SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
PRECLINICAL RESEARCH
DRUG THERAPY

Received
22 July 2014

Accepted
17 September 2014

Published
10 October 2014

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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 $AUC_{0\text{-}540}$ of EVL by 70.6% and 71.5%, respectively, and both dosages reduced the C_{max} 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.

Curcumin (Diferuloylmethane), a polyphenol constituent in spice turmeric, is a specific secondary metabolite of *Curcuma longa*, *C.* zedoaria, *C. aromatica*, *C. wenyujin* and *C. kwangsiensis*[1]. Numerous beneficial health effects of curcumin have been reported such as vascular protection[2], cancer prevention[3] and anti-Alzheimer's disease[4], etc. Therefore, curcumin is nowadays used as a dietary supplement. Pharmacokinetic studies of oral curcumin indicated that the major molecules in the systemic circulation were the glucuronides and sulfates of curcumin, whereas only trace of the parent form of curcumin was detected in rats and humans[5,6]. Besides, demethoxycurcumin and bis-demethoxycurcumin, the phase I metabolites of curcumin, as well as their conjugated metabolites have also been detected in mice[7].

Everolimus (EVL), a macrolide immunosuppressant, is usually prescribed with corticosteroids and cyclosporine to prevent kidney, liver or heart transplant rejection in clinical practice[8]. In recent years, EVL has also been used to treat advanced breast cancer[9] and advanced renal cell carcinoma[10]. The recommended therapeutic range of EVL is 3–8 ng/mL in renal and cardiac transplant patients. The higher acute rejection rate was reported when the trough EVL level was below 3 ng/mL. On the other hand, higher EVL trough level had been associated with increasing incidence of thrombocytopenia[11]. In addition, pulmonary toxicity is a potentially life - threatening complication of EVL[12]. Commonly reported adverse effects of EVL included stomatitis, hyperglycemia, rash and fatigue[13,14]. Most of the adverse effects are dose-related, and dosage reduction or discontinuation of EVL is usually adopted to avoid severe adverse reactions[12–14].

In regard to the pharmacokinetics of EVL, the oral bioavailability was only 15–16% with peak concentration being reached within 0.5–4.0 h, and the half-life is 18–35 h[15]. Owing to its narrow therapeutic index, regular blood concentration monitoring of EVL is required to ensure therapeutic efficacy[15–17]. EVL is a substrate of P-glycoprotein (P-gp), a drug-efflux transporter, and primarily metabolized by CYP3A4, an important drug-metabolizing enzyme[12]. Any potential modulation effects on P-gp or CYP 3A4 may alter the oral bioavailability of EVL, which might affect the efficacy or toxicity of EVL in patients.

Numerous *in vitro* studies have reported that curcumin inhibited not only P-gp[18–20], but also CYP3A4[21–23]. Based on these *in vitro* results, coadministration of curcumin should increase the oral bioavailability of substrates

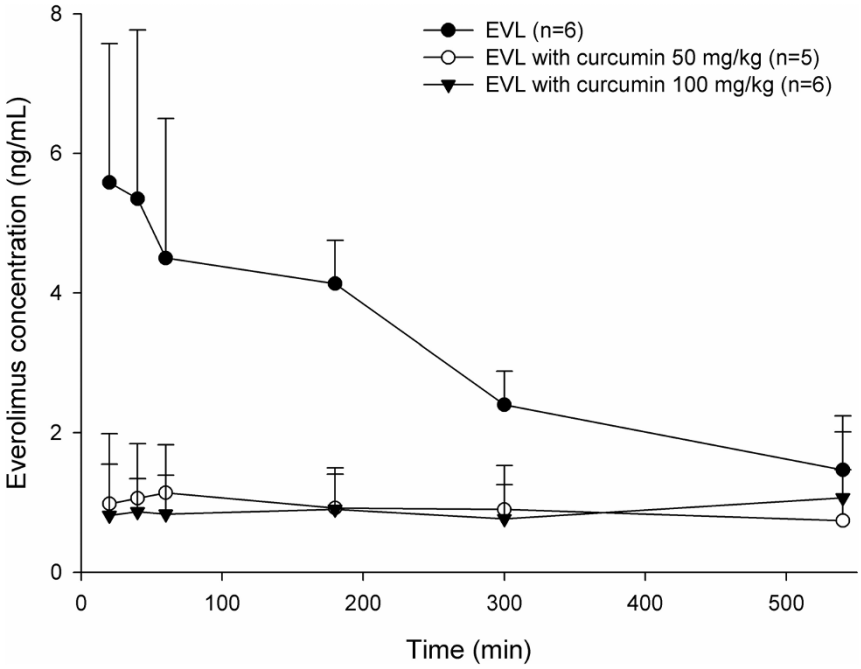

Figure 1 | Mean (±S.D.) blood concentration-time profiles of everolimus (EVL) after oral administration of EVL alone (●, 0.5 mg/kg) and coadministration with 50 mg/kg (○, n=5) and 100 mg/kg (▼) of curcumin to rats (n = 6).

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 C_{max} of EVL by 76.7%, and reduced the $AUC_{0\text{-}540}$ 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 172.9%, 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 sulfataser/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 \ Treatments	EVL alone	EVL + curcumin (50 mg/kg)	EVL+ curcumin (100 mg/kg)
C_{max}	6.0 ± 1.8	1.4 ± 0.9 *** (−76.7%)	1.4 ± 1.2 *** (−76.7%)
AUC_{0-540}	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.
C_{max} (ng/mL): peak blood concentration.
AUC_{0-540} (ng·min/mL): area under the blood concentration–time curve from 0 to 540 min.
MRT (min): mean residence time.

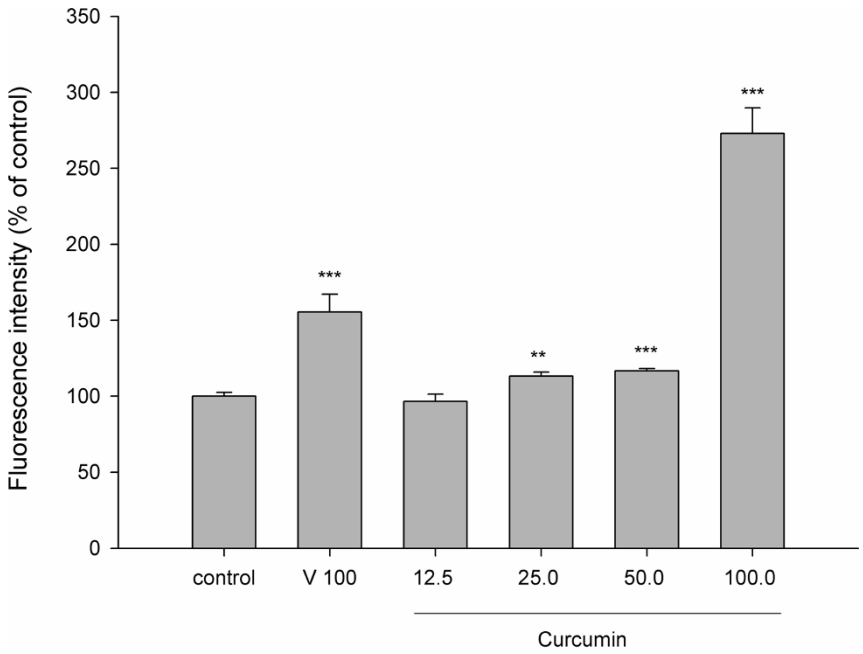

Figure 2 | Effect of curcumin (μg/mL) on the intracellular accumulation of rhodamine 123 in LS 180 cells. C: control, V: verapamil (positive control of P-gp inhibitor, 100 μg/mL). **p < 0.01, *** p < 0.001.

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 C_{max} and $AUC_{0\text{-}540}$ 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[12]. 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 reports[18–20], 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 curcumin[5–7]. 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 intake[5–7]. 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* studies[21–23]. 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 reactions[5–7].

Taken together, our mechanism studies showed that curcumin inhibited P-gp, whereas CSM markedly activated CYP 3A4. Based on the effect of oral corcumin 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

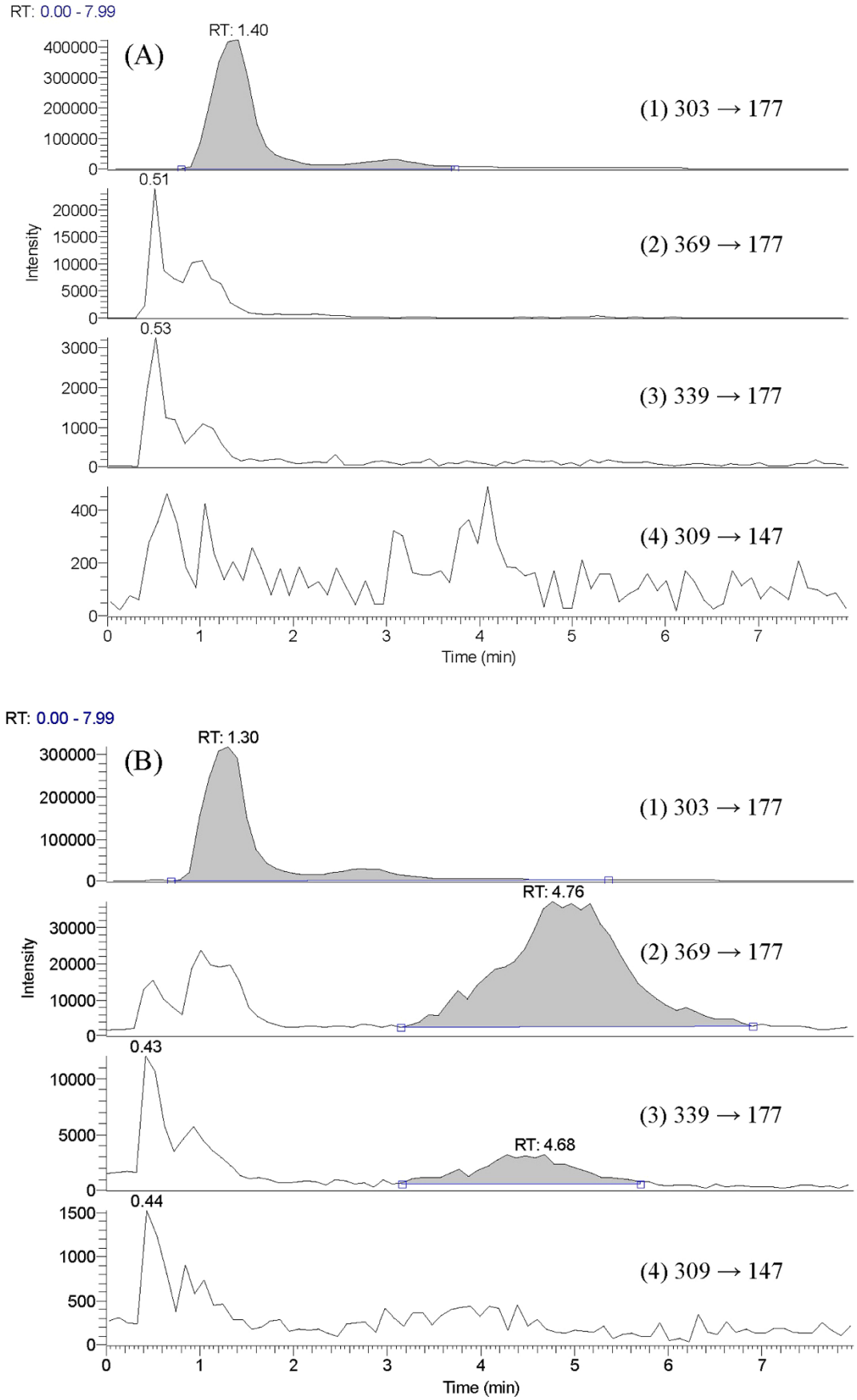

Figure 3 | LC-MS/MS chromatograms of hesperetin (1, internal standard), curcumin (2), demethoxycurcumin (3) and bis-demethoxycurcumin (4) in serum prior to (A) and after hydrolysis with sulfatase/glucuronidase (B).

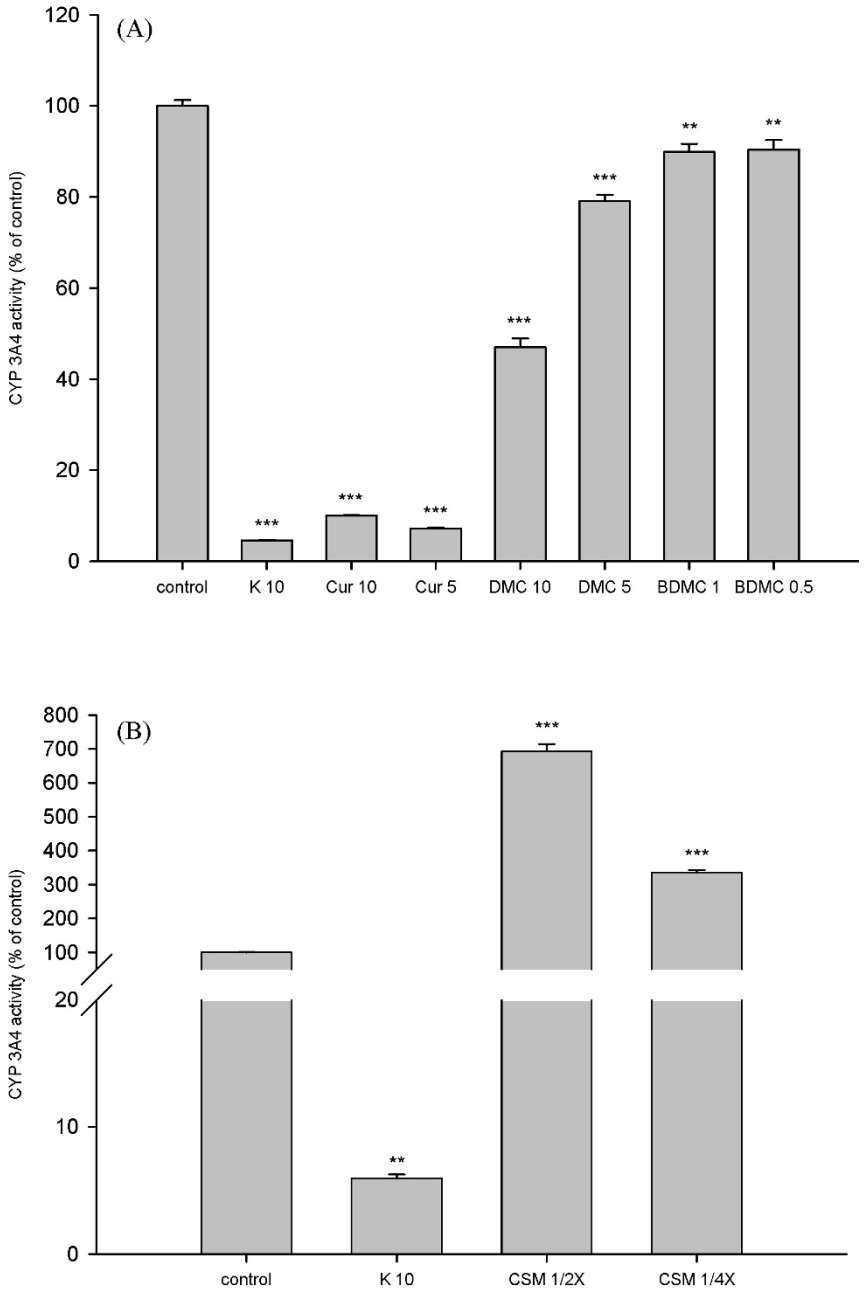

Figure 4 | Effects of curcumin (Cur, μM), demethoxycurcumin (DMC, μM), bis-demethoxycurcumin (BDMC, μM) (A) and CSM (1/2- and 1/4-fold serum concentrations) (B) on CYP 3A4 activity. K: ketoconazole (positive control of CYP 3A4 inhibitor, μM). *p <0.05, **p < 0.01, *** p < 0.001.

(carbamazepine), anti-depressants (quetiapine, sertraline) and anti-cancer agents (paclitaxel, vinblastine), etc[24]. Moreover, it has been well recognized that CYP 3A4 was involved in numerous clinical life-threatening drug-drug interactions, such as ketoconazole-terfenadine[25] and simvastatin-cisapride[26]. In addition, grapefruit-felodipine interaction was arisen from inhibition on intestinal CYP 3A4[27]. On the contrary, several food - drug interactions such as mulberry-cyclosporine[28], resveratrol-cyclosporine[29] and soymilk-cyclosporine[30], 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),

trypsin/EDTA, nonessential amino acid, Hank's Buffered Salt Solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Vivid® CYP450 screening kits were obtained from Invitrogen (Grand Island, NY, USA). 3-(4′,5′-Dimethylthiazol-2′-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Alfa Aesar (Lancaster, UK).Methanol was LC grade and was purchased from Echo (Miaoli, Taiwan). Milli-Q plus water (Millipore, Bedford, MA, USA) was used for all preparations.

Animals and drug administration. Male Sprague-Dawley rats were supplied by National Laboratory Animal Center (Taipei, Taiwan) and kept in the Animal Center of China Medical University (Taichung, Taiwan). The animal study was under "The Guidebook for the Care and Use of Laboratory Animals (2002)" published by the Chinese Society of Animal Science, Taiwan. The protocol was approved by the Animal Management Committee, China Medical University (Permit Number: CMU-102-144-N). The narcotization was performed under isoflurane, and all efforts were made to minimize suffering. A total of 17 rats weighing 280–350 g (n = 5–6 in each group) were fasted for 12 h before drug administration.

Certican® 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 gavages 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.

Determination of blood EVL concentration. The blood EVL concentration was measured by a QMS® Everolimus Immunoassay kit supplied by Thermo Fisher Scientific (Fremont, CA, USA), which had been reported comparable with LC-MS method[31]. The assay was calibrated for concentration range from 1.5 to 20 ng/mL. The lower limit of quantitation (LLOQ) of this assay is 1.3 ng/mL.

Cell line and culture conditions. LS-180, human colon adenocarcinoma cell line, was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in DMEM medium with 10% fetal bovine serum (Biological Industries Ltd., Kibbutz Beit Haemek, Israel), 0.1 mM nonessential amino acid, 100 units/mL of penicillin, 100 μg/mL of streptomycin and 292 μg/mL of glutamine. Cells were grown at 37°C in a humidified incubator containing 5% CO_2. The medium was replaced every two days and cells were subcultured when 80 to 90% confluency was reached.

Cell viability assay. The effects of curcumin, verapamil and DMSO on viability of LS 180 were evaluated by MTT assay[32]. 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 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 (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 modification[33–35]. LS-180 cells (1×10^5) 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 correspondent wells and incubated at 37°C. 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 quantitate 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 after protein correction.

Preparation of curcumin serum metabolite (CSM). Based on a previous study reporting the biological fate of curcumin[5–7], 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[31]. Briefly, 100 μL serum sample was mixed with 50 μL pH 5 acetate buffer or sulfatase (type H-1 from *Helix pomatia*, containing 1000 units/mL of sulfatase and 39,861 units/ml of β-glucuronidase), 50 μL ascorbic acid (200 mg/mL) and incubated at 37 °C for 30 min. After hydrolysis, the serum sample was partitioned with 200 μL ethyl acetate (containing 2.0 μg/mL hesperetin as internal standard). The ethyl acetate layer was evaporated under N_2 to dryness and reconstituted with an appropriate volume of mobile phase prior to LC-MS/MS analysis.

The HPLC system was equipped with Accela 1250 pump and auto-sampler (Thermo Fisher Scientific Inc. U.S.A.). Chromatographic separation of analytes was achieved using a Thermo Hypersil GOLD C18 analytical column (50 mm × 2.1 mm, 1.9 μm) with a prefilter. The mobile phase consisted of 0.01% formic acid (A) and acetonitrile containing 0.01% formic acid (B) (A : B=60 : 40), and eluted isocratically for 8 min. The flow rate was 0.2 mL/min. The column effluent was detected by H-ESI (heated-electrospray ionization) -II probe with Quantum Access MAX triple stage quadrupole (TSQ) mass spectrometer (Thermo Fisher Scientific Inc. U.S.A.). Nitrogen was used as sheath gas at 40 arbitrary units and auxiliary gas at 10 arbitrary units. The collision energy was set at 10 V, spray voltage at 4700 V, capillary temperature at 325°C, vaporizer temperature at 300°C and tube lens offset at 114 V. The following mass transitions were used for selected reaction monitoring analysis (SRM): curcumin (369/177), demethoxycurcumin (339/177), bis-demethoxycurcumin (309/147) and the internal standard hesperetin (303/177). The ESI-MS spectra were recorded in positive ion mode.

Effects of curcumin, demethoxycurcumin, bis-demethoxycurcumin and CSM on CYP3A4 activity. Vivid® CYP450 screening kit was used for evaluating the effects of curcumin, demethoxycurcumin, bis-demethoxycurcumin and CSM on the activity of CYP3A4. All the procedures were performed according the manual provided by Invitrogen. Briefly, after incubating CYP450 recombinant 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 CYP3A4 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 (C_{max}) was obtained from experimental observation. The pharmacokinetic parameters of EVL were analyzed by noncompartment model of the program WinNonlin® (version 1.1 SCI software, Statistical Consulting, Inc., Apex, NC). The area under the blood concentration - time curve (AUC_{0-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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Acknowledgments

The work was in part supported by the Ministry of Science and Technology, Taipei, Taiwan, R.O.C. (MOST 103-2320-B-039-025, NSC102-2320-B-039-014-MY2 and NSC102-2320-B-039-008); and China Medical University, Taichung, Taiwan, R.O.C. (CMU103-105, CMU102-TC-03).

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

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Hsieh, Y.-W. *et al.* Oral intake of curcumin markedly activated CYP 3A4: *in vivo* and *ex-vivo* studies. *Sci. Rep.* **4**, 6587; DOI:10.1038/srep06587 (2014).