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Possible inhibitory mechanism of *Curcuma* drugs on CYP3A4 in 1α,25 dihydroxyvitamin D_3 treated Caco-2 cells

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Abstract

Curcuma longa and *C. zedoaria*, belonging to genus *Curcuma*, have become prevalent as supplements in East Asia. Curcumin is the most well-studied bioactive component isolated from rhizomes of *C. longa* and other *Curcuma* species except *C. zedoaria*. In this study, we investigated the affects of *C. longa*, *C. zedoaria* from Japan and curcumin on CYP3A4. Caco-2 cells, in which CYP3A4 expression was induced by 1α,25-$(OH)_2$-D_3, were used to mimic the metabolism of small intestine. Caco-2 cells were treated with methanol extracts from two *Curcuma* rhizomes (0.1 mg/ml) or curcumin (30 μM) for 72 h. Both extracts significantly decreased the activity of CYP3A4 by about 85–98%. The 50% inhibitory concentrations of *C. longa* and *C. zedoaria* extracts were 0.019 and 0.014 mg/ml, respectively. They caused a 60–70% decrease in CYP3A4 protein. Otherwise, curcumin treatment caused a 30–40% decrease in CYP3A4 catalytic activity and a 38% decrease in CYP3A4 protein expression. Moreover, it was found that both *Curcuma* extracts and curcumin treatment had no influence on CYP3A4 mRNA expression. Our results suggested that administration of *Curcuma* drugs might inhibit the catalytic activity of intestinal CYP3A4. However, curcumin was not the major compound responsible for this inhibitory effect.

Keywords: *C. longa*; *C. zedoaria*; Curcumin; CYP3A4; Caco-2

1. Introduction

In East Asia, *Curcuma* species (*Curcuma longa*, *Curcuma zedoaria*) have been used as traditional medicines because of their various pharmacological activities including wound healing, digestion promotion, anti-cancer, anti-microbial, etc. (Araujo and Leon, 2001). Recent pharmacological studies have demonstrated that the rhizomes of *C. longa* and *C. zedoaria* expressed antiallergic (Ram et al., 2003) and hepatoprotective activities (Matsuda et al., 1998). Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the major yellow pigment from rhizomes of *C. longa* rhizome and other *Curcuma* species. However, no curcumin content was detected in Japanese *C. zedoaria*. Since curcumin possesses wide range of pharmacological activity, bulky volume of curcumin is administrated to keep health (Maheshwari et al., 2006; Chainani-Wu, 2003; Cheng et al., 2001). With the prevalence of *Curcuma* as supplements, case reports about the side effect of *Curcuma* supplements appeared in Japan (Kumashiro et al., 2006). In these cases, fatal liver disorder appeared after *Curcuma* administration. However, the cause still keeps unclear.

Abbreviations: *C. longa*, *Curcuma longa*; *C. zedoaria*, *Curcuma zedoaria*; TST, testosterone; 6β-OH TST, 6β-hydroxytestosterone; 1α,25-$(OH)_2$-D_3, 1α,25 dihydroxyvitamin D_3; HPLC, high performance liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum; KTZ, ketoconazole; RFP, rifampicin; IC_{50}, 50% inhibitory concentrations; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

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As herbal medicinal products have become prevalent throughout the world, the fatalness of herb–drug interactions stands out (Mills et al., 2005; Fugh-Berman and Ernst, 2001). Cytochrome P450 (CYP) 3A4 enzyme is the most abundant in human cytochromes. CYP3A4 is responsible for the metabolism of about 60% of the drugs in current clinical use (Guengerich, 1999). The inhibition/induction of CYP3A4 has been reported as a significant reason for herb–drug interaction (Wrighton et al., 2000).

Caco-2 cell model is a well-established model to study the absorption and related mechanism of drugs (Hilgers et al., 1990; Neuhaus et al., 2006). Schmiedlin-Ren et al. (1997) showed that expression of CYP3A4 could be up-regulated by 1α,25-$(OH)_2$-D_3 treatment in Caco-2 cells, and that these cells might be a promising model to simulate the absorption and metabolism of small intestine. Following that, some researchers have verified the activity of CYP3A4 in the modified Caco-2 cells, and showed that the expression and activity of CYP3A4 were inhibited by grapefruit juice addition (Aiba et al., 2005; Hara et al., 2002; Paine et al., 2005). These modified cells have become an accepted tool to detect inhibition/induction of CYP3A4. Furthermore, Caco-2 cell model has been used to demonstrate the intestinal first-pass metabolism of baicalein from Radix Scutellariae (Zhang et al., 2005).

In this study, Caco-2 cells treated with 1α,25-$(OH)_2$-D_3 were utilized to detect the *Curcuma* drugs interaction mediated by intestinal CYP3A4. The major goals are as following: (1) To show some information for the correct application of *Curcuma* alternative medicines. (2) To investigate the relationship between curcumin and the correct application of *Curcuma* alternative medicines.

2. Materials and methods

2.1. Materials and chemicals

Caco-2 cells (American Type Culture Collection HTB37) were obtained at passage 18 from American Type Culture Collection (Manassas, VA). *C. longa* (Okinawa, Japan) and *C. zedoaria* (Okinawa, Japan) used in this study were correctly identified by the molecular biological method previously reported (Cao et al., 2001). All drugs are stored in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama, Japan. Dulbelcco's modified Eagle's medium (DMEM), non-essential amino acids (NEAA), antibiotic–antimycotic mixed stock solution, glucose, DL-α-tocopherol, sodium selenite, zinc sulfate, ferrous sulfate, ethlenediaminertetracetic acid (EDTA) and trypsin were obtained from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was from ICN Biomedicals, Inc. (Aurora, Ohio, USA). 1α,25-$(OH)_2$-D_3, TST, 6β-OH TST, nifedipine, oxidized nifedipine and ketoconazole were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Transwell polycarbonate cell culture inserts (24 mm diameter, 0.4 μm pore size) were from Costar Corp. (Bedford, MA, USA). Millicell ERS device was obtained from Millipore (Bedford, MA, USA). Stock solution of *Curcuma* extracts and curcumin were prepared at 50 mg/ml and 100 mM in DMSO and stored in −20 °C.

2.2. Cell culture conditions

Caco-2 cells at passage 28–41 were used for all experiments. Cell cultures were maintained in a humidified 37 °C incubator with a 5% carbon dioxide in air atmosphere. Caco-2 cells were grown in plastic tissue culture dishes in a maintenance medium consisting of DMEM containing 25 mM glucose, 4 mM L-glutamine, 0.1 mM non-essential amino acids, 100 units/ml penicillin, 100 units/ml streptomycin, 250 nM amphotericin and supplemented with 10% heat-inactivated FBS. When cells reached 80% confluence, they were removed using 0.2% trypsin/EDTA, diluted with 1:4 and reseeded onto fresh tissue culture dishes. Medium was changed at 2–3 days intervals.

2.3. Preparation of Curcuma extracts

The methanol extracts of *Curcuma* rhizomes were prepared as previously described (Sasaki et al., 2003). Samples were analyzed using a HPLC apparatus consisting of pumps (Shimadzu LC-10AT, Japan), a degasser (Shimadzu DGU-12A, Japan), an autosampler (Shimadzu SIL-10A, Japan), and a UV–VIS detector (Shimadzu SPD-10A, Japan). The reversed-phase separation was performed in Inertsil® ODS-3 column (4.6 mm × 150 mm, 5 μm particles, GL Sciences Inc., Japan). The eluate was CH_3CN–H_2O–AcOH (45:55:1, by vol.) and elution was performed at a rate of 1 ml/min. Curcumin was detected at 410 nm and identified according to the retention time. A methanolic stock solution of curcumin was prepared at 100 μM. Concentrations were obtained by extrapolation of peak area from a standard curve.

2.4. MTS assay

The cytotoxicity of *Curcuma* extracts/curcumin on Caco-2 cell proliferation was determined by MTS assay using CellTiter 96® aqueous one solution reagent (Promega Corporation). Caco-2 cells at approximately 10^5 cells/cm^2 were seeded in 96-well plate. After the cells had reached 80–90% confluence, cells were treated with 0–0.5 mg/ml *Curcuma* extracts/0–200 μM curcumin for 72 h. Following removal of the extracts/curcumin, cells were washed with phosphate-buffered saline (PBS) for 3 times. The cells were then incubated in serum free maintenance medium (100 μl) with one solution reagent (20 μl) for a further 4 h. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells at 490 nm.

2.5. 1α,25-$(OH)_2$-D_3 treatment

Caco-2 cells were seeded at 5×10^5 cells/cm^2 onto the culture insert and cultured in complete growth medium, which was prepared based on maintenance medium supplied with 45 nM DL-α-tocopherol and 20% heat-inactived FBS. After 10 days, the medium was additionally supplemented with sodium selenite (100 nM), zinc sulfate (3 nM), ferrous sulfate (5 μM) and

1α,25-$(OH)_2$-D_3 (250 nM). Simultaneously, the content of FBS was decreased to 5%. Cells were incubated for a further 3 weeks.

2.6. Monolayer integrity

Cell monolayer integrity was evaluated with transepithelial electrical resistance (TEER). Resistance was measured using a Millicell-ERS resistance system after the medium was changed. An insert that did not contain cells was used as background resistance. TEER was calculated from the background-corrected resistance and the surface area of the insert (4.76 cm^2).

2.7. Induction of CYP3A4 catalytic activity by rifampicin

After 1α,25-$(OH)_2$-D_3 treatment, the cells were incubated with the presence of rifampicin (50 μM) for 72 h. After medium was aspirated the monolayers were washed with warm EBSS buffer twice. EBSS buffer containing TST (250 μM) was added to the apical compartment (1.5 ml). After incubating the cells at 37 °C for 4 h, basolateral (2.6 ml) media was collected and stored at −80 °C for analysis.

2.8. Inhibition of CYP3A4 catalytic activity by Curcuma extracts and curcumin

After treated with 1α,25-$(OH)_2$-D_3 for 3 weeks, *Curcuma* extracts (0.1 mg/ml) or curcumin (30 μM) were added to the apical compartment and the incubation lasted for 72 h. After medium was aspirated, the monolayers were washed with warm EBSS buffer twice. EBSS buffer containing TST (250 μM) or nifedipine (200 μM) was added to the apical compartment. After incubating the cells at 37 °C for 4 h, basolateral media was collected and stored at −80 °C for analysis.

2.9. Analysis of 6β-OH TST by HPLC

Media (50 μl) was injected into a HPLC equipment the same as mentioned in the method of preparation of *Curcuma* extracts. The reversed-phase separation was performed in Inertsil® ODS-3 column (4.6 mm × 150 mm, 5 μm particles, GL Sciences Inc., Japan). Elution was performed at a rate of 1 ml/min with eluate (acetonitrile:H_2O = 40:60). The eluate was monitored at 254 nm. Standards of 6β-OH TST were made in methanol. Concentrations were obtained by extrapolation of peak area from a standard curve.

2.10. Analysis of oxidized nifedipine by HPLC

EtOAc (3 ml) was added to media (800 μl) in an amber vial. Then, the content of each vial was mixed using a vortex device and the two layers were separated by centrifugation at 3000 *g* for 5 min. From each upper organic layer, 2.5 ml was transferred to another amber vial and dried using a centrifugation evaporator. The content was dissolved in 200 μl of eluate (methanol:H_2O = 55:45) and 50 μl was injected into the HPLC equipment the same as mentioned in the method of preparation of *Curcuma* extracts. The reversed-phase separation was performed in Inertsil® ODS-3 column (4.6 mm × 150 mm, 5 μm particles, GL Sciences Inc., Japan). Elution was performed at a rate of 1 ml/min and monitored at 254 nm. Standards of oxidized nifedipine were made in methanol. Concentrations were obtained by extrapolation of peak area from a standard curve.

2.11. Cell lysis

Cell monolayers were washed with cold PBS for three times. Total cellular proteins were extracted in lysis buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid and protease inhibitor mixture. Protein concentrations were determined by the BCA protein assay reagent kit (Pierce), using bovine serum albumin as a standard. Total cell lysate was stored at −80 °C for analysis.

2.12. Western blot analysis

Proteins (12 μg) from the total cell lysate were analyzed by SDS-PAGE (12.5% gel). After blotting, the Immobilon-P membrane (Millipore) was blocked with 5% skim milk in PBS containing 0.5% Tween 20 at room temperature for 1 h. Immunoblots were incubated at room temperature for 1 h with the specific primary antibody to CYP3A4 (BD Biosciences). After further washing, the membranes were incubated for 1 h with the secondary antibody (Santa Cruz Biotechnology). Blots were reprobed with antibody to GAPDH as a loading control. Quantitative analysis of immunoblotted bands was performed by computer program (NIH Image, version 1.61).

2.13. RNA extraction and SYBR® GREEN I real time RT-PCR

Total RNA was extracted from the treated cells using TRIzol reagent. First strand cDNA was generated from 1 μg total RNA by using the oligo(dT) first strand primer. Real time PCR was performed with 50,000-fold diluted SYBR® GREEN I dye (Invitrogen). For CYP3A4, the forward primer sequence used was 5′-ACTGAGTCCCACAAAGCTCTGTC-3′ and the reverse primer sequence used was 5′-AACTGCATCAATTTCCTCCTGC-3′; for β-actin, the forward primer sequence used was 5′-GGTCATCACCATTGGCAATGA-3′ and the reverse primer sequence used was 5′-GTAGTTTCGTGGATGCCACAGG-3′. Aliquots of the reverse-transcription reaction mixture (1 μl) were amplified and detected using a ABI PRISM 7700 sequence Detector System (Applied Biosystems) with the following profile: 1 cycle of 95 °C for 5 min, and 45 cycles each of 95 °C for 15 s and 60 °C for 1 min. The CYP3A4 mRNA levels were normalized relative to β-actin mRNA level in each sample.

2.14. Statistics

Results were expressed as the means ± S.D. Statistical significance was determined using Student's *t*-test or one-way

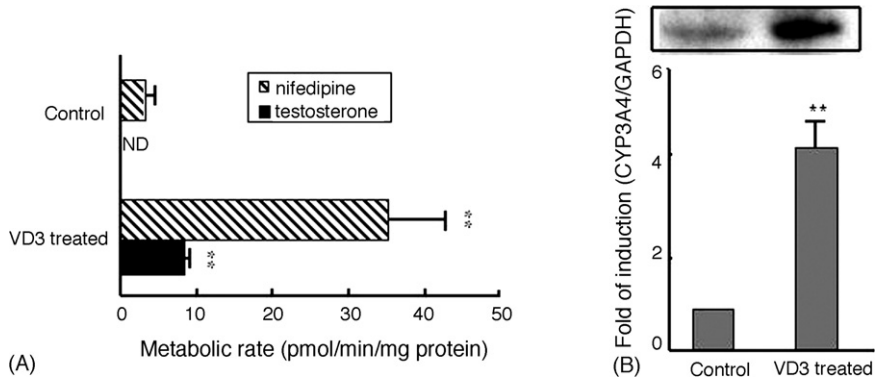

Fig. 1. Induction of CYP3A4 catalytic activity and immunoactive protein by 1α,25-$(OH)_2$-D_3. Caco-2 cells grown on culture inserts were treated with 1α,25-$(OH)_2$-D_3 (250 nM) for 3 weeks after confluence. The medium was then replaced with medium free of 1α,25-$(OH)_2$-D_3 for 72 h. TST (250 μM) or nifedipine (200 μM) was added to the apical compartment. After 4 h, basolateral media was collected and analyzed for 6β-OH TST or oxidized nifedipine concentration. (A) Metabolic rate of TST and nifedipine. (B) Results of immumoblots of cell lysates. Results are means ± S.D. from triplicate experiments. $^{**}P < 0.001$. "ND" stands for "not detected".

ANOVA followed by Bonferroni/Dunn multiple range test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Induction of CYP3A4 by 1α,25-$(OH)_2$-D_3 and the response of 1α,25-$(OH)_2$-D_3 treated Caco-2 cells to rifampicin

After the cells were treated with 1,25-$(OH)_2$-D_3 and then incubated in maintenance medium for 72 h, the activity of CYP3A4 in modified Caco-2 cells was compared with that of untreated Caco-2 cells (Fig. 1A). In untreated Caco-2 monolayers, no 6β-OH TST formation was detected in the basolateral compartment and oxidized nifedipine was formed at a rate of 3.19 ± 1.31 pmol/min mg protein. In contrast, in the basolateral compartment of treated Caco-2 cells, the formation rates of 6β-OH TST and oxidized nifedipine were detected as 8.20 ± 0.96 pmol/min mg protein and 35.20 ± 7.52 pmol/min mg protein, respectively (Fig. 1A). The expression of immunoactive CYP3A4 protein was proved to be significantly induced by about 5-fold after 1α,25-$(OH)_2$-D_3 treatment (Fig. 1B).

RFP (50 μM) treatment did not lead to any damage to Caco-2 cell morphology (Fig. 2A). The catalytic activity of modified Caco-2 cells was significantly increased by about 15 fold when TST was used as CYP3A4 substrate (Fig. 2B). The mRNA and immunoactive protein levels were induced by about 2-fold and 4-fold, respectively (Fig. 2C and D).

3.2. Cytotoxicity of Curcuma extracts and curcumin to Caco-2 cells

After 72 h incubation, both *Curcuma* extracts and curcumin exhibited dose-dependent inhibition on cell growth compared to control group. Both extracts showed no cytotoxicity to Caco-2 cells when the concentration was below 0.1 mg/ml. The 50% cytotoxicity concentrations (CC_{50}) of *C. longa* and *C. zedoaria* extracts were determined to be 0.14 and 0.19 mg/ml, respectively. As shown in Table 1, the content of curcumin in *C. longa* extract was 3.9%, while there was no curcumin content in *C. zedoaria* extract. As the main component of *C. longa*, curcumin did not affect the viability of Caco-2 cells when the concentration was below 30 μM, and the CC_{50} of curcumin was 73 μM (Table 1). In this research, we picked out the largest dose without cytotoxicity (*Curcuma* extracts: 0.1 mg/ml; curcumin: 30 μM) to carry out our experiments.

Table 1
Comparison between *Curcuma* extracts and curcumin in cytotoxicity and CYF3A4 inhibitory activity

	Curcumin content (% of extract)	CC_{50} (mg/ml)	IC_{50} (mg/ml)
C. longa	3.9	0.14	0.019
C. zedoaria	ND	0.19	0.014
Curcumin	–	0.027 (73 μM)	ND

The CC_{50} was calculated from MTS assay. Cells were grown and treated with extracts or curcumin for up to 72 h as described in the methods. Percentage viability was determined by setting the absorbance of control, non-treated cells as 100%. The IC_{50} was calculated from the inhibitory activity of *Curcuma* extracts on nifedipine oxidization. Results are from triplicate experiments. "ND" stands for "not detected".

Affects of *Curcuma* extracts and curcumin on Caco-2 cell monolyers were further validated. It seemed that *Curcuma* extracts and curcumin treatments had no influence on Caco-2 monolayers morphology (Fig. 3A). There were no significant changes in the TEER (Table 2) and protein content (Fig. 3B) when Caco-2 monolayers were treated with *Curcuma* extracts/curcumin for 72 h compared to control.

3.3. Inhibitory potential of Curcuma extracts and curcumin on CYP3A4

Testosterone and nifedipine were utilized as the probes of CYP3A4 catalytic activity. As an inhibitor of CYP3A4 catalytic activity, KTZ (1 μM) significantly inhibited 6β-hydroxylation of TST and oxidization of nifedipine by almost 100% and 60%, respectively. After 72 h treatment, *C. longa* extract

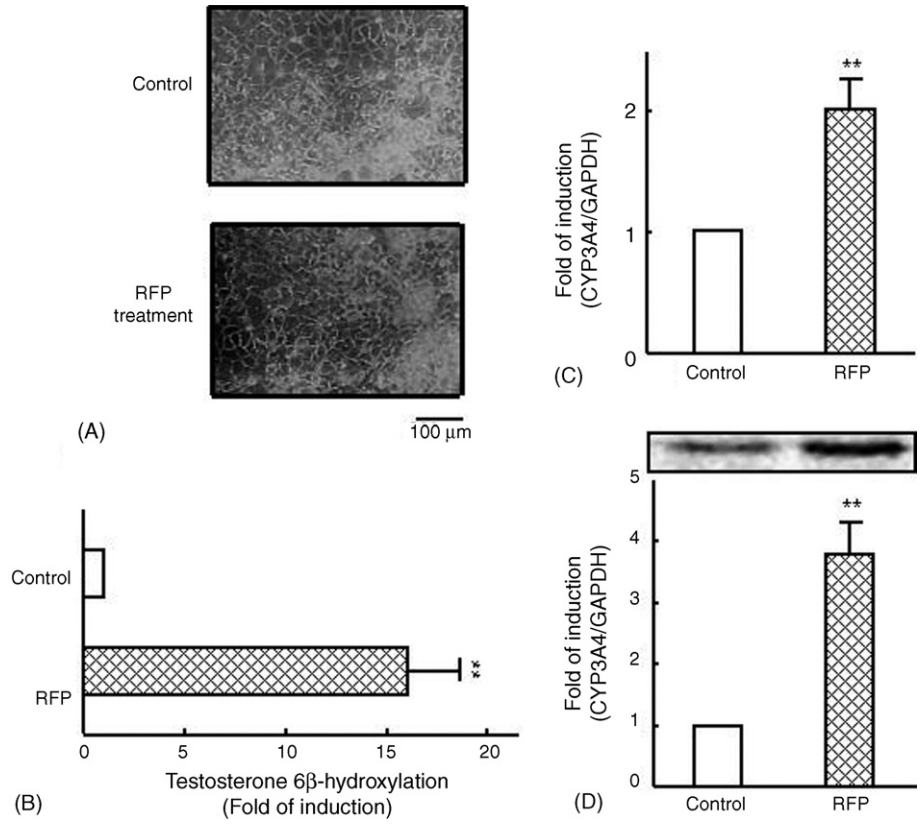

Fig. 2. Induction of CYP3A4 by RFP (50 μM) in 1α,25-$(OH)_2$-D_3 treated Caco-2 cells. (A) Influence of RFP on Caco-2 morphology. (B) Induction of CYP3A4 activity by RFP. After 1α,25-$(OH)_2$-D_3 treatment for 3 weeks, the medium was replaced with medium free of 1α,25-$(OH)_2$-D_3 and RFP was applied to the apical compartment. After 72 h, TST (250 μM) was added to the apical compartment and incubated for 4 h. The amount of 6β-OH TST in the basolateral compartment was measured. Results are means ± S.D. from triplicate experiments. $^{**}P < 0.001$. (C) Induction of CYP3A4 mRNA by RFP. After treatment by RFP for 72 h, total mRNA was extracted for real-time RT-PCR. Results are means ± S.D. from triplicate experiments. $^{**}P < 0.001$. (D) Induction of CYP3A4 immunoactive protein by RFP. After treatment by RFP for 72 h, cell lysates were prepared for western immunoblot. Results are means ± S.D. from triplicate experiments. $^{**}P < 0.001$.

(0.1 mg/ml) and *C. zedoaria* extract (0.1 mg/ml) decreased the 6β-hydroxylation formation by 86% and 98%, respectively. Especially, only trace amount of 6β-OH TST was detectable after *C. zedoaria* extract treatment. On the other hand, when the substrate was changed to nifedipine, *C. longa* extract and *C. zedoaria* extract inhibited nifedipine oxidation by 86% and 85%, respectively (Fig. 4). Both *Curcuma* extracts exhibited dose-dependent inhibitory effect on nifedipine oxidization. The IC_{50} of *C. longa* and *C. zedoaria* extracts were 0.019 and 0.014 mg/ml, respectively (Table 1). The IC_{50}s for both *Curcuma* extracts were about 10-fold lower than their CC_{50}s. About 2 μM of curcumin was comprised in the IC_{50} of *C. longa* extract. Inter-

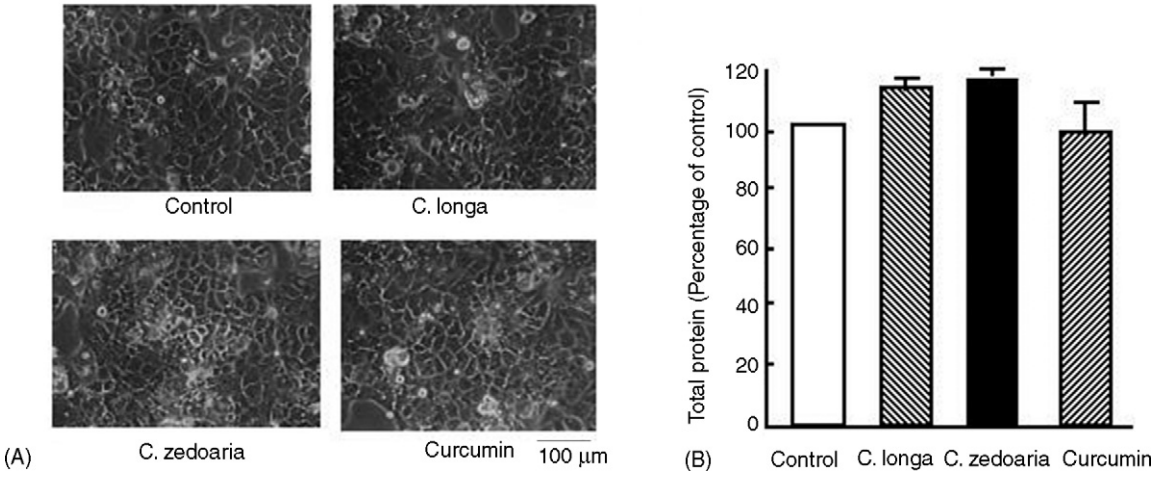

Fig. 3. Affects of methanol extracts of *Curcuma* rhizomes or curcumin on Caco-2 monolayer. (A) Affects on cell morphology after 72 h treatment. (B) Changes in total protein content of Caco-2 cells after treatment for 72 h. Results are means ± S.D. from triplicate experiments.

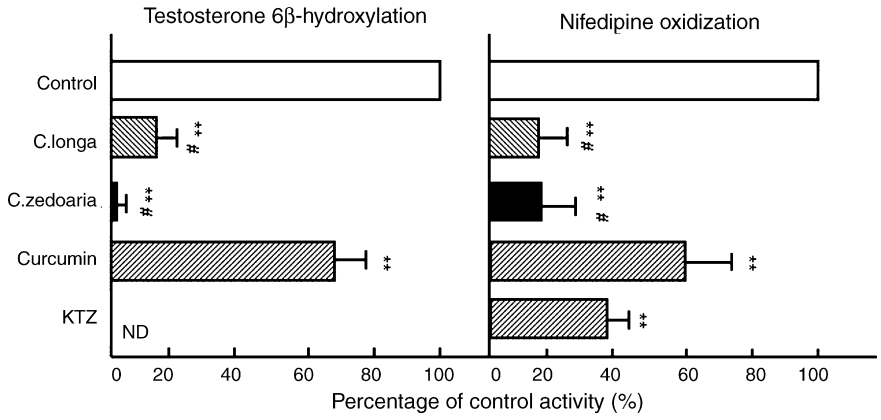

Fig. 4. Inhibition of TST 6β-hydroxylation and nifedipine oxidation in 1α,25-$(OH)_2$-D_3 treated Caco-2 cells by methanol extracts of *Curcuma* rhizomes (0.1 mg/ml) or curcumin (30 μM). Extracts or curcumin were applied to the apical compartment and incubated for 72 h. After removing extracts/curcumin, testosterone (250 μM) or nifedipine (200 μM) was added to the apical compartment and incubated for 4 h. The amount of 6β-OH TST or oxidized nifedipine in the basolateral compartment was measured. Results are means ± S.D. from triplicate experiments. $^{**}P < 0.001$ compared with control. $^{\#}P < 0.01$ compared to curcumin treatment. "ND" stands for "not detected".

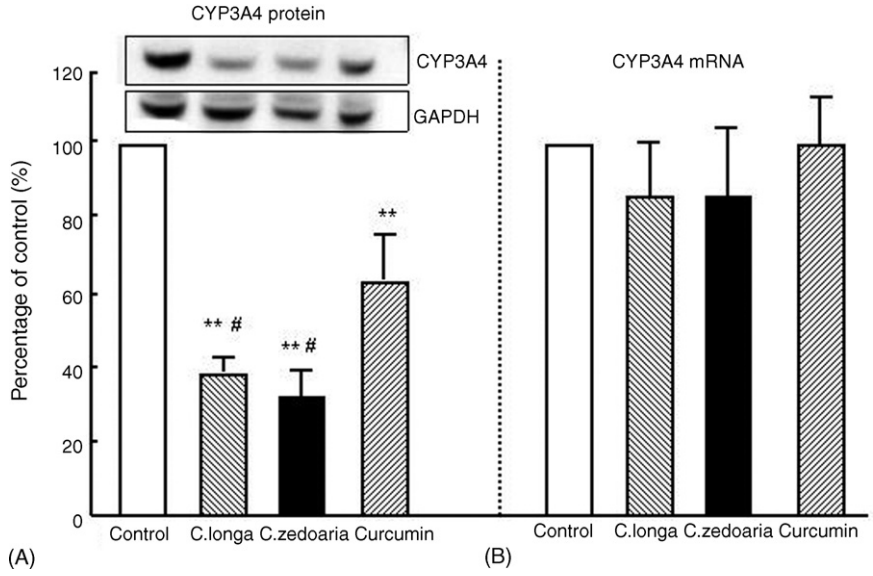

Fig. 5. Affects on CYP3A4 immunoactive protein and mRNA following methanol extracts of *Curcuma* rhizomes (0.1 mg/ml) or curcumin (30 μM) treatment. Extracts or curcumin were applied to the apical compartment and incubated for 72 h. After removing extracts/curcumin, cell lysates or total mRNA were prepared for western immunoblot or real-time RT-PCR. (A) Representative western immunoblot for CYP3A4 and quantitative analysis of CYP3A4 immunoactive protein. The band intensities were normalized with that of GAPDH. Results are means ± S.D. from triplicate experiments. $^{**}P < 0.001$ compared to control. $^{\#}P < 0.01$ compared to curcumin treatment. (B) Quantitative analysis of CYP3A4 mRNA. Results are means ± S.D. from triplicate experiments.

Table 2
Affect of *Curcuma* extraxts/curcumin on transepithelial electrical resistance (TEER) of Caco-2 cell monolayers

	TEER ($\Omega\,cm^2$)			
	0	12 h	24 h	72 h
Control	1151 ± 165	1008 ± 176	1084 ± 85	974 ± 120
C. longa	1054 ± 92	1008 ± 109	1042 ± 131	941 ± 171
C. zedoaria	1159 ± 100	1084 ± 83	1071 ± 75	962 ± 112
Curcumin	1067 ± 103	958 ± 133	1058 ± 86	899 ± 98

TEER of Caco-2 cell monolayers were recorded during the treatment of *Curcuma* extraxts/curcumin. Resistance was measured using a Millicell-ERS resistance system after the medium was changed. TEER was calculated from the background-corrected resistance and the surface area of the insert (4.76 cm^2). Results are means ± S.D. from triplicate experiments.

estingly, when 30 μM of curcumin was administrated for 72 h, TST 6β-hydroxylation and nifedipine oxidization were inhibited by 34% and 38%, respectively. It was found that the CYP3A4 catalytic activity of *Curcuma* extracts treated Caco-2 cells was apparently lower than that of cells treated by curcumin (Fig. 4).

Cell lysates from the Caco-2 monolayers were analyzed for GAPDH and CYP3A4 immunoreactive protein by western blot (Fig. 5A). After treatment for 72 h, both *C. longa* extract and *C. zedoaria* extract decreased CYP3A4 protein content by 60–70% compared to control, while curcumin had only modestly inhibitory effect (30–40%) on CYP3A4 protein expression. In contrast to curcumin, both extracts led to a greater loss of CYP3A4 immunoactive protein. On the other hand, there were no significant changes on the CYP3A4 mRNA following treatment with *Curcuma* extracts and curcumin (Fig. 5B).

4. Discussion

Almost all herbs are administrated orally. The first major barrier for them to across is the intestinal epithelium. Large dose of herb components administration might change the activity and/or expression of CYP3A4 in intestinal epithelium cells, but have no affect on the CYP3A4 in liver because of low blood concentration (Evans, 2000; Murray, 2006). The most typical example was grapefruit juice (Fuhr, 1998; Veronese et al., 2003). It is of great means to predict the possibility of herb–drug interaction in small intestine. There are always two limitations for the *in vitro* model of herb–drug interaction: metabolism ability and species differences. In this study, we confirmed that 1α,25-$(OH)_2$-D_3 modified Caco-2 cell monolayers offered an applicable system for testing the potential of herb–drug interaction mediated by CYP3A4.

There have been some reports showing that 1α,25-$(OH)_2$-D_3 modified Caco-2 cell system exhibited detectable CYP3A4 metabolism ability for a short term after 1α,25-$(OH)_2$-D_3 was removed (Fisher et al., 1999; Paine et al., 2005). However, since consumption of herb products was normally a long-last doings (Sharma et al., 2001), it is important for the herb–drug interaction testing system to sustain drug treatment for a relatively long term. In our study, after modified Caco-2 monolayers were incubated in medium free of 1α,25-$(OH)_2$-D_3 for 72 h, the metabolites appeared in basolateral compartment were detected by HPLC. The formation rates of 6β-OH TST and oxidized nifedipine were about 8 pmol/min mg protein and 35 pmol/min mg protein, respectively. In the report of Schmiedlin-Ren et al. midazolam was utilized as CYP3A4 probe, and the detection of 1′-hydroxymidazolam was performed by gas chromatography selective ion mass spectrometry (Schmiedlin-Ren et al., 1997). Their system might exhibit high metabolism activity because they have obtained the genetic homogeneity among Caco-2 cells. Another group using CYP3A4 transfected Caco-2 cell system indicated the metabolic properties as follows: testosterone, 7–32 pmol/min mg protein; nifedipine, 2–10 pmol/min mg protein (Hu et al., 1999). It was indicated that our system exhibited ample metabolism ability since it was not weaker than that of another well-used model, CYP3A4 transfected Caco-2 system.

Species difference is an acknowledged problem for the application of animals in metabolism field (Guengerich, 1997; Bogaards et al., 2000). In this study, rifampicin, a strong inducer of CYP3A4 in human hepatocytes but not CYP3A in rat hepatocytes (Waxman, 1999; Lu and Li, 2001), was administrated to our modified Caco-2 cell monolayers. Although it has been reported that rifampicin cannot induce the expression of CYP3A4 in wild Caco-2 cells (Schmiedlin-Ren et al., 2001), our results showed that the expression and activity of CYP3A4 were further up-regulated by rifampicin in modified Caco-2 cells. To our knowledge, this is the first report about the induction of CYP3A4 by rifampicin in 1α,25-$(OH)_2$-D_3 treated Caco-2 cells. Above all, these results support that modified Caco-2 cell is a suitable model to study herb–drug interaction.

Using our modified Caco-2 monolayers, we demonstrated that both intestinal CYP3A4 enzyme activity and immunoactive protein levels were significantly decreased (>50%) following 72 h of *Curcuma* extracts treatment. This is the first time to indicate the potential of herb–drug interaction caused by methanol extracts of *Curcuma* rhizomes. Curcumin is the most famous bioactive component isolated from *Curcuma* rhizomes. In 1996, Firozi et al. demonstrated the inhibition of CYP isoenzymes by curcumin in a reconstituted microsomal monooxygenase system (Firozi et al., 1996). The inhibition effects of curcumin on benzo[a]pyrene induced CYP1A1, 1A2 and 2B1 activities were verified in rat liver microsome and in rat (Thapliyal and Maru, 2001). Furthermore, recently, Valentine et al. reported that curcumin modulates drug metabolism enzymes in the female Swiss Webster mouse (Valentine et al., 2006). They demonstrated that curcumin treatment would cause a 25% decrease in CYP1A activity, but not in immunoactive protein level. Significantly, their results indicated that 2 weeks of curcumin (400 mg/kg) treatment led to a 20% decrease in the activity and a 28% decrease in immunoactive protein level of liver CYP3A. Similarly, we also demonstrated that curcumin treatment (30 μM) for 72 h modestly down-regulated the CYP3A4 enzyme activity (about 30–40%) and immunoactive protein level (about 35%). Indeed, we observed that both *C. longa* and *C. zedoaria* exhibited strong inhibitory affect on CYP3A4 activity (about 90%) and strong down-regulation effect on CYP3A4 immunoactive protein expression (about 60%). However, curcumin content in *C. longa* extract (0.1 mg/ml) occupied only 3.9% (10.6 μM). Interestingly, there was no curcumin content in Japanese *C. zedoaria* extract. Our results suggested that curcumin was not the major compound responsible for the inhibitory effect of *Curcuma* on CYP3A4.

Curcuma rhizomes are multi-component systems. Except for curcumin, some essential oils, such as turmerones and zingiberene, have been isolated from *Curcuma* rhizomes. Reports showed that these essential oils exhibited multiple biological activities (Lai et al., 2004; Syu et al., 1998). From our results, we speculated that besides curcumin, there must be at least one compound owning CYP3A4 inhibitory activity in *Curcuma* rhizomes. The inhibitory mechanism of *Curcuma* must be very complicated because it might be a combined effect of several compounds.

The decreased Caco-2 CYP3A4 protein levels observed after *Curcuma* extracts/curcumin treatment implied that the interaction was not simple competitive inhibition. However, we found that the treatment did not decrease the level of CYP3A4 mRNA. Similarly, Raucy reported that CYP3A4 mRNA levels in primary human hepatocytes were not changed after curcumin (5 μM) treatment for 48 h (Raucy, 2003). Cheng et al. also demonstrated that curcumin (1 mg/kg) did not alter CYP3A gene expression in male rat (Cheng et al., 2003). Our result indicated that *Curcuma* extracts/curcumin did not decrease CYP3A4 protein content by a transcriptional or posttranscriptional way. Rather, it suggested that this inhibitory effect might result from reduced translation or enhanced degradation of CYP3A4 protein. Our results suggested that the case of *Curcuma* extracts/curcumin was very similar to that of grapefruit juice, which exhibited a mechanism-based inhibition on intestinal CYP3A4 (Dresser and Bailey, 2003). Further study is under way to explain the inhibition mechanism

of the methanol extracts of *Curcuma* rhizomes and curcumin on CYP3A4 by our group.

In summary, our study showed that 1α,25-$(OH)_2$-D_3 treated Caco-2 cell was an available model for screening of herb–drug interaction. Using this model, we provided strong evidence that both *C. longa* and *C. zedoaria* significantly inhibited the activity and protein expression of CYP3A4, although they have no affect on the expression of CYP3A4 mRNA. We also showed that curcumin played a minor role in the inhibitory potential of *Curcuma* rhizomes on CYP3A4. Further studies are needed to clarify the impact of *Curcuma* rhizomes on human CYP3A4 and other drug-metabolizing enzymes.

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References

Aiba, T., Susa, M., Fukumori, S., Hashimoto, Y., 2005. The effects of culture conditions on CYP3A4 and MDR1 mRNA induction by 1alpha,25-dihydroxyvitamin D(3) in human intestinal cell lines, Caco-2 and LS180. Drug Metab. Pharmacokinet. 20, 268–274.

Araujo, C.C., Leon, L.L., 2001. Biological Activities of *Curcuma L.* Memórias do Instituto Oswaldo Cruz 96, 723–728.

Bogaards, J.J., Bertrand, M., Jackson, P., Oudshoorn, M.J., Weaver, R.J., Van Bladeren, P.J., Walther, B., 2000. Determining the best animal model for human cytochrome P450 activities: a comparison of mouse, rat, rabbit, dog, micropig, monkey and man. Xenobiotica 30, 1131–1152.

Cao, H., Sasaki, Y., Fushimi, H., Komatsu, K., 2001. Molecular analysis of medicinally-used Chinese and Japanese *Curcuma* based on 18S rRNA gene and trnK gene sequences. Biol. Pharm. Bull. 24, 1389–1394.

Chainani-Wu, N., 2003. Safety and anti-inflammatory activity of curcumin: a component of tumeric (*Curcuma longa*). J. Altern. Complement. Med. 9, 161–168.

Cheng, A.L., Hsu, C.H., Lin, J.K., Hsu, M.M., Ho, Y.F., Shen, T.S., Ko, J.Y., Lin, J.T., Ming-Shiang, W., Yu, H.S., Jee, S.H., Chen, G.S., Chen, T.M., Chen, C.A., Lai, Pu, Y.S., Pan, M.H., Wang, Y.J., Tsai, C.C., Hsieh, C.Y., 2001. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res. 21, 2895–2900.

Cheng, P.Y., Wang, M., Morgan, E.T., 2003. Rapid transcriptional suppression of rat cytochrome P450 genes by endotoxin treatment and its inhibition by curcumin. J. Pharmacol. Exp. Ther. 307, 1205–1212.

Dresser, G.K., Bailey, D.G., 2003. The effects of fruit juices on drug disposition: a new model for drug interaction. Eur. J. Clin. Invest. 33, 10–16.

Evans, A.M., 2000. Influence of dietary components on the gastrointestinal metabolism and transport of drugs. Ther. Drug Monit. 22, 131–136.

Firozi, P.F., Aboobaker, V.S., Bhattacharya, R.K., 1996. Action of curcumin on the cytochrome P450 system catalyzing the activation of aflatoxin B1. Chem. Biol. Interact. 100, 41–51.

Fisher, J.M., Wrighton, S.A., Wrighton, S.A., Watkin, P.S., Schmiedlin-Ren, P., Calamia, J.C., Shen, D.D., Kunze, K.L., Thummel, K.E., 1999. First-pass midazolam metabolism catalyzed by 1α,25-dihydroxyvitamin D_3-modified Caco-2 cell monolyers. J. Pharmacol. Exp. Ther. 289, 1134–1142.

Fugh-Berman, A., Ernst, E., 2001. Herb–drug interactions: review and assessment of report reliability. Br. J. Clin. Pharmacol. 52, 587–595.

Fuhr, U., 1998. Drug interactions with grapefruit juice. Extent, probable mechanism and clinical relevance. Drug Saf. 18, 251–272.

Guengerich, F.P., 1997. Comparisons of catalytic selectivity of cytochrome P450 subfamily enzymes from different species. Chem. Biol. Interact. 106, 161–182.

Guengerich, F.P., 1999. Cytochrome P-450 3A4: regulation and role in drug metabolism. Annu. Rev. Pharmacol. Toxicol. 39, 1–17.

Hara, H., Yasunami, Y., Adachi, T., 2002. Alteration of cellular phosphorylation state affects vitamin D receptor-mediated CYP3A4 mRNA induction in Caco-2 cells. Biochem. Biophys. Res. Commun. 296, 182–188.

Hilgers, A.R., Conradi, R.A., Burton, P.S., 1990. Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. Pharm. Res. 7, 902–910.

Hu, M., Li, Y., Davitt, C.M., Huang, S.M., Thummel, K., Penman, B.W., Crespi, C.L., 1999. Transport and metabolic characterization of Caco-2 cells expressing CYP3A4 and CYP3A4 plus oxidoreductase. Pharm. Res. 16, 1352–1359.

Kumashiro, R., Hisamochi, A., Sata, M., 2006. Liver injury by health food. SouGo RinSyou 55, 50–51.

Lai, E.Y., Chyau, C.C., Mau, J.L., Chen, C.C., Lai, Y.J., Shih, C.F., Lin, L.L., 2004. Antimicrobial activity and cytotoxicity of the essential oil of *Curcuma zedoaria*. Am. J. Chin. Med. 32, 281–290.

Lu, C., Li, A.P., 2001. Species comparison in P450 induction: effects of dexamethasone, omeprazole, and rifampin on P450 isoforms 1A and 3A in primary cultured hepatocytes from man, Sprague-Dawley rat, minipig, and beagle dog. Chem. Biol. Interact. 134, 271–281.

Maheshwari, R.K., Singh, A.K., Gaddipati, J., Srimal, R.C., 2006. Multiple biological activities of curcumin: a short review. Life Sci. 78, 2081–2087.

Matsuda, H., Ninomiya, K., Morikawa, T., Yoshikawa, M., 1998. Inhibitory effect and action mechanism of sesquiterpenes from Zedoariae Rhizoma on D-galactosamine/lipopolysaccharide-induced liver injury. Bioorg. Med. Chem. Lett. 8, 339–344.

Mills, E., Montori, V., Perri, D., Phillips, E., Koren, G., 2005. Natural health product–HIV drug interactions: a systematic review. Int. J. STD ATDS 16, 181–186.

Murray, M., 2006. Altered CYP expression and function in response to dietary factors: potential roles in disease pathogenesis. Curr. Drug Metab. 7, 67–81.

Neuhaus, W., Bogner, E., Wirth, M., Trzeciak, J., Lachmann, B., Gabor, F., Noe, C.R., 2006. A novel tool to characterize paracellular transport: the APTS–dextran ladder. Pharm. Res. 23, 1491–1501.

Paine, M.F., Criss, A.B., Watkins, P.B., 2005. Two major grapefruit juice components differ in time to onset of intestinal CYP3A4 inhibition. J. Pharmacol. Exp. Ther. 312, 1151–1160.

Ram, A., Das, M., Ghosh, B., 2003. Curcumin attenuates allergen-induced airway hyperresponsiveness in sensitized guinea pigs. Biol. Pharm. Bull. 26, 1021–1024.

Raucy, J.L., 2003. Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. Drug Metab. Dispos. 31, 533–539.

Sasaki, Y., Goto, H., Tonda, C., Hatanaka, F., Shibahara, N., Shimada, Y., Terasawa, K., Komatsu, K., 2003. Effects of *Curcuma* drugs on vasomotion in isolated rat aorta. Biol. Pharm. Bull. 26, 1135–1143.

Schmiedlin-Ren, P., Thummel, K.E., Fisher, J.M., Paine, M.F., Lown, K.S., Watkins, P.B., 1997. Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1α,25-dihydroxyvitamin D3. Mol. Pharmacol. 51, 741–754.

Schmiedlin-Ren, P., Thummel, K.E., Fisher, J.M., Paine, M.F., Watkins, P.B., 2001. Induction of CYP3A4 by 1 alpha,25-dihydroxyvitamin D_3 is human cell line-specific and is unlikely to involve pregnane X receptor. Drug Metab. Dispos. 29, 1446–1453.

Sharma, R.A., McLelland, H.R., Hill, K.A., Ireson, C.R., Euden, S.A., Manson, M.M., Pirmohamed, M., Marnett, L.J., Gescher, A.J., Steward, W.P., 2001. Pharmacodynamic and pharmacokinetic study of oral *Curcuma* extract in patients with colorectal cancer. Clin. Cancer Res. 7, 1894–1900.

Syu, W.J., Shen, C.C., Don, M.J., Ou, J.C., Lee, G.H., Sun, C.M., 1998. Cytotoxicity of curcuminoids and some novel compounds from *Curcuma zedoaria*. J. Nat. Prod. 61, 1531–1534.

Thapliyal, R., Maru, G.B., 2001. Inhibition of cytochrome P450 isozymes by curcumins in vitro and in vivo. Food Chem. Toxicol. 39, 541–547.

Valentine, S.P., Le Nedelec, M.J., Menzies, A.R., Scandlyn, M.J., Goodin, M.G., Rosengren, R.J., 2006. Curcumin modulates drug metabolizing enzymes in the female Swiss Webster mouse. Life Sci. 78, 2391–2398.

Veronese, M.L., Gillen, L.P., Burke, J.P., Dorval, E.P., Hauck, W.W., Pequignot, E., Waldman, S.A., Greenberg, H.E., 2003. Exposure-dependent inhibition of intestinal and hepatic CYP3A4 in vivo by grapefruit juice. J. Clin. Pharmacol. 43, 831–839.

Waxman, D.J., 1999. P450 gene induction by structurally diverse xenochemicals: centrtal role of nuclear receptors CAR, PXR, and PPAR. Arch. Biochem. Biophys. 369, 11–23.

Wrighton, S.A., Schuetz, E.G., Thummel, K.E., Shen, D.D., Korzekwa, K.R., Watkins, P.B., 2000. The human CYP3A subfamily: practical considerations. Drug Metab. Rev. 32, 339–361.

Zhang, L., Lin, G., Chang, Q., Zuo, Z., 2005. Role of intestinal first-pass metabolism of baicalein in its absorption process. Pharm. Res. 22, 1050–1058.