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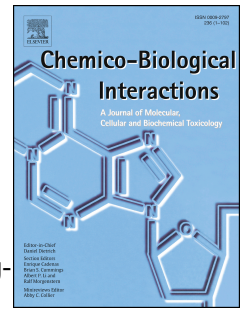

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Research paper

Inhibition of human carboxylesterases by magnolol: kinetic analyses and mechanism

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Abstract

Magnolol, the most abundant bioactive constituent of the Chinese herb *Magnolia officinalis*, has been found with multiple biological activities, including anti-oxidative, anti-inflammatory and enzyme-regulatory activities. In this study, the inhibitory effects and inhibition mechanism of magnolol on human carboxylesterases (hCEs), the key enzymes responsible for the hydrolytic metabolism of a variety of endogenous esters as well as ester-bearing drugs, have been well-investigated. The results demonstrate that magnolol strongly inhibits hCE1-mediated hydrolysis of various substrates, whereas the inhibition of hCE2 by magnolol is substrate-dependent, ranging from strong to moderate. Inhibition of intracellular hCE1 and hCE2 by magnolol was also investigated in living HepG2 cells, and the results showed that magnolol could strongly inhibit intracellular hCE1, while the inhibition of intracellular hCE2 was weak. Inhibition kinetic analyses and docking simulations revealed that magnolol inhibited both hCE1 and hCE2 in a mixed manner, which could be partially attributed to its binding at two distinct ligand-binding sites in each carboxylesterase, including the catalytic cavity and the regulatory domain. In addition, the potential risk of the metabolic interactions of magnolol *via* hCE1 inhibition was predicted on the basis of a series of available pharmacokinetic data and the inhibition constants. All these findings are very helpful in deciphering the metabolic interactions between magnolol and hCEs, and also very useful for avoiding deleterious interactions *via* inhibition of hCEs.

Keywords: Magnolol; Human carboxylesterases (hCEs); Inhibition potential; Herb-drug interactions (HDIs)

1. Introduction

Magnolia officinalis (also named Houpo in Chinese), an edible herbal medicine, has been frequently used for the treatment of gastrointestinal disorders, anxiety, asthma and cough, in Asian countries. In China, a variety of prescriptions containing Houpo are used in modern clinical practice for their sedative, anti-inflammatory, antibiotic, and antispastic effects [1]. In Japan, some herbal medicines containing *Magnolia* bark, such as Hange-Koboku-To and Saiboku-To, are also used in modern clinical practice [2, 3]. Furthermore, the concentrated *Magnolia* bark extracts (MBEs, containing more than 92% magnolol) have been approved as an active ingredient for preparing dietary supplements and cosmetics [4, 5]. Although MBEs and its major constitute magnolol (5,5'-diallyl-2,2'-biphenyldiol; **Fig 1**) displayed good safety profile, recent investigations have demonstrated that magnolol could affect the pharmacokinetic behavior of some therapeutic drugs *via* inhibition of a panel of human drug metabolizing enzymes, including cytochrome P450 enzymes (CYPs) and UDP-glucuronosyltransferases (UGTs) [6, 7]. Considering that MBEs are commonly added to foods (such as mints and chewing gums), as well as cosmetic products [5, 8], human could be easily exposed to magnolol at high doses in daily life. Therefore, it is important to carefully investigate the interactions of magnolol with drug metabolizing enzymes before the combined use of MBEs and clinical drugs.

As mentioned above, regulatory effects of magnolol on CYPs and UGTs have been reported already [6, 7], but the interactions of magnolol with other key drug metabolizing enzymes in human, such as the esterases, have not been well-examined. Mammalian esterases are pivotal serine hydrolases that are expressed in metabolic organs and catalyze the cleavage

of important ester, amide or thioester bonds within a wide variety of compounds [9, 10]. Esterases contribute to the metabolism of about 10% of the total clinical drugs that contain ester or amide bonds [11]. In mammals, carboxylesterases (CEs) are the most abundant esterases in metabolic organs (such as liver, intestine and kidney), and play key roles in the hydrolytic metabolism of a variety of endogenous esters and xenobiotics bearing ester bonds. In the human body, human carboxylesterase 1 (hCE1) and carboxylesterase 2 (hCE2) have been identified as the predominant CEs isoforms and they are involved in detoxification of ester toxins and the hydrolysis of many ester drugs or prodrugs, including clopidogrel, oseltamivir, irinotecan and capecitabine [12, 13]. Inhibition of hCEs may slow down the hydrolysis of their substrate drugs *in vivo*, thereby modulating the outcomes of these drugs. For instance, the primary metabolite of clopidogrel (one of the most frequently prescribed antiplatelet drugs) is the inactive clopidogrel carboxylic acid that is generated by hepatic hCE1 [14]. Upon addition of hCE1 inhibitors, clopidogrel hydrolysis may be partially blocked, while the plasma levels of both clopidogrel and its active metabolite that is activated by several hepatic CYPs could rise to too high levels [15]. Another interesting and important example is CPT-11 (also named irinotecan), an anti-cancer prodrug that can be activated by hCE2 to the active metabolite SN-38. Accumulation of SN-38 in the gastrointestinal system can trigger severe delayed diarrhea that may even be life threatening. It is suggested that co-administration of irinotecan with potent hCE2 inhibitors may ameliorate its associated life-threatening diarrhea, and thereby improve the quality of the patient's life [16-19].

In addition to xenobiotic metabolism, human CEs, particularly hCE1, play crucial roles in endogenous metabolism. Recent investigations have demonstrated that hCE1 is a key enzyme

88 responsible for hydrolysis of endogenous esters (such as cholesteryl esters), thus playing
89 central roles in energy metabolism-related processes, such as lipid metabolism and
90 cholesterol homeostasis [20, 21]. Moreover, it was found that both hCE1 levels and its
91 hydrolytic activities in the adipose tissues from type 2 diabetic and obese patients are
92 elevated when compared with lean healthy subjects [22]. Upon addition of CE1 inhibitors,
93 some beneficial effects to metabolic features were observed in type 2 diabetic mice [22].
94 These findings highlight the importance of hCEs in both endogenous and xenobiotic
95 metabolism and raise much interest in the discovery of more potent hCEs modulators with
96 good safety profiles to regulate hCEs-associated endogenous metabolism, or improve
97 treatment outcomes of hCEs substrate drugs.

98 In the present study, the metabolic interactions of magnolol with the human CEs were
99 carefully investigated in human liver microsomes (HLM) and in living cells. For this purpose,
100 two isoform-specific optical substrates, including D-luciferin methyl ester (DME, a hCE1
101 substrate) and fluorescein diacetate (FD, a hCE2 substrate), were used for screening the
102 inhibition of both hCE1 and hCE2 by magnolol. Subsequently, mechanistic insights into the
103 interactions of magnolol with the hCEs were obtained by carefully characterizing the
104 inhibition kinetics for both enzymes, using a panel of hCEs probe substrates (**Table S1**).
105 Finally, docking simulations were also performed to gain deeper insights into the interactions
106 of magnolol with both hCE1 and hCE2. These new findings added considerable information
107 on the metabolic interactions between magnolol and hCEs, which was useful for the clinical
108 pharmacists to reasonably use magnolol-containing products in order to avoid possible
109 hCEs-mediated drug interactions.

2. Materials and methods

2.1 Materials

Magnolol was obtained from Dalian Meilun Biotech Co., Ltd (Dalian, China). The probe substrates for hCEs, including 3-O-*p*-ethylbenzoylflavone (3-EBF), N-(2-butyl-1,3-dioxo-2,3-dihydro-1*H*-phenalen-6-yl)-2-chloroacetamide (NCEN), 2-(2-benzoyloxy-3-methoxyphenyl) benzothiazole (BMBT), DME and 3,5-Dimethyl BODIPY acid methyl ester (BME), were synthesized by the authors and the synthetic schemes have been dipicted previously [23-27]. Fluorescein diacetate (FD), nevadensin and loperamide (LPA) were purchased from TCI (Shanghai, China). Clopidogrel and clopidogrel carboxylic acid (CCA) were obtained from Shanghai Boylechem Co., Ltd (Shanghai, China). Irinotecan and its hydrolytic metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) were obtained from Tianjin Heowns Biochem LLC (Tianjin, China). The luciferin detection reagent (LDR) was purchased from Promega Biotech (Madison, USA). Magnolol and each substrate were dissolved in dimethyl sulfoxide (LC grade, Tedia, USA) to prepare stock solutions. Pooled human liver microsomes (HLMs, from 50 donors, lot no. X008067) were obtained from Bioreclamation IVT (Baltimore, MD, USA). Cell culture medium and fetal bovine serum were acquired from Hylcone (Logan, UK). HepG2 cell was purchased from the American Type Culture Collection (Teddington, Middlesex, UK). Phosphate buffered saline (0.1 M, pH 6.8 and pH 7.4), Millipore water and LC grade acetonitrile (Tedia, USA) were used for all experiments.

2.2 hCE1 inhibition assays

2.2.1 Inhibition of hCE1-mediated DME hydrolysis by magnolol

A bioluminescent probe (DME) was used as an optical substrate for evaluating the inhibitory effects of magnolol on hCE1, while nevadensin (a specific hCE1 inhibitor) was used as a positive control [28]. Briefly, 91 μL PBS (pH 6.8) was pre-mixed with 2 μL magnolol at different concentrations and 5 μL HLM (1 ug/mL), and then pre-incubated at 37 °C for 10 minutes. Subsequently, 2 μL DME (3 μM, final concentration) were added to the incubation system to start the reaction. After incubating at 37 ^{o}C in a shaking bath for 10 minutes, all incubations were stopped by the addition of LDR (100 μL). A fluorescence microplate reader (SpectraMax® iD3, Molecular Devices, Austria) was used for luminescence measurements. The chemical structures of DME and its hydrolytic metabolite (D-luciferin), as well as the detection conditions are depicted in **Table S1**.

2.2.2 Inhibition of hCE1-mediated BME hydrolysis by magnolol

BME, a newly developed fluorescent substrate for hCE1, was also used for evaluating the inhibitory effects of magnolol on hCE1. In brief, 194 μL PBS (pH 7.4) was pre-mixed with 2 μL HLM (2 μg/mL) and 2 μL magnolol at varying concentrations, and then pre-incubated at 37 °C for 10 minutes in a shaking bath. BME (5 μM) was added to initiate the hydrolytic reaction. The hydrolytic metabolite of BME were real-time analyzed by a fluorescence microplate reader using excitation and emission wavelengths of 505 nm and 560 nm, respectively (Gain = 500). The kinetic parameters were set at 30 reads, with an interval of 60 s and orbital shakes every 10 s before each read, at 37 °C. The chemical structures of BME and its hydrolytic metabolite, as well as the detection conditions are also depicted in **Table S1**.

2.2.3 Inhibition of hCE1-mediated BMBT hydrolysis by magnolol

In brief, 194 µL PBS (pH 7.4) was pre-mixed with 2 µL HLM (2 µg/mL) and 2 µL magnolol at multiple concentrations, which was pre-incubated for 10 minutes at 37 °C. The reaction was initiated by the addition of BMBT (2 µM, final concentration) and the mixture was further incubated for 20 min at 37 °C in a shaking bath. The reaction was terminated by the addition of ice-cold acetonitrile (200 µL), and the mixture was centrifuged at 20,000g for 20 min at 4 °C. After that, aliquots of the supernatants were analyzed by LC-FD. The chemical structures of BMBT and its hydrolytic metabolite HMBT combined with the detection conditions of these two compounds were depicted previously [25].

2.2.4 Inhibition of hCE1-mediated clopidogrel hydrolysis by magnolol

Considering that clopidogrel is a substrate drug for hCE1 and this drug has been frequently used in the clinic, the inhibitory effect of magnolol on hCE1-mediated clopidogrel hydrolysis was also investigated. Briefly, 194 µL PBS (pH 7.4) was pre-mixed with 2 µL HLM (50 µg/mL, final concentration) and 2 µL magnolol at various concentrations, which was then pre-incubated for 10 minutes at 37 °C. The hydrolytic reaction was started by adding clopidogrel (6 µM, final concentration) and the mixture was further incubated for 30 min at 37 °C in a shaking bath. The reaction was terminated by adding 200 µL of ice-cold acetonitrile containing 5-hydroxyflavone as the internal standard (IS, 0.5 µM). After centrifuged at 20,000 × g for 20 min at 4 °C, and the supernatant was diluted 10 times with acetonitrile for further analysis by LC-MS/MS. LC-MS/MS parameter for quantification of clopidogrel carboxylic acid as shown in **Table S2**. The detection conditions of clopidogrel and its hydrolytic metabolite CCA were depicted previously [28].

2.3 hCE2 inhibition assays

2.3.1 Inhibition of hCE2-mediated FD, 3-EBF and NCEN hydrolysis by magnolol

Considering that hCE2 has multiple ligand-binding sites, different probe substrates, including FD, 3-EBF and NCEN, were used for studying the substrate-dependent inhibition of hCE2 by magnolol [29, 23, 24]. LPA was used as a positive hCE2 inhibitor in this study [30]. The chemical structures of FD, 3-EBF and NCEN and their site of hydrolysis by hCE2, as well as the detection conditions that were used in this study, are presented in **Table S1**. The incubation conditions and the detection conditions of these hydrolytic metabolites have been reported previously [29, 23, 24].

2.3.2 Inhibition of hCE2-mediated Irinotecan hydrolysis by magnolol

In brief, 194 μL phosphate buffer (pH 7.4) was pre-mixed with 2 μL HLM (200 μg/mL, final concentration) and 2 μL magnolol at various concentrations. The mixtures were pre-incubated at 37 °C for 10 min, and then the hydrolytic reactions were initiated by adding irinotecan (5 μM, final concentration). Following 50 min incubation at 37 °C in a shaking bath, all incubations were quenched by adding 200 μl of ice-cold acetonitrile. After centrifuged at 20,000 × g for 20 min at 4 °C, 10 μL of the supernatant was injected into the LC-FD system for further analysis. Both irinotecan and its metabolite SN38 were quantified by a liquid chromatography system (Shimadzu, Kyoto, Japan) coupled with a florescence detector, the method was modified according to a previous study [31]. Chromatographic separation of irinotecan and its metabolite SN-38 were performed using a Shim-pack ODS column (150 mm × 2.0 mm, 2 μm, Shimadzu). Acetonitrile (A) and ammonium acetate (50 mM, pH=4.0, B) were used as the mobile phase, and the gradient was as follow, 0.01-1.50 min, 25% A; 1.50-6.00 min, 25-40% A; 6.00-8.00 min, 40-90% A; 8.00-12.00 min, 90% A;

12.00-12.50 min, 90-25% A; 12.50-15.00 min, 25% A. The column temperature was kept at 40 °C and the flow rate was 0.6 mL/min.

2.4 Inhibition kinetic analyses

To determine the inhibition constant (K_i) and inhibition kinetic types of magnolol against hCEs, the inhibition kinetics of both hCE1 and hCE2 by magnolol were carefully investigated using varying concentrations of probe substrates in the presence of different concentrations of magnolol (inhibitor). The details for determining the inhibition constants have been reported previously [32-34].

2.5 Cell culture and fluorescence imaging analyses

The inhibitory effects of magnolol on both hCE1 and hCE2 were also investigated in living HepG2 cells. To this end, HepG2 cells were cultured at 37 °C in 5% CO_2 in Modified Eagle's Medium (MEM) containing 0.1% antibiotic-antimycoticmix antibiotic, supplemented with 10% fetal bovine serum (FBS). For fluorescence imaging, HepG2 cells were seeded in 96-well plates (8000 cells / well) in complete medium and then incubated for 24 hours. Afterwards, the cells were washed twice with FBS-free culture medium and incubated in the medium containing magnolol (prepared in FBS-free at various concentrations) for 30 min at 37 °C under 5% CO_2. To assess the intracellular hCE1 and hCE2 function, HepG2 cells were then co-incubated with BME (final concentration, 5 μM) or NCEN (final concentration, 10 μM) for another 30 min and 50 min, respectively. The living cells were imaged and analyzed using an ImageXpress® Micro Confocal High-Content Imaging system (Molecular Devices, Austria).

2.6 Prediction of herb-drug interaction potentials from in vitro data

The potential risk of the metabolic interactions of magnolol on hCE1 was predicted *via* evaluation of the changes in the area under the plasma concentration-time curve (AUC) in the presence of magnolol. The AUC_{ratio} was predicted using the equation 1 [55].

$$AUCratio = \frac{1}{f_{hep}\ (\frac{1/E_h}{(1/E_h - 1)(1 + I/K_i) + 1}) + (1 - f_{hep})} \quad (1)$$

$$E_h = CL_{hep} / Q_h = \frac{1}{\frac{Q_h \times f_m \times K_m}{V_{max} \times MSP \times f_u} + 1} \quad (2)$$

Where f_{hep} is the percentage of hepatic clearance mediated by hCE1; E_h is the hepatic extraction ratio; I is the maximum plasma concentration of magnolol, while K_i (μM) is the inhibitory constant of magnolol against hCE1; f_m is the fraction metabolized by hCE1; f_u is the unbound fraction of magnolol; K_m (μM) is the substrate affinity constant; V_{max} (nmol·min·mg) is the maximum reaction velocity; Q_h (ml/min) is the liver blood flow; and *MSP*(mg) is the total microsomal proteins.

2.7 Molecular docking simulations of magnolol into hCEs

For docking simulations, Discovery Studio (BIOVIA Discovery Studio 2016, Dassault Systèmes, San Diego, USA) was used to mimic the interactions of magnolol with both hCE1 and hCE2. The whole process for docking simulation was depicted previously [28].

2.8 Statistical analysis

All assayed were performed in triplicate and the results presented here were expressed as mean ± SD. The IC_{50} values were determined using nonlinear regression by GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, USA).

3. Results

3.1 Assessments of human carboxylesterases inhibition by magnolol

Firstly, the inhibition potentials of magnolol on both hCE1 and hCE2 were screened using three inhibitor concentrations (1 μM, 10 μM and 100 μM, final concentrations), while DME and FD were used as isoform-specific probes for hCE1 and hCE2 in HLM, respectively. As shown in **Fig. S1**, magnolol displayed potent inhibition of both hCE1-mediated DME hydrolysis and hCE2-mediated FD hydrolysis. Upon addition of magnolol (1 μM), the residual activities of hCE1 or hCE2 were less than 50%, suggesting that the IC_{50} values of magnolol against both hCE1-mediated DME hydrolysis and hCE2-mediated FD hydrolysis in HLM were less than 1 μM. To quantify the half maximal inhibition concentration (IC_{50}) of magnolol more accurately, the dose-response curves were plotted using different inhibitor concentrations. As shown in **Fig. 2A** and **Fig. 3A**, magnolol inhibited the catalytic activities of both hCE1 and hCE2 in a dose-dependent manner. The IC_{50} values of magnolol toward hCE1-mediated DME hydrolysis and hCE2-mediated FD hydrolysis in HLM were determined as 0.35 μM and 0.90 μM, respectively. These findings stimulated us to further investigate the mechanism of hCEs inhibition by magnolol, the most abundant natural constituent from *M. officinalis*.

3.2 Kinetic analyses of human carboxylesterase 1 inhibition by magnolol

As shown in **Fig. S2**, inhibition of hCE1-mediated DME hydrolysis in HLM by magnolol was not time-dependent, suggesting that magnolol was a reversible inhibitor of hCE1 [35]. Subsequently, the kinetics of hCE1 inhibition by magnolol was carefully studied using a panel of hCE1 substrates, including three optical substrates (DME, BMBT, BME) and a substrate drug (clopidogrel). As shown in **Fig. 2** and **Table 1**, magnolol exhibited significant

inhibitory effects toward hCE1-mediated hydrolysis of various substrates, all of them with IC_{50} values below 1.2 μM. The inhibition modes and the K_i values of magnolol of the hCE1-mediated hydrolysis inhibition were then carefully characterized in HLM, using these four different substrates. As shown in **Fig. 2** and **Fig. S11**, the inhibition kinetic plots clearly showed that magnolol inhibited hCE1-mediated hydrolysis of all the tested hCE1 substrates (including DME BMBT, BME and clopidogrel) in HLM *via* mixed inhibition mode, with the K_i values ranging between 0.23 μM and 1.36 μM (**Table 1**). These findings indicate that magnolol is a potent hCE1 inhibitor for all the tested substrates.

3.3 Kinetic analyses of human carboxylesterase 2 inhibition by magnolol

Similarly, a time-dependent inhibition assay was also performed to ascertain the inhibition type of magnolol on hCE2. As shown in **Fig**. **S3**, prolonging the pre-incubation time did not affect the inhibition of hCE2-mediated FD hydrolysis in HLM by magnolol, suggesting that magnolol was a reversible inhibitor toward hCE2, too [35]. Subsequently, the inhibition kinetics of hCE2 by magnolol was investigated using three optical substrates for hCE2 substrates, namely FD, 3-EBF, and NCEN, as well as the drug substrate irinotecan. The IC_{50} values of magnolol in the hCE2-mediated hydrolysis of these substrates were 0.90 μM, 2.74 μM, 10.21 μM and 24.16 μM for FD, 3-EBF, NCEN and irinotecan, respectively (**Table 1**). These findings revealed a rather large variability among the IC_{50} values of magnolol against the tested hCE2 substrates, in contrast to the situation with hCE1 (**Table 1**).

Kinetic analyses of hCE2 inhibition by magnolol using Lineweaver-Burk plots suggested that this natural compound was a mixed inhibitor against hCE2-mediated hydrolysis of all the tested hCE2 substrates (including FD, 3-EBF, NCEN and irinotecan) in HLM (**Fig. 3 and Fig.**

S11). The large variability in IC_{50} values in the case of hCE2 was also reflected in the K_i values for magnolol, when hCE2 substrates were used. The K_i values for the hydrolysis of FD, 3-EBF, NCEN, and irinotecan were 1.02 μM, 0.86 μM, 17.13 μM and 29.91 μM, respectively. These results suggested that the inhibition of hCE2 by magnolol might be substrate-dependent.

3.4 Inhibition of hCEs by magnolol in living cells

Considering that hCEs were intracellular enzyme localized within the lumen of the endoplasmic reticulum, it was important to assay the inhibition potentials of magnolol, given on the outside, on intracellular hCEs. To this end, BME and NCEN were selected as highly specific fluorescent probe substrates for hCE1 and hCE2, respectively. This selection was due to the inherent advantages of these two fluorescent probe substrates, including high fluorescence quantum yield and good biocompatibility, as well as high chemical and photochemical stability [27, 24]. Prior to test the inhibitory effects of magnolol on intracellular hCEs in HepG2 cells, the cytoxicity of magnolol towards HepG2 cells was assayed. As shown in **Fig S5**, magnolol exhibited weak cytoxicity on HepG2 when the final concentration was lower than 60 μM. But the HepG2 cells could be damaged at high dosage (100 μM), with the cell viability of 37%. The inhibition assays were carried out and the results are shown in **Fig. S6**. Magnolol could inhibit intracellular hCE1-mediaed BME hydrolysis and reduce the fluorescence intensity in the green channel (for the hydrolytic metabolite of BME) in living HepG2 cells in a dose-dependent manner. The IC_{50} value of this inhibition was measured and found to be 8.59 μM. The inhibition of hCE2-mediated NCEN hydrolysis in living HepG2 cells by magnolol, on the other hand was weaker, exhibiting an

IC_{50} value higher than 60 μM, the highest concentration of magnolol that was used in the assay (**Fig. S7)**. These findings suggest that magnolol is cell membrane permeable and capable of inhibiting the endogenous hCE1 in living cells.

3.5 Docking simulations of magnolol in hCE1 and hCE2

A different approach for further understanding the interactions of magnolol with hCEs was docking simulations. As shown in **Fig. 4**, magnolol could be well-docked into hCE1 at two distinct ligand-binding sites, one located inside the catalytic cavity and another on the regulatory domain (Z site). This finding suggests that magnolol could bind on hCE1 at two distinct ligand-binding sites, one of which overlaps and competes with DME for its binding site within the catalytic cavity of hCE1, while the other magnolol-binding site is located on the regulatory domain that is situated far away from the catalytic triad of the enzyme. Hence, the mixed-type inhibition of magnolol in hCE1 probably resulted from competitive inhibition by binding at the catalytic cavity and non-competitive inhibition from binding at the regulatory domain.

The key residues for the interactions between magnolol and hCE1 were also analyzed by docking simulations. As depicted in **Fig. 4** and **Fig. S8**, magnolol could interact with residues around the catalytic cavity of hCE1 mainly *via* hydrophobic interactions, while at the regulatory domain of hCE1 magnolol would mainly bind through hydrogen bonding and hydrophobic interactions. As a result, the docking simulations of magnolol on both the active site and the Z site in the best binding conformations yielded the lowest binding free energies (-95.82 kcal/mol for magnolol binging at the catalytic site of hCE1, and -90.06 kcal/mol for binging at the Z site).

As depicted in **Fig. 5**, magnolol could also be well-docked into both the catalytic cavity and the regulatory domain (Z site) of hCE2, suggesting that magnolol could compete with FD for an overlapping site in the catalytic cavity. The latter and the 2nd binding site at the regulatory domain, far away from the catalytic triad of hCE2, could well explain the mixed-type kinetics of magnolol in hCE2 inhibition. As shown in **Fig. 5** and **Fig. S9**, magnolol appears to be tightly bind at residues around the catalytic cavity of hCE2, predominantly *via* hydrophobic interactions, while the binding energy of the best binding mode (magnolol on the active site of hCE2) was estimated as -85.92 kcal/mol. It was also found that magnolol could strongly bind at the regulatory domain of hCE2 through both hydrogen bonding and hydrophobic interactions. The binding energy of the best binding mode of magnolol on the Z site of hCE2 was as low as -109.99 kcal/mol. It thus appears that the docking results agree with the inhibition kinetics results, for both hCE1 and hCE2 inhibition by magnolol.

3.6 Quantitative prediction of magnolol-associated HDI risks via hCE1 inhibition

Considering that magnolol could inhibit intracellular hCE1 in living cells and its inhibition potency was really strong, it was necessary to evaluate the potential risks of magnolol *via* hCE1 inhibition. In this case, the magnitudes of the metabolic interactions between magnolol and hCE1 substrate drug was predicted by estimating the changes in the AUCs of the hCE1 substrate drugs (or the drugs predominantly metabolized by hCE1) co-administrated with magnolol-containing marketed products. The AUC ratios were calculated based on a series of available pharmacokinetic data and inhibition constants, including the hepatic extraction ratio (E_h), the percentage of hepatic clearance mediated by hCE1 relative to the total clearance of

the substrates (f_{hep}), the K_i values for magnolol against hCE1 and the predicted maximum concentration of magnolol in human plasma (C_{max}). As shown in **Table S3**, magnolol might increase the AUCs of the clopidogrel by 28%-142% when clopidogrel was co-administrated with magnolol-containing products. This finding implied that magnolol might modulate the pharmacokinetic behaviors of the ester-bearing drugs that predominantly metabolized by hCE1, such as clopidogrel.

4. Discussion

Over the past decade, increasing evidence has demonstrated that human carboxylesterases (hCEs) play crucial roles in both endogenous and xenobiotic metabolism [20, 22, 36]. The biological roles of hCEs have raised great interest in the discovery of potent hCEs modulators that can be used to regulate lipid metabolism or to improve the treatment outcomes of ester drugs [37]. In recent years, many groups have tried to identify potent inhibitors of hCEs from edible herbs, since most edible herbs have displayed good safety profiles during long history of use in medical applications. Our preliminary studies have found that crude extracts of some herbs (such as *Fructus Psoraleae* and *Magnolia officinalis*) strongly inhibit the activity of hCEs [38]. Unfortunately, the major constituents of *Fructus Psoraleae* can trigger hyperbilirubinemia and liver injury *via* strong inhibition of human UDP-glucuronosyltransferase 1A1 [39]. Therefore, it is of great importance to find more readily available ingredients or natural compounds with good safety profiles as hCEs inhibitors [32, 40, 56]. In the present study, the results clearly demonstrate that magnolol (the most abundant constitute in *Magnolia officinalis*) displays potent inhibition on

hCE1-mediated hydrolysis of a panel of substrates. Magnolol also exhibits strong to moderate inhibition of hCE2-mediated hydrolysis of various substrates in HLM. The initial observations prompted us to deepen and extend our investigation on the inhibition mechanism of magnolol against both hCEs.

As an abundant drug-metabolizing enzyme in the liver, hCE1 can effectively hydrolyse a variety of ester drugs, as well as prodrugs with ester bonds or amide bonds, including clopidogrel, temopril, midazolam, oseltamivir and enalapril [12, 13]. Thus, co-administration of one of the above listed hCE1 substrate drugs with magnolol-containing products, may trigger clinically relevant drug/herb-drug interactions [17]. Notably, inhibition of hCE1 is a double-edged sword for patients administrated with hCE1-substrate drugs. Strong inhibition of hepatic hCE1 may slow down the hydrolytic rates of hCE1 substrates *in vivo*, which may affect the pharmacokinetic properties of co-administrated ester drugs and thus bring beneficial effects (such as enhancement of efficacy) or unbeneficial effects (such as herb-drug interactions). For example, hCE1 catalyses oseltamivir hydrolysis to form the active metabolite oseltamivir carboxylate, thus inhibition of hCE1 may result in decreased plasma exposure of oseltamivir carboxylate and reduce efficacy of this hCE1-substrate drug [41]. By contrast, clopidogrel, one of the most frequently prescribed antiplatelet agents, can be rapidly hydrolysed to an inactive metabolite by hepatic hCE1, whereas only about 15% of this ester drug is activated by CYPs to form 2-oxo-clopidogrel, followed by conversion to the active metabolite [42]. Dysfunction or inhibition of hCE1 may partially block the hydrolysis of clopidogrel and accelerated the formation rate of the active metabolite *via* the CYP-mediated pathway [14]. Thus, magnolol-containing products or MBEs could modulate the efficacy of

clopidogrel in the clinic. This result of our study, reasonable use magnolol-containing products, may be helpful for clinical pharmacists.

In addition to the involvement of xenobiotic metabolism, hCE1 also participates in the hydrolytic metabolism of some key endogenous esters, such as cholesterol esters and triglycerides [43]. This indicates that hCE1 inhibitors (such as magnolol) may regulate lipid metabolism and cholesterol homeostasis *via* hCE1 inhibition. Recently, Dominguez et al has found that the levels and enzymatic activities of hCE1 in subcutaneous adipose tissue and visceral adipose tissue from obese and type 2 diabetic patients are much higher than that of health individuals, while treatment with hCE1 inhibitors may benefit the patients with obesity and type 2 diabetes through improving metabolic features [22]. It should be noted that magnolol has been found to have anti-diabetic effects and other beneficial effects on a panel of metabolic diseases. For example, a recent investigation has demonstrated that oral administration of this compound to type 2 diabetic rats could reduce fasting blood glucose and plasma insulin levels, as well as induce glucose uptake in adipocytes [44]. Interestingly, in the present study, we found that magnolol is a potent and cell permeable inhibitor of hCE1, it could strongly inhibit intracellular hCE1 in living cells. This finding indicates that magnolol may inhibit some key intracellular hCE1-mediated endogenous esters hydrolysis, which in turn alleviate the metabolic disorders of type 2 diabetes. At least, intracellular hCE1 may be one of the targets of magnolol for the prevention and treatment of diabetes or other metabolic diseases.

Considering that hCE2 plays a key role in the hydrolysis of some important ester drugs, such as irinotecan and capecitabine [45, 46], the inhibition potentials of magnolol on hCE2

was also investigated in HLM and living cells. The results demonstrate that magnolol displays strong inhibition on hCE2-mediated hydrolysis of FD and 3-EBF, but the inhibition of hCE2-mediated hydrolysis of NCEN and irinotecan was moderate (**Table 1**). The substrate-dependent inhibition of hCE2-mediated hydrolysis reactions by magnolol could be partially explained by the larger catalytic cavity of this enzyme. As shown in **Fig. S10**, the volume of the catalytic cavity of hCE2 is about 2-fold larger than that of hCE1, which suggests that the catalytic cavity of hCE2 can simultaneously accommodate more than one small molecule ligands. Furthermore, evidence from X-ray crystal structure of hCE1 revealed that under physiological conditions hCE1 exists as a mixture of monomeric and trimeric, as well as hexameric forms [36]. By contrast, hCE2 exists as a monomer. Thus, it is conceivable that the conformation of hCE2 could be easily changed by binding of ligands but the changes in the conformation of hCE1 in such a way is relatively difficult, owing to the major forms of hCE1 are trimer or hexamer, which may partially block the free access of the ligands.

Taking into account that hCE2 is an intracellular target that is localized in the lumen of ER, it is necessary to ascertain the inhibition potential of magnolol on intracellular hCE2. In these cases, we use NCEN (a fluorescent probe substrate for hCE2) to replace irinotecan in order to assay the inhibitory effects of magnolol on hCE2 inside living cells. This is because both NCEN and its hydrolytic metabolite exhibit excellent optical properties [24], as well as similar inhibition tendency as the hCE2 substrate drug irinotecan. Notably, the results demonstrate that magnolol hardly inhibits hCE2-mediated NCEN hydrolysis in living HepG2 cells. These findings suggest that the inhibition potency of magnolol on intracellular hCE2 is relatively weak in living HepG2 cells, which may partially be attributed to the metabolic

clearance of magnolol in HepG2 cells [47].

Although magnolol displayed strong inhibition of hCE1, its potency and selectivity is not so high. Therefore, it is desirable to further develop more potent hCE1 inhibitors using this natural compound as lead compound. As mentioned above, the content of magnolol in *Magnolia officinalis* is very high [4], the medicinal chemists can easily get this natural compound from this herb and then to semi-synthesize a variety of structurally diverse magnolol derivatives for systematic pharmacological and toxicological studies. To develop a potent and highly specific hCE1 inhibitor, it is necessary to explore the structure-activity relationships of magnolol derivatives as hCE1 inhibitors, as well as to assay their specificity towards hCE1 over other serine hydrolases. Considering that the crystal structure and the catalytic triad of hCE1 have been reported [48], hCE1 inhibitors could be rationally designed and developed with the help of computer-assisted virtual screening and design. Considering that magnolol can be readily metabolized by hepatic drug metabolizing enzyme [47], attention should be paid to further optimization of the metabolic stability of magnolol derivatives during lead to candidate optimization. In addition, in the future, the medicinal chemists could consider designing dual inhibitors against both hCE1 and other target(s) for the treatment of metabolic diseases [49, 50]. All these investigations will be very helpful for the discovery of novel efficacious hCE1 inhibitors for potential medical applications.

5. Conclusion

In summary, the inhibitory effects of magnolol on human carboxylesterases (hCEs), key enzymes participating in the hydrolytic metabolism of a wide range of endogenous and

xenobiotic esters, were carefully investigated using a panel of hCEs substrates including optical substrates and substrate drugs. Our results demonstrated that magnolol was a strong inhibitor of hCE1-mediated hydrolysis of all the tested substrates. By contrast, magnolol displayed strong inhibition of hCE2-mediated hydrolysis of FD and 3-EBF, but moderate inhibition of NCEN and irinotecan hydrolysis. The inhibition mechanism of magnolol against both hCE1 and hCE2 were carefully investigated by a panel of kinetic analyses and docking simulations, the results showed that magnolol could tightly bind on hCE1 or hCE2 at both the catalytic cavity and the regulatory domain, which was consistent with the experimental data in which this compound functions as a potent hCEs inhibitor *via* mixed-inhibition manner. Further investigation demonstrated that magnolol could also strongly inhibit intracellular hCE1 but hardly inhibit intracellular hCE2-mediated NCEN hydrolysis. All these findings provided new insights into the interactions between magnolol and hCEs, which was very useful for avoiding deleterious interactions *via* modulating the pharmacokinetic behaviors of hCEs-substrate drugs. Additionally, these findings clearly suggested that magnolol could be used as a good lead compound for the development of more efficacious hCEs inhibitors with good safety profile.

Declaration of interest

The authors have no conflicts of interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http:

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Table 1. The inhibition parameters and inhibition modes of magnolol against hCEs.

Target enzyme	Enzyme source	Substrate	IC_{50} (μM)	f_u	f_u*IC_{50} (μM)	K_i (μM)	Inhibition mode	Goodness of fit (R^2)
hCE1	HLM	DME	0.35±0.01	0.98	0.34	0.34	Mixed	0.99
hCE1	HLM	BMBT	0.70±0.05	1.00	0.70	0.52	Mixed	0.99
hCE1	HLM	BME	0.21±0.02	0.96	0.20	0.23	Mixed	0.99
hCE1	HLM	Clopidogrel	1.14±0.10	0.50	0.57	1.36	Mixed	0.92
hCE2	HLM	FD	0.90±0.04	0.96	0.86	1.02	Mixed	0.97
hCE2	HLM	3-EBF	2.74±0.24	0.84	2.30	0.86	Mixed	0.99
hCE2	HLM	NCEN	10.21±3.15	0.84	8.58	17.13	Mixed	0.97
hCE2	HLM	Irinotecan	24.16±5.21	0.20	4.83	29.91	Mixed	0.95

Note. Estimate the in vitro fraction unbound from drug's properties. Equations published by Austin are being used for this calculation. For microsomes: % unbound = 100/[MicrosConc*[10^[0.56*logD/P-1.41]]+1] [51]; logP value of magnolol was set to 4.83.

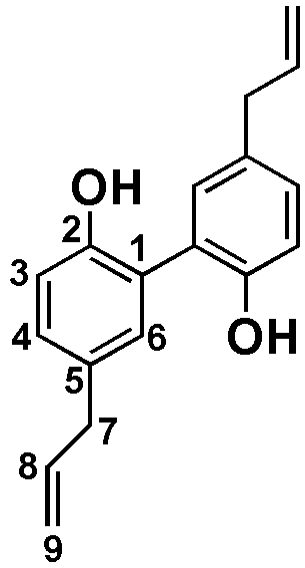

Fig. 1. The chemical structure of magnolol

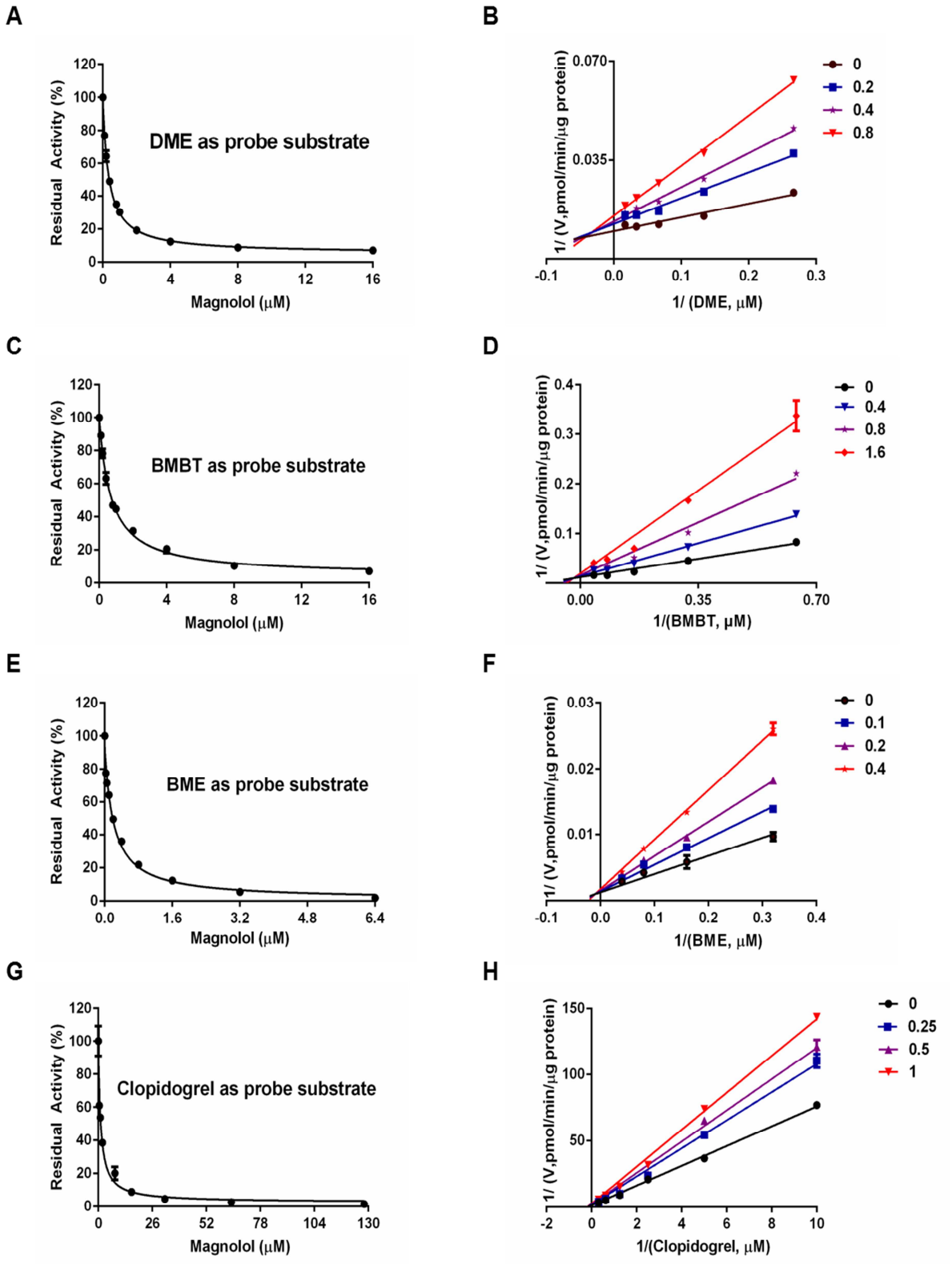

Fig. 2. The dose-response inhibition curves of magnolol on hCE1 using DME (A), BMBT (C), BME (E) and clopidogrel (G) as probe substrate, respectively. The Lineweaver-Burk plots for the inhibition of magnolol on hCE1 using DME (B), BMBT (D), BME (F) and clopidogrel (H) as probe substrate, respectively. All data were shown as mean ± SD of triplicate determinations.

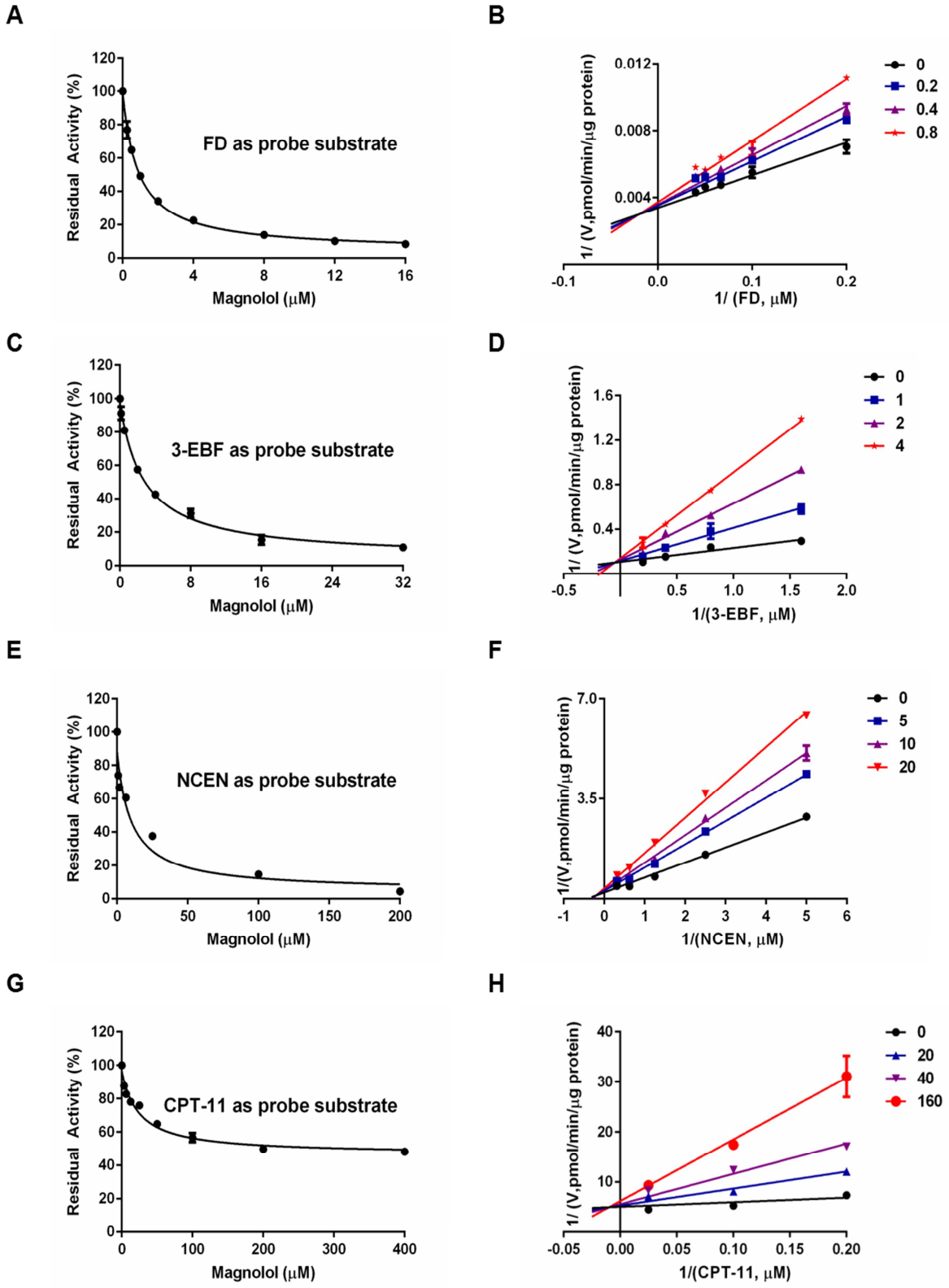

Fig. 3. The dose-response inhibition curves of magnolol on hCE2 using FD (A), 3-EBF (C), NCEN (E) and CPT-11 (G) as probe substrate, respectively. The Lineweaver-Burk plots for the inhibition of magnolol on hCE2 using FD (B), 3-EBF (D), NCEN (F) and CPT-11 (H) as probe substrate, respectively. All data were shown as mean ± SD of triplicate determinations.

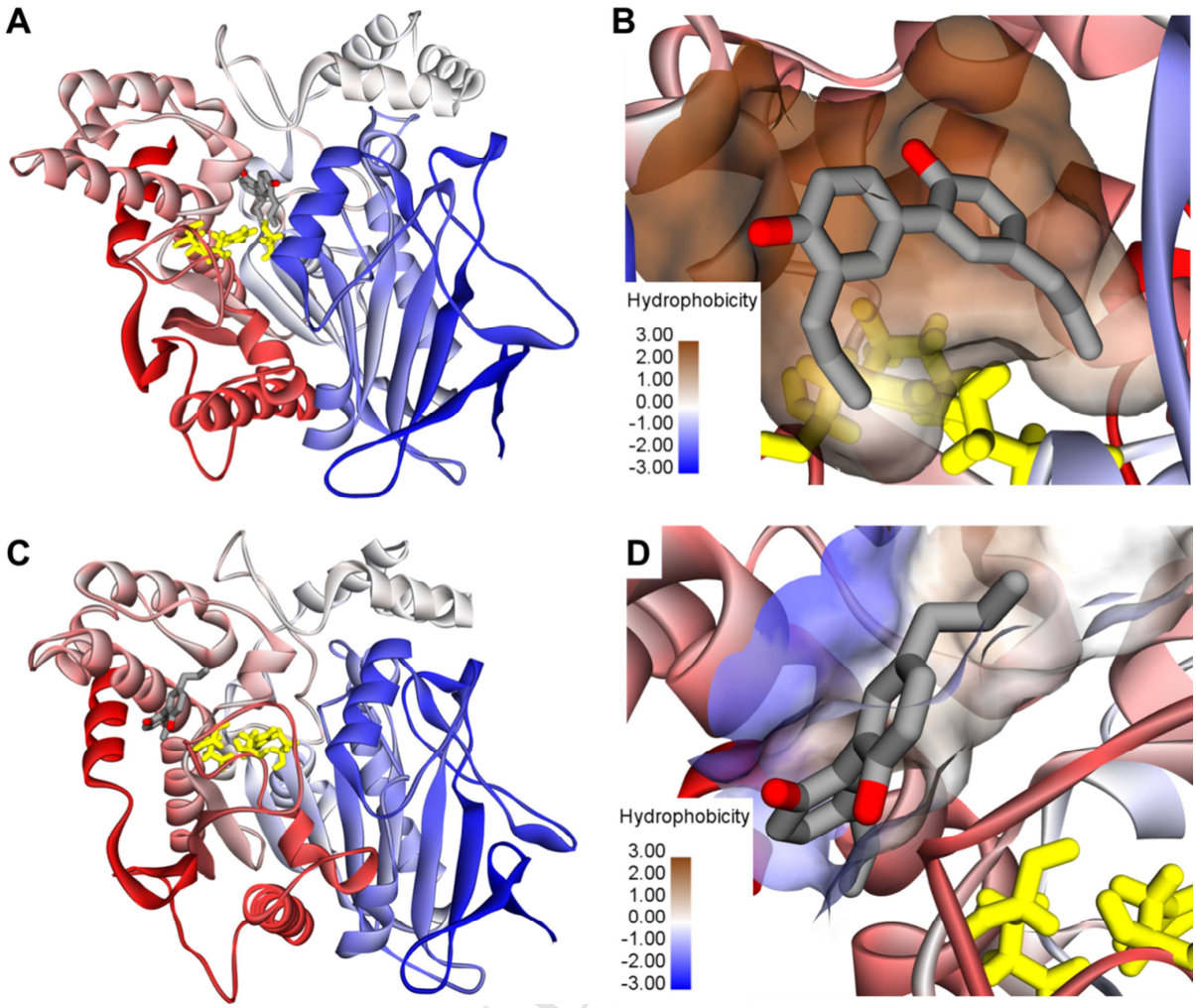

Fig. 4. The docking simulations of magnolol into hCE1 (PDB ID: 1MX5). The stereodiagram of magnolol aligned in the active site (A) or the Z-site (C) of hCE1. A detailed view of the binding area of magnolol on the active site (B) or the Z-site (D) of hCE1 with surrounding residues. Note that the catalytic triad of hCE1 (Ser^{221}, Glu^{354} and His^{468}) are colored in yellow, while the surface hydrophobicity scale in right panel is given from brown (3.0) to blue (-3.0).

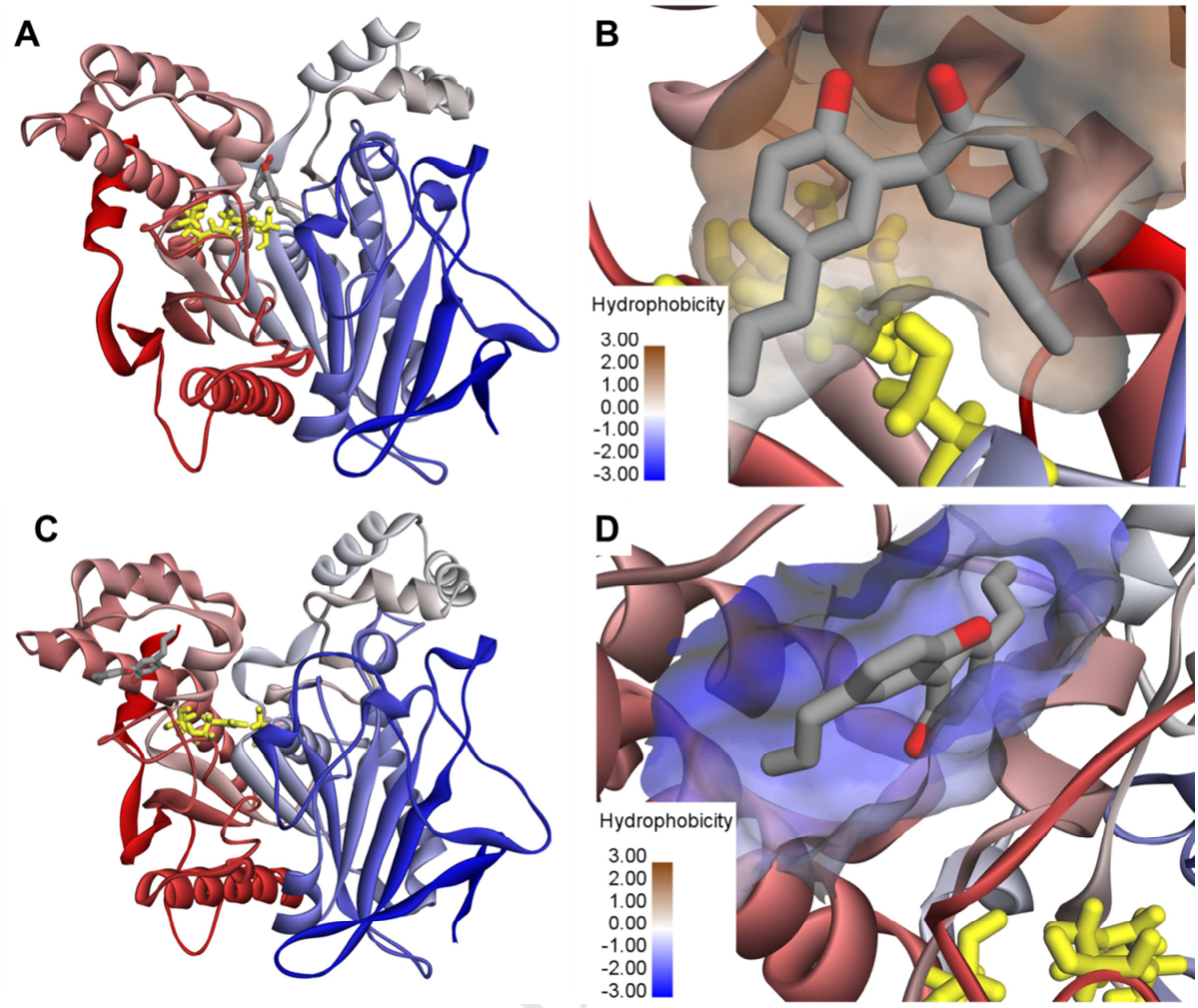

Fig. 5. The docking simulations of magnolol into hCE2 (UniProt O00748, build by Swiss-model homology model). The stereodiagram of magnolol aligned in the active site (A) or the Z-site (C) of hCE2. A detailed view of the binding area of magnolol on the active site (B) or the Z-site (D) of hCE2 with surrounding residues. Note that the catalytic triad of hCE2 (Ser^{228}, Glu^{345} and His^{457}) are colored in yellow, while the surface hydrophobicity scale in right panel is given from brown (3.0) to blue (-3.0).

Highlights

1. Magnolol strongly inhibits hCE1-mediated hydrolysis of various substrates but inhibits hCE2 in a substrate-dependent manner.
2. Magnolol is cell membrane permeable and capable of inhibiting the intracellular hCE1 in living cells.
3. Magnolol inhibits both hCE1 and hCE2 in a mixed manner.
4. Magnolol could bind on hCE1 at two distinct ligand-binding sites.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The authors declare no conflict of interest