Inhibition of Human Efflux Transporter ABCC2 (MRP2) by Self-emulsifying Drug Delivery System: Influences of Concentration and Combination of Excipients

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ABSTRACT - PURPOSE: This study investigated influences of concentration and combination of excipients, commonly used in self-emulsifying drug delivery systems (SEDDS), on inhibition of human efflux transporter ABCC2 (MRP2). **METHODS:** Ten commonly used excipients of SEDDS with inhibitory effect on MRP2 including Cremophor® EL, Cremophor® RH, Pluronic® F127, Maisine® 35-1, β-cyclodextrin, Labrasol®, Pluronic® F68, PEG 2000, PEG 400 and Transcutol® were studied with the Caco-2 cell model. Six excipients with inhibitory effect including Cremophor® EL, Cremophor® RH, Pluronic® F127, PEG 2000, PEG 400 and Transcutol® were further analyzed using the MRP2 vesicle assay and ATPase activity assay. Ultra-performance liquid-chromatography tandem mass spectrometry was used to measure scutellarin as the MRP2 substrate. **RESULTS:** In studying concentration-dependent effects, five excipients including Cremophor® EL, Cremophor® RH, Pluronic® F127, Maisine® 35-1 and β-cyclodextrin showed concentration-dependent decrease in efflux ratio of scutellarin. The other five excipients did not show such phenomenon, and their inhibitory effects were restricted to be above to certain critical or minimum concentrations. In studying combined effects, PEG 2000 and Pluronic® F127 both showed combined effect with Cremophor® EL on inhibiting MRP2. However, some combinations of excipients such as PEG 400 and Transcutol® with Cremophor® EL increased the scutellarin efflux ratio and decreased the transport of scutellarin and ATPase activity, compared to Cremophor® EL alone. **CONCLUSION**: The above results suggest that appropriate choice of excipients according to their concentration-dependent and combined effects on MRP2 inhibition can facilitate formulation of SEDDS for improving the bioavailability of drugs that are MRP2 substrates.

INTRODUCTION

Human multidrug resistance related protein 2 (MRP2/ABCC2) is a member of the ATP-binding cassette (ABC) transporter family. MRP2 is expressed on the brush border membrane of intestinal and renal epithelial cells, and also on the bile canalicular membrane of hepatocytes (1-4). Research has previously focused on its ability to promote resistance to chemotherapy, but recently another main reason for studying MRP2 is to inhibit its efflux effect for improving the absorption of a series of drugs (5,6). In inhibition studies, the choice of MRP2 substrates relied mainly on endogenous, sulfate and glutathione conjugates (7,8). More and more recent studies have turned to multidrug-resistance associated proteins. The reason for this may be due to the finding of many flavonoids which could be used as the substrate of MRP2 (6,7,9). For example, scutellarin, an effective compound for treating cardiovascular diseases, was demonstrated to be both a substrate and inhibitor of MRP2, and the efflux activity of MRP2 could be one of the main factors limiting scrutellarin's oral bioavailability (9, 10).

The *in vitro* assessments of MRP2 inhibition have relied mostly on cellular models. Permeability analysis in Caco-2 monolayers, MDCK-MRP2 transfected cells, transport assays with MRP2

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vesicles, and measurement of ATPase activity on MRP2 membranes have been commonly used models in MRP2 efflux evaluation (11-13). Caco-2 cells are from a human colorectal carcinoma. It can be cultured to form a differentiated monolayer for investigating drug permeability (14-16). The Caco-2 cell assay is a cell-based epithelial monolayer assay that can measure MRP2 inhibition effect on the tested compounds (6,17). Many studies of Caco-2 permeability have focused on the detection of substrates by liquid chromatography tandem mass spectrometry (LC-MS). Among the LC-MS methods, ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) has many advantages including higher sensitivity and higher throughput of analysis (18-20). Therefore it is appropriate for analyzing substrates in Caco-2 permeability studies. However, considering the complex environment that might interfere with MRP2 in Caco-2 cell monolayers, purified membranes and vesicles from *Spodoptera frugiperda* (Sf9) insect cells expressing high level of MRP2 proteins can provide more specific validation (21,22). The ATPase activity and transport assays can respectively be studied using purified MRP2 membranes and MRP2 vesicles. ATPase activity can be measured by visible-range spectrophotometry for detection of phosphoric acid. For improved consistency, the detection method of transport assay by MRP2 vesicles can also use visible-range spectrophotometry. It is simpler than the previous fluorescent and radioisotope methods in transport assay of MRP2 vesicles (8, 23).

More and more studies have shown that absorption enhancement in SEDDS is not only related to their effective solubilization and particle size effects, but also attributed to the excipients in their formulation (10, 24, 25). Many kinds of SEDDS excipients such as surfactants, co-surfactants, oils and solid carriers were reported to show inhibitory effects on some efflux proteins. However, compared to the efflux protein P-glycoprotein, there have been fewer studies on the relations between MRP2 and excipients (26-29). Some researchers have reported that among the excipients used in SEDDS, some nonionic surfactants such as Cremophor® EL and Cremophor® RH showed inhibitory effect on MRP2 (12). In our previous study (10), we have screened different kinds of excipients that are commonly employed in SEDDS for the inhibitory effect on MRP2. Our previous study has shown that 11 excipients could reduce efflux ratio in Caco-2 models (10). They included five surfactants (Cremophor® EL, Cremophor® RH, Labrasol®, Pluronic® F68, and Pluronic® F127), three oils (Capmul® MCM, Maisine® 35-1 and Labrafac Lipophile® WL 1349), three co-surfactants (PEG 400, PEG 2000 and Transcutol®), as well as four solid carriers (HPMC K4M, HPMC K100, β-Cyclodextrin and Lactose). Among them, six excipients including Cremophor® EL, Cremophor® RH, Pluronic® F127, PEG 400, PEG 2000 and Transcutol® showed inhibition effect on MRP2 in the Caco-2 cell and MRP2 vesicle models. Our previous report has also stated that Cremophor® EL possessed the most potent inhibition effect on MRP2 (10).

However, our previous study has only demonstrated the inhibitory effects of excipients on MRP2. Further studies on whether these effects are concentration-dependent, and how do excipients interact with each other in the inhibitory effects of MRP2 could be helpful for optimizing the formulation of SEDDS. We hypothesized that some specific excipients such as Cremophor® RH, Pluronic® F127, PEG 400, PEG 2000 and Transcutol® may have concentration-dependent and combined inhibition effects when used together with Cremophor® EL. The aim of this study was to further investigate this hypothesis. For optimizing SEDDS formulation, the concentration-dependent inhibition effects of ten excipients on MRP2 were firstly analyzed. Subsequently, in order to assess interactions among excipients, this study also investigated the combined effects between Cremophor® EL and other excipients in MRP2 inhibition.

For the above purposes, Caco-2 permeability study, MRP2 vesicle transport assay and ATPase activity measurement were applied together in assessing concentration-dependent and combined effects of excipients interacting with MRP2. Accordingly, the protocol of this study was as follows. Firstly, UPLC-MS/MS quantification method of scutellarin and two spectrophotometric methods of scutellarin and phosphoric acid were established. Secondly, concentration-dependent inhibition effects of ten excipients were investigated using the Caco-2 permeability assay. Six excipients were also evaluated by MRP2 vesicles transport and ATPase activity analyses. Thirdly, concentration-dependent inhibition of MRP2 and combined effects of excipients with Cremophor® EL were assessed using the three assays.

MATERIALS AND METHODS

Materials

Pluronic® F127, Pluronic® F68, Cremophor® EL and Cremophor® RH were obtained from BASF Wyandotte Corp., Parsippany, NJ. USA. Labrasol®, Transcutol® and Maisine® 35-1 were purchased from Gattefossé Co. Ltd., Cedex, France. β-Cyclodextrin was obtained from J&K Scientific Ltd., Hong Kong, China. Polyethylene glycol (PEG) 2000 and PEG 400 were purchased from Damao Chemical Regent Co., Ltd., Tianjin, China.

Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co., Deisenhofen, Germany. 5-(3-(2-(7-chloroquinolin-2-yl)ethenyl)phenyl)-8-di methyl-carbamyl-4,6-dithiaoc -tanoic acid sodium salt hydrate, $C_{26}H_{26}CIN_2NaO_3S_2 \cdot xH2O$ (MK 571, a known inhibitor of MRP2), HPLC grade acetonitrile and acetic acid was obtained from Merck Co. Ltd., Darmstadt, Germany. The standard sample of scutellarin was purchased from the State Food and Drug Administration of China (SFDA), Beijing, China. Deionized water was purified by the Ultra-purification Water System from Millipore Co. Ltd., Bedford, MA, USA.

MRP2 membrane vesicles were obtained from Becton Dickinson & Co., Mountain View, CA, USA. Fetal bovine serum (FBS), Hank's balanced salts solution (HBSS), non-essential amino acids (NEAA), Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, phosphate buffered saline, 0.25% trypsin-EDTA and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) were purchased from Gibco Laboratories, Grand Island, NY, USA. Millicell 24-well cell culture insert plates (pore size 3.0 μm and Polyethylene Terephthalate filter) for Caco-2 monolayer cultivation and 96-well filter plates (pore size 0.7 μm in Polyethylene Terephthalate filter) for vesicle transport inhibition assay of MRP2 were obtained from Millipore Company.

Cell culture

Caco-2 cells were obtained from American Tissue Culture Collection (ATCC), Rockville, MD, USA. The cell lines employed in this study were cultured between passages 15 and 35. They were cultured at 37 ◦C in an atmosphere of 5 % CO_2, 90 % relative humidity. Caco-2 cells were cultured in the medium of DMEM added with 10 % FBS, 1 % NEAA, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES.

UPLC-MS/MS method for scutellarin

An Agilent UPLC-MS/MS (Agilent Technologies, Palo Alto, CA, USA) was used for analysis of scutellarin. The concentrations of scutellarin prepared for the seven-point standard curve of UPLC-MS/MS were diluted from 100 ng/ml to 1 ng/ml. The analytical system comprised an Agilent 1290 Infinity Auto-sampler (G4226), binary pump with integrated degrasser (G4220A), thermostat (G1330B) and Thermo Column Compartment (G1316C) coupled with Agilent 6460 triple quadruple mass spectrometer. The column employed in the quantification was Agilent ZORBAX Extend-C18 column (4.6 mm × 50 mm, 1.8 μm). The column temperature was 25 ◦C and the injection volume was 5 μl. Flow rate of the mobile phase was 350 μl/min. It was composed of solvent A and solvent B, which were 0.1 % acetic acid in water and 0.1 % acetic acid in acetonitrile, respectively. For liquid chromatography, the linear gradient was started at 25 % solvent B progressing to 35 % within an analysis time span of 5 min. In the mass spectrometry, electro spray ionization (ESI) interface was performed at 3900 V. The positive mode of selected reaction monitoring (SRM) was used in detecting mass ions of scutellarin, the precursor ion was measured at *m/z* 463.0 and product ion was measured at *m/z* 287.0. The collision energy (CE) was 17 eV with nitrogen applied as the collision gas. Scutellarin in the UPLC had the retention time of 2.83 min within the 5-min period of UPLC-MS/MS analysis. The standard curve was $Y = 23989X-14959$, $R^2 = 0.999$. The linear range of this standard curve was from 1 to 100 ng/ml. The recovery rate of scutellarin was from 91.1 to 104.7 %, and precisions of the method and the equipment were 0.38 and 0.54 % respectively.

Permeation study in Caco-2 monolayers

In this study, Caco-2 cells were cultured for 21 days until they were stable, and seeded on 24-well insert filters with a density of 1×10^5 cells/well. Previous studies have evaluated Caco-2 cell monolayers by analyzing its integrity, impermeability and differentiation (10).

The transport study on the Caco-2 cell monolayers was to determine the apparent permeability coefficient (*Papp*) in two directions of the cell monolayers. $Papp_{ab}$ was the permeability value from the apical (AP) to the basolateral (BL) side, and $Papp_{ba}$ was in the direction from BL to AP side. The permeability tests were performed at 37

◦C with 5 % CO_2 after equilibration. The monolayers were equilibrated by adding of 400 µl HBSS (pH 7.4) in AP side and 600 µl HBSS in BL side for 30 min after washing the cultured cells three times with HBSS buffer in 37 ◦C. One hundred µl samples were drawn from the apical and basolateral chambers at 30, 60 and 90 min, followed immediately by adding 100 µl HBSS. Before quantification of scutellarin by UPLC-MS/MS, the samples were centrifuged at 10000 rpm for 10 min and filtered through a 0.22 µm filter membrane. *Papp* was calculated as below in cm/s.

$$Papp = (dC/dt) \times (V_r / A\, C_0)$$

In this equation, A was the area of the cell monolayers surface (cm^2), C_0 the initial concentration in the donor chamber, dC / dt the permeability rate (µg/(ml × s)), and V_r the volume of the receiver chamber (ml) (30). And the efflux ratio of scutellarin is a division of $Papp_{BA}$ to $Papp_{AB}$.

In the concentration-dependent assay, ten excipients were divided into five analytical groups in the Caco-2 model. The first group was 100 µM scutellarin without or with the addition of 20 µM MK571, this group was evaluated as the blank and positive comparative group. The second, third, fourth and fifth sample groups were 100 µM scutellarin added respectively with five surfactants, three co-surfactants, one oil and one solid carrier. The surfactants were Pluronic® F127, Pluronic® F68, Cremophor® EL, Cremophor® RH and Labrasol®, the co-surfactants were Transcutol®, PEG 2000 and PEG 400, the oil was Maisine® 35-1 and the solid carrier was β-cyclodextrin. Each of the excipients had four concentrations of 100, 10, 1 and 0.1 µg/ml. Sterilized DMSO was used to dissolve the samples. In the combined-effect analysis, 100 µM scutellarin was also added in each test. The transport experiments of Cremophor® EL alone and the mixed samples (with one of the nine excipients) were analyzed following similar procedures as in the concentration-dependent assays.

Transport inhibition assay with MRP2 vesicles

The MRP2 vesicle transport inhibition assay used 1 mg/ml sf9 over-expressed MRP2 vesicles. After preparation of the tested reagents and samples, inhibition reactions were started with the addition of 20 µl MRP2 vesicles and 100 µl different groups of tests samples. The tests were performed at 37 ◦C for 40 min. With the addition of the stop solution (cold HBSS buffer), the transport solutions in the 96-well transparent plates were transferred to 96-well filtration plates (0.7 µm pore size). After vacuum filtration and washing with assay buffer, the diluted solutions were collected with the existence of ATP or AMP, respectively. Compared with the radioactive method in previous studies, this study detected absorbance at 335 nm in Micro-plate Reader for the MRP2 substrate scutellarin.

In the concentration-dependent assay using the MRP2 transport vesicles, the sample groups were just surfactants and co-surfactants group. The surfactants group included Cremophor® EL, Cremophor® RH and Pluronic® F127, and the co-surfactants were Transcutol®, PEG 2000 and PEG 400. In the combined-effect analysis, scutellarin at 100 µM was added to each sample. The concentration of excipients used in the study was 100 µg/ml. The experiments could be divided into two assay groups: the comparative group and the mixed sample group. The comparative group was composed of scutellarin and 100 µg/ml Cremophor® EL. The mixed excipient groups in the MRP2 transport assay included five pairs: Cremophor® EL+ Cremophor® RH, Cremophor® EL+ Pluronic® F127, Cremophor® EL+ PEG 2000, Cremophor® EL+ PEG 400, and Cremophor® EL+ Transcutol®. All of them were dissolved in sterilized DMSO.

Activity assay of ATPase with MRP2 membranes

ATPase activity was assayed using 1 mg/ml sf9 over-expressed MRP2 membranes in 96-well transparent plates. Following the revised protocols of BD Company, experiments were conducted in the following steps. Firstly, the standard curve of phosphoric acid was established at 800 nm in Spectra Max 190 Microplate reader (Molecular Device, Sunnyvale, CA, USA). Secondly, the solutions of MgATP and MRP2 membranes were prepared. Thirdly, ATPase activity was assayed by measurement of absorbance at 800 nm. According to the protocol, absorbances of each group with or without vanadic acid salts were compared. Each sample was analyzed for three times in parallel, and data obtained in ATPase assay were compared with the vanadic acid group for three times.

Beside the assay probe of 100 µM scutellarin, samples under evaluation were 20 µM MK571, 6

excipients with 4 concentrations in concentration - dependent assay, and 5 mixed excipients groups in combined effect analysis.

STATISTICAL ANALYSIS

Experimental data were obtained from triplicate analyses. All results in the Caco-2 cell model and MRP2 transport model were expressed as means ± SD. Statistical comparisons of more than two groups of data (Figures 1-5 and Table 1) were performed by one-way analysis of variance (ANOVA), while the Student's t test was used for comparing two groups of data (e.g. excipient + Cremophor® EL vs Cremophor® EL alone).

RESULTS

Concentration-dependent and combined effects of excipients with Cremophor® EL in the Caco-2 monolayer model

The ten excipients studied in Caco-2 models comprised five surfactants (Pluronic® F127, Pluronic® F68, Cremophor® EL, Cremophor® RH and Labrasol®), three co-surfactants (Transcutol®, PEG 2000 and PEG 400), one oil (Maisine® 35-1) and one solid carrier (β-cyclodextrin). All excipients showed reducing effects on efflux ratio of scutellarin as in our previous studies (10). **Table 1** shows the concentration-dependent inhibition effects with variations in *Papp* value, efflux ratio and concentration-dependence of the ten excipients.

Table 1 further illustrates that Pluronic® F127 (10, 1, 0.1 μg/ml) and Pluronic® F68 (1, 0.1 μg/ml) in surfactants, PEG 400 (100, 10, 1, 0.1 μg/ml) in co-surfactants, and Maisine® 35-1 (100, 10, 1, 0.1 μg/ml) in oils all had $Papp_{ab}$ values lower than 1.81×10^{-6} cm·s^{-1} in the scutellarin alone group ($p<0.05$). At the concentration of 100 μg/ml scutellarin, the three excipients of Cremophor® EL, PEG 2000 and β-cyclodextrin had $Papp_{ab}$ values higher than 2.74×10^{-6} cm·s^{-1} seen for the positive group of 20 μM MK571 ($p<0.05$). These results suggest that Cremophor® EL, PEG 2000 and β-cyclodextrin exhibited the enhancing transport effect on scutellarin better than the positive group of 20 μM MK571 in Caco-2 cell models.

Results from study of efflux ratios showed that the nine excipients except β-cyclodextrin had lower efflux ratio than the scutellarin alone group ($p<0.05$) at all concentrations of 100, 10, 1 and 0.1 μg/ml. Only at concentrations of 1 and 0.1 μg/ml, β-cyclodextrin had efflux ratios similar to the scutellarin alone group.

The decrease in efflux ratios was due to increase in $P_{app,AP-BL}$ and decrease in $P_{app,BL-AP}$ for a majority of excipients including Cremophor® EL, Cremophor® RH, Labrasol®, Transcutol®, Pluronic® F127 at the concentration of 100 μg/ml, Pluronic® F68 at concentrations of 100 and 10 μg/ml, PEG 2000 at 1 and 0.1 μg/ml, and β-cyclodextrin at 100 and 10 μg/ml. However, for PEG 2000 at 100 and 10 μg/ml and β-cyclodextrin at 1 μg/ml, increases in $P_{app,AP-BL}$ were more than corresponding increases in $P_{app,BL-AP}$, resulting in decreases in efflux ratios. In contrast, decreases in $P_{app,BL-AP}$ were more than corresponding decreases in $P_{app,AP-BL}$ resulting in decreases in efflux ratios for PEG 400, Maisine® 35-1, Pluronic® F127 at concentrations of 10, 1 and 0.1 μg/ml and Pluronic® F68 at 1 and 0.1 μg/ml.

Comparing with the positive control, Cremophor® EL, PEG 2000, PEG 400 and Maisine® 35-1 at concentrations of 100, 10 and 1μg/ml had lower efflux ratios. In these Caco-2 cell monolayers, even the minimal concentration of PEG 2000 and PEG 400 showed better efflux reduction on scutellarin than 20 μM MK571. These comparisons indicate that inhibitory effects of the ten excipients are in line with our previous study in Caco-2 cells (10).

Table 1 also demonstrates the concentration-dependent effects of the ten excipients. With the decreasing concentration trend of 100, 10, 1 and 0.1 μg/ml, the efflux ratios of scutellarin in three surfactants (Cremophor® EL, Cremophor® RH and Pluronic® F127), the oil (Maisine® 35-1) and the solid carrier (β-cyclodextrin) were correspondingly increased. The other 5 excipients did not show this concentration-dependent phenomenon. These results suggest that the five excipients had reducing effects on effluxing scutellarin in a concentration dependent manner, which may relate to energy consumption and concentration saturation. For those excipients that did not show the concentration-dependent phenomenon, there were critical concentrations in some excipients. For example, the concentration of 1 μg/ml could be the critical concentration for Pluronic® F68, Labrasol® and Transcutol®; below this concentration, the efflux ratio of scutellarin may not show concentration dependence as in 100, 10 and 1 μg /ml.

Table 1. Concentration-dependent effects of 10 excipients in Caco-2 model (*p < 0.05 in each excipient group.)

Excipients		$Papp_{AB}$ (10^{-6} cm·s^{-1})	$Papp_{BA}$ (10^{-6} cm·s^{-1})	Efflux Ratio
Control group	scutellarin alone	1.81±0.05	9.70±0.16	5.36±0.13
	scutellarin with MK571	2.74±0.13	5.43±0.02	1.98±0.03
Cremophor® EL	100μg /ml	4.34±0.18*	5.29±0.13*	1.22±0.02*
	10μg /ml	3.71±0.08*	5.12±0.11*	1.38±0.03*
	1μg /ml	2.59±0.05*	4.12±0.15*	1.59±0.03*
	0.1μg /ml	2.41±0.07*	6.31±0.34*	2.62±0.07*
Cremophor® RH	100μg /ml	2.73±0.04*	4.56±0.11*	2.03±0.04
	10μg /ml	2.45±0.06*	4.99±0.20*	2.04±0.03
	1μg /ml	2.22±0.07*	5.70±0.06*	2.57±0.06*
	0.1μg /ml	2.09±0.07*	6.00±0.07*	2.87±0.05*
Pluronic® F68	100μg /ml	2.05±0.12	4.39±0.08*	2.14±0.04*
	10μg /ml	2.07±0.11	4.74±0.06*	2.29±0.02*
	1μg /ml	1.15±0.02*	3.07±0.18*	2.67±0.06*
	0.1μg /ml	1.53±0.03*	3.86±0.19*	2.52±0.08*
Pluronic® F127	100μg /ml	1.87±0.02*	5.09±0.17*	2.72±0.02*
	10μg /ml	1.66±0.04*	4.86±0.10*	2.93±0.01*
	1μg /ml	1.58±0.03*	4.98±0.15*	3.15±0.05*
	0.1μg /ml	1.38±0.06*	4.58±0.11*	3.32±0.03*
Labrasol®	100μg /ml	2.26±0.13*	4.43±0.09*	2.40±0.02*
	10μg /ml	2.74±0.18*	7.32±0.08*	2.67±0.02
	1μg /ml	2.75±0.17*	7.47±0.14*	2.72±0.03
	0.1μg /ml	2.64±0.08	6.90±0.10*	2.61±0.03*
Transcutol®	100μg /ml	2.66±0.14	5.77±0.08	2.17±0.04
	10μg /ml	2.75±0.04*	6.19±0.15*	2.25±0.04
	1μg /ml	2.60±0.10*	7.25±0.13*	2.79±0.07*
	0.1μg /ml	2.21±0.11*	5.94±0.11	2.69±0.06*
PEG2000	100μg /ml	10.05±0.14*	15.08±0.13*	1.50±0.01*
	10μg /ml	9.52±0.27*	13.61±0.10*	1.43±0.04
	1μg /ml	5.33±0.16*	7.73±0.13*	1.45±0.03
	0.1μg /ml	3.89±0.18*	5.80±0.04*	1.49±0.01
PEG 400	100μg /ml	1.54±0.06*	2.26±0.18	1.47±0.02
	10μg /ml	1.39±0.09	2.02±0.24*	1.45±0.06
	1μg /ml	1.22±0.11	2.15±0.19	1.76±0.04
	0.1μg /ml	1.36±0.12	2.41±0.17*	1.77±0.04
Maisine® 35-1	100μg /ml	1.10±0.13	1.49±0.04*	1.35±0.05*
	10μg /ml	1.07±0.06	2.02±0.09*	1.89±0.04
	1μg /ml	1.13±0.11	2.19±0.07*	1.94±0.06
	0.1μg /ml	1.06±0.15	3.15±0.16*	2.97±0.10*
β-cyclodextrin	100μg /ml	4.00±0.09*	9.00±0.11	2.25±0.03*
	10μg /ml	3.38±0.11*	9.13±0.07	2.70±0.08*
	1μg /ml	2.91±0.07*	15.22±0.16*	5.23±0.08*
	0.1μg /ml	2.22±0.06*	12.68±0.10*	5.71±0.27*

The concentration of 0.1 μg/ml could also be the minimal concentration for PEG 2000, because there was no statistically significant difference in the efflux ratio of scutellarin when the

concentration of PEG 2000 varied from 0.1 μg/ml to 100 μg/ml. The concentration-dependent effects and the critical or minimal concentrations of excipients should provide useful information for SEDDS formulation. For instance，0.1 μg/ml of the minimal concentration of PEG 2000 showed better efflux reduction effect on scutellarin, so the formulation needs not contain excess of PEG 2000. The physiological relevance of these concentrations used is also important, it will be discussed below.

Nine excipients were assessed for their combined inhibition effects when used together with Cremophor® EL. The results of efflux ratio are shown in **Fig. 1**. In the Caco-2 cell monolayer model, there were three groups that showed joint enhancement effects better than Cremophor® EL alone ($p < 0.05$). The three groups were Cremophor® EL+ Pluronic® F127, Cremophor® EL + PEG 2000 and Cremophor® EL + β-cyclodextrin. These results demonstrate that the surfactant (Pluronic® F127), co-surfactant (PEG 2000) and solid carrier (β-cyclodextrin) all had the positive combined effect in reducing efflux of scutellarin, and this may indicate that the three combined-excipient groups could have the combined inhibition effect on MRP2.

Concentration-dependent and combination effects of excipients with Cremophor® EL in the MRP2 vesicle transport model

After confirming the concentration-dependent effect in Caco-2 cell monolayers, MRP2 vesicle transport assay was performed to study the efflux-reducing mechanisms of scutellarin. Absorbance at 335 nm was used as the detection method, and each sample in the MRP2 vesicle transport assay was tested in triplicate. After adding ATP or AMP to the reaction wells, data were obtained by subtracting contents of scutellarin in ATP group with those in AMP group. In this part of study, each scutellarin result was derived from 18 absorbance measurements at 335 nm. The standard curve of scutellarin analysis was Y=21.32X-6.91, R^2=0.999.

Fig. 2 shows the concentration-dependent inhibition effects of the six excipients. Except for Cremophor® EL, all of 0.1 μg/ml group in the other five excipients did not manifest inhibition effect on MRP2. Through increasing the transport of scutellarin, the surfactants Cremophor® EL (100 and 10 μg/ml) and co-surfactant PEG 2000 (100 μg/ml) both demonstrated a better inhibition effect on MRP2 than the positive group of 20 μM MK571.

For the study of concentration-dependent effect, results in **Fig. 2** indicate that the concentrations of scutellarin detected in Cremophor® EL, Cremophor® RH, Pluronic® F127, Transcutol®, PEG 2000, PEG 400 decreased as excipient concentrations decreased. It can be concluded that these excipients inhibited MRP2 in a concentration dependent manner in the MRP2-vesicle transport assay. Considering the concentration-dependent results in the Caco-2 cell model, it can be generalized that Cremophor® EL and Cremophor® RH decreased the efflux of scutellarin via the mechanism of inhibiting MRP2, while Pluronic® inhibition was not through this mechanism. Although PEG 2000 and PEG 400 showed inhibitory effect on MRP2, none of them decreased the efflux of scutellarin in Caco-2 cells concentration-dependently.

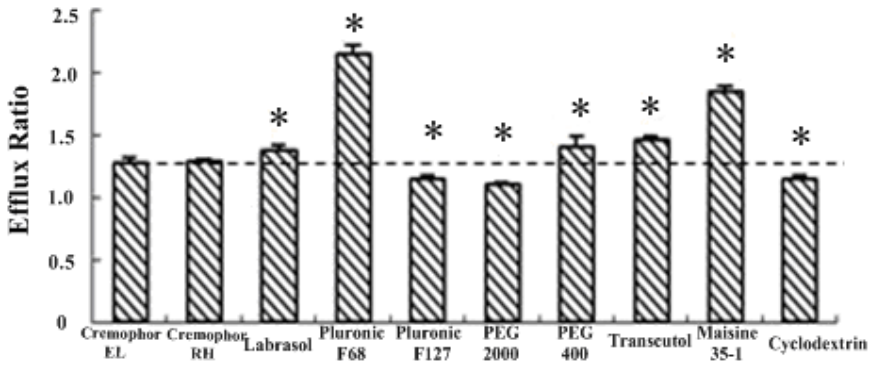

Figure 1. Combined effects of 9 excipients used together with Cremophor® EL in the Caco-2 model (*$p < 0.05$, related to efflux of scutellarin with Cremophor® EL).

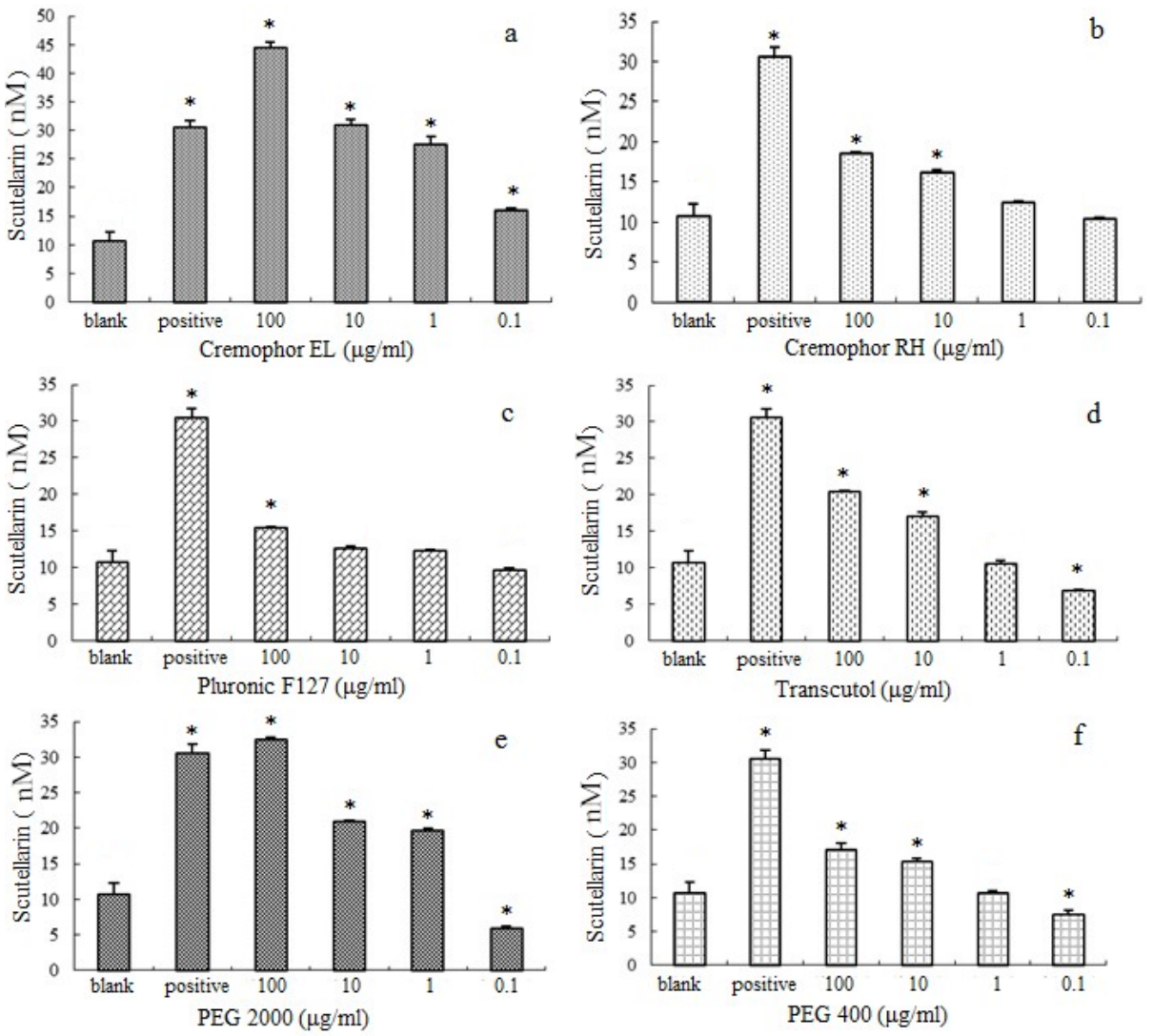

Figure 2. Concentration-dependent inhibition effects of 6 excipients in MRP2 vesicle assay (data groups a, b, c, d, e and f are from Cremophor® EL , Cremophor® RH, Pluronic® F127, Transcutol®, PEG 2000 and PEG 400, respectively. *$p < 0.05$, compared to scutellarin alone. "Blank" refers to scutellarin alone and "positive" refers to scutellarin with MK571.).

Fig. 3 illustrates the combined inhibition effects among Cremophor® EL used together with five other excipients Cremophor® RH, Pluronic® F127, PEG 2000, PEG 400 and Transcutol®. From the listed transported quantities of scutellarin, Pluronic® F127 increased the inhibitory effect on MRP2 ($p < 0.05$). Compared with results in the Caco-2 cell model, data in **Fig. 3** show that Pluronic® F127 could decrease efflux of scutellarin when used with Cremophor® EL.

Concentration-dependent and combination effects of excipients with Cremophor® EL on ATPase activity

As one of membrane-based assay systems, the ATPase activity assay has been used to assess interactions between inhibitors and efflux transporters by measuring phosphoric acid released during the transport process (31). In ATPase activity assay of MRP2, the quantities of phosphoric acid can reflect activation of ATPase activity. With measurement of absorbance at 800 nm (Y), the standard curve of phosphoric acid concentration (X) was $Y=0.024X+0.031$, $R^2 = 0.997$.

As shown in **Fig. 4**, phosphoric acid concentrations were used for comparing the degree of phosphoric acid release from ATP. Membranes over-expressing MRP2 were added to the reaction mixture, so the concentrations of phosphoric acid represented activation effect on ATPase that existed in the MRP2 membranes. Compared with the positive control group of 20 μM MK571, all concentration groups in the six excipients did not show higher activation effect on ATPase activity of MRP2. **Fig. 4** illustrates that phosphoric acid concentration of Cremophor® EL, Cremophor® RH and PEG 2000 decreased as excipient concentrations were reduced. Therefore, comparing with findings in the Caco-2 model and MRP2 vesicle transport assay, it can be deduced that Cremophor® EL, Cremophor® RH and PEG 2000 decreased the efflux of scutellarin via the mechanism of inhibiting MRP2.

Fig. 5 shows that the combined effects on ATPase of five other excipients with Cremophor® EL. Compared with Cremophor® EL alone as the standard, Cremophor® EL + Pluronic® F127 and Cremophor® EL + PEG 2000 both increased phosphoric acid concentration by 19.22 % and 13.24 %.

DISCUSSION

In this study, we compared the concentration-dependent and combined effects of excipients commonly used in SEDDS, and evaluated their combined interactions with MRP2 by using three assay systems. For the bi-directional transporter assay (apical-to-basal and vice versa), Caco-2 polarized epithelial cell lines are considered to be more reflective of a drug's efflux and inhibition (31). Although limitations of this system include variability in transporter expression profiles with the small intestine， it is a mechanistic assessment and the standard method for evaluating drug transport across the small intestine. Results in the concentration-dependent analyses demonstrated that Cremophor® EL, PEG 2000 and β-cyclodextrin exhibited the enhancing transport effect on scutellarin better than the standard inhibitor MK571. From the study of combined effects, surfactant (Pluronic® F127), co-surfactant (PEG 2000) and solid carrier (β-cyclodextrin) all showed synergism in reducing efflux of scutellarin when used together with Cremophor® EL. These results may indicate that the three combined-excipient groups exert more inhibition effect on MRP2.

In membrane-based assay systems, the combined assay of membrane vesicle transport and ATPase activity analysis is a simpler and more practical method for high-throughput assays (31). Since drugs are directly added into the cytoplasmic compartment of infected insect cells and influx, rather than efflux, is measured, the membrane vesicle transport assay can be used to analyze kinetically the interaction of inhibitors with the transporter (31).

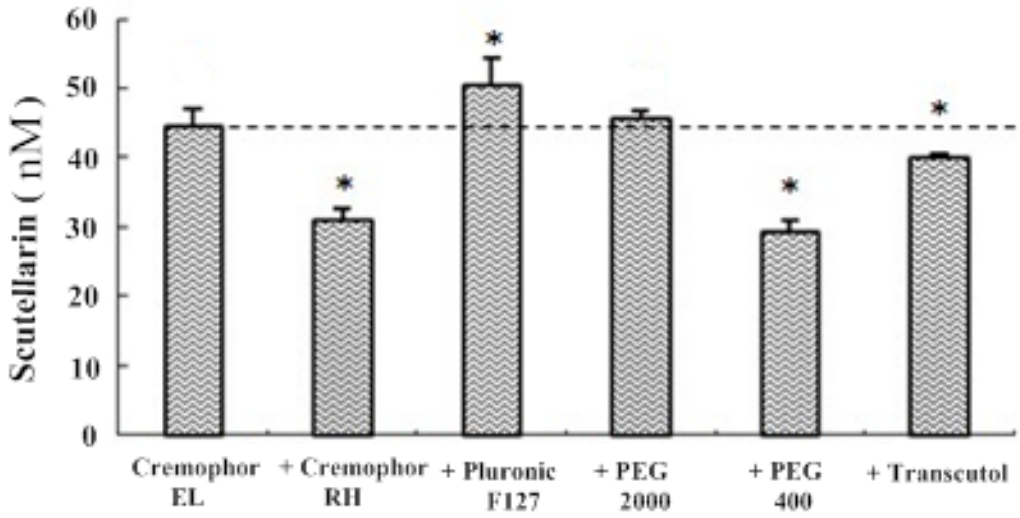

Figure 3. Combined inhibition effects of 5 excipients used together with Cremophor® EL in the MRP2 vesicle model (*$p < 0.05$, related to Cremophor® EL alone).

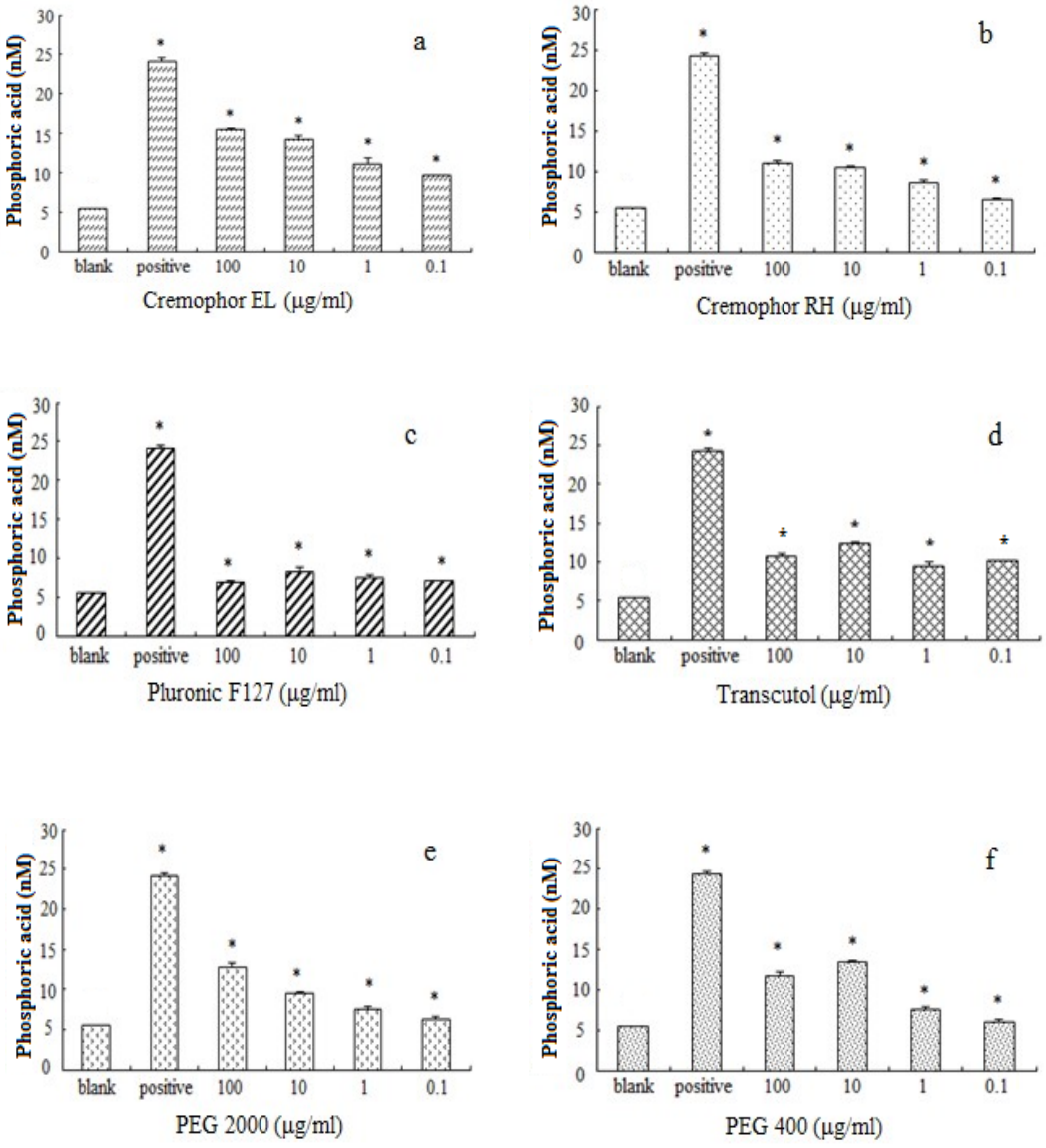

Figure 4. Concentration-dependent activating effects of 6 excipients in ATPase activity assay (data groups a, b, c, d, e and f are from Cremophor® EL , Cremophor® RH, Pluronic® F127, Transcutol®, PEG 2000 and PEG 400, respectively. *$p < 0.05$, compared to scutellarin alone. "Blank" refers to scutellarin alone and "positive" refers to scutellarin with MK571.).

In the concentration-dependent effect analysis, Cremophor® EL and Cremophor® RH were shown to have inhibitory effect on MRP2 via the mechanism of activating ATPase activity. Results from the study of combined effects indicated that Pluronic® F127 and PEG 2000 could be used in the SEDDS prescriptions together with Cremophor® EL for effective inhibition of MRP2. However, compared with the Caco-2 model, solid carrier (β-cyclodextrin) did not show the inhibitory effect on MRP2 in the ATPase assay. This may be due to the drawback of this technique entailing false positive and false negative assay results (31), and also the low permeability of scutellarin (11,32). The ATPase assay does not differentiate between inhibitors and substrates (33), and drugs with low permeability can have underestimated results (34).

The results in Figures 2d, 2e and 2f indicate

that some excipients (Transcutol®, PEG 2000 and PEG 400) at the lowest tested concentration of 0.1 μg/ml decreased the scutellarin transport in comparison to the blank (scutellarin alone). This may be attributed to scutellarin was not only the substrate of MRP2, but also its inhibitor (10). At low concentration of 0.1 μg/ml, inhibition effects of Transcutol®, PEG 2000 and PEG 400 on MRP2 were weaker than that of scutellarin; whereas their affinities to MRP2 were stronger than that of scutellarin (Figures 4d, 4e and 4f). Therefore, these excipients at the low concentration may block the inhibition of scutellarin on MRP2, resulting in reduced transport of scutellarin.

Compared with Cremophor® EL alone, some excipients together with Cremophor® EL increased the efflux ratio of scutellarin and decreased the transport of scutellarin instead of increasing it. Reasons for this phenomenon are complicated. Firstly, this may be due to differences in the mechanisms of efflux reduction and increased transport. For Cremophor® EL, increase in $P_{app,AP-BL}$ and decrease in $P_{app,BL-AP}$ resulted in efflux reduction of scutellarin. In contrast, PEG 400 and Maisine® 35-1 caused reduction in both $P_{app,AP-BL}$ and $P_{app,BL-AP}$, which may counteract the increasing effect of Cremophor® EL on $P_{app,AP-BL}$. Consequently, the transports of scutellarin for PEG 400 and Maisine® 35-1 together with Cremophor® EL were less than that for Cremophor® EL alone. In addition, our previous study showed that Cremophor® EL possessed the most potent inhibitory effect on MRP2, while Labrasol®, Maisine® 35-1 and β-cyclodextrin might have the ability to decrease scutellarin efflux in another mechanism other than inhibiting MRP2 (10). Another explanation may be related to the property of the membrane vesicle transport assay and competitive inhibition on MRP2.

In the MRP2 membrane vesicle transport assay, the transporter-mediated uptake of scutellarin was measured by the uptake difference in the presence or absence of ATP for MRP2 transporter (34). Being similar to Cremophor® EL in structure, Cremophor® RH probably has the same inhibition mechanism and binding sites of MRP2 as Cremophor® EL. When Cremophor® RH and Cremophor® EL were both added to the MRP2 membrane vesicle transport assay, Cremophor® RH could competitively interfere with the inhibition effect of Cremophor® EL on MRP2, resulting in the decreased transport of scutellarin compared to Cremophor® EL alone.

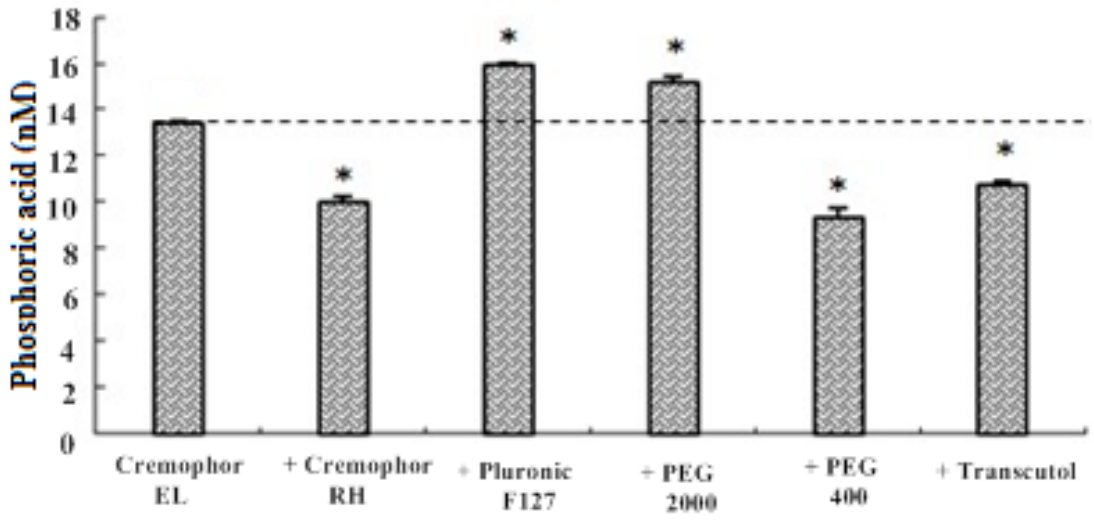

Figure 5. Combined inhibitory effects of 5 excipients used together with Cremophor® EL in the ATPase activity assay (*$p < 0.05$, compared to Cremophor® EL alone).

In this study, some excipients showed the concentration-dependent inhibition effect on MRP2, while others without concentration-dependent effect exhibited significantly increased inhibitory effect above certain critical concentrations. According to the mean fluid volume of 118 ml in

the stomach and 212 ml in the small intestine (35), the concentrations of excipients administered in the gastrointestinal tract could be approximately calculated. Consequently, we can conceive the optimized dose of excipients in SEDDS formulation for achieving appropriate inhibition of MRP2. For example, based on the *in vitro* results of this study, excipient combinations of Cremophor® EL with Pluronic® F127 and PEG 2000 are expected to improve the *in vivo* absorption of scutellarin. In order to arrive at the excipient concentration of 100 μg/ml in the gastrointestinal tract, the typical SEDDS formulation of scutellarin should comprise each excipients (Cremophor® EL, Pluronic® F127 and PEG 2000) of 33 mg, based on amount in 330 mL of gastrointestinal fluids (35). While these may be true for the excipients, predictions of their intestinal concentrations can help to determine whether the results of the *in vitro* experiments may be observed in *vivo*. However, there are practical problems in evaluating *in vivo* inhibitory effects of excipients that inhibit MRP2 *in vitro*. One of them is the physiological relevance of the concentrations of excipients used *in vitro*. The fluid in the gastrointestinal tract is not homogeneously distributed but exists as fluid pockets (36), which likely contributes to the variability of excipient concentration. In fact, the concentration of excipients in the intestine may be influenced by many physiological factors including fluid volume, fluid composition, food intake and digestion of lipid excipients. Therefore, the optimized dose of excipients in SEDDS formulation should be further evaluated by *in vivo* study.

CONCLUSION

In this study of the influences of concentration and combined effects of excipients on inhibition of MRP2 in SEDDS, Caco-2 permeability analysis, MRP2 vesicle transport assay and ATPase activity measurement were performed. Five commonly used excipients including Cremophor® EL, Cremophor® RH, Pluronic® F127, Maisine® 35-1 and β-cyclodextrin showed concentration-dependent effects in decreasing the efflux ratio of scutellarin. The other five excipients did not show such phenomenon. Combined used of PEG 2000 and Pluronic® F127 with Cremophor® EL showed enhanced MRP2 inhibition in the three *in vitro* models. However, other combinations of excipients in the Caco-2 assay increased the scutellarin efflux ratio compared to Cremophor® EL alone. In the MRP2 vesicle transport assay and the ATPase assay, some combinations decreased the transport of scutellarin and the activity of ATPase. These findings should be helpful in the formulation of SEDDS for increasing bioavailability of drugs that are MRP2 substrates. For those excipients that did not show the concentration-dependent and combined effects with Cremophor® EL, their critical or minimal concentrations should also be useful for optimizing the formulation of SEDDS. Based on the results in this *in vitro* study, excipient combinations of Cremophor® EL with Pluronic® F127 and PEG 2000 are expected to improve the *in vivo* absorption of scutellarin. The control of excipient concentrations could not only maximize MRP2 inhibition, but it could also save on the dosage of excipients. We should be alert that combined use of excipients might increase or decrease MRP2 inhibition, and optimize such combination in the formulation of SEDDS.

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