in MOC by HPLC showed that the content of MN was higher than HK, which was consistent with previous studies [37,38]. The results of MOC-MTX interaction study in rats revealed that single-dose MOC either at 2.0 or 4.0 g/kg markedly increased

Table 1. Comparison of pharmacokinetic parameters of MTX after oral administration of 5.0 mg/kg of MTX alone (n = 6) and coadministered with 2.0 g/kg (n = 6), 4.0 g/kg (n = 6) and seven doses of 2.0 g/kg (n = 5) of MOC.

Parameters	Treatment			
	MTX + water	MTX + MOC (2.0 g/kg)	MTX + MOC (4.0 g/kg)	MTX + MOC (2.0 g/kg, 7th dose)
C_{max}	$0.2 \pm 0.0_3$	$0.3 \pm 0.0_1$	$0.2 \pm 0.0_3$	$0.3 \pm 0.0_4$
AUC_{0-t}	44.6 ± 5.0^a	201.5 ± 39.2^b (+352%)	206.8 ± 35.9^b (+362%)	250.2 ± 31.2^b (+461%)
MRT	287.6 ± 26.4^a	1174.1 ± 255.8^b (+308%)	1124.1 ± 189.6^b (+291%)	1248.8 ± 242.3^b (+334%)

Values are means ± S.E.
Mean in a row without a common superscript differ, $P < 0.05$.
A mean with a symbol "a" was significantly different from a mean with a symbol "b", and the symbol "ab" means that this treatment group show no significant difference when compared to other groups.
T_{max} (min): time to reach peak serum concentration.
C_{max} (μmol·mL^{-1}): peak serum concentration.
AUC_{0-t} (μmol·min·mL^{-1}): area under the blood concentration–time curve.
MRT (min): mean residence time.

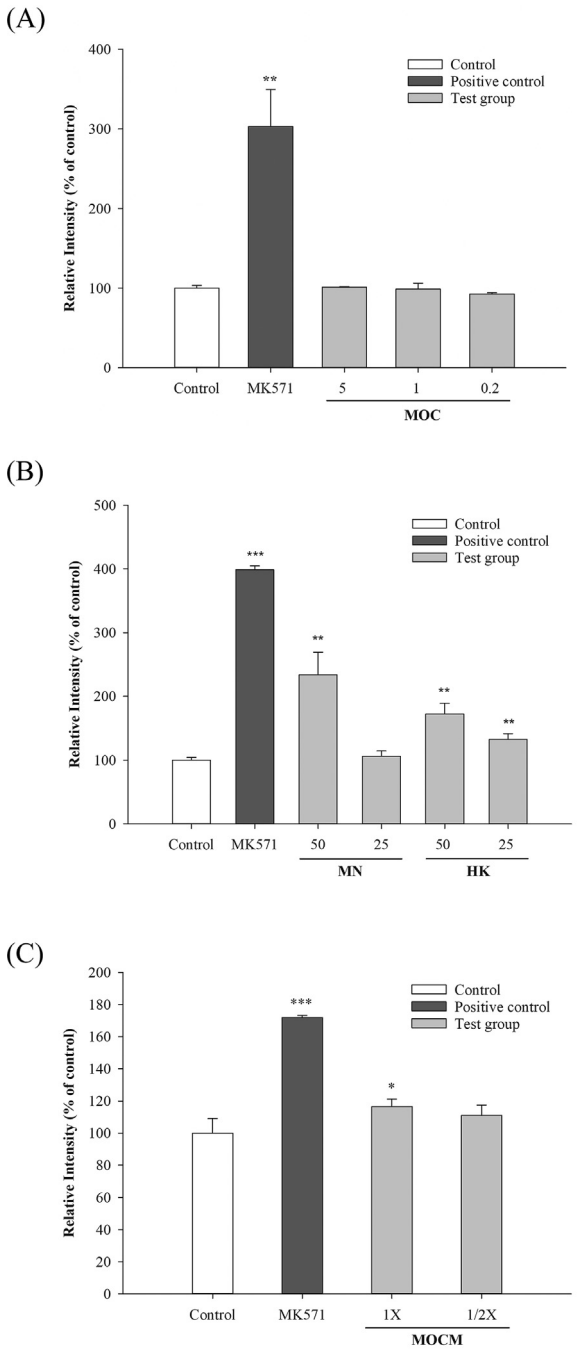

*Fig. 3. Effects of (A) MOC (mg/mL), (B) MN (μM), HK (μM) and MK 571 (50 μM, a specific inhibitor of MRP2) on the intracellular accumulation of GSMF (mean ± S.D.) in MDCKII-WT cells. (C) Effects of MOCM (1- and 1/2-fold serum concentration) and MK 571 (100 μM, spiked in blank serum) on the intracellular accumulation of GSMF (mean ± S.D.) in MDCKII-MRP2 cells. (*P < 0.05, **P < 0.01, ***P < 0.001).*

the AUC_{0-t} and MRT of MTX, indicating that the oral bioavailability of MTX was greatly enhanced by the coadministration of MOC. Likewise, when rats were given the 7th dose of MOC at 2.0 g/kg before MTX, the oral bioavailability of MTX was increased in comparably great extent with the single-dose treatments. Observation of the serum profiles among four treatments revealed that the four curves in the very short absorption phase were essentially overlapping, and the post-absorption profile of control group was much lower than other three groups coadministered with MOC, indicating that either single dose or multiple doses of MOC markedly decreased the elimination of MTX. Therefore, the increased bioavailability of MTX was apparently resulted from the inhibited elimination of MTX.

Referring to relevant pharmacokinetic studies, MN and the glucuronides/sulfates of MN were existing in the serum after ingestion of MN, while after administration of HK, only the glucuronides/sulfates of HK were present in the plasma, but no parent form of HK [11,12], indicating that MN and HK underwent extensive phase II metabolism, especially HK. Accordingly, for mimicking the virtual molecules contacting with transporters at the apical membranes of liver and kidney, MOCM was prepared from the serum of rats administered with MOC. Then, MOCM was used in cell studies to evaluate the modulation effects on MRP2 and BCRP.

In recent decades, several polyphenolics and their conjugated metabolites were verified as substrates and/or inhibitors of MRPs and BCRP [31,39–41], and MTX is also a substrate of MRP2 and BCRP, therefore, MRP2 and BCRP were proposed to be involved in the mechanism of MOC-MTX interaction. Subsequently, cell lines including MDCKII-WT, MDCKII-MRP2 and MDCKII-BCRP were employed for the mechanism elucidation.

Regarding the involvement of MRP2 in this MOC-MTX interaction, cell studies showed that the intracellular accumulation of GSMF, a typical substrate of MRP2, was increased by MN, HK and MOCM, indicating that MN, HK and MOCM all inhibited the MRP2-mediated efflux transport. Based on previous pharmacokinetic findings, we could assume that the free form MN, MN glucuronides/sulfates and HK glucuronides/sulfates were the major causative molecules of MOCM in inhibitions on MRP2 [11,12]. Because MTX was predominantly eliminated *via* kidney, once the MRP2 on the apical membrane of kidney was inhibited by MOCM, the excretion of MTX would be reduced. On other hand, MOC did not exert influence on MRP2 activity, we speculated that beyond MN and HK, some other compositions in MOC might activate MRP2 and cancel out the inhibition effects caused by MN and HK. Taken together, we concluded that the MRP2-mediated renal excretion

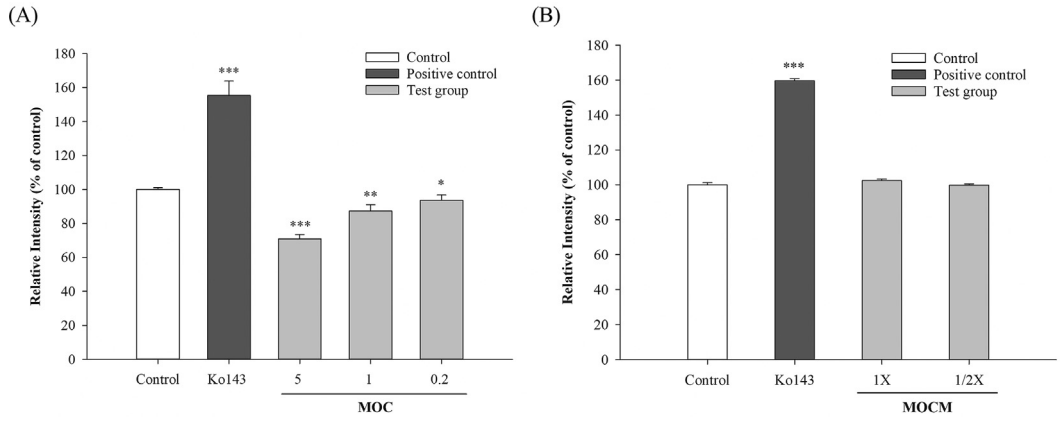

*Fig. 4. Effects of (A) MOC (mg/mL) and Ko143 (0.25 μM, a specific inhibitor of BCRP) on the intracellular accumulation of MXR (mean ± S.D.) in MDCKII-BCRP cells. (B) MOCM (1- and 1/2-fold serum concentration) and Ko143 (0.25 μM, spiked in blank serum) on the intracellular accumulation of MXR (mean ± S.D.) in MDCKII-BCRP cells. (*P < 0.05, **P < 0.01, ***P < 0.001).*

of MTX was inhibited by MN, MN glucuronides/sulfates and HK glucuronides/sulfates, which could in part account for the decreased elimination and enhanced systemic exposure of MTX after MOC coadministration.

Concerning the probable involvement of BCRP in this MOC-MTX interaction, our previous study found that MN and HK at concentrations of 12.5−100 μM significantly inhibited BCRP [16], conversely, this study showed that MOC slightly activated BCRP. We speculated that beyond MN and HK, some other constituents of MOC strongly activated BCRP and overwhelmed the inhibition effects caused by MN and HK. On other hand, MOCM at 1-fold serum concentration did not influence the function of BCRP. These results indicated that both MOC and MOCM did not inhibit the efflux transport of BCRP. Accordingly, we could infer that MOC ingestion did not inhibit the BCRP-mediated elimination of MTX. Therefore, BCRP was not involved in the mechanism of MOC-MTX interaction.

In brief, coadministration of MOC inhibited the MRP2-mediated renal excretion of MTX and resulted in marked increases of the systemic exposure and mean residence time of MTX. Besides, during the pharmacokinetic study, we also noticed that the mortalities of rats in the three groups coadministered with MOC were higher than that of control group. The control rats administered MTX alone all survived very well throughout the study. Apparently, the enhanced mortalities of MTX in rats was arisen from the markedly increased systemic exposure of MTX when MOC was coadministered.

In clinical settings, if patients are given low dose of MTX for treating rheumatoid arthritis or psoriasis [42−44], coadministration of MOC may lead to better efficacy. Nevertheless, when cancer patients are treated with high dose of MTX [45], the coadministration of MOC may result in serious adverse effects. Therefore, clinicians are suggested to be cautious for the blood concentration monitoring and dose adjustment of MTX when high dose of MTX is coadministered with MOC. Furthermore, the concurrent use of MOC with any critical substrate drugs of MRP2 should be avoided.

In conclusion, coadministration of MOC markedly increased the systemic exposure and mean residence time of MTX through inhibiting the MRP2-mediated renal excretion of MTX. We suggest that the concomitant use of MOC with MTX should be with great caution.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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