Role of catechol-O-methyltransferase in the disposition of luteolin in rats

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Non-standard abbreviations:

AUC_{0-t}, area under the plasma concentration versus time curve from 0 to t post-dose; COMT, catechol-O-methyltransferase; CYP, cytochrome P450 monooxygenase; DAD, diode array detector; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; MB-COMT, membrane-bound COMT; MRT_{0-t}, Mean residence time from 0 to t post-dose; MS, mass spectrometry; MS/MS, tandem mass spectrometry; Papp, apparent permeability coefficients; S-COMT, soluble COMT; SULT, sulfotransferase; UGT, uridine diphosphate glucuronosyltransferase.
Abstract

Luteolin is mainly metabolized by phase II enzymes in animals and humans with glucuronidation and sulfation as the two known metabolic pathways. Although methylation of luteolin was reported previously, the structure of the methylated metabolites and the enzymes involved in the process are not clarified. In our study, two methylated metabolites, M1 (chrysoeriol) and M2 (diosmetin), were identified in the urine after i.v. administration of luteolin to rats and the data also suggested the methylation was mediated by the catechol-O-methyltransferase (COMT). When luteolin was co-administered with a specific COMT inhibitor, entacapone, the formation of M1 and M2 were significantly reduced while the plasma concentration of luteolin increased. Methylation of luteolin was also studied in vitro using rat tissue homogenates. The apparent kinetic parameters associated with the formation of M1 and M2 in vitro were estimated, and region-selectivity of methylation of luteolin was observed. In the in vitro experiment, there was a preference in the formation of M2 over M1. In contrast, M1 was preferred to be accumulated in vivo in both rat plasma and urine following i.v. dose of luteolin. In conclusion, COMT played a crucial role in the disposition of luteolin in rats. Our results indicated that the methylation pathway in rats was significantly reduced when luteolin was co-administered with a specific COMT inhibitor. Therefore, COMT-associated drug-drug interaction need be considered in the future in luteolin clinical trials because the plasma concentrations and related therapeutic effects may be altered in vivo in the presence of a COMT inhibitor.
Introduction

Luteolin (3', 4', 5, 7-tetrahydroxyflavone) is one of the most widely occurring flavonoids in the plant kingdom. Several epidemiological studies have shown that a high consumption of food containing luteolin is associated with a reduced risk of developing chronic diseases (Lopez-Lazaro, 2009). Numerous experimental data have revealed that luteolin possesses a wide range of pharmacological effects, including anti-oxidation, anti-inflammation, anti-microbial activity, and anti-carcinogenic activity (Seelinger et al., 2008a; Seelinger et al., 2008b; Lopez-Lazaro, 2009).

Since the knowledge of the potential benefits of luteolin on health is increasing, it is essential to study the ADME process (absorption, distribution, metabolism and elimination) of luteolin in vivo. Luteolin is present in plants as aglycone and glycosides (Lopez-Lazaro, 2009). In general, aglycone of luteolin can be directly absorbed in the intestine through passive diffusion, while the glycosides of luteolin will be absorbed after being hydrolyzed in the intestine by micro-bacteria or lactase phlorizin hydrolase (Lu et al., 2010; Shimoi et al., 1998; Sesink et al., 2003; Kottra and Daniel, 2007). It has been proved that luteolin was mainly absorbed in the jejunum passively in a single-pass perfused rat intestinal model (Zhou et al., 2008), and luteolin was relatively permeable (with a Papp, 5.8±0.1×10^{-6} cm/sec) in a Caco-2 cell model (Ng et al., 2004), which indicated a moderate (20-70%) absorption (Yee, 1997). Because of its polyphenol structure, luteolin is a good substrate of uridine diphosphate glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) and mainly undergoes glucuronidation and sulfation during passing through the intestinal
mucosa or the liver (Shimoi et al., 1998). It is consistent with our previous studies, that luteolin occurred mainly as conjugated forms *in vivo* when *Chrysanthemum morifolium* extract, containing luteolin 7-*O*-glucoside, was orally administrated to rat, dog and human (Li et al., 2005; Li and Jiang, 2006; Chen et al., 2007).

In our previous excretion studies after administration of *Chrysanthemum morifolium* extract containing luteolin 7-*O*-glucoside to rat and human (Li and Jiang, 2006; Chen et al., 2007), we focused on the free luteolin or glucuronide and sulfate conjugates of luteolin, and the total excreted luteolin (free and conjugated forms) in rat urine, bile and feces were 6.60%, 2.05% and 31.3% of the dose, respectively, while the excreted luteolin (free and conjugated forms) in human urine were 2.03%. Apart from the glucuronidation and sulfation, few reports focused on the other metabolism pathways responsible for disposition of luteolin. Based on the structure of catechol, luteolin might be a substrate of catechol-*O*-methyltransferase (COMT; EC 2.1.1.6), however it has not been clearly clarified. In our preliminary experiment, two metabolites with longer retention time than luteolin on HPLC chromatograms were found in acidic hydrolyzed urine from rats dosed with luteolin, and we deduced they were methylated metabolites of luteolin. Therefore the present study was designed to indentify methylated metabolites of luteolin, to characterize the methylation of luteolin systemically and to evaluate the effect of methylation in disposition of luteolin in rats. The results will undoubtedly contribute to the better understanding of the disposition and pharmacological effects of luteolin.
Materials and methods

Chemicals. Luteolin was purchased from Hangzhou Skyherb technologies Co., Ltd, China (purity >99%). Diosmetin (3’, 5, 7-tri hydroxy-4’-methoxyflavone) was purchased from Shaanxi Huangteng Biotechnology Co., Ltd, China (purity >95%). Chrysoeriol (4’, 5, 7-trihydroxy-3’-methoxyflavone) was synthesized by Department of Medicinal Chemistry, College of Pharmaceutical Sciences, Zhejiang University (purity >99%). S-adenosyl-L-methionine p-toluenesulfonate salt was obtained from Aladdin-reagent, China (purity >80%). Entacapone was obtained from Jinan Wedo Industrial Co., Ltd., China (purity >99%). Methanol, acetonitrile, formic acid, and water were HPLC-grade. All other chemicals were from standard commercial sources and were of the highest quality.

Animal. Male Sprague-Dawley rats weighing 200-250 g were obtained from Experimental Animal Center of Zhejiang Academy of Medical Sciences. They were housed in cages at 23 °C with a 12 h light/dark cycle and given ad libitum access to food and water. All animals were handled according to an approved animal use protocol of Zhejiang University. Animals were free access to water but fasted for 12 h before experiment and for 2 h after drug administration.

Preparation of COMT fractions from rat tissues. Liver, kidney, small intestine (jejenum) and lung tissue homogenates were prepared as previously described (Bonifacio et al., 2009). Briefly, 6 male Sprague-Dawley rats were sacrificed by cervical dislocation after fasting for 12 h. Tissues (liver, kidney, small intestine and
lung) were homogenized in phosphate buffer (5 mM, pH 7.4) respectively, and then centrifuged at 15000 g for 20 min. The supernatant was used as total COMT fraction (containing both soluble-COMT and membrane bound-COMT).

Erythrocyte total COMT fraction was prepared from haemolyzed erythrocyte as previously described (Soares-da-Silva et al., 2003) with minor modifications. Briefly, erythrocytes were separated by centrifugation for 10 min (2000 g, 4 °C), plasma and the uppermost cell layer were removed, while the remaining erythrocytes were washed twice with 3 volumes of ice-cold 0.9% NaCl solution. Then erythrocytes were lysed with 4 volumes of ice-cold water and the membranes were separated by centrifugation for 20 min (15000 g, 4 °C). And the lysate was applied for total COMT assay.

The protein concentration was assayed with the BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China).

**Preparation of rat liver microsomes.** Rat liver microsomes were prepared by ultracentrifugation method. Male rats were sacrificed by cervical dislocation after fasting for 12 h, livers were perfused with 4 °C of 0.9% NaCl and homogenized in 4 volumes of 0.25 M sucrose. After centrifugation for 20 min (9000 g, 4 °C), the supernatant was collected and centrifuged for another 20 min (19000 g, 4 °C), then the supernatant was centrifuged for 55 min (100000 g, 4 °C) to precipitate the microsomes. After removing the supernatant, the pellet was re-homogenized with 0.15 M KCl, and then centrifuged for another 45 min (100000 g, 4 °C). The pellet of microsomes was re-suspended in 20 mM Tris-sucrose (pH 7.4). The protein
concentration was assayed with the BCA protein assay kit.

**HPLC-DAD analysis of two metabolites of luteolin in rat urine.** Urine samples from rat were hydrolyzed with hydrochloric acid and extracted with ethyl acetate after i.v. administration of 6.5 mg/kg luteolin to rats (Chen et al., 2007). The HPLC analysis was performed as described previously by Chen (Chen et al., 2007), while the mobile phase was optimized to separate luteolin and its two metabolites. Briefly, the HPLC analysis was carried out using an Agilent 1200 DAD HPLC system (Agilent Technologies, Waldbronn, Germany). The separation was performed on an Agilent Zorbax SB-C$_{18}$ column (250 mm$\times$4.6 mm, 5 $\mu$m) with a mobile phase consisted of 0.1% phosphoric acid, acetonitrile and methanol (60: 16: 24, v/v/v) at 30±1 °C with a constant rate 1 ml/min. The inject volume was 50 $\mu$l and the DAD detector was set at a wavelength of 350 nm for quantitative analysis. The UV-spectra were obtained with DAD detector from 200 to 400 nm.

**MS/MS analysis of two metabolites of luteolin.** Two metabolites from the rat urine were separated and collected on HPLC with the chromatographic conditions described above except that 0.2% formic acid was used instead of 0.1% phosphoric acid to adapt to the mass spectrometry. The collected elutions were dried under vacuum and re-dissolved with methanol. MS and MS/MS analysis was performed on ACQUITY™ TQD triple quadrupole instrument (Waters, Milford, MA, USA) with a Z-spray electrospray ionization source. Metabolites and standard compounds were infused continuously into the ESI source and the deprotonated molecular ions were detected in mass spectrometry with the following parameters: negative mode,
capillary voltage 3.7 kV, cone voltage 68 V, source temperature 120 °C, desolvation gas temperature 350 °C and collision energy of 40 eV for the MS/MS spectra.

**Methylation of luteolin in rat tissue homogenates with or without COMT inhibitor.** COMT reaction was performed as described previously with some modifications (Bonifacio et al., 2009). In brief, reaction mixture containing homogenate protein (1.0 mg/ml), 100 µM MgCl₂, 1.0 mM EGTA, 20 µM luteolin, with or without 30 nM entacapone in 150 µl phosphate buffer (5 mM, pH 7.8) was pre-incubated for 3 min at 37 °C, then 1.5 µl of 25 mM s-adenosyl methionine dissolved in phosphate buffer (5 mM, pH 7.8) was added to initiated the reaction. Reactions were terminated by adding 150 µl of ice-cold methanol after incubation for 0, 20, 40 and 60 min at 37 °C. The mixture was centrifuged (15000 g for 10 min) and luteolin and its metabolites in the supernatant were analyzed by HPLC method described above.

**Kinetics study of formation of two methylated metabolites of luteolin with rat tissue homogenates.** Protein concentration of homogenates (0.25 mg/ml for liver, 0.50 mg/ml for kidney and 1.0 mg/ml for small intestine, lung and erythrocyte homogenates, in which initial formation rate of M1 and M2 could be detected within 10 min incubation) and incubation time (10 min for all tissue homogenates) were used in the kinetics study. COMT reaction was conducted in the same conditions as described above. The formation kinetics of both M1 and M2 was determined by incubating luteolin at a series of concentrations (1, 2.5, 5, 10, 20, 40, 80 µM for liver, kidney, lung and erythrocytes, while 0.5, 1, 2.5, 5, 10, 20, 40, 80 µM for small
The experiments were performed in triplicate, and the reaction velocities were calculated in the unit of nanomoles of product formed per minute and milligram of homogenate protein. Apparent kinetic parameters $K_m$ and $V_{max}$ values were obtained from nonlinear regression by Prism 5 software (Graphpad software Inc., San Diego, CA) with Michaelis-Menten equation.

**The effect of entacapaone on pharmacokinetics of luteolin and two methylated metabolites in vivo.** To evaluate the influence of methylation on the pharmacokinetics of luteolin in rat in vivo, entacapaone was used as a specific COMT inhibitor. Ten male Sprague-Dawley rats were allocated into control group and entacapaone group with 5 rats each. 5 mg/kg entacapone (dissolved in 20% hydroxypropyl-β-cyclodextrin (w/v) containing 5% glucose (w/v)) and relevant vehicle were given to entacapone group and control group by i.v. through the tail vein 30 min prior to administration of 6.5 mg/kg of luteolin (dissolved in 20% hydroxypropyl-β-cyclodextrin (w/v) containing 5% glucose (w/v)) by i.v. through the tail vein. The blood samples from orbital venous sinus were collected into heparinized tubes at 0.083, 0.25, 0.5, 1, 2, 3, 5, 7, 9, 12 h post-dose, and centrifuged at 2000 g for 10 min under 4 °C. Plasma samples were stored at -20 °C until analysis. Plasma samples were prepared according to the method developed in our laboratory (Chen et al., 2007). Briefly, 100 µl plasma samples were hydrolyzed by hydrochloric acid (2 M) at 80 °C for 1.5 h, then the mixture was extracted by ethyl acetate. After the extraction was evaporated to dryness, the residue was reconstituted in mobile phase and the concentration of luteolin and two methylated metabolites were determined by
HPLC methods described above.

**The effect of entacapaone on urinary excretion of luteolin and two methylated metabolites.** 10 male Sprague-Dawley rats were allocated into control group and entacapaone group with 5 rats each, and each rat was housed individually in metabolic cage at 23 °C with a humidity level of 50%. Rats were administered of luteolin (control group) or entacapone and luteolin as the method described above. Urine samples were collected in glass flasks containing 200 μl ascorbic acid (0.1%, w/v) at the intervals of 0-4, 4-8, 8-12, 12-24 h after dose. Residual urine adhering to the metabolic cages was carefully washed with a small volume of saline and pooled with the collected urine. Urine samples were prepared the same as the plasma samples, and luteolin and two methylated metabolites were determined by HPLC methods described above.

**Phase I metabolism of M1 and M2 in rat microsomes.** The phase I metabolism of M1 and M2 in rat microsomes was conducted using the method described by Xia (Xia et al., 2007). In brief, a 100 μl of mixture containing 14.9 mM trinatric isocitric acid, 0.035 unit/ml isocitric dehydrogenase, 0.15 M magnesium chloride (MgCl₂), 10 μM substrate (M1 or M2), 0.4 mg/ml microsomal protein, 0.1 M (pH 7.4) Tris-HCl buffer, was pre-incubated at 37 °C for 3 min, the reaction was initiated by adding NADP and NADPH (0.9 mM/0.2 mM). After 10 or 30 min incubation at 37°C, the reaction was terminated by adding 100 μl of ice-cold methanol. After centrifugation at 15000 g for10 min, the supernatant was analyzed by HPLC.

**Data analysis.** Data were expressed as mean and standard deviations. Student’s
$t$-test (unpaired, two-tail) was utilized to evaluate the significance of the COMT inhibitor ($P \leq 0.05$). Pharmacokinetic parameters of luteolin and its two metabolites were estimated by the non-compartmental method using software DAS 2.0 (Chinese Pharmacologic Society, Beijing, China).
Results

Identification of two methylated metabolites in urine after intravenous administration of luteolin to rats. Urine samples were collected after i.v. administration of 6.5 mg/kg luteolin to rats. With a hydrolysis and extraction preparation, urine samples were analyzed by RP-HPLC, and luteolin and two metabolites were separated from endogenous substances in the chromatography with retention time of 15.46 min (luteolin), 29.35 min (M1) and 30.85 min (M2), respectively (Fig. 2A). The retention time of M1 and M2 was matched with the standard of chrysoeriol and diosmetin, respectively under the same HPLC condition (Fig. 2A). In addition, the two metabolites were also detected in rat tissue homogenates from in vitro incubation study with luteolin.

The M1 and M2 metabolites shared the same UV and MS/MS spectra with chrysoeriol and diosmetin, respectively (Fig. 2B and Fig. 2C). In the MS/MS analysis, a characteristic fragmentation of flavonoids was obtained, in which [M-H-CH$_3$]$^-$ ion of 284 represented a methylated luteolin, ion m/z of 107 and 151 represented A ring of luteolin, and ion m/z 132 represented B ring of luteolin, ion m/z 255 and 284 represented [M-H-CH$_3$-CO]$^-$ and [M-H-CH$_3$], respectively. Based on the HPLC chromatograms, UV spectra and MS/MS analysis, M1 and M2 were identified to be 3’-O-methylated luteolin (chrysoeriol) and 4’-O-methylated luteolin (diosmetin), respectively.

Methylation of luteolin in rat tissue homogenates. Methylated metabolites, M1 and M2, were detected after incubation with luteolin in rat liver, kidney, lung, small
intestine, erythrocyte homogenates in the presence of s-adenosyl methionine. The time profiles of luteolin elimination and metabolites formation were shown in Fig. 3. It revealed that the methylation contributed almost all the elimination of luteolin in rat tissues homogenates after incubation. The elimination of luteolin and formation of M1 and M2 both dramatically decreased in the presence of 30 nM entacapone, the specific COMT inhibitor (Fi. 3). In all the tissue homogenate incubations investigated, there was a preference in formation of M2 over M1, with a M2/M1 ratio of 2.2 to 2.6 in liver, kidney and erythrocyte and 1.2 in small intestine and lung (incubation for 20 min). The M2/M1 ratio did not change significantly except a slight decrease in the small intestine and lung in the presence of entacapone. Of all tissues studied, liver showed the highest methylation activity, followed by kidney, small intestine, lung, and erythrocyte (Fig. 3).

Kinetics study of M1 and M2 formation in rat tissue homogenates. M1 and M2 were formed based on the methylation of luteolin, and the initial formation rate could be obtained within 10 min incubation. Both of M1 and M2 followed Michaelis-Menten kinetics well (Fig. 4). The formation rate of M1, M2 almost reached apparent $V_{\text{max}}$ at 40 μM of luteolin in all the tissue homogenates. The apparent $V_{\text{max}}$ of M2 were approximately 2-fold higher than that of M1 in all the tissue homogenates, and both M1 and M2 showed the highest apparent $V_{\text{max}}$ in the liver, followed by kidney, small intestine, lung and erythrocyte (Table 1). M1 and M2 had a similar apparent $K_m$ (3.57~3.82μM) in liver and kidney homogenates, while with different apparent $K_m$ in other tissues (Table 1). The estimated values of $V_{\text{max}}/K_m$ for
M2 were about twice higher than that of M1 in all the tissues except for the lung (Table 1).

**Pharmacokinetics of M1, M2 and luteolin in rats with or without entacapone.**

The plasma concentration-time profiles of M1, M2 and luteolin after i.v. administration of 6.5 mg/kg luteolin to rats in the control (without entacapone) and entacapone treated groups were presented in Fig. 5A. In the control group, the intact profiles of luteolin, M1 and M2 could be obtained, the maximal concentration of M1 and M2 reached at 5 min after dosing, and the total AUC\(_{0-\infty}\) of two metabolites were 33.6 \% of that of luteolin (Table 2 and Table 3). *In vivo*, the M2/M1 ratio in AUC\(_{0-t}\) was 0.20, while the *in vitro* ratio ranged from 1.4 to 2.6 using AUC\(_{0-t}\), calculated from the formation profiles of M1 and M2 (Fig. 3). In the entacapone treated group, the plasma concentration of luteolin were significantly higher than that of the control group, and the AUC\(_{0-\infty}\) of luteolin increased by 52\% and the MRT\(_{0-t}\) was longer than that of the control group (Table 2). Meanwhile, plasma concentrations of M1 and M2 dramatically decreased (Fig. 5A). M1 was only detected at 0.083h, 0.25h, 0.5h, and 1h post-dose, while M2 was not detected at any time points.

**Excretion of M1, M2 and luteolin in rat urine.** After intravenous administration of luteolin to rats, luteolin and its two methylated metabolites were detected in rat hydrolyzed urine and the cumulative excretion did not increase obviously after 12 h (Fig. 5B). In the control group, the cumulative excretion of luteolin, M1 and M2 in 24 h urine were 5.12\%, 1.92\% and 1.22\% of the dose, respectively, hence the total methylated metabolites were 3.11\% of the dose, which were about half of the luteolin
excreted in urine. In the entacapone treated group, the total excretion of luteolin increased to 7.32% of dose, while the total excretion of M1 and M2 decreased to 0.451% and 0.254% of dose, respectively. The ratio of M2/M1 in the urine collected in each period (0-4, 4-8, 8-12, 12-24 h) in the two groups was about 0.71, which was in line with the results from plasma.

The stability of M1 and M2 in rat microsomes. In the in vitro experiment, there was a preference in the formation of M2 over M1. In contrast, the concentrations of M1 were significantly higher than that of M2 in vivo in both rat plasma and urine following i.v. dose of luteolin. The data suggest that M1 and M2 might be converted back to luteolin in vivo, especially in the liver. To confirm this deduction, an experiment was conducted using rat liver microsomes, and the result demonstrated that M1 and M2 could be de-methylated by the phase I enzymes and converted back to luteolin. However, luteolin formed from M2 was about 2-fold higher than that formed from M1 (Fig. 6B) and this might suggest that M1 was more stable than M2 in rat microsomes (Fig. 6A).
Discussion

In the present study, two metabolites (M1: chrysoeriol and M2: diosmetin) were identified in rat urine after intravenous administration of luteolin (Fig. 1). It was the first time to clearly identify and enzymatically characterize the two methylated metabolites in rats. We may speculate that 3’- or 4’-hydroxyl of luteolin are subjected to methylation based on its structure, however it has not been confirmed. Since 3’- and 4’- methylated metabolites of luteolin have a similar partition coefficient, it was hard to separate them in the chromatography; and this might be the reason why only one methylated metabolite was reported in the previous studies of luteolin (Gradolatto et al., 2004). In addition, the two methylated metabolites could be further metabolized by UGTs or SULTs in vivo, which made it difficult to be identified and quantitated. To simplify the analysis of methylation, samples, such as blood and urine, were hydrolyzed in the preparation before HPLC analysis, in which total methylation (conjugated and free forms) were assayed.

COMT is a phase II enzyme, which is responsible for O-methylation and involved in the inactivation of the catecholamine neurotransmitters, such as dopamine, epinephrine, and norepinephrine. COMT introduces a methyl group to the catechol ring, which is donated by a co-substrate s-adenosyl methionine. Luteolin has a catechol ring and was identified to be a substrate of COMT in rat in the present study. It is known there are two isoforms of COMT from one gene in mammals, membrane-bound COMT (MB-COMT) and soluble COMT (S-COMT). In our study,
the rat tissue homegenates containing both MB-COMT and S-COMT were utilized in kinetics study, so the $K_m$ and $V_{max}$ for M1 and M2 were all apparent parameters.

In our *in vitro* study, COMT showed a preference in methylation of 4'- over 3'-hydroxyl of luteolin in all the rat tissues investigated, with a 4'/3'- ratio (about 2.5 in $V_{max}/K_m$) (*Table 1*). In fact, region-selectivity of COMT reaction was already reported for compounds, such as L-dopa, dopamine and quercetin, however 3-hydroxyl (3- and 4-position in catechol are equivalent to 3'- and 4'-position in luteolin) was preferential to be methylated in these substrates (Zhu et al., 1994; Lotta et al., 1995; de Boer et al., 2005). Since the rat S-COMT has been crystallized and the critical atomic structure has been described in detail (Vidgren, 1998). Region-selectivity of methylation could be further explained by the protein-substrate interaction. According to the molecular dynamic simulation studies (Lau and Bruice, 1998), the catechol ring displays a tilt of about 30 degrees compared with the active site of COMT, which directs the substituent at the 1-position of catechol ring (*Fig. 1*, equivalent to 1’-position in luteolin) into a hydrophobic pocket. Thus hydrophobic substituents accommodated in this pocket make a preference in 4-O-methylation, whereas polar substituents repelled by this hydrophobic pocket make 3-O-methylation favorable. According to this hypothesis, methylation of L-dopa, dopamine, and quercetin would prefer to 3-hydroxyl in catechol since they are relatively polar with the calculated LogP (software, ChemOffice 2004) of substituents -2.83, -0.32 and -0.57, respectively. While a preferential 4-O-methylation of catechol (4’-O-methylation in luteolin) will take place on luteolin because of its relative hydrophobicity with the calculated LogP.
Unexpectedly, the concentration of 3'-O-methylated luteolin (chrysoeriol) was significantly higher than that of 4'-O-methylated luteolin (diosmetin) in plasma and urine after intravenously dosing luteolin to rats, which was contradictory to the \textit{in vitro} result. Such different region-selectivities of O-methylation of by COMT \textit{in vivo} versus \textit{in vitro} conditions were already reported for other substrates but not clearly explained (Palma et al., 2003). We supposed that further metabolism might cause different degradation to each metabolite (Palma et al., 2003). For example, some CYPs, such as CYP1A and CYP2C, selectively demethylate the 4-O-methyl (Androutsopoulos et al., 2010; Busse et al., 1995), which may result in accumulation of 3-O-methylation \textit{in vivo}. The present study indicated that diosmetion (4'-O-methylation of luteolin) was more unstable than chrysoeriol (3'-O-methylation of luteolin) and the demethylation of diosmetin was significantly faster than that of chrysoeriol in rat microsomal phase I metabolism (\textbf{Fig. 6}). However, the demethylation only explained about 39% of reduced substrates (in 30 min reaction), and other metabolism pathways for the other 61% still to be disclosed. It is proposed that luteolin may indeed be methylated with a preference in 4'-O-methylation over 3'-O-methylation but the subsequent metabolic pathways were preferential to metabolize the 4'-O-methyated luteolin, which led to the apparent preference in 3'-O-methylation over 4'-O-methylation \textit{in vivo} in rat.

COMT is widely distributed throughout the organs of the body in mammals. It is consistent with our preliminary study that methylation of luteolin was detected in all
the incubations with rat tissue homogenates investigated, including liver, kidney, small intestine, lung, erythrocyte, brain, spleen, heart. Then the first five tissues were selected for further study because of their important role in metabolism in vivo. The in vitro study revealed that liver showed the highest COMT activity to luteolin, followed by the kidney, small intestine, lung and erythrocyte. Therefore, the liver is considered to play the major role in methylation of luteolin in rats. However, contribution of other tissues should not be neglected due to the wide distribution of COMT in non-liver tissues. For example, erythrocyte displays relatively low COMT activity, but it composes the majority of the blood and drugs expose to the erythrocyte for a relative long time, which indicates a potential contribution to pharmacokinetics of catechol compounds. Recently, the platelet was reported to have COMT activity (Wright et al., 2010), which might mediate the methylation of luteolin in vivo. Though the small intestine may hardly play a role in methylation of luteolin when we intravenously administrated luteolin to rats, but COMT will methylate oral drugs once absorbed into the enterocytes. Almost all of the tissues are able to methylate luteolin in vivo, therefore the methylated metabolites detected in plasma or urine may result from not only liver but also other non-liver tissues.

Because the catechol structure is always associated with many biological activity, such as anti-oxidant activity, free radical scavenger (Heim et al., 2002), it is essential to study whether the pharmacodynamics of luteolin will be influenced by COMT. Interestingly, it is reported that some catechol containing flavonoids, such as luteolin and querctin, displayed mutagenic activity in vitro (Rueff et al., 1995), but in fact they
did not play this role in vivo because of widely methylation in the body (Zhu et al., 1994). On the other hand, metabolites (chrysoeriol and diosmetin) of luteolin not only possessed many biological activity as well as luteolin, but also showed their specific activities (Hsu and Kuo, 2008; Cha et al., 2009; Liu et al., 2009). So it will become more complex to study the pharmacodynamics of luteolin in vivo when methylation occurs, and we should distinguish the effects from luteolin and metabolites in the mechanism studies.

It is a typical therapy to utilize COMT inhibitor to prolong elimination half-life of L-dopa in Parkinson’s disease (Piccini et al., 2000), and nitrocatechol COMT inhibitors, entacapone and tolcapone, are widely used in currently clinical therapy. And a further point of concern is to discover completely new COMT inhibitors without a nitrocatechol structure possibly associated with some side effects (Mannisto and Kaakkola, 1999). Recently, some flavonoids, such as quercetin and tea catechins, were reported to efficiently decrease COMT activity, which might be potential COMT inhibitors (Lu et al., 2003; Singh et al., 2003). In our current study, luteolin also revealed a relatively high affinity to COMT, thus it is essential to verify the inhibition role of luteolin on COMT in the further study.

In conclusion, the present study mainly identified two methylated metabolites of luteolin both in vitro and in vivo and characterized the enzymology of methylaion of luteolin in rat tissue homogenates. Region-selectivity of methylation of luteolin revealed the reaction mechanism of COMT and different region-selectivities of O-methylation of luteolin in vivo versus in vitro conditions could be explained by
further metabolisms. Moreover, contribution of methylation on disposition of luteolin in rat was revealed, and COMT was another crucial phase II enzyme for luteolin, which indicates a potential COMT-based luteolin-drug interaction. Since the methylated metabolites (chrysoeriol and diosmetin) of luteolin and have their own pharmacological effect, it is essential to take these metabolites into consideration when we study the pharmacological mechanism of luteolin in vivo.
Authorship Contributions

Participated in research design: Zhongjian Chen, Huidi Jiang and Su Zeng.

Conducted experiments: Zhongjian Chen and Meng Chen.

Contributed new reagents or analytic tools: Zhongjian Chen Hao Pan, Siyuan Sun, and Liping Li.

Performed data analysis: Zhongjian Chen, Liping Li, Hao Pan, Siyuan Sun, Su Zeng, and Huidi Jiang.

Wrote or contributed to the writing of the manuscript: Zhongjian Chen, Liping Li, Meng Chen and Huidi Jiang.
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Footnotes

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Legends for figures

**Fig. 1.** Proposed COMT-mediated \(O\)-methylation of luteolin. Metabolites: chrysoeriol (3’-\(O\)-methylation) and diosmetin (4’-\(O\)-methylation).

**Fig. 2.** Identification of two methylated metabolites of luteolin in hydrolyzed urine after intravenous administration of 6.5 mg/kg luteolin to rats. (A) HPLC chromatograms of blank rat urine (A1), 0-4h urine (A2) after intravenous administration of 6.5 mg/kg luteolin to rat, standard compounds, chrysoeriol (A3) and diosmetin (A4); (B) UV-spectra of M1, M2, chrysoeriol and diosmetin; (C) MS/MS spectra of M1, M2, chrysoeriol and diosmetin.

**Fig. 3.** Luteolin metabolism and metabolites formation by incubation of rat tissue homogenates (liver, kidney, small intestine (jejunum), lung and erythrocytes) with 20 \(\mu\)M luteolin in the absence (dashed line) or in the presence (solid line) of 30 nM entacapone. Incubation for 0, 20, 40 and 60 min at 37 °C. Protein concentration of tissue homogenates were 1 mg/ml. Values are expressed as means ± S.D. (\(n=3\)); Comparison between control and experiment groups with Student’s \(t\)-test (unpaired, two-tail), *: \(P<0.05\), **: \(P<0.01\).

**Fig. 4.** Apparent formation rates of M1 (3’-\(O\)-methylation) and M2 (4’-\(O\)-methylation)
in rat tissue homogenates (liver, kidney, lung, small intestine (jejunum), erythrocyte). Formation rates of M1 and M2 were determined with a series of concentrations of luteolin (1, 2.5, 5, 10, 20, 40, 80 μM for liver, kidney, lung and erythrocytes, while 0.5, 1, 2.5, 5, 10, 20, 40, 80 μM for small intestine. Protein concentrations (0.25 mg/ml for liver, 0.5 mg/ml for kidney and 1.0 mg/ml for small intestine, lung and erythrocyte homogenates) and incubation time (10 min) were used. The formation rate is the average of three determinations and the error bar represents the S.D. of the mean. The points were observed formation rates of M1 and M2, and the curves were estimated base on nonlinear regression by Prism 5 software with Michaelis-Menten equation.

**Fig. 5.** The plasma concentration-time curves (A) and urinary excretion-time profiles (B) of luteolin and metabolites after intravenous administration of 6.5 mg/kg luteolin to vehicle-treated (dashed line, control group) or entacapone-treated (solid line, experimental group) rats. Data are expressed as mean ± S.D. (n=5), and data are compared between control and experiment groups with Student’s t-test (unpaired, two-tail), *, P<0.05, **, P<0.01.

**Fig. 6.** Phase I metabolism of M1 and M2 in rat microsomes. Remaining of M1 and M2 after incubation for 10 min and 30 min (A); luteolin demethylated from M1 and M2 after incubation (B). Data are expressed as mean ± S.D. (n=3). Comparison between two groups with Student’s t-test, *, P<0.05; **, P<0.01. A: the remaining of
M1 and M2 are compared at 10 min and 30 min, respectively. B: luteolin from demethylation of M1 and M2 are compared at 10 min and 30 min, respectively.
Table 1  Apparent kinetics parameters for M1 and M2 by rat tissue homogenates with luteolin. Data are expressed as mean ±S.D., n=3.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (nmol mg protein$^{-1}$ min$^{-1}$)</th>
<th>$V_{max}/K_m$ (mL mg protein$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M1</td>
<td>M2</td>
<td>M1</td>
</tr>
<tr>
<td>liver</td>
<td>3.54±0.34</td>
<td>3.38±0.51</td>
<td>271±6</td>
</tr>
<tr>
<td>kidney</td>
<td>3.45±0.37</td>
<td>3.57±0.44</td>
<td>144±4</td>
</tr>
<tr>
<td>lung</td>
<td>2.39±0.24</td>
<td>4.10±0.28</td>
<td>36.7±0.8</td>
</tr>
<tr>
<td>small intestine (jejenum)</td>
<td>1.06±0.09</td>
<td>1.31±0.11</td>
<td>24.7±0.4</td>
</tr>
<tr>
<td>erythrocyte</td>
<td>2.92±0.44</td>
<td>2.38±0.17</td>
<td>12.3±0.4</td>
</tr>
</tbody>
</table>
Table 2 Pharmacokinetics parameters of luteolin after intravenous administration of 6.5 mg/kg luteolin to vehicle-treated or entacapone-treated rats. Data are expressed as mean ±S.D., n=5.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>IV vehicle+luteolin</th>
<th>IV entacapone+luteolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC0-t (μg/mL*h)</td>
<td>7.60±1.10</td>
<td>11.4±2.3**</td>
</tr>
<tr>
<td>AUC0-∞ (μg/mL*h)</td>
<td>8.18±0.87</td>
<td>12.5±2.5**</td>
</tr>
<tr>
<td>MRT0-t (h)</td>
<td>1.41±0.17</td>
<td>1.65±0.09*</td>
</tr>
<tr>
<td>MRT0-∞ (h)</td>
<td>2.32±0.43</td>
<td>2.68±0.31</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>3.20±0.70</td>
<td>3.31±0.55</td>
</tr>
<tr>
<td>CL(L/h/kg)</td>
<td>0.80±0.076</td>
<td>0.537±0.097**</td>
</tr>
<tr>
<td>V (L/kg)</td>
<td>3.74±1.10</td>
<td>2.57±0.63</td>
</tr>
</tbody>
</table>

Comparison between two groups with Student’s t-test, *, P<0.05; **, P<0.01.

Vehicle: 20% hydroxypropyl-β-cyclodextrin (w/v) containing 5% glucose (w/v). Both entacapone and luteolin for i.v. administration were dissolved in this vehicle.
Table 3 Pharmacokinetic parameters of M1 and M2 following intravenous of 6.5 mg/kg luteolin to rats. Data are expressed as mean ±S.D., n=5.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$AUC_{0-t}$ ($\mu$g/mL*h)</td>
<td>2.09±0.33**</td>
<td>0.427±0.054</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ ($\mu$g/mL*h)</td>
<td>2.28±0.49**</td>
<td>0.466±0.086</td>
</tr>
<tr>
<td>$MRT_{0-t}$ (h)</td>
<td>1.24±0.14**</td>
<td>0.597±0.035</td>
</tr>
<tr>
<td>$MRT_{0-\infty}$ (h)</td>
<td>1.76±0.52**</td>
<td>0.767±0.140</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>1.57±0.75*</td>
<td>0.514±0.140</td>
</tr>
<tr>
<td>CL (L/h/kg)</td>
<td>2.94±0.54**</td>
<td>14.3±2.5</td>
</tr>
<tr>
<td>V (L/kg)</td>
<td>6.34±2.50*</td>
<td>10.3±1.6</td>
</tr>
<tr>
<td>$C_{max}$ ($\mu$g/mL)</td>
<td>2.06±0.25**</td>
<td>0.560±0.070</td>
</tr>
</tbody>
</table>

Comparison between two groups with Student’s t-test, *, $P<0.05$; **, $P<0.01$. 
Figure 1

Luteolin

M1

M2
Figure 2

A

1

2

3

4

luteolin

M1

M2

chrysoeriol

diosmetin

B

mAU

M1

M2

chrysoeriol

diosmetin

C

107 132 151

[M-H-CH₃]⁻ 255 284

107 132 151

[M-H-CH₃]⁻ 255 284

107 132 151

[M-II-CH₃]⁻ 255 284

107 132 151

[M-H-CH₃]⁻ 255 284
Figure 3

- Liver
- Kidney
- Small intestine

Concentration (µM) vs. Time (min)

Symbols:
- Luteolin
- Luteolin (with entacapone)
- M1
- M1 (with entacapone)
- M2
- M2 (with entacapone)
Figure 4

Graphs show the relationship between concentration and enzyme activity for different tissues:
- Liver
- Kidney
- Small intestine
- Lung
- Erythrocyte

The graphs depict enzyme activity (v) in nmol mg protein⁻¹ min⁻¹ as a function of concentration (µM). The graphs are labeled with M1 and M2 markers.
Figure 5

--- Control group (i.v. luteolin)  Experimental group (i.v. entacapone+luteolin)

A

Luteolin concentration in plasma (μg/mL)

M1 concentration in plasma (μg/mL)

M2 concentration in plasma (μg/mL)

time (h)

B

Cumulative urinary excretion of dose (%)

Cumulative urinary excretion of dose (%)

Cumulative urinary excretion of dose (%)

time (h)
Figure 6

A

Remaining of substrate (%)

Incubation time (min)

10  30  10  30

M1  M2

B

Luteolin from demethylation (% of substrate)

Incubation time (min)

10  30  10  30

luteolin from M1  luteolin from M2

**