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Research paper

Pharmaceutical excipients inhibit cytochrome P450 activity in cell free systems and after systemic administration

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Abstract

Excipients are largely used as inert vehicles in formulation. Recent studies indicated that some excipients could affect drug transport and disposition. But the effects of most excipients on drug metabolism are yet to be unveiled. To evaluate the actual action of pharmaceutical excipients in biotransformation, we examined the effects of 22 common excipients on cytochrome P450 3A4, the main CYP in intestinal and liver, using midazolam as the probe. The results showed that 15 of 22 (68.2%) tested excipients could inhibit the activity of CYP3A4 more than 50% *in vitro*, particularly the surfactants and polymers. To further understand these effects *in vivo*, five excipients were selected to study the effects on CYP3A4 in rats through the pharmacokinetics of midazolam and its primary metabolite 1′-hydroxymidazolam. In *in vivo* studies, most selected excipients significantly inhibited the activity of CYP3A4 by increasing the midazolam $AUC_{0-\infty}$ and decreasing the midazolam CL/F as well as decreasing the ratio of $AUC_{0-\infty}$ (1′-hydroxymidazolam)/$AUC_{0-\infty}$ (midazolam). For examples, single and multiple dose administration of PEG400 increased intraduodenally dosed midazolam $AUC_{0-\infty}$ to 1.78- and 1.51-fold, decreased midazolam CL/F from 8.86 to 5.25 and 6.28 L/h/kg and decreased the ratio of $AUC_{0-\infty}$ (1′-hydroxymidazolam)/$AUC_{0-\infty}$ (midazolam) from 1.14 to 0.34 and 0.39, respectively ($p < 0.05$). This study indicated that some excipients could change drug metabolism through the effects on cytochrome P450 activity, such as CYP3A4, and thus this kind of inhibition should be taken into consideration in drug formulation and administration.

Keywords: Cytochrome P450; Excipient; Midazolam; 1′-Hydroxymidazolam; Pharmacokinetics

1. Introduction

Pharmaceutical excipients are substances other than the pharmacologically active drugs that are included in the final pharmaceutical products. These substances are used as binders, diluents, lubricants, coloring, flavoring or coating agents for the drugs. Often these substances are regarded as inert from a therapeutic sense. More recently, however, there are great concerns that excipients may influence the absorption, disposition, metabolism, or elimination of the active drugs [1–5].

One mechanism by which excipients may impact the metabolism of the active drug is through inhibiting the cytochrome P450 enzymes in cellular microsomes, a major player in drug metabolism. The cytochrome P450 are membrane-bound enzymes metabolizing endogenous and exogenous compounds through oxidation, and the cytochrome P450 system is the major route of oxidative metabolism for pharmaceutical compounds [6]. Substances that inhibit or enhance cytochrome P450 activity can alter metabolism of drugs, which lead to either decrease the efficacy or increase the bioavailability of a drug. For example, bioac-

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tive compounds in grapefruit juice inhibit CYP 450 activity and influence drug activity [7]. Currently, over 41 human cytochrome homologs spanning 21 families have been identified [8–10]. These homologs vary in their organ distribution and their spectra of activities. Enzymes of the cytochrome P450 3A (CYP3A) family constitute more than 70% of small intestinal cytochrome P450. The major player of the CYP3A family is CYP3A4, which is mainly located in the small intestine and liver, and is the most important intestinal drug metabolizing enzyme [11], and is involved in the metabolism of more than 50% of the currently marketed drugs [12,13]. In addition, CYP3A4 is involved in the oxidation of a variety of endogenous substrates, such as steroids and bile acids [6]. Thus, the effects on CYP3A4 activity by inactive ingredients would be of importance for oral drug bioavailability.

Here, we have surveyed a sample of common pharmaceutical excipients for their effects on the activity of cytochrome P450 enzymes. As a model system, we have studied CYP3A4 given its abundance and importance in drug metabolism with midazolam (MDZ) as our CYP3A4 substrate. Midazolam is well characterized in its metabolism by CYP450 and used widely as a probe for CYP3A4. Moreover, it is completely excreted and is not a substrate of P-glycoprotein [14]. The aim of this study was to assess the effects of pharmaceutical excipients on CYP3A4 activity in cell free systems and after systemic administration *in vivo*.

2. Materials and methods

2.1. Chemicals

Ketoconazole (KTZ) was purchased from Jiade pharmaceutical Co. (Beijing, China). 1′-Hydroxymidazolam (1′-OH-MDZ) was generously provided by Dr. Ulrich Klotz (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany). Midazolam injectable solution (Dormicum) was purchased from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Midazolam (MDZ), Nicotinamide adenine dinucleotide phosphate (NADP), Glucose-6-phosphate (G-6P), and Glucose-6-phosphate dehydrogenase (G-6-PDH) were purchased from Sigma–Aldrich (St. Louis, MO). Propylene glycol and glycerin were purchased from Shanghai Chemicals Ltd., Shanghai, China; Olive oil and lecithin were purchased from Avatar Corporation, University Park, IL, USA; PEG200, PEG400, PEG1000, PEG2000, PEG4000, and PEG6000 were purchased from Dow Chemical Ltd., Midland, MI, USA; Poloxamer 188 (F68) was purchased from BASF China Ltd., Shanghai, China; Triton X-100, Polyoxyl 35 castor oil (EL35), polyoxyl 40 hydrogenated castor oil (RH40), Tween20, and Tween80 were purchased from Cognis UK Ltd., Southampton, Hampshire, UK; polyoxyl 40 stearate (S40) was purchased from Adina Chemicals Ltd., Tunbridge Wells, Kent, UK; Sodium lauryl sulfate (SLS) and Sodium alginate was purchased from Blagden Speciality Chemicals Ltd., Liverpool, UK; Oleic Acid were purchased from Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK; Sodium bisulfite ($NaHSO_3$) and ascorbic acid (Vit.C) were purchased from Hubei pharmaceutical Corporation, Wuhan, Hubei, China.

These excipients were classified as six groups based on their chemical and physical characteristics: (1) co-solvents, which included Propylene Glycol, glycerin, PEG200, and PEG400; (2) oils, which included olive oil; (3) surfactants, which included Tween 20, Tween 80, S40, F68, Triton X-100, EL35, RH40, and SLS; (4) Polymers, which included PEG1000, PEG2000, PEG4000, PEG6000, and Sodium alginate; (5) absorption enhancers which included lecithin and oleic acid; (6) antioxidant which included Vit.C and $NaHSO_3$.

2.2. Cytochrome P450

Recombinant CYP3A4 (rCYP3A4) was expressed in baculovirus infected *Sf*9 cells and was purchased from Sigma–Aldrich (St. Louis, MO, USA). This 3A4 isozyme microsome was supplemented with recombinant P450 reductase and cytochrome b_5.

2.3. Animals

Male Sprague–Dawley rats with body weight in a range of 200–250 g were purchased from the Experimental Animal Supply Center, Tongji Medical College (Wuhan, China). All the rats were housed individually for at least three days under controlled conditions with free access to food and water. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

2.4. In vitro study

The basic incubation medium contained 100 mM potassium phosphate buffer (pH 7.4), a NADPH-regenerating system (1.3 mM NADP, 3.3 mM G-6P, 0.4 U ml^{-1} G-6-PDH, and 3.3 mM $MgCl_2$) and MDZ (5.0 μM). The final incubation volume was 200 μl. The formation of metabolites with rCYP3A4 was linear with respect to the incubation time and microsomal protein concentration over ranges relevant to this study. To maximize the activity of the enzyme, the reaction except the enzyme was preincubated at 37 °C for 5 min. After that 10 pmol of recombinant CYP3A4 was added to the mixture and the incubation was carried out at 37 °C for 10 min [15]. Adding 200 μl of cold methanol containing 480 ng/ml diazepam (internal standard) stopped the reaction. The mixture was centrifuged at 10,000 rpm for 10 min, 50 μl of the supernatant was injected in HPLC system. The terminated incubation mixtures, as well as standard curve and quality control samples, composed of the same matrix materials but without microsomes, were passed through.

To evaluate the effect of excipients on rCYP3A4 activity, the excipient (50 μl) was co-added to MDZ in the incubation mixture (200 μl) instead of 50 μl of potassium phosphate buffer (control). The procedure was then performed as described in the previous section. Metabolite formation with the co-addition of an excipient was expressed as a percentage ratio relative to the control incubated without excipient. KTZ, the positive inhibitor of CYP3A4 activity, was 0.4 μM. The concentrations of the tested excipients were 50 mM except that EL35, RH40, lecithin, and Sodium alginate were 75 mg/ml.

To determine the IC_{50} values of the selected excipients, the inhibition of MDZ hydroxylation was conducted by preincubating with selected excipients at various concentrations at 37 °C for 5 min. The concentration range of each excipient was as follows: RH40 (0–30 mg/ml), PEG400 (0–64 mg/ml), SLS (0–0.32 mg/ml), Vit.C (0–7 mg/ml), lecithin (0–60 mg/ml). The concentration range of KTZ was 0–0.8 mg/ml. The procedure was then performed as described in the previous section.

2.5. In vivo study

To further understand the effects of the excipients in *in vivo* system, five selected excipients were administrated by single or multiple dosing to male Sprague–Dawley rats. These rats were randomly divided into 13 groups with six each: One group was administered saline and served as a negative control. Six groups were administered intraduodenally (i.d.) with single dose of KTZ 75 mg/kg, RH40 150 mg/kg, PEG400 60 mg/kg, SLS 15 mg/kg, Vit.C 75 mg/kg, and lecithin 300 mg/kg, respectively. Six other groups were administered intragastrically with multiple doses of KTZ and selected excipients with the same doses as in the single dosing regimens. Rats were treated once a day for five consecutive days. All the rats were administered i.d. with MDZ 10 mg/kg 20 min after the last treatment of each excipient or KTZ.

Each group of rats, fasted overnight for at least 12 h, was anesthetized with an intraperitoneal injection of 20% urethane (1.2 g/kg). They were placed in a supine position on a heating pad under a surgical lamp to maintain constant normal body temperature. A central venous catheter was inserted into the right jugular artery for blood sampling. All rats undergo abdominal operation and administered intraduodenally with various excipients and MDZ injection.

Blood samples (0.5 ml) were collected at 0.05, 0.1, 0.17, 0.33, 0.5, 0.75, 1.00, 1.5, 2.0, 2.5, 3.0, 3.5, and 4 h from the arterial cannula after MDZ administration. The isolated serum was stored at −80 °C before HPLC analysis.

2.6. HPLC assay of MDZ and 1′-OH-MDZ

In *in vitro* study, 1′-OH-MDZ production was determined by HPLC. The mobile phase for the analysis of 1′-OH-MDZ was methanol/20 mM PPB (pH 7.0) (75:25, v/v) at a flow rate of 1.0 ml/min. The analytical column was a reversed-phase Eurospher-100 C_{18} (250 mm × 4.6 mm, 5 μm). The detector was set at 230 nm. The calibration curve for 1′-OH-MDZ was linear within the concentration ranges of 18.2–1820 ng/ml ($r^2 = 0.999$). Low-, medium-, and high-quality controls (QC) for 1′-OH-MDZ were prepared and analyzed. The recovery rates of 1′-OH-MDZ were 97.86 ± 5.49% at 36.4 ng/ml, 103.29 ± 3.87% at 364 ng/ml, and 102.80 ± 4.82% at 1082 ng/ml, respectively ($n = 5$). The within-day and between-day coefficients of variation (CV) of the different QC samples were in the range of 2.6% to 7.4%.

In *in vivo* study, plasma samples were analyzed for MDZ and 1′-OH-MDZ concentrations by HPLC essentially as previously described analytical method with a minor modification [16]. Briefly, 100 μl of plasma was mixed with phosphate buffer (1 M, pH 9.8) and diazepam as an internal standard. The samples were extracted with 4 ml of hexane, and the upper organic layer was transferred to clean conical tubes. The solvent was evaporated to dryness under a stream of nitrogen. The dry residue was reconstituted with 100 μl of mobile phase and 50 μl was applied to the HPLC system. The mobile phase consisted of methanol/20 mM PPB (pH 7.0) (70:30, v/v); and the flow rate was 1.0 ml/min. With this HPLC setting, the calibration curve of MDZ were linear over a concentration range of 25–2500 ng/ml ($r^2 = 0.997$). The recovery rates of MDZ was 89.37 ± 9.89% at 50 ng/ml, 85.69 ± 5.56% at 800 ng/ml, and 79.13 ± 5.82% at 2000 ng/ml, respectively ($n = 5$). The quantification limit for MDZ was 25 ng/ml, and the CV range was from 3.20% to 11.73%. At the same time, the calibration curve for 1′-OH-MDZ was linear within the concentration ranges of 18.2–1820 ng/ml ($r^2 = 0.998$). The recovery rates of 1′-OH-MDZ were 65.45 ± 5.49% at 36.4 ng/ml, 67.89 ± 2.87% at 364 μg/L, and 61.49 ± 4.82% at 1082 ng/ml, respectively ($n = 5$). The interassay CV values were lower than 15%. In accordance with the guidance for Analytical Methods Validation, this sensitive HPLC method can thus be used for the quantification of MDZ and 1′-OH-MDZ.

2.7. Data analysis

The formation of 1′-OH-MDZ in rCYP3A4 was calculated and expressed as picomoles per minute per picomole protein. All the analyses were performed using the mean values obtained from triplicate incubations. The P450-mediated activities in the presence of excipients were expressed as percentages of the corresponding control values. The IC_{50} values were determined by plotting the percentage of control values of each test article versus log concentration using GraFit 3.0 (Erithacus Software Ltd., Horley, Surrey, UK).

Pharmacokinetics parameters for MDZ and its metabolite 1′-OH-MDZ were estimated from the plasma concentration–time data by a noncompartmental approach using the Software WinNonlin (Pharsight, Mountain View, CA). The peak concentration in serum (C_{max}) and the corresponding time of maximum concentration (T_{max}) were

obtained from the original data. The area under the serum concentration–time curve from time 0 to 4 h (AUC_{0-4h}) was calculated by the trapezoidal rule and the $AUC_{0-\infty}$ with extrapolation to infinity by dividing the last measured concentration by λ. The elimination rate constant (λ) was determined as the slope of linear regression for the terminal log-linear portion of the concentration versus time curve, and the elimination half-life ($t_{1/2}$) was calculated from $0.693/\lambda$. The mean residence time (MRT) value was determined as the ratio of the area under the first moment curve over AUC_{0-4h}. The apparent clearance (CL/F) was calculated from $D_{intraduodenal}/AUC_{0-4h}$.

Standard curve fitting was accomplished with Quanlynx (ver4.0) Software (Waters Co., Milford, Massachusetts, USA). Assay run acceptance was defined by the accuracy and precision of independently prepared quality control samples at three concentrations. The statistical differences between the groups were tested using two compared-samples *t*-test of Sign. Statistical significance was admitted for a $p < 0.05$ for both *in vitro* and *in vivo* experiments. The ratio of $AUC_{0-\infty}$ (1′-OH-MDZ)/$AUC_{0-\infty}$ (MDZ) was calculated to assess the CYP3A4 activity.

3. Results

3.1. Effect of excipients on rCYP3A4 activity in vitro

Pharmaceutical excipients may influence drug metabolism, therefore, we tested the effects of 22 excipients on the activity of CYP450 in a cell-free system. Ketoconazole (KTZ), the positive control for CYP3A4 inhibition, could inhibit the MDZ metabolism as in the previous reports. Of the 22 tested excipients, 15 (68.2%) inhibited the activity of CYP3A4 at least 50%, particularly the group of surfactants and PEG analogies (Fig. 1). Surfactants could completely inhibit CYP3A4 activity, for example, the inhibition rates were 99.40%, 99.50%, and 99.80% for RH40, SLS, and Triton X-100, respectively. Olive oil was the only excipient having no effects on the CYP3A4 activity.

Five excipients were selected for IC_{50} determination through 1′-OH-MDZ formation. The IC_{50} values *in vitro* of selected excipients were 0.25, 0.29, 4.10, 6.61, and 10.77 mg/ml for RH40, SLS, Vit.C, lecithin, and PEG400, respectively (Fig. 2). The IC_{50} value of KTZ was 0.10 mg/ml, which is consistent with previous findings [17].

3.2. Effects of excipients on MDZ and 1′-OH-MDZ pharmacokinetics in single-dose treated rats

Given the effects of excipients on CYP3A4 activity in a cell-free assay, we asked whether systemic administration of the excipients would also impact drug metabolism. To address this question, rats were treated with single doses of selected excipients followed by MDZ administration. Blood samples were taken at different times and MDZ as well as 1′-OH-MDZ concentrations were measured by HPLC. The pharmacokinetic parameters were evaluated by fitting it to a noncompartmental model. Compared to the saline control, single dose of the KTZ raised the concentrations of MDZ over time

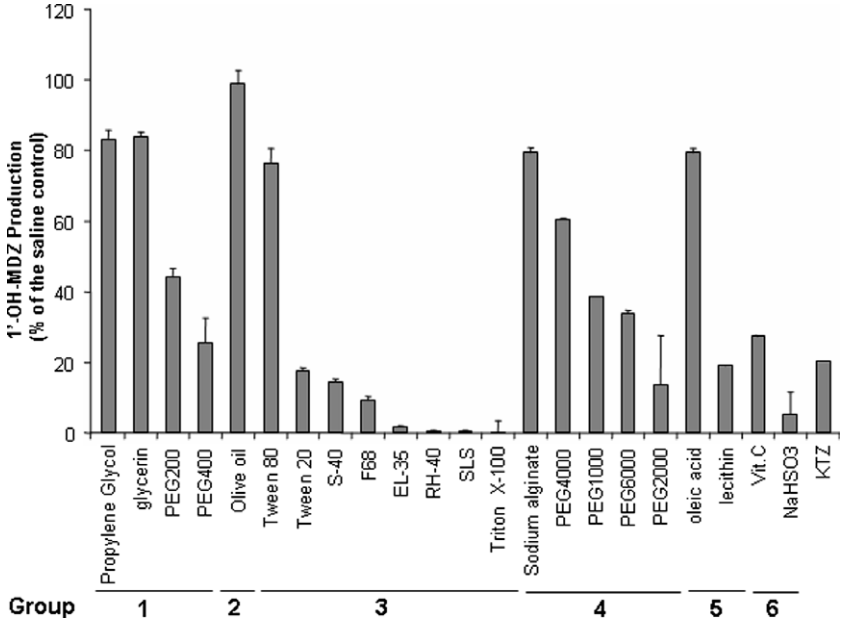

Fig. 1. The effects of different excipients on rCYP3A4 *in vitro*. Recombinant human CYP3A4 and MDZ were incubated with individual excipients for 10 min. The metabolite 1′-OH-MDZ production was determined by HPLC. Each column was expressed as the activity of CYP3A4 by the 1′-OH-MDZ production compared to the negative control. The results represent the averages of the three independent experiments. The concentrations of the test excipients were 50 mM except that EL-35, RH-40, lecithin and Sodium alginate concentrations were 75 mg/ml. Group 1, co-solvents; Group 2, oils; Group 3, surfactants; Group 4, Polymers; Group 5, absorption enhancers; Group 6, antioxidants.

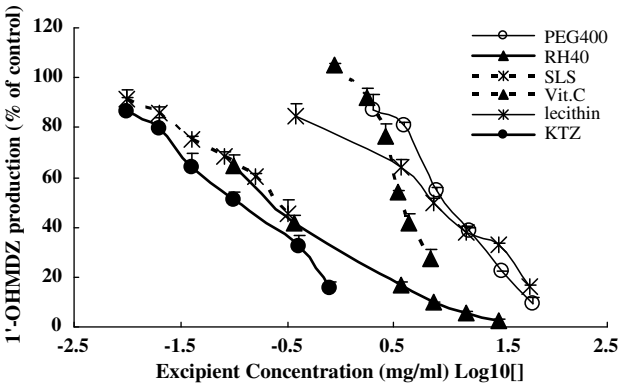

Fig. 2. The IC_{50} determination of selected excipients on the inhibition of CYP3A4 activity. rCYP3A4 microsomes were incubated with 5 μM MDZ in the presence of a series of various concentrations of excipients for 10 min at 37 °C. The concentration range of each excipient was as follows: RH40 (0–30 mg/ml), PEG400 (0–64 mg/ml), SLS (0–0.32 mg/ml), Vit.C (0–7 mg/ml), lecithin (0–60 mg/ml). The concentration range of KTZ was 0–0.8 mg/ml. Activities were expressed as a percentage of the 1′-OH-MDZ production compared with the negative control. The results represent the averages of the three independent experiments.

and decreased the 1′-OH-MDZ formation (Fig. 3). Similar to KTZ, most excipients but SLS raised the MDZ while decreased 1′-OH-MDZ concentrations, indicating that MDZ biotransformation was inhibited by these excipients through inhibiting CYP3A4 activity. These findings were further confirmed by the changes of $AUC_{0-\infty}$ values of plasma MDZ (Table 1) and 1′-OH-MDZ (Table 2). The ratio of AUC_i/AUC of MDZ was used to assess the degree of potential interactions with CYP3A4 [18]. And measurement of MDZ pharmacokinetics could be a sensitive probe by which to detect changes in CYP3A4 activity resulting from CYP3A4 inhibitors or inducers [19]. For example, PEG400 was able to raise the $AUC_{0-\infty}$ up to 1.78-folds and decreased 1′-OH-MDZ production to 0.52-folds. Thus, these excipients could inhibit the metabolism of MDZ by inhibiting the activity of CYP3A4. In contrast, sodium lauryl sulfate (SLS), a commonly used surfactant, decreased both the plasma concentrations of MDZ and 1′-OH-MDZ production in the single-dose treatment. The $AUC_{0-\infty}$ values for MDZ and 1′-OH-MDZ in the plasma were decreased to 59% (676.96 ng/ml h in the SLS group compared to 1144.84 ng/ml h in the saline control) and 22% (from 1274.71 down to 285.91 ng/ml h), respectively (Tables 1 and 2). This suggests that SLS possibly also activated other metabolizing enzymes for MDZ.

Other PK parameters were also altered by these excipients. The significant changes could be seen in CL/F and

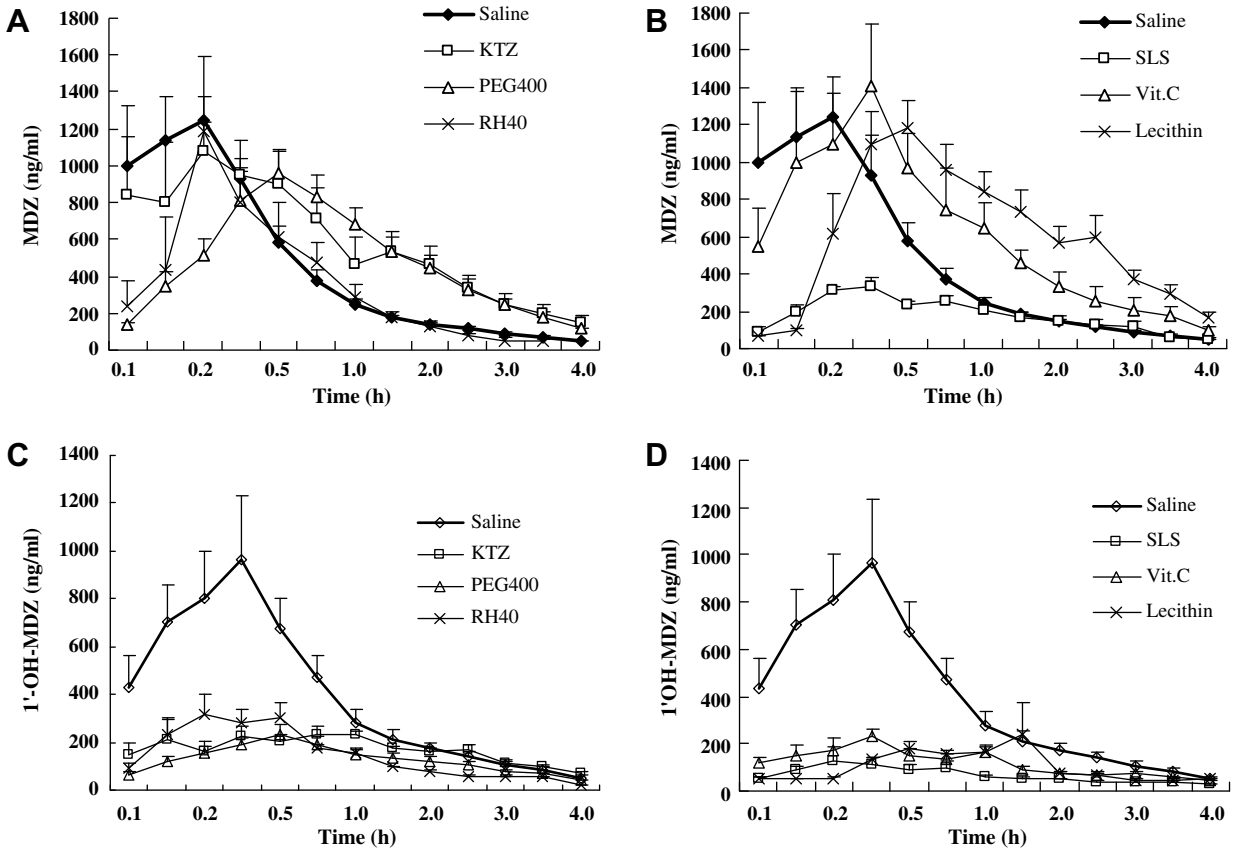

Fig. 3. Excipients altered MDZ metabolism in single-dose treatment in rats. An intraduodenal dose of MDZ 10 mg/kg was given along with single dose of KTZ or excipients (see Section 2). The results represent the average concentration of MDZ in six rats. (A and B) The MDZ concentration change; (C and D) the production of 1′-OH-MDZ change.

Table 1
Pharmacokinetic parameters of MDZ after single-dose administration of excipients in rats

Parameters	Saline	KTZ	PEG400	RH40	SLS	Vit.C	Lecithin
$AUC_{0-\infty}$ (ng/ml h)	1144.84 ± 149.64	2176.89 ± 572.49*	2042.37 ± 547.74*	1264.22 ± 268.93	676.96 ± 120.20*	2101.63 ± 740.29*	2758.72 ± 834.10*
MRT (h)	1.48 ± 0.47	2.15 ± 0.81*	2.12 ± 0.43*	0.96 ± 0.13*	1.86 ± 0.16	1.85 ± 0.60	2.20 ± 0.19*
$t_{1/2}$ (h)	1.33 ± 0.61	1.41 ± 0.68	1.32 ± 0.38	0.62 ± 0.11*	1.01 ± 0.61	1.43 ± 0.23	1.36 ± 0.29
T_{max} (h)	0.17 ± 0.13	0.51 ± 0.26*	0.44 ± 0.09*	0.25 ± 0.09	0.39 ± 0.29*	0.23 ± 0.12	0.49 ± 0.15*
CL/F (L/h/kg)	8.86 ± 1.13	5.14 ± 1.72*	5.25 ± 1.31*	8.29 ± 1.67	15.63 ± 3.37	5.29 ± 1.56*	4.02 ± 1.37*
C_{max} (ng/ml)	1664.32 ± 461.51	1474.37 ± 475.61	967.42 ± 282.38*	1849.00 ± 365.68	372.37 ± 79.68*	1869.11 ± 644.76	1289.87 ± 229.18

Values represented the mean ± SD, $n = 6$.
C_{max}, maximum plasma concentration; T_{max}, time to C_{max}; $AUC_{0-\infty}$, area under the plasma concentration–time from zero to infinity; $t_{1/2}$, plasma elimination half-life; CL/F, duodenal clearance.
* Indicated significant difference ($p < 0.05$) between saline control and KTZ or excipients based on paired t-test on MDZ content.

Table 2
Pharmacokinetic parameters of 1′-OH-MDZ after single-dose administration of excipients in rats

Parameters	Saline	KTZ	PEG400	RH40	SLS	Vit.C	Lecithin
$AUC_{0-\infty}$ (ng/ml h)	1274.71 ± 319.69	752.90 ± 104.12*	659.22 ± 161.94*	555.62 ± 71.27*	285.91 ± 51.83*	645.23 ± 45.57*	521.50 ± 123.98*
MRT (h)	1.46 ± 0.42	2.31 ± 0.14*	2.52±0.64*	2.20 ± 0.76*	2.61 ± 0.44*	4.42 ± 1.30*	3.15 ± 1.25*
$t_{1/2}$ (h)	1.20 ± 0.39	1.12 ± 0.04*	1.92 ± 0.88	1.72 ± 1.04	1.71 ± 0.96	3.65 ± 2.14*	1.91 ± 1.07*
T_{max} (h)	0.27 ± 0.15	0.68 ± 0.30*	0.46 ± 0.07*	0.23 ± 0.21	0.42 ± 0.30*	0.22 ± 0.09	0.83 ± 0.41*
CL/F (L/h/kg)	8.39 ± 2.63	13.60 ± 1.86*	16.12 ± 3.97*	17.28 ± 1.98*	34.29 ± 6.05*	15.78 ± 1.80*	20.14 ± 4.32*
C_{max} (ng/ml)	1187.57 ± 570.17	338.74 ± 99.24*	268.54 ± 67.09*	433.97 ± 64.85*	142.36 ± 43.60*	238.57 ± 53.96*	318.96 ± 214.62*

Values represented the mean ± SD, $n = 6$.
* Indicated significant difference ($p < 0.05$) between saline control and KTZ or excipients based on paired t-test on 1′-OH-MDZ production.

MRT. The clearance of MDZ correlated with CYP3A activity well [20]. CL/F of MDZ showed a significant decrease in KTZ, PEG400, Vit.C and lecithin treatment groups, indicating that PEG400, Vit.C and lecithin had the same inhibitory potential as KTZ. MRT was extended by most excipients in terms of MDZ contents and the production of 1′-OH-MDZ (Tables 1 and 2). For example, Vit.C extended MRT for 1′-OH-MDZ to 4.42 h compared to 1.46 h in the saline control. For 1′-OH-MDZ, peak concentrations (C_{max}) were significantly decreased by all excipients, e.g. it was decreased from 1187.57 to 238.57 ng/ml in Vit.C treatment.

In the overall analysis of the changes of MDZ and 1′-OH-MDZ in the single dose systemic administration of excipients, the ratios of $AUC_{0-\infty}$ (1′-OH-MDZ)/$AUC_{0-\infty}$ (MDZ) were significantly decreased. In saline control rats, the ratio is 1.14, which was decreased to less than 0.5 in all excipient-treated rats (Table 5). The ratio of metabolite/parent drug AUC could be used to index the change of metabolic enzymes [21]. Thus, these results further confirmed that single-dose excipient administration could inhibit the bioactivity of CYP450.

3.3. Effects of excipients on MDZ and 1′-OH-MDZ pharmacokinetics in multiple-dose treated rats

Clinically, it is a common practice for patients to take multiple doses of drugs during the illness period. To check the potential effects of such regimens on the activity of cytochrome P450 by excipients, we designed an experiment to treat rats with one dose daily of each excipients for five consecutive days followed by one dose of MDZ. Plasma concentrations of MDZ and 1′-OH-MDZ were determined by HPLC. In this setting, the CYP3A4 inhibitor KTZ raised the MDZ concentration in a time-dependent manner. Similar to KTZ, PEG400 and RH40 also raised the MDZ concentration curve in a time-dependent manner (Fig. 4A). In contrast, Vit.C and lecithin decreased the concentration of MDZ (Fig. 4B). Compared to the MDZ concentration in saline controls, SLS raised the concentration curve of MDZ at 30 min later after MDZ administration (Fig. 4B). PK parameters analyses indicated that all excipients but Vit.C increased the $AUC_{0-\infty}$ and decreased the CL/F values of MDZ (Table 3), such as in PEG400- and RH40-treated rats. In terms of 1′-OH-MDZ, its concentration was decreased by all excipients, similar to that of KTZ (Fig. 4C and D), but $AUC_{0-\infty}$ values had no significant difference in KTZ, RH40, SLS and lecithin treatment groups compared to the saline group. Furthermore, CL/F of 1′-OH-MDZ showed increases in all excipient-treated rats (Table 4). Different from the single-dose treatment, there were no significant changes in MRT for MDZ for most of the excipients, similar to that of KTZ. But MRT was increased in SLS- or lecithin-treated rats. For example, lecithin could extent the MRT from 1.48 to 3.36 h. However, in terms of 1′-OH-MDZ, the MRT was prolonged

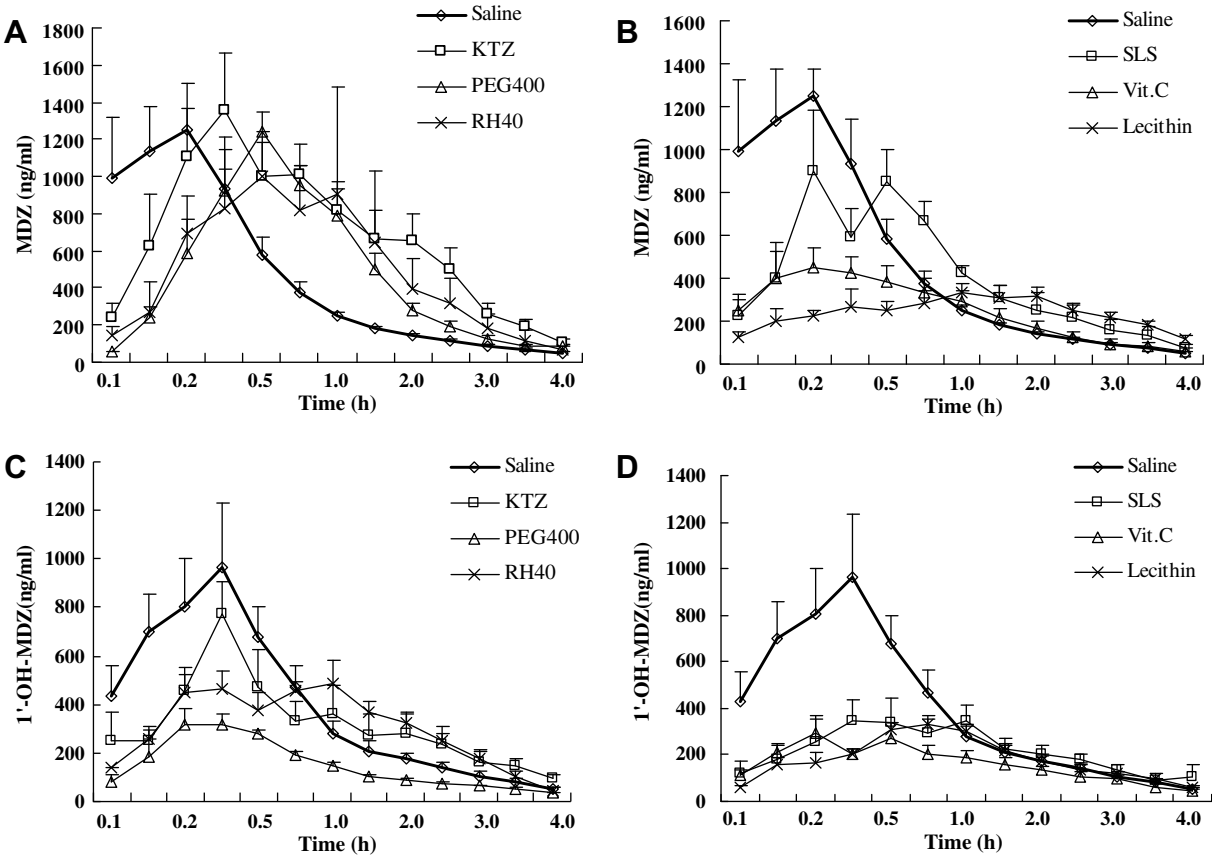

Fig. 4. Excipients altered MDZ metabolism in multiple-dose treatment in rats. An intraduodenal dose of MDZ 10 mg/kg was given after five consecutive administrations of KTZ or excipients (see Section 2). The results represent the average concentration of MDZ in six rats. (A and B) The MDZ concentration change; (C and D) the production of 1′-OH-MDZ change.

Table 3
Pharmacokinetic parameters of MDZ after multiple-dose administration of excipients in rats

Parameters	Saline	KTZ	PEG400	RH40	SLS	Vit.C	Lecithin
$AUC_{0-\infty}$ (ng/ml h)	1144.84 ± 149.64	2452.971 ± 497.81*	1726.07 ± 477.43*	1931.34 ± 508.81*	1489.92 ± 550.91	815.00 ± 315.18*	1391.09 ± 290.92
MRT (h)	1.48 ± 0.47	1.56 ± 0.20	1.41±0.15	1.52 ± 0.24	1.87 ± 0.49	1.41 ± 0.16	3.36 ± 1.05*
$t_{1/2}$ (h)	1.33 ± 0.61	0.75 ± 0.07*	0.92 ± 0.21	0.78 ± 0.20*	1.36 ± 0.37	0.79 ± 0.28*	1.95±1.10
T_{max} (h)	0.17 ± 0.13	0.42 ± 0.31*	0.49 ± 0.15*	0.64 ± 0.29*	0.31 ± 0.16*	0.32 ± 0.17*	0.61 ± 0.31*
CL/F (L/h/kg)	8.86 ± 1.13	4.62 ± 1.64*	6.28 ± 1.77	5.70 ± 1.58*	7.48 ± 2.21	14.59 ± 6.38*	7.58 ± 1.73
C_{max} (ng/ml)	1664.32 ± 461.51	1870.25 ± 660.78	1303.55 ± 179.06	1163.35 ± 361.60*	1260.37 ± 333.60*	598.38±203.26*	428.23 ± 82.43*

Values represented the mean ± SD, $n = 6$.
* Indicated significant difference ($p < 0.05$) between saline control and KTZ or excipients based on paired t-test on MDZ content.

by all excipients, along with that of KTZ group (Table 4). To our surprise, although MDZ biotransformation was inhibited by some excipients such as PEG400 and RH40, C_{max} was not increased in all excipient-treated rats (see Table 3).

Thus, the effects of excipients on CYP P450 activities were more complicated in multiple-dose regimen than that in single-dose counterparts. Analyses on $AUC_{0-\infty}$ indicated that most excipients but Vit.C could increase MDZ contents in plasma and inhibit the 1′-OH-MDZ production, the overall result suggested that all excipients but Vit.C would decrease the biotransformation of MDZ through inhibiting CYP450 activity (Table 5).

4. Discussion

Excipients are generally regarded as inactive ingredients in the drug formulation and are added to facilitate administration of the active ingredients. However, some excipients are also potential toxicants. Tween 80 has been previously reported to augment the toxicity of drugs such as Amiodarone causing acute hepatotoxicity and also shown to decrease the glutathione levels [22,23]. One important point that was overlooked is the effects of excipients to susceptible groups such as infants and asthma patients. The relevance to paediatric medicine has to be emphasized since most commonly used excipients are not

Table 4
Pharmacokinetic parameters of 1′-OH-MDZ after multiple-dose administration of excipients in rats

Parameters	Saline	KTZ	PEG400	RH40	SLS	Vit.C	Lecithin
$AUC_{0-\infty}$ (ng/ml h)	1274.71 ± 319.69	1210.72 ± 283.14	640.31 ± 170.28*	1227.35 ± 337.45	880.98 ± 229.81	606.84 ± 126.89*	876.46 ± 181.07
MRT (h)	1.46 ± 0.42	1.86 ± 0.58	2.40 ± 0.66*	1.72 ± 0.20	1.86 ± 0.13	1.83 ± 0.28	2.44 ± 0.73*
$t_{1/2}$ (h)	1.20 ± 0.39	1.03 ± 0.32	2.35 ± 1.39*	0.72 ± 0.27*	0.77 ± 0.15*	0.90 ± 0.30	1.51 ± 0.78
T_{max} (h)	0.27 ± 0.15	0.29 ± 0.16	0.27 ± 0.15	0.51 ± 0.33*	0.53 ± 0.25*	0.33 ± 0.15	0.56 ± 0.16*
CL/F (L/h/kg)	8.39 ± 2.63	10.83 ± 6.40	16.62 ± 3.83*	8.65 ± 1.85	12.12 ± 2.94*	17.28 ± 3.85*	12.21 ± 3.15
C_{max} (ng/ml)	1187.57 ± 570.17	907.23 ± 294.68*	424.60 ± 75.30*	658.35 ± 125.43*	556.73 ± 177.99*	407.88 ± 135.70*	381.27 ± 20.12*

Values represented the mean ± SD, $n = 6$.
* Indicated significant difference ($p < 0.05$) between saline control and KTZ or excipients based on paired t-test on 1′-OH-MDZ.

Table 5
$AUC_{0-\infty}$ changes of MDZ and 1′-OH-MDZ after single/multiple-dose administration of excipients in rats

	Single dose			Multiple dose		
	AUC_i/AUC (MDZ)	AUC_i/AUC (1′-OH-MDZ)	AUC(1′-OH-MDZ)/AUC (MDZ)	AUC_i/AUC (MDZ)	AUC_i/AUC (1′-OH-MDZ)	AUC(1′-OH-MDZ)/AUC (MDZ)
Saline	1.00 ± 0.13	1.00 ± 0.25	1.14 ± 0.36	1.00 ± 0.13	1.00 ± 0.25	1.14 ± 0.36
KTZ	1.83 ± 0.51*	0.59 ± 0.09*	0.40 ± 0.20*	2.14 ± 0.87*	0.95 ± 0.54	0.50 ± 0.23*
PEG400	1.78 ± 0.48*	0.52 ± 0.11*	0.34 ± 0.09*	1.51 ± 0.42*	0.50 ± 0.13*	0.39 ± 0.14*
RH40	1.10 ± 0.23	0.44 ± 0.06*	0.45 ± 0.08*	1.69 ± 0.48*	0.96 ± 0.26	0.70 ± 0.12*
SLS	0.59 ± 0.10*	0.22 ± 0.04*	0.44 ± 0.13*	1.30 ± 0.48	0.69 ± 0.18	0.65±0.24*
Vit.C	1.84 ± 0.65*	0.51 ± 0.04*	0.33 ± 0.09*	0.71 ± 0.27	0.47 ± 0.10*	0.91 ± 0.53
Lecithin	2.41 ± 0.73*	0.41 ± 0.08*	0.20 ± 0.05*	1.22 ± 0.25	0.69 ± 0.14	0.66 ± 0.21*

Values represented the mean ± SD, $n = 6$.
* Indicated significant difference ($p < 0.05$) between saline control and different excipients based on paired t-test on AUC_i/AUC (MDZ), AUC_i/AUC (1′-OH-MDZ), the ratio of AUC(1′-OH-MDZ)/AUC(MDZ), "i" represents treatment group of KTZ or various excipients.

tolerated by infants. For example, E-ferol could cause many infant deaths and sulfites could lead to paradoxical broncospasm in asthma patients and some colorants (such as the azoics) could cause death [4]. In addition to the direct toxicity, excipients may also alter the pharmacokinetics of active ingredients through altering the pharmacokinetics of the drug thus affecting the drug efficacy. Recently, Buggins et al. had reviewed the effects of the common pharmaceutical excipients including DMSO, Propylene glycol, PEGs, Cremophor EL on drug disposition. For example, PEGs could inhibit intestinal transporter (P-gp) and enzymes (CYP3A) [5]. Another common excipient Cremophor could affect *in vivo* Cyclosporin A disposition [24]. Moreover, the inhibition of CYP450 activity was observed with amphiphilic ingredients (for example, mixed micellar solutions, Tween 80, and oleic acid) [24–26]. Here, we evaluated 22 common excipients for their effects on cytochrome P450 3A4-mediated metabolism both in cell free systems and after systemic administration. These ingredients cover various excipients, including co-solvents, oils, surfactants, polymers, absorption enhancers and antioxidants (see Section 2 for details). The results indicated that most (15 of 22) of the tested excipients could inhibit the metabolic activity of CYP3A4 in a cell-free system. This is beyond our expectation because excipients generally are regarded as inert substance and have little effects on drug activity. However, it is possible because the active site of CYP3A4 is generally considered to be the multiple-binding sites as evidenced by its ability to oxidize a wide range of structurally diverse molecules and contributes to the biotransformation of approximately 50% of drugs currently on the market [12,13,27]. This is a double-edged sword and these multiple activating sites let CYP3A4 to be exposed and to be inhibited easily and, thus we could see that CYP3A4 could be inhibited by most excipients. Previous studies had demonstrated that the excipients Tween 80 and Cremophor EL inhibit drug metabolism and block the CYP450 enzyme [25]. However, it is unknown whether the effects of excipients on drug metabolism are only restricted to those selected substrates or broad issue. Our results suggested that many excipients from a variety of classes could impact drug metabolism.

Using KTZ, a specific inhibitor for CYP3A4, as a positive control, we further examined 5 excipients in a systemic administration model. Owing to the less intestinal absorption of PEGs with high molecular, we did not select the PEGs polymers to study its impact on CYP3A4 *in vivo* [28]. Although the effects on CYP3A4 activity were not completely consistent in the single-dose and multiple-dose models, it was clear that most excipients could inhibit CYP3A4 activity according to their AUC_i/AUC and CL/F of MDZ or the ratios of $AUC_{0-\infty}$ (1′-OH-MDZ)/$AUC_{0-\infty}$ (MDZ).

According to our results, the inhibiting capacity depended on the chemical and physical nature of each excipient. Surfactants were the most potent inhibitors

of CYP3A4, which probably contributed the ability of surfactants that could disrupt enzyme activity. Vit.C and $NaHSO_3$, which were known to be antioxidants, also were very strong inhibitors for CYP3A4. This kind of chemicals might influence the oxidation process of MDZ 1′-hydroxylation. Secondly, the inhibition on CYP activity was also possibly dependent on the substrate used for the test. In our study, oleic acid was less toxic to CYP3A4 activity with only less than 20% inhibition, which was different from the previous report where 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) was used as the substrate of CYP3A4. In their study, the IC_{50} of oleic acid on BFC metabolism was 2 mM [25], where oleic acid had no effects on CYP3A4 activity in terms of substrate MDZ in our experiment at 50 mM concentration. This result suggested that the action of excipients on biotransformation was associated with the specific drugs, which was consistent with previous hypothesis [29]. Thus, the excipient has no effects on one drug metabolism but possibly has effects on another drug. Due to this drug-excipient interaction, we have to study each formulation before going to clinic.

MDZ undergo extensive first-pass metabolism by both hepatic and intestinal CYP3A4 and was rapidly converted to 1′-OH-MDZ [30]. However, there are more than 41 CYP members spanning 21 subfamilies. And because of the structure similarity, excipients that affect CYP3A4 activity could also alter other CYP activities. In this study, most selected excipients would significantly inhibit CYP3A4 activity after a single/multiple dose administration. However, some excipients, such as Vit.C failed to increase MDZ concentration although 1′-OH-MDZ was decreased in multiple-dose administration. The AUC ratios for MDZ and 1′-OH-MDZ in multiple-dose treatment were 0.71 and 0.47, respectively. We believed that Vit.C might activate or enhance another metabolizing pathway for MDZ. Firstly, 1′-OH-MDZ was the main metabolite (via CYP3A4), the oxidation of MDZ also lead to the production of 4-OH-MDZ and 1′, 4-dihydroxymidazolam [17,31]. Secondly, several enzymes might be involved in the metabolism of MDZ. The oxidation of MDZ was predominantly contributed to CYP3A4; however, CYP3A3 and CYP3A5 could also be largely involved, especially in rat microsomes [32,33]. Therefore, in addition to inhibit CYP3A4 activity, Vit.C might have an influence on other MDZ metabolic enzymes in as extended treatment *in vivo* over 5 days.

However, there are several issues to be further understood. Firstly, we only examined CYP3A4, one of the 41 members of the CYP family, and we are not certain how the excipients will interact with the other enzymes. Especially, we have not excluded the hepatic CYP450s from intestinal ones. Further evaluation of the inhibition of CYP3A4 with an ex-vivo perfusion model with rat intestines will eliminate the hepatic metabolism of the drug and thus provide more useful information on oral formulation. Secondly, we only selected one CYP3A4 substrate probe. There are many routes of drug elimination. Potentially the ‘‘benign’’ excipients such as oleic acid could alter these routes of the elimination and thereby alter drug activity. Based on a study of the modulatory effect of 34 compounds on 10 commonly used CYP3A4-mediated reactions, Kenworthy et al. reported that the effect was substrate-dependent [29]. Consequently, the selection of appropriate substrates for investigating the potential inhibition of CYP3A4 is critical as the magnitude of effect was often substrate-dependent, and a weak correlation was often observed among different CYP3A4 substrates. Thus, other probe substrates may be used in the inhibitory study of pharmaceutical excipients on the CYP3A4 enzyme in the future. Thirdly, although several studies have suggested that MDZ is stable in some oral formulations up to 100 days [34,35], the stability of MDZ in individual excipients should be paid attention. Lastly, our results are conducted to investigate the effects of individual excipients on CYP450 activity, further studies and extra cautions are necessary when extending to the combined effects of 2 or more excipients used together or patients who are receiving more than two medications simultaneously because such individual excipients probably act synergistically. In addition, because we only evaluated the effects of excipients in rat models, cautions should be taken in extrapolating these results to human beings.

In summary, this report raises awareness of the ability of excipients to inhibit drug metabolism. And this kind of inhibition should be taken into consideration in drug formulation and administration.

5. Conclusion

Our study showed that: (1) most excipients including surfactants, PEG analogies and antioxidants could inhibit the activity of CYP3A4 in both a cell-free system and the systemic administration. (2) Excipients could alter the pharmacokinetics of active ingredients in a systemic administration model, but the patterns are different between single-dose and multiple-dose treatment. (3) Most excipients could inhibit the biotransformation enzymes cytochrome P450, such as CYP3A4, thus changed the drug metabolism and the drug efficacy. Therefore, this kind of inhibition should be taken into consideration in drug formulation and administration.

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