Oral intake of curcumin markedly activated CYP 3A4: in vivo and ex-vivo studies

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Curcumin, a specific secondary metabolite of Curcuma species, has potentials for a variety of beneficial health effects. It is nowadays used as a dietary supplement. Everolimus (EVL) is an immunosuppressant indicated for allograft rejection and cancer therapy, but with narrow therapeutic window. EVL is a substrate of P-glycoprotein (P-gp) and cytochrome P450 3A4 (CYP3A4). This study investigated the effect of coadministration of curcumin on the pharmacokinetics of EVL in rats and the underlying mechanisms. EVL (0.5 mg/kg) was orally administered without and with 50 and 100 mg/kg of curcumin, respectively, in rats. Blood samples were collected at specific time points and EVL concentrations in blood were determined by QMS® immunoassay. The underlying mechanisms were evaluated using cell model and recombinant CYP 3A4 isozyme. The results indicated that 50 and 100 mg/kg of curcumin significantly decreased the AUC0-540 of EVL by 70.6% and 71.5%, respectively, and both dosages reduced the Cmax of EVL by 76.7%. Mechanism studies revealed that CYP3A4 was markedly activated by curcumin metabolites, which apparently overrode the inhibition effects of curcumin on P-gp. In conclusion, oral intake of curcumin significantly decreased the bioavailability of EVL, a probe substrate of P-gp/CYP 3A4, mainly through marked activation on CYP 3A4.

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of P-gp or CYP 3A4. However, till now no in vivo relevant evidence has been reported in literature. Therefore, this study was set to investigate the effect of coadministration of curcumin on the pharmacokinetics of EVL, a probe substrate of P-gp/CYP 3A4, in rats. Furthermore, the underlying mechanisms of interaction were evaluated using cell model and recombinant CYP 3A4 isozyme.

Results

Effect of curcumin on EVL pharmacokinetics in rats. The blood EVL concentration – time profiles after oral administration of EVL alone and coadministrations with 50 or 100 mg/kg of curcumin are shown in Figure 1. The pharmacokinetic parameters of EVL after various treatments are given in Table 1. The results showed that 50 and 100 mg/kg of curcumin both significantly decreased the Cmax of EVL by 76.7%, and reduced the AUC0-540 by 70.6% and 71.5%, respectively. The MRT of EVL was significantly increased by 100 mg/kg of curcumin by 35.3%, but not affected by 50 mg/kg of curcumin.

Effect of curcumin on P-gp activity. Figure 2 shows the effect of curcumin on the intracellular accumulation of rhodamine 123, a typical substrate of P-gp. The results indicated that 25, 50 and 100 μM of curcumin significantly decreased the efflux function of P-gp by 13.2%, 16.7% and 173.9%, respectively. As a positive control of P-gp inhibitor, verapamil inhibited the intracellular accumulation of rhodamine 123 by 55.5%.

Characterization of curcumin serum metabolites (CSM). An LC-MS/MS method using selective reaction monitoring (SRM) technique was performed to analyze CSM prior to and after treatment with sulfatase/glucuronidase. The results shown in Figure 3(A) indicated that curcumin, demethoxycurcumin and bis-demethoxycurcumin were not detected before enzymatic hydrolysis of CSM. After treatment with sulfatase/glucuronidase, the peaks of curcumin and demethoxycurcumin emerged, indicating that the glucuronides/sulfates of curcumin and demethoxycurcumin were present in CSM. Apparently, the concentration of curcumin glucuronides/sulfates was higher than that of demethoxycurcumin glucuronides/sulfates.

Effects of curcumin, demethoxycurcumin, bis-demethoxycurcumin and CSM on CYP 3A4 activity. The effects of curcumin, demethoxycurcumin, bis-demethoxycurcumin and CSM on CYP 3A4 activity are shown in Figure 4(A) and 4(B), respectively. Curcumin at 5 and 10 μM significantly inhibited the activity of CYP 3A4 by 93% and 90%, respectively. Demethoxycurcumin at 5 and 10 μM significantly inhibited the activity of CYP 3A4 by 21% and 53%, respectively. Bis-demethoxycurcumin at 0.5 and 1.0 μM significantly inhibited the activity of CYP 3A4 by 10%.

Table 1 | Pharmacokinetic parameters of EVL in rats after various treatments

| Parameter | EVL alone | EVL + curcumin (50 mg/kg) | EVL + curcumin (100 mg/kg) |
|-----------|-----------|---------------------------|---------------------------|
| Cmax      | 6.0 ± 1.8 | 1.4 ± 0.9 *** [−76.7%]    | 1.4 ± 1.2 *** [−76.7%]    |
| AUC0−540  | 1637.7 ± 256.8 | 481.8 ± 327.8 *** [−70.6%] | 466.0 ± 330.2 *** [−71.5%] |
| MRT       | 207.8 ± 23.8 | 251.2 ± 28.5              | 281.2 ± 37.6 ** [35.3%]  |

Data are expressed as the mean ± S.D.
*p < 0.05, **p < 0.01, ***p < 0.001.
Cmax [ng/mL]: peak blood concentration.
AUC0−540 [ng min/mL]: area under the blood concentration–time curve from 0 to 540 min.
MRT [min]: mean residence time.
On the contrary, CSM at 1/4- and 1/2-fold serum concentrations significantly increased the activity of CYP 3A4 by 235% and 593%, respectively, when compared to those of correspondent concentrations of blank serum specimen. As a positive control of CYP 3A4 inhibitor, ketoconazole significantly decreased CYP 3A4 activity by 95%.

**Discussion**

In this study, EVL was used as a probe substrate of P-gp/CYP 3A4. The results showing that the Cmax and AUC0-540 were markedly decreased by coadministration of curcumin at both dosages of 50 and 100 mg/kg indicated that the oral bioavailability of EVL in rats was significantly reduced by concurrent intake of curcumin. Observation on the blood profiles of EVL revealed that the absorption of EVL was apparently hampered by curcumin. In regard to the magnitudes of interactions, two dosages of curcumin demonstrated comparable influences, implying that the interaction machinery had been saturated at the lower dosage.

EVL is a substrate of P-gp and CYP 3A4. The absorption of EVL should be highly correlated with the function and expression of P-gp and CYP 3A4. In order to delineate the underlying mechanism of the acute inhibition effect of curcumin on EVL absorption, in vitro and ex-vivo models were employed to investigate the effects of oral curcumin on the activities of P-gp and CYP 3A4, respectively.

In P-gp mediated transport study of rhodamine 123, the result showing that curcumin significantly increased the intracellular accumulation of rhodamine 123 indicated that the efflux activity of P-gp was inhibited by curcumin, which was in good agreement with several previous reports14–18, although a different cell model was used in the present study. We thus can infer that this inhibition effect of curcumin on P-gp did not play an important role in the mechanism of decreased absorption of EVL in rats.

For evaluating the in vivo effect of curcumin on CYP 3A4 activity, CSM was prepared from rats receiving curcumin to mimic the virtual molecules interacting with CYP 3A4 in the enterocytes and hepatocytes based on the consideration of metabolic fate of curcumin19–21. After characterization by LC-MS/MS method, CSM was found containing curcumin metabolites including the glucuronides/sulfates of curcumin and demethoxycurcumin, whereas the free forms of curcumin, demethoxycurcumin and bis-demethoxycurcumin were not detected. This finding further confirmed that curcumin was rapidly and extensively metabolized after oral intake22–24. The results of CYP 3A4 assay showed that 1/2- and 1/4-fold serum concentrations of CSM remarkably increased the activity of CYP 3A4, which could account for the markedly decreased absorption of EVL via enhancing the first pass effect during the early phase. Accordingly, the activation effect of CSM on CYP 3A4 could be attributed to the conjugated metabolites of curcumin and/or demethoxycurcumin. We thus strongly suggest that using the metabolites of curcumin in in vitro studies to investigate the bioactivities was important for understanding the virtual effects and mechanism of curcumin in the in vivo system.

In order to investigate the structure-activity relationship of curcuminoids regarding their modulation on CYP 3A4, three pure compounds including curcumin, demethoxycurcumin and bis-demethoxycurcumin were in parallel evaluated with CSM using this specific method. The results showing that curcumin inhibited CYP 3A4 was in good agreement with previous in vitro studies25–27. Like curcumin, demethoxycurcumin and bis-demethoxycurcumin also inhibited the activity of CYP 3A4, but in lesser extent. Apparently, the inhibition effects of curcuminoid free forms on CYP 3A4 were opposite to the activation effect by CSM. In fact, free forms of these curcuminoids might have no chance to interact with CYPs located in the enterocytes and hepatocytes judged from their rapid metabolism by conjugation reactions28–30.

Taken together, our mechanism studies showed that curcumin inhibited P-gp, whereas CSM markedly activated CYP 3A4. Based on the effect of oral curcumin on the pharmacokinetics of EVL in rats, it clearly implied that the activation effect on CYP 3A4 by CSM had overwhelmed the inhibition effect on P-gp by curcumin, which resulted in a net effect of decreased absorption of EVL.

CYP 3A4 is an important human metabolizing enzyme present in intestine and liver. Clinically, more than 50% of drugs are metabolized by CYP3A4, including proton pump inhibitors (esomeprazole, omeprazole), antihyperlipidemic (atorvastatin, simvastatin), anti-HIV agents (indinavir, ritonavir), anti-infection agents (erythromycin, ketoconazole), immunosuppressants (cyclosporine, tacrolimus), anti-hypertensive agents (amlodipine, felodipine), anticonvulsants...
Figure 3 | LC-MS/MS chromatograms of hesperetin (1, internal standard), curcumin (2), demethoxycurcumin (3) and bis-demethoxycurcumin (4) in serum prior to (A) and after hydrolysis with sulfatase/glucuronidase (B).
(carbamazepine), anti-depressants (quetiapine, sertraline) and anti-cancer agents (paclitaxel, vinblastine), etc. Moreover, it has been well recognized that CYP 3A4 was involved in numerous clinical life-threatening drug-drug interactions, such as ketoconazole-terfenadine and simvastatin-cisapride. In addition, grapefruit-felodipine interaction was arisen from inhibition on intestinal CYP 3A4. On the contrary, several food - drug interactions such as mulberry-cyclosporine, resveratrol-cyclosporine and soymilk-cyclosporine, which resulted in decreased blood levels of cyclosporine, were stem from activation on CYP 3A4.

Given CSM is a strong activator of CYP 3A4, curcumin would be a promising chemoprevention agent for numerous xenobiotics. If a CYP 3A4 substrate drug is taken concomitantly with curcumin, we assumed that the efficacy of this medication might be ameliorated owing to greatly enhanced metabolism, even it was a P-gp substrate like EVL. On the other hand, for a drugs which is a P-gp substrate but not metabolized by CYP 3A4, such as digoxin and talinolol, the blood levels might be elevated by coadministered curcumin due to P-gp inhibition. Therefore, it is suggested that curcumin and curcumin-containing dietary supplements are not recommended for chronic patients using medications regularly. In conclusion, oral intake of curcumin significantly decreased the absorption of EVL mainly through marked activation on CYP 3A4 by curcumin metabolites.

**Methods**

**Chemicals and reagents.** Everolimus (Certican®, 0.5 mg/tab) was kindly provided by Novartis (Taiwan) Co. Ltd. Curcumin (purity 94%), demethoxycurcumin (purity 98%), hesperetin (purity 95%), rhodamine 123, sodium dodecyl sulfate (SDS), Triton X-100, verapamil and sulfatase (type H-1 from Helix pomatia) were purchased from Sigma (St. Louis, MO, USA). bis-demethoxycurcumin (purity 99%) was obtained from ChromaDex (Irvine, CA, USA). Dulbecco’s Modified Eagle Medium (DMEM),

Figure 4 | Effects of curcumin (Cur, µM), demethoxycurcumin (DMC, µM), bis-demethoxycurcumin (BDMC, µM) (A) and CSM (1/2- and 1/4-fold serum concentrations) (B) on CYP 3A4 activity. K: ketoconazole (positive control of CYP 3A4 inhibitor, µM). *p < 0.05, **p < 0.01, *** p < 0.001.
Acid, 100 units/mL of penicillin, 100 units/mL of streptomycin (Hsinchu, Taiwan). Cells were cultured in DMEM medium with 10% fetal bovine serum purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The lower limit of quantitation (LLOQ) of this assay is 1.3 ng/mL.

Sci. Total Environ. 43x113: 31. Briefly, 100 µL of cell lysate was added into each well and incubated for 4 h. In this period, MTT was turned to formazan crystal by live cells. SDS solution (10%) was added to liquefy the purple crystal and the optical density was detected at 570 nm by a microplate reader.

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The blood EVL concentration was measured by a QMS® ESI/TOF (heated-electrospray ionization) -II probe with Quantum Access MAX triple stage quadrupole mass spectrometer (Thermo Fisher Scientific Inc., U.S.A.). NADP⁺ was used as an internal standard in all experiments. The peptidylglycine α-amidating enzyme (PHE) was purchased from the Chinese Society of Animal Science, Taiwan. The protocol was approved by the Animal Management Committee, China Medical University (Permit Number: CMU-102-144). The necrosis was performed under isoflurane, and all efforts were made to minimize suffering. A total of 17 rats weighing 280–350 g were anesthetized for drug administration.

Cerican® was ground into fine powders and solubilized with PEG 400 to afford 0.5 mg/mL of EVL. In a parallel design, a dose of 0.5 mg/1.0 mL/kg of EVL was orally given to rats via gastric gavage without and with 50/1.0 mL/kg or 100 mg/2.0 mL/kg of curcumin, which was also solubilized with PEG 400 to afford concentrations of 25 and 50 mg/mL.

Cell viability assay. The effects of curcumin, verapamil and DMSO on viability of LS 180 were evaluated by MTT assay. Cells were seeded into a 96-well plate. After overnight incubation, the tested agents were added and incubated for 24 h. MTT (5 mg/mL) was added into each well and incubated for 4 h. In this period, MTT was turned into formazan crystal by live cells. SDS solution (10%) was added to liquefy the purple crystal and the optical density was detected at 570 nm by a microplate reader (BioTex, Highland Park, Winooski, VT, U.S.A.).

Effect of curcumin on P-gp activity. The effects of curcumin on P-gp - mediated transport of rhodamine 123 were evaluated by following previous studies with some modifications. LS-180 cells (1×10⁶) were cultured in 96-well plate. The medium was removed and washed with ice-cold PBS after overnight incubation. One hundred microliter of rhodamine 123 in HBSS (10 µM) was put into each well and incubated at 37°C. After 1-h incubation, the supernatant was removed, and cells were washed twice with ice-cold PBS. Curcumin, verapamil (as a positive control of P-gp inhibitor) and DMSO were added into corresponding wells and incubated for another 4 h. After 4-h incubation, the medium was removed and the cells were washed twice with ice-cold PBS again. Then, 0.1% Triton X-100 (100 µL) was added to lyse the cells, and the fluorescence was measured with excitation at 485 nm and emission at 528 nm.

In order to quanitate the content of protein in each well, 10 µL of cell lysate was added to 200 µL of diluted protein assay reagent (Bio-Rad, Hercules, CA, U.S.A.) and the optical density was measured at 570 nm. The relative intracellular accumulation of rhodamine 123 was calculated by comparing with that of control protein correction.

Preparation of curcumin serum metabolite (CSM). Based on a previous study reporting the biological fate of curcumin (1'), CSM of rats was prepared to mimic the molecular forms which interacted with CYP 3A4 in the enterocytes. After overnight fasting, six rats were orally administered curcumin (100 mg/kg) and blood was collected via cardiopuncture at 30 min after dosing. The blood was centrifuged to obtain serum.

Characterization of CSM. A portion of serum was characterized prior to and after hydrolysis with sulfatase/glucuronidase following a previous method using LC-MS/MS with some modifications. Briefly, 100 µL serum sample was mixed with 30 µL pH 5 acetate buffer or 30 µL of 2 mM acetaldehyde (type H-1 from Fisher, CYP3A4. All the procedures were performed according to the manual provided by Invitrogen. Briefly, after incubating CYP450 recombiant BACULOSOMES®, glucose-6-phosphate and glucose-6-phosphate dehydrogenase with CSM (1/2- and 1/4-fold serum concentrations) in 96-well black plate at room temperature for 20 min, a specific CYP4A substrate (Vivid® BOMR) and NADP⁺ were added, and incubated at room temperature for another 30 min. At the end of incubation, ketoconazole was added to stop the reaction and the fluorescence was measured with excitation at 530 nm and emission at 590 nm.

Data analysis. The peak blood concentration (Cmax) was obtained from experimental observation. The pharmacokinetic parameters of EVL were analyzed by non-compartmental model of the program WinNonlin® (version 1.1 SCI software, Statistical Consulting, Inc., Apex, NC). The area under the blood concentration - time curve (AUC0-t) was calculated using trapezoidal rule to the last point. One way ANOVA with Scheffe’s test was used for statistical comparison. Statistical significance level was set at p < 0.05 as significant.

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Author contributions
Y.-W.H. contributed clinical opinion and study design. C.-Y.H., S.-Y.Y., C.-P.Y. and Y.-H.P. performed experimental work, data analysis and formulation of the article. P.-D.L.C. and Y.-C.H. made contributions to conception, study design and revision of the manuscript.

Additional information
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