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Inhibition of human liver catechol-*O*-methyltransferase by tea catechins and their metabolites: Structure–activity relationship and molecular-modeling studies

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Abstract

(–)-Epigallocatechin-3-gallate (EGCG) is the major polyphenol present in green tea. We previously demonstrated that EGCG was both a substrate and potent inhibitor of human liver cytosolic catechol-*O*-methyltransferease (COMT). We now report the structure–activity relationship for the inhibition of COMT-catalyzed *O*-methylation of catecholestrogens in human liver cytosol by tea catechins and some of their metabolites. The most potent inhibitors were catechins with a galloyl-type D-ring, including EGCG (IC_{50} = 0.07 μM), 4″-*O*-methyl-EGCG (IC_{50} = 0.10 μM), 4′,4″-di-*O*-methyl-EGCG (4′,4″-DiMeEGCG) (IC_{50} = 0.15 μM), and (–)-epicatechin-3-gallate (ECG) (IC_{50} = 0.20 μM). Catechins without the D-ring showed two to three orders of magnitude less inhibitory potency. Enzyme kinetic analyses revealed that EGCG behaved as a mixed inhibitor, whereas 4′,4″-di-*O*-methyl-EGCG exhibited competitive kinetics for the *S*-adenosylmethionine (SAM), and noncompetitive kinetics for the catechol binding site. These compounds may represent a new type of COMT inhibitor. In silico molecular-modeling studies using a homology model of human COMT were conducted to aid in the understanding the catalytic and inhibitory mechanisms. Either D-ring or B-ring of EGCG could be accommodated to the substrate binding pocket of human COMT. However, the close proximity (2.6 Å) of 4″-OH to the critical residue Lys144, the higher acidity of the hydroxyl groups of the D-ring, and the hydrophobic interactions between the D-ring and residues in the binding pocket greatly facilitated the interaction of the D-ring with the enzyme, and resulted in increased inhibitory potency. These results provide mechanistic insight into the inhibition of COMT by commonly consumed tea catechins.

Keywords: Catechol-*O*-methyltransferase; Tea catechins; Epigallocatechin-3-gallate; Catecholestrogens; Methylation; Molecular modeling

1. Introduction

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) catalyzes the transfer of methyl groups from *S*-adenosylmethionine (SAM) to one of the hydroxyl groups of catechol or substituted catechols in the presence of Mg^{2+}, and produces *O*-methylated catechol and *S*-adenosyl-L-homocysteine (SAH) [1]. In mammals, COMT is widely distributed in brain and peripheral tissues. The highest COMT activity in both rats and humans is found in the liver, kidney, and gastrointestinal tract [2]. COMT exists in a soluble form in most tissues, but a membrane-bound form is predominant in the human brain [3]. The primary function of COMT is to deactivate biologically-active endogenous and exogenous catechols. Important endogenous substrates of COMT include catecholestrogens, catecholamine neurotransmitters (dopamine, norepinephrine, and epinephrine), and 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) [4,5]. Certain dietary and medicinal products are also COMT substrates, e.g., flavonoids, carbidopa, and dihydroxyphenyl serine [2,6,7]. In current therapies for Parkinson's disease, selective COMT inhibitors, mainly synthetic nitrocatechol com-

Abbreviations: EGCG, (–)-epigallocatechin-3-gallate; ECG, (–)-epicatechin-3-gallate; EGC, (–)-epigallocatechin; EC, (–)-epicatechin; EGCG-7-Gluc, (–)-EGCG-7-*O*-glucuronide (and similar abbreviations for other glucuronidates); 4′-MeEGC, 4′-*O*-methyl-EGC; 4″-MeEGCG, 4″-*O*-methyl-EGCG; 4′,4″-DiMeEGCG, 4',4″-di-*O*-methyl-EGCG; 2-OH-E_2, 2-hydroxyestradiol; 4-OH-E_2, 4-hydroxyestradiol; COMT, catechol-*O*-methyltransferase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosyl-L-homocysteine; L-DOPA, 3,4-dihydroxy-L-phenylalanine; HPLC–ECD, high-performance liquid chromatography–electrochemical detector

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pounds, are used in combination with L-DOPA and dopa decarboxylase inhibitors [8,9]. By reducing COMT activity, these inhibitors increase the bioavailability and clinical efficacy of L-DOPA.

The crystal structure of rat S-COMT, bound to SAM, Mg^{2+} and an inhibitor (3,5-dinitrocatechol), shows that the catalytic site is situated on the surface of the enzyme and consists of two pockets to accommodate the substrate and SAM [10]. The substrate pocket contains an Mg^{2+} ion, which is essential for the catalytic activity. The binding pocket for SAM is embedded somewhat deeper in the enzyme. Kinetic analysis with recombinant COMT suggested that SAM binds first to the enzyme, then Mg^{2+}, and finally the substrate [11]. Rat and human S-COMT share an 81% similarity in amino acid sequences, and the active site is highly conserved [12].

Tea (*Camellia sinensis*) is a popular beverage worldwide, and its consumption has been proposed to have many beneficial health effects [13,14]. Many of these biological activities have been attributed to tea catechins of which (–)-epigallocatechin-3-gallate (EGCG), (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin (EGC), and (–)-epicatechin (EC) are the most abundant (Fig. 1). In our previous studies, we have shown that EGCG, the major tea catechin, was methylated by COMT to form 4′,4″-di-*O*-methyl-EGCG (4′,4″-DiMeEGCG), both in vitro and in vivo [15,16]. Furthermore, EGCG and its metabolites were potent inhibitors of COMT-mediated *O*-methylation of EGC and L-DOPA [16]. The inhibitory effect of tea catechins on the *O*-methylation of endogenous catecholestrogens has also been proposed [17–20] and recently observed in vitro [20].

In the present study, we characterized the inhibition of *O*-methylation of catecholestrogens by tea catechins and some of their metabolites in detail. The structure–activity relationship of different catechins and their methylated and glucuronidated metabolites in inhibiting COMT was determined as well. Molecular-modeling experiments were performed in an effort to provide mechanistic insight of the inhibitory action of catechins and their metabolites on COMT.

2. Materials and methods

2.1. Chemicals and reagents

EGCG was a gift from Dr. Yukihiko Hara (Tokyo Food Techno Co. Ltd.). EGC and ECG were purified in Food Science Department of Rutgers University. 4′-*O*-methyl-EGC (4′-MeEGC); 4″-*O*-methyl-EGCG (4″-MeEGCG); 4′,4″-di-*O*-methyl-EGCG (4′,4″-DiMeEGCG); EGCG-7-*O*-glucuronide (EGCG-7-Gluc); EGCG-4″-*O*-glucuronide (EGCG-4″-Gluc); EGCG-3″-*O*-glucuronide (EGCG-3″-Gluc); EGCG-3′-*O*-glucuronide (EGCG-3′-Gluc) were synthesized and purified in our laboratory [15,16]. EC, dithiothretiol, and other chemicals were purchased from Sigma Co. 2-Hydroxyestradiol (2-OH-E_2), 4-hydroxyestradiol (4-OH-E_2), and their methylated products were purchased from Steraloids Inc. Pooled human liver cytosol samples were obtained from BD Biosciences in frozen state.

2.2. Inhibition of human liver COMT-catalyzed O-methylation of 2-OH-E_2 and 4-OH-E_2

The *O*-methylation of 2- and 4-OH-E_2 was carried out as described previously with minor modifications [16]. To determine the IC_{50} of COMT inhibition, the reactions were

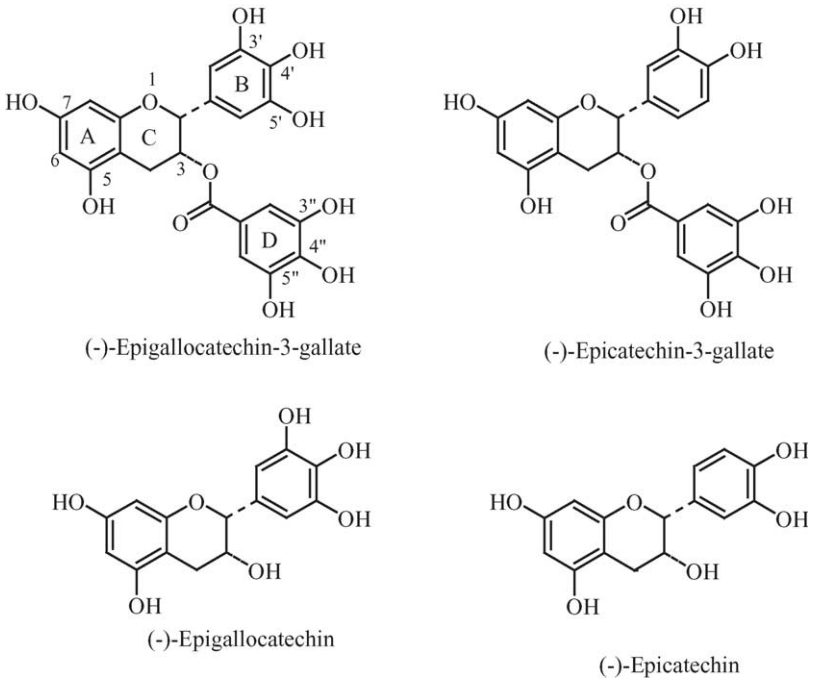

Fig. 1. Chemical structures of the tea catechins.

performed in the mixture containing 0.2 mg human liver cytosol, 50 μM 2- or 4-OH-E_2, 60 μM SAM, 1.0 mM dithiothreitol, 1.2 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4), and varying concentrations of test compounds (0–100 μM). For the determination of inhibition kinetics, either the concentration of the substrate catecholestrogen was varied between 5 and 200 μM at saturating SAM concentration (200 μM), or the concentration of SAM was varied between 2 and 200 μM at saturating catecholestrogen (200 μM) in the presence of different concentrations of EGCG (0, 0.1, 0.25, and 0.5 μM) or 4′,4″-DiMeEGCG (0, 0.1, 0.2 μM) as the inhibitor. After preincubation at 37 °C for 3 min, SAM was added to start the reaction, and the incubation was carried out at 37 °C for 30 min. The final volume of the incubation mixture was 100 μL. The incubation was stopped by the addition of 100 μL of ice-cold methanol containing 1% ascorbic acid. After centrifugation at 10,000 × *g* for 10 min, 50 μL of the supernatant was analyzed by high-performance liquid chromatography coupled with electrochemical detector (HPLC–ECD).

2.3. HPLC–ECD analysis of methylated metabolites of 2- and 4-OH-E_2

The samples were analyzed using an HPLC system consisting of a Waters model 510 solvent delivery system (Waters Corp.). A Supelcosil C-18 reversed-phase column (15 cm × 4.6 mm, particle size 5 μm; Supelco Inc.) was used with an isocratic mobile phase consisting of 33% acetonitrile, 6% THF, and phosphate buffer (15 mM) at pH 3.4. The flow rate was 1.0 mL/min. The eluant was monitored by a model 5200 ESA Coulochem detector (ESA Inc.) with potential settings of 100 and 300 mV. Data were processed using Millenium^{32} software (Waters Corp.).

2.4. Data analysis

The kinetic analysis were performed by fitting the initial velocity as a function of concentration to the following equations using the non-linear regression analysis program in GraFit 5.0 software (GraFit, Erithacus):

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad \text{competitive}$$

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + \left(1 + \frac{[I]}{K_i'}\right)[S]} \quad \text{mixed type}$$

$$v = \frac{V_{max}[S]\frac{1}{1+[I]/K_i'}}{K_m + [S]} \quad \text{non-competitive}$$

V_{max} is the maximal velocity; K_i is the inhibition constant, indicating the dissociation of the enzyme-inhibitor complex (EI); and K_i' is the inhibition constant when the inhibitor binds to an enzyme-substrate complex, indicating the dissociation constant of the enzyme-substrate-inhibitor complex (ESI); in a pure noncompetitive inhibition, $K_i = K_i'$ [21]. The fitting is global, simultaneously all substrate and inhibitor concentrations. The goodness of fit was inspected visually and then evaluated statistically by the *F*-test (GraFit, Erithacus). The mean inhibitory concentration (IC_{50}) values were the mean ± S.E. of three independent experiments with duplicate determinations for each assay. Statistical differences in IC_{50} values were determined using a two-tailed Student's *t*-test. Data from kinetic studies were mean ± S.D. from three independent experiments with single determination for each assay. Statistical differences in K_m and V_{max} values were determined using one-way ANOVA and Tukey's multiple comparison test. Significance was achieved with $p < 0.05$.

2.5. In silico molecular-modeling studies of COMT

The protein sequence of human catechol *O*-methyltransferase (accession no. BC000419) was retrieved from the National Center for Biotechnology Information Reference Sequence (RefSeq) Collection. A structural model of human COMT was constructed using the Insight II Homology Module (Accelrys Inc.) from the published crystal structure of rat COMT (RCSB Protein Data Bank: http://www.rcsb.org/pdb; PDB ID = 1VID) as the modeling template. The quality of the model was confirmed by the WHATIF-Check program (http://www.cmbi.kun.nl/gv/servers/WIWWWI). The coordination of Mg^{2+} and the surrounding residues were deduced directly from the rat crystal structure and superimposed onto the homology model. Since the catechol binding sites between the rat and human COMT are highly conserved, the initial geometries and coordinates (distances and charges) from the rat crystal structure were taken as the criteria for the EGCG and its derivatives in binding to the human COMT model. To calculate the effective charge of the Mg ion due to electron delocalization via interactions with surrounding residues, we applied the AM1 quantum mechanical procedure accessed through Spartan 02 (Wavefunction Inc.). Initial structures of EGCG and its analogues, quercetin, and isoflavones were built using the Sketch Molecule module in Sybyl (Tripos Inc.) and subsequently energy minimized to yield a stable conformation. Each ligand was docked in the ligand-enzyme binding domain using FlexX, a fast flexible docking method using an incremental construction algorithm that allows maximal rotation and conformation of ligands in the binding pocket [22]. Based on the idea that Lys144 acts as the base to abstract the proton from the enzyme-bound catechol, the distance between the Lys144 residue and hydroxyl groups was measured to predict the rate of deprotonation [23]. The pK_a values of EGCG, ECG, EGC and EC were obtained from the literature [24]. Following these docking studies to elucidate stable ligand-binding modes, values of the ligand-enzyme

binding energy ($\Delta E_{binding}$) were calculated for each of the 10 ligands using molecular mechanics procedures. Residues within 7.4 Å of the Mg^{2+} were defined as the ''active site'' pocket and were permitted full relaxation, while holding all of the other residues fixed. We defined $\Delta E_{binding}$ as the difference between the potential energy of the ligand-enzyme ($E_{complex}$) and the sum of potential energies of the ligand (E_{ligand}) and enzyme (E_{enzyme}).

$$\Delta E_{binding} = E_{complex} - (E_{ligand} + E_{enzyme})$$

A favorable (more negative) binding energy was taken as evidence that the ligand possesses high affinity for COMT.

3. Results

3.1. Inhibition of O-methylation of catecholestrogens by catechins and their metabolites

To elucidate the structure–activity relationship of the inhibition of COMT by tea polyphenols, we compared the inhibitory activities of EGCG, ECG, EGC, and EC along with some of their methylated and glucuronidated metabolites. All catechins tested inhibited human liver COMT-catalyzed *O*-methylation of 2-OH-E_2 and 4-OH-E_2 activities in a concentration-dependent manner. EGCG was the most potent inhibitor. The dose-dependent inhibitory effects of EGCG and ECG were shown in Fig. 2, and IC_{50} values of all test compounds were listed in Table 1. The IC_{50} values for inhibition of 2-OH-E_2 methylation by EGCG and ECG were 0.07 and 0.20 μM, respectively. In contrast, EGC and EC, which only differ structurally from EGCG and ECG by lacking the galloyl group at C-3, were two to three orders of magnitude less potent, with the IC_{50} values of 44 and 60 μM, respectively. 4″-MeEGCG (IC_{50} = 0.1 μM) and 4′,4″-DiMeEGCG (IC_{50} = 0.15 μM) was slightly less active than EGCG. Glucuronidation of EGCG at the 7-position decreased the inhibitory activity of EGCG by 10-fold (IC_{50} = 0.6 μM), whereas glucuronidation at the 3′-, 3″-, and 4″-position decreased the inhibitory activity by 20- to 30-fold (IC_{50}: 1.8–2.5 μM). For the substrate 4-OH-E_2, there was no significant difference observed in the IC_{50} values of all test compounds compared to those when 2-OH-E_2 was used as the substrate.

Table 1
Median inhibitory concentration (IC_{50}) for the methylation of catecholestrogens by catechins and their metabolites

Inhibitor	2-OH-E_2 as substrate	4-OH-E_2 as substrate
EGCG	0.07 ± 0.0084	0.08 ± 0.0090
4″-MeEGCG	0.10 ± 0.010*	0.10 ± 0.014*
4′,4″-DiMeEGCG	0.15 ± 0.0075*	0.15 ± 0.010*
EGCG-7-Gluc	0.60 ± 0.064*	0.80 ± 0.12*
EGCG-3′-Gluc	1.80 ± 0.42*	2.3 ± 0.33*
EGCG-3″-Gluc	2.0 ± 0.26*	2.5 ± 0.20*
EGCG-4″-Gluc	2.5 ± 0.23*	4.0 ± 0.33*
ECG	0.20 ± 0.020*	0.30 ± 0.020*
EGC	44 ± 6.2*	50 ± 5.5*
4′-MeEGC	32 ± 4.0*	40 ± 6.3*
EC	60 ± 7.3*	80 ± 6.0*
Quercetin	8.5 ± 1.2*	8.5 ± 1.5*
Genistein	NE	NE
Daidzein	NE	NE
Biochanin A	NE	NE

Methylation reaction was conducted using human liver cytosol and 2-OH-E_2 and 4-OH-E_2 as substrates under the conditions described in the Section 2. IC_{50} values (μM) were statistically significant from those of EGCG when $^*p < 0.05$ (two-tailed Students' *t*-test); NE, no effect. All the values were the mean ± S.E. of three duplicated determinations.

To characterize the inhibition of human liver COMT by EGCG, inhibition kinetic studies were performed using increasing concentrations of 2-OH-E_2 and 4-OH-E_2 as the

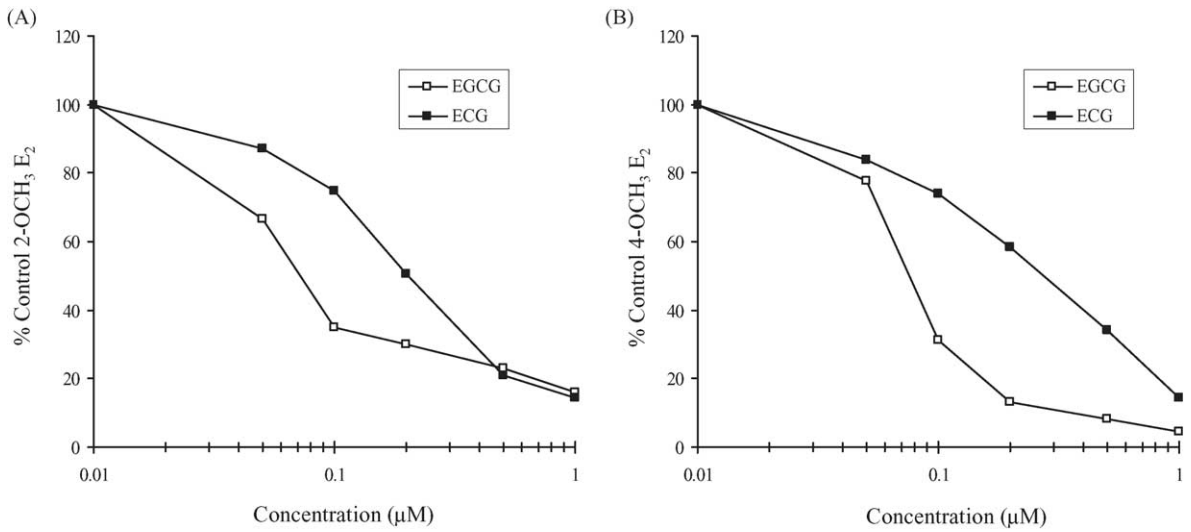

Fig. 2. Inhibition of human liver COMT catalyzed *O*-methylation of 2-OH-E_2 (A) and 4-OH-E_2 (B) by EGCG and ECG. The reaction mixture contained 0.2 mg human liver cytosol, 60 μM SAM, 50 μM of 2- or 4-OH-E_2, 0–1 μM EGCG or ECG, 1.0 mM dithiothreitol, 1.2 mM $MgCl_2$, and 10 mM Tris–HCl (pH 7.4) in a total volume of 100 μL, and was incubated at 37 °C for 30 min. All the values were the mean of three independent experiments each performed in duplicate. The variations among the replicates were within 10%.

Table 2
Kinetic parameters: inhibition of human liver COMT catalyzed *O*-methylation of 2-OH-E_2 and 4-OH-E_2 by EGCG

Substrate	Inhibitor EGCG (μM)	K_m (μM)	V_{max} (pmol/mg/min)	K_i (μM)	K_i' (μM)
2-OH-E_2 (5–200 μM)	–	13.7 ± 0.22	397.6 ± 15.2	0.32 ± 0.04	0.22 ± 0.01
	0.1	16.3 ± 0.60[a]	221.1 ± 13.7[a]		
	0.25	21.7 ± 0.57[b]	140.7 ± 22.1[b]		
	0.5	33.9 ± 2.38[c]	132.1 ± 16.3[b]		
4-OH-E_2 (5–200 μM)	–	15.4 ± 0.61	294.4 ± 14.5	0.37 ± 0.02	0.23 ± 0.02
	0.1	19.6 ± 0.34[a]	165.4 ± 15.7[a]		
	0.25	25.1 ± 0.65[b]	106.6 ± 11.2[b]		
	0.5	37.3 ± 1.10[c]	101.3 ± 9.7[b]		

Kinetic studies were conducted at 37 °C for 30 min using 2 mg/mL of human liver cytosolic protein in the presence of 200 μM SAM. The kinetic values (K_m, V_{max}, K_i, and K_i') were calculated using the GraFit 5.0 software. K_i and K_i' represent the competitive and noncompetitive inhibition constant, respectively. For the K_m and V_{max} values, different letters represent a significant ($p < 0.05$) difference between two groups after one-way ANOVA and Tukey's multiple comparison test. Values represent best-fit values ± standard deviation from three independent determinations.

substrates. SAM was maintained at a saturating concentration (200 μM). In the absence of the inhibitor, the K_m values for 2-OH-E_2 and 4-OH-E_2 were 13.7 and 15.4 μM, respectively, and the corresponding V_{max} values were 397.6 and 294.4 pmol/mg/min, respectively. In the presence of 0.1, 0.25 and 0.5 μM EGCG, the V_{max} values for the *O*-methylation of 2-OH-E_2 and 4-OH-E_2 decreased in a concentration-dependent manner, whereas the corresponding K_m values increased, suggesting a mixed (competitive and noncompetitive) inhibition (Table 2 and Fig. 3). The values of the competitive inhibition constants K_i were of 0.32 and 0.37 μM for 2-OH-E_2 and 4-OH-E_2, respectively; and the values of the non-competitive constants K_i' were of 0.22 and 0.23 μM for 2-OH-E_2 and 4-OH-E_2, respectively.

4′,4″-DiMeEGCG displayed a noncompetitive-type inhibition of COMT activity with respect to 2-OH-E_2 (Table 3 and Fig. 4(A)) at a saturation concentration of SAM (200 μM); with the increase of 4′,4″-DiMeEGCG concentrations, the V_{max} values for 2-OH-E_2 methylation decreased, whereas the corresponding K_m values were unchanged. The K_i' value was approximately 0.27 μM. To determine the inhibition mechanism with respect to the SAM binding site, the concentration of SAM was varied with saturated 2-OH-E_2. The increased K_m values and unchanged V_{max} values indicated a competitive inhibitory pattern with respect to the SAM binding site (Table 3 and Fig. 4(B)). The K_i value averaged 0.11 μM.

In addition to the tea catechins and their metabolites, we also tested the COMT-inhibitory effect of quercetin and isoflavones (genistein, daidzein, and biochanin A). Quercetin, which only structurally differs from EC by having a double bond between C-2 and C-3 and a carbonyl group at C-4, was found to be a weaker inhibitor than EGCG, but a stronger inhibitor than EGC and EC (IC_{50} = 8.5 μM).

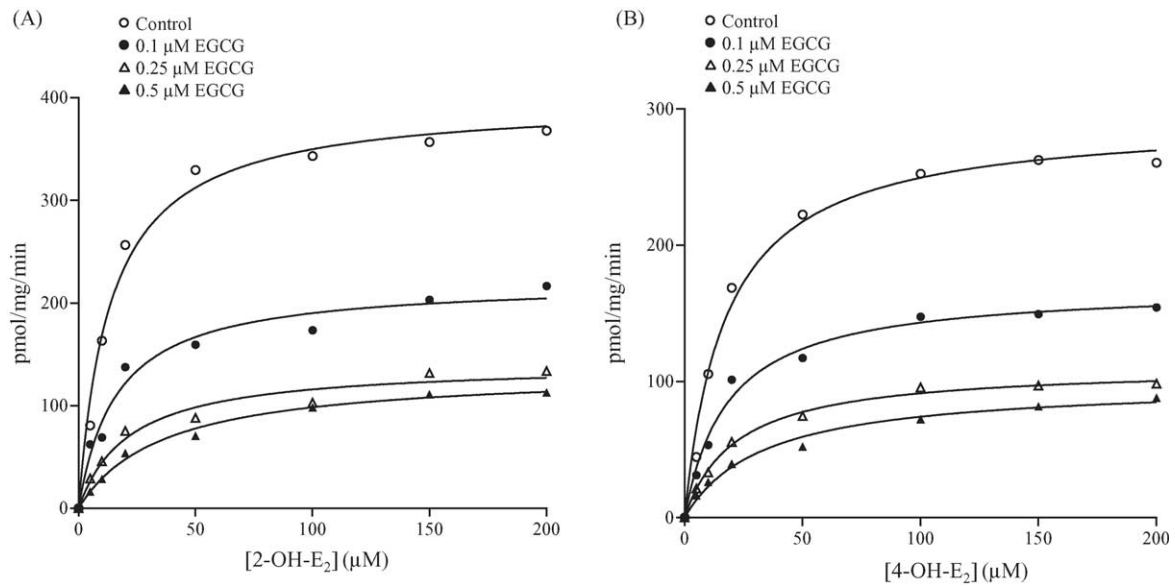

Fig. 3. Kinetic studies on the inhibition of human liver COMT by EGCG with respect to 2-OH-E_2 (A) and 4-OH-E_2 (B). The reaction mixture contained 0.2 mg human liver cytosol, 200 μM SAM, 5–200 μM of 2- or 4-OH-E_2, 0, 0.1, 0.25 and 0.5 μM of EGCG, 1.0 mM dithiothreitol, 1.2 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4) in a total volume of 100 μL, and was incubated at 37 °C for 30 min. All the values were the means of three independent experiments with single determination for each assay. The variations among the replicates were within 12%. The *graphs* represent the best global fit of the data to the mixed inhibition mode after analyzing the data by using equations described in the Section 2.

Table 3
Kinetic parameters: inhibition of human liver COMT catalyzed *O*-methylation of 2-OH-E_2 by 4′,4″-DiMeEGCG

Substrate	Inhibitor 4′,4″-DiMeEGCG (μM)	K_m (μM)	V_{max} (pmol/mg/min)	K_i (μM)	K_i' (μM)
2-OH-E_2 (5–200 μM)	–	13.1 ± 0.08	404.6 ± 14.2		0.27 ± 0.03
	0.1	12.5 ± 0.15	281.5 ± 13.7[a]		
	0.2	10.2 ± 0.11	204.6 ± 12.6[b]		
SAM (2–200 μM)	–	17.2 ± 0.95	456.7 ± 20.1	0.11 ± 0.01	
	0.1	38.8 ± 1.90[a]	453.5 ± 10.2		
	0.2	64.9 ± 4.43[b]	450.7 ± 30.7		

Kinetic studies were conducted at 37 °C for 30 min using 2 mg/mL of human liver cytosolic protein in the presence of 5–200 μM 2-OH-E_2 and 200 μM SAM, or 2–200 μM SAM and 200 μM 2-OH-E_2. The kinetic values (K_m, V_{max}, K_i, and K_i') were calculated using the GraFit 5.0 software as described under Section 2. K_i and K_i' represent the competitive and noncompetitive inhibition constant, respectively. For the K_m and V_{max} values, different letters represent a significant ($p < 0.05$) difference between two groups after one-way ANOVA and Tukey's multiple comparison test. Values represent best-fit values ± standard deviation from three independent determinations.

The isoflavones, which lack a catechol group, showed no inhibitory activity against COMT.

3.2. Molecular-modeling and docking studies of the mode of binding of catechins and their metabolites with human COMT

To investigate the mechanism and structure–activity relationship of the inhibition by the test compounds, we constructed a three-dimensional homology model of human COMT based on the X-ray crystal structure of rat COMT (Fig. 5(A)). This model revealed that the catalytic pocket of COMT is closely conserved between the rat and human. The substrate pocket contains an Mg^{2+} ion, which forms a hexa-coordinated complex with residues of Asp 141, Asp 169, and Asn 170; one conserved water molecule; and the two vicinal hydroxyl groups of the catecholic substrate (Fig. 6). The coordination of Mg^{2+} orients one of the hydroxyls close to the amino group of Lys144. The other hydroxyl forms a hydrogen bond with the negatively charged carboxyl acid group of Glu199. The effective charge of the Mg ion yielded by the AM1 quantum mechanical procedure was +1.23e (where 'e' refers to the elementary electronic charge). Ligand docking studies using our homology model of human COMT (Fig. 5B) permitted us to measure the distances between the ligand's hydroxyl groups that are subject to deprotonation and the amino group of Lys144 (Fig. 6). FlexX docking studies indicated that EGCG can be accommodated inside the binding site with either the D-ring or B-ring occupying the catalytic pocket. Docking of EGCG showed that Lys144-NH_2 was much closer to the 4″-OH (2.6 Å) when the D-ring occupied the catalytic pocket than to the alternative 4′-OH (4.5 Å) when the B-ring occupied the catalytic pocket. Docking of 4″-MeEGCG placed the B-ring in the catalytic pocket whereby the O···N distance between 4′-OH and Lys144-NH_2 shortened (4.0 Å). The pK_a values of the D-ring of EGCG (pK_a = 7.55) and ECG

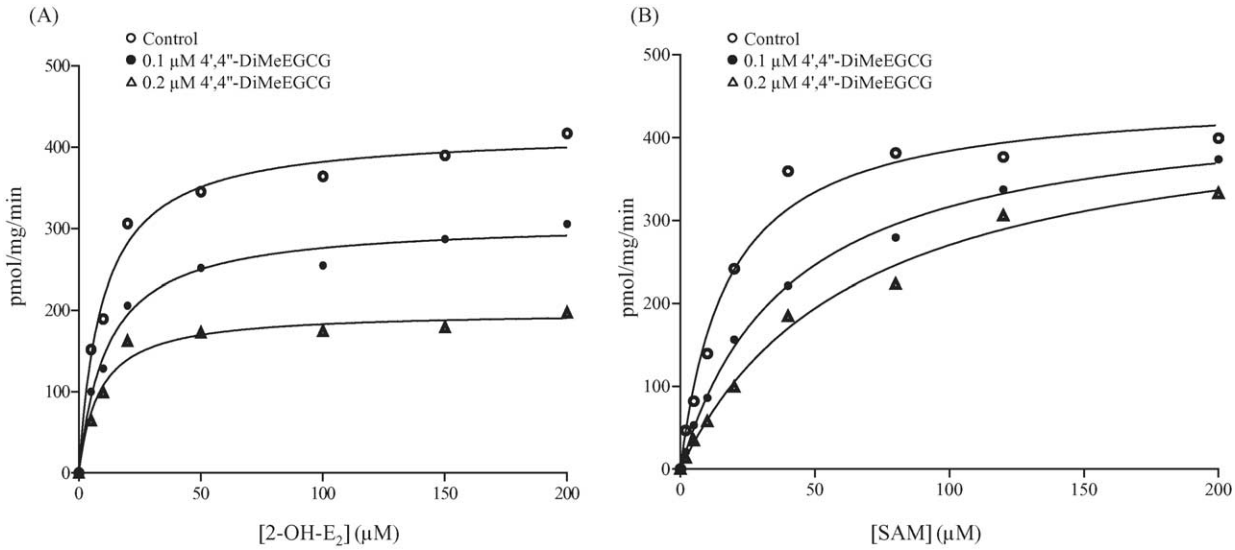

Fig. 4. Kinetic studies on the inhibition of human liver COMT by 4′,4″-DiMeEGCG with respect to 2-OH-E_2 (A) and SAM (B). The reaction mixture contained 0.2 mg human liver cytosol, 200 μM SAM and 5–200 μM 2-OH-E_2 (A) or 2–200 μM SAM and 200 μM 2-OH-E_2 (B), 0, 0.1, and 0.2 μM of 4′, 4″-DiMeEGCG, 1.0 mM dithiothreitol, 1.2 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4) in a total volume of 100 μL, and was incubated at 37 °C for 30 min. All the values were the means of three independent experiments with single determination for each assay. The variations among the replicates were within 12%. The *graphs* represent the best global fit of the data to the non-competitive inhibition mode (*A*) and competitive inhibition mode (*B*) after analyzing the data by using equations described in the Section 2.

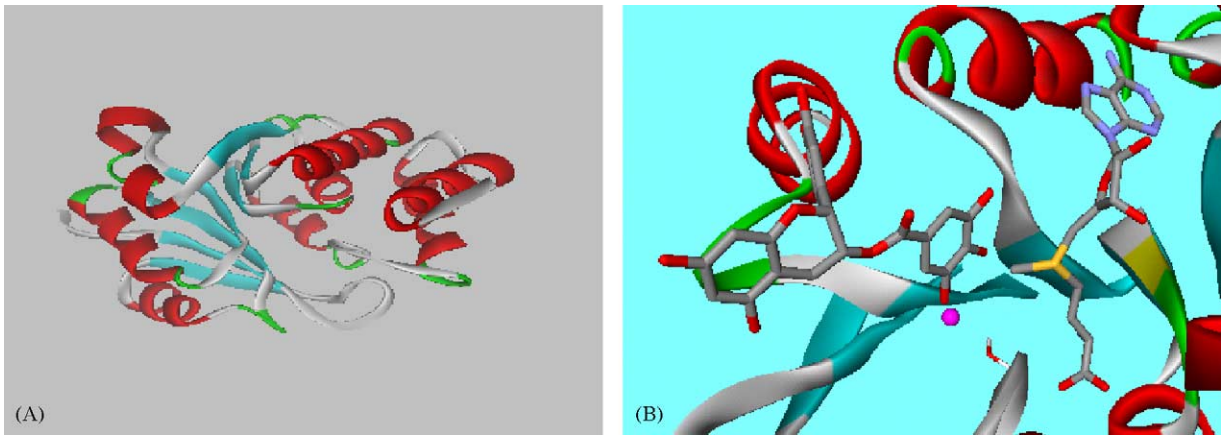

Fig. 5. Molecular modeling of the interaction between EGCG and human COMT. (A) Three-dimensional structural model of human COMT. (B) The close-up view of the consensus orientation for EGCG inside the catalytic site. The protein was depicted in ribbon representation and colored by secondary structures (i.e., helix, strand, and loop). Both EGCG and ligand contact residues were represented in stick form and colored by atom type with carbon in gray, oxygen in red, sulfur in yellow, and magnesium in magenta.

($pK_a = 7.6$) are significant lower than those of the B-ring of these two catechins ($pK_a > 8.7$) and those of EGC ($pK_a > 8.5$) and EC ($pK_a > 8.7$) [24], suggesting that the hydroxyl groups on the galloyl moiety lose protons more easily under the experimental condition (pH 7.4), and would require less assistance from Lys144. A consensus-binding mode was obtained from 30 independent docking procedures for EGCG, each of the 10 analogues, and quercetin bound to human COMT. Calculated values of $\Delta E_{binding}$ (shown in parentheses, in units of kcal/mol) for each ligand in its consensus pose yielded the following order of binding affinity to human COMT: EGCG (−48) = 4″-MeEGCG (−48) > ECG (−47) > 4′,4″-DiMeEGCG (−44) > 4′-MeEGC (−43) > EGC (−39) > EC (−31). These results were generally consistent with the experimental data with exception of 4′,4″-DiMeEGCG, which was further shown in the biological assay to be noncompetitive with respect to the catechol binding site. Values for EGCG and ECG represented the Boltzmann average of two possible poses, i.e., with either the D-ring or the B-ring occupying the catalytic pocket.

4. Discussion

The present studies elucidate a structure–activity relationship for the inhibition of *O*-methylation of catecholestrogens by tea catechins and some of their metabolites. The most potent inhibitors were found to be the catechins with a galloyl-type D-ring. EGCG showed the strongest inhibitory activity against COMT, with IC_{50} value in the nanomolar range. The importance of the D-ring in the COMT inhibition is clearly demonstrated by the significant loss of potency when this ring is absent or glucuronidated, which is represented by the 100–1000-fold difference in the potency between gallated (EGCG and ECG) and

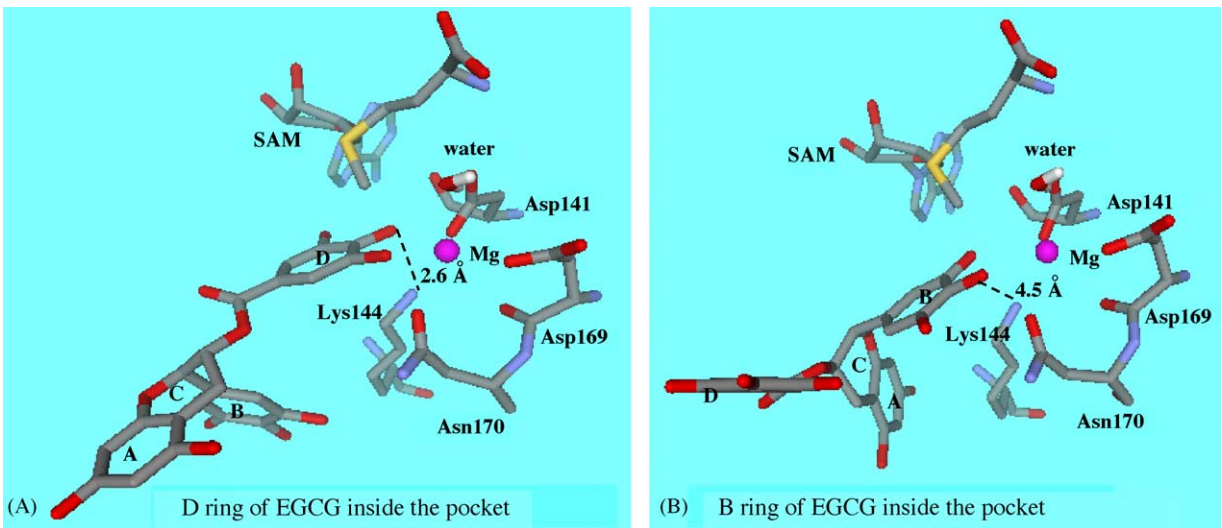

Fig. 6. FlexX docking studies of EGCG with the D-ring (A) or B-ring (B) inside the substrate binding pocket. The distances between the hydroxyl groups and Lys144 were measured and labeled in dashed lines. The D-ring or B-ring of EGCG formed a hexa-coordination complex with Mg^{2+} and surrounding residues.

non-gallated (EGC and EC) catechins, and 40–50-fold difference in the potency between EGCG and EGCG-4″-Gluc. The three-fold difference in the potency between EGCG and ECG suggests that B-ring also contributes to the COMT-inhibiting activity of EGCG.

Results from our molecular-modeling studies also suggest that the galloyl group plays a vital role in the enhanced inhibitory effects of EGCG and ECG on human COMT. Docking studies using FlexX software and a homology model of human COMT showed that either the D-ring or the B-ring of EGCG could be accommodated to the substrate binding pocket of COMT. The measurement of the distances between the reacting hydroxyl groups for methoxylation and Lys144-NH_2 was based on the idea that the enzyme-bound catechol interacts with Lys144 in the active site of COMT via a general acid–base reaction, in which Lys144 acts as the base to abstract the proton from the enzyme-bound catechol as the first step to the nucleophilic attack of the catecholate oxygen on the methyl carbon of SAM [23]. The function of Lys as the general base has also been suggested in other enzymatic systems [25]. The much closer distance of Lys144 to 4″-OH (2.6 Å) than 4′-OH (4.5 Å), and the higher acidity of the D-ring than that of the B-ring [24] would cause the D-ring to interact more strongly with the binding pocket of COMT and would thus result in increased inhibitory potency. This is also consistent with our previous observations that EGCG was readily methylated by human liver cytosolic COMT to 4″-MeEGCG and then to 4′,4″-DiMeEGCG [16]. The D-ring of EGCG would be preferably deprotonated by Lys144, yielding 4″-MeEGCG as the initial product. Methylation on 4″-OH helped to orient the B-ring closer (4.0 Å) to Lys144 than before (4.5 Å), thus 4″-MeEGCG could be further methylated to 4′,4″-DiMeEGCG.

The stability of the deprotonated inhibitor in the active site of the COMT is also important for the potency of the inhibitor. It has been shown that EGCG and its derivatives are stabilized by engaging in hydrophobic interactions with Trp38, Leu198, Pro174, and Trp143, located at the entrance of the binding pocket [26,27]. The present molecular modeling suggests that EGCG encounters these hydrophobic interactions after the D-ring occupies the pocket, and this in turn secures it in a tight binding complex with Mg^{2+}. In the case of 4″-MeEGCG, the D-ring can participate in π–π interactions with the side chain of the hydrophobic residue Trp38. In contrast, 4′-MeEGC without this stabilizing effect has less inhibitory potency.

For quercetin, the high degree of conjugation between the A and B rings stabilizes the hydroxyl group of the B-ring after deprotonation and thereby increases the potency in comparison to EGC and EC that lack this structural feature. However, due to the absence of the critical galloyl group, quercetin has much less inhibitory potency than EGCG.

The inhibition kinetics showed that EGCG inhibited the human liver cytosolic COMT-catalyzed *O*-methylation of catecholestrogens in a mixed (competitive and noncompetitive) manner. We believe that the competitive component of COMT inhibition relies on the binding affinity of EGCG to the active site of COMT [16–18], whereas the noncompetitive component of COMT inhibition may result from the feedback noncompetitive inhibition by the methylation product 4′,4″-DiMeEGCG. It is unexpected that 4′,4″-DiMeEGCG is a competitive inhibitor at the SAM-binding site, because 4′,4″-DiMeEGCG and SAM are structurally different. These compounds may represent a new type of COMT inhibitor. We are unable to model the docking of 4′,4″-DiMeEGCG to the SAM binding site due to the lack of the apo-form of COMT crystal structure (a crystal structure of COMT without any bound cofactor (SAM), Mg^{2+}, and substrate). Our calculations predict that 4′,4″-DiMeEGCG should bind to the catechol binding site with slightly low binding affinity than EGCG. This result is somewhat inconsistent with our experimental results which indicate a noncompetitive inhibition with respect to the catechol binding site. The reason for this discrepancy is not known; it may reflect unknown factors and assumptions implicit in our modeling approach.

Inhibition of *O*-methylation of endogenous catecholestrogens may lead to elevated tissue levels of the 2- and 4-OH-E_2 and decreased tissue levels of their methoxy products. It has been suggested that 4-OH-E_2 has carcinogenic activity, and 2-methoxyestradiol has anticarcinogenic activity [28,29]. Nevertheless, the clinical use of COMT inhibitors (entacapone and tolcapone) in the treatment of Parkison's disease has not been associated with changes in cancer risk [8]. COMT-knockout mice have been reported to be fertile and healthy [30]. EGCG and its methylated metabolites have potent inhibitory activities against the methylation of L-DOPA in vitro [16], and also likely against the methylation of other catecholamines. The role of COMT deficiency in human hormone-related and neurodegenerative diseases is not clearly known. The effects of tea consumption on the metabolism of catecholestrogens and catecholamines in vivo, as well as their biological consequences need further investigation.

In summary, we have demonstrated the efficacy of a series of galloyl-containing catechins and their metabolites as inhibitors of COMT. Using a combined classical enzymology and molecular-modeling approach, we have elucidated the importance of the galloyl-type D-ring of EGCG in the inhibition. The distance between Lys144 and the reacting catecholic hydroxyl group, the pH of the catechol moiety, and the stability of the deprotonated inhibitor are the key factors in the inhibition of COMT. Future studies in vivo are needed to better characterize the effects of the compounds presently studied.

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References

[1] Axelrod J, Tomchick R. Enzymatic *O*-methylation of epinephrine and other catechols. J Biol Chem 1958;233:702–5.
[2] Guldberg HC, Marsden CA. Catechol-*O*-methyl transferase: pharmacological aspects and physiological role. Pharmacol Rev 1975;27:135–206.
[3] Tenhunen J, Salminen M, Lundstrom K, Kiviluoto T, Savolainen R, Ulmanen I. Genomic organization of the human catechol *O*-methyltransferase gene and its expression from two distinct promoters. Eur J Biochem 1994;223:1049–59.
[4] Axelrod J. Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. Pharmacol Rev 1966; 18:95–113.
[5] Ball P, Knuppen R. Catecholoestrogens (2-and 4-hydroxyoestrogens): chemistry, biogenesis, metabolism, occurrence and physiological significance. Acta Endocrinol Suppl (Copenh) 1980;232:1–127.
[6] Mannisto PT, Kaakkola S. Catechol-*O*-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacol Rev 1999;51: 593–628.
[7] Lautala P, Ulmanen I, Taskinen J. Molecular mechanisms controlling the rate and specificity of catechol *O*-methylation by human soluble catechol *O*-methyltransferase. Mol Pharmacol 2001;59:393–402.
[8] Bonifati V, Meco G. New, selective catechol-*O*-methyltransferase inhibitors as therapeutic agents in Parkinson's disease. Pharmacol Ther 1999;81:1–36.
[9] Dingemanse J. Catechol-*O*-methyltransferase inhibitors: clinical potential in the treatment of Parkinson's disease. Drug development research 1997;42:1–25.
[10] Vidgren J, Svensson LA, Liljas A. Crystal structure of catechol *O*-methyltransferase. Nature 1994;368:354–8.
[11] Lotta T, Vidgren J, Tilgmann C, Ulmanen I, Melen K, Julkunen I, et al. Kinetics of human soluble and membrane-bound catechol *O*-methyltransferase: a revised mechanism and description of the thermolabile variant of the enzyme. Biochemistry 1995;34:4202–10.
[12] Vidgren J, Ovaska M. Structural aspects in the inhibitor design of catechol *O*-methyltransferase. In: Veerapandian P, editor. Structure-based drug design. New York: Marcel Dekker Inc; 1997. p. 343–63.
[13] Yang CS, Maliakal P, Meng X. Inhibition of carcinogenesis by tea. Annu Rev Pharmacol Toxicol 2002;42:25–54.
[14] Higdon JV, Frei B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Crit Rev Food Sci Nutr 2003; 43:89–143.
[15] Meng X, Sang S, Zhu N, Lu H, Sheng S, Lee MJ, et al. Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats. Chem Res Toxicol 2002;15:1042–50.
[16] Lu H, Meng X, Yang CS. Enzymology of methylation of tea catechins and inhibition of catechol-*O*-methyltransferase by (−)-epigallocatechin gallate. Drug Metab Dispos 2003;31:572–9.
[17] Zhu BT, Patel UK, Cai MX, Conney AH. *O*-Methylation of tea polyphenols catalyzed by human placental cytosolic catechol-*O*-methyltransferase. Drug Metab Dispos 2000;28:1024–30.
[18] Zhu BT, Patel UK, Cai MX, Lee AJ, Conney AH. Rapid conversion of tea catechins to monomethylated products by rat liver cytosolic catechol-*O*-methyltransferase. Xenobiotica 2001;31:879–90.
[19] Zhu BT. Catechol-*O*-Methyltransferase (COMT)-mediated methylation metabolism of endogenous bioactive catechols and modulation by endobiotics and xenobiotics: importance in pathophysiology and pathogenesis. Curr Drug Metab 2002;3:321–49.
[20] Nagai M, Conney AH, Zhu BT. Strong inhibitory effects of common tea catechins and bioflavonoids on the o-methylation of catechol estrogens catalyzed by human liver cytosolic catechol-o-methyltransferase. Drug Metab Dispos 2004;32:497–504.
[21] Segel IH. Enzyme kinetics: Behavior and analysis of rapid equilibrium and steady-state enzyme systems. New York: John Wiley & Sons, 1975. p. 100–202.
[22] Rarey M, Kramer B, Lengauer T, Klebe G. A fast flexible docking method using an incremental construction algorithm. J Mol Biol 1996;261:470–89.
[23] Zheng YJ, Bruice TC. A theoretical examination of the factors controlling the catalytic efficiency of a transmethylation enzyme: catechol *O*-methyltransferase. J Am Chem Soc 1997;119:8137–45.
[24] Inoue MB, Inoue M, Fernando Q, Valcic S, Timmermann BN. Potentiometric and (1)H NMR studies of complexation of Al(3+) with (−)-epigallocatechin gallate, a major active constituent of green tea. J Inorg Biochem 2002;88:7–13.
[25] Paetzel M, Strynadka NC. Common protein architecture and binding sites in proteases utilizing a Ser/Lys dyad mechanism. Protein Sci 1999;8:2533–6.
[26] Bonifacio MJ, Archer M, Rodrigues ML, Matias PM, Learmonth DA, Carrondo MA, et al. Kinetics and crystal structure of catechol-*O*-methyltransferase complex with co-substrate and a novel inhibitor with potential therapeutic application. Mol Pharmacol 2002;62:795–805.
[27] Palma PN, Bonifacio MJ, Loureiro AI, Wright LC, Learmonth DA, Soares-da-Silva P. Molecular modeling and metabolic studies of the interaction of catechol-*O*-methyltransferase and a new nitrocatechol inhibitor. Drug Metab Dispos 2003;31:250–83.
[28] Liehr JG. Is estradiol a genotoxic mutagenic carcinogen? Endocr Rev 2000;21:40–54.
[29] Zhu BT, Conney AH. Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis 1998;19:1–27.
[30] Gogos JA, Morgan M, Luine V, Santha M, Ogawa S, Pfaff D, et al. Catechol-*O*-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior. Proc Natl Acad Sci USA 1998;95:9991–6.