



Discovery of cardiovascular drugs as effective inhibitors of human carboxylesterase

Chang-Sheng Lv^{a,1}, Bin Wang^{b,1}, Yan-Ze Zheng^b, Yu-Heng Wang^a, Jing Zhang^b, Ze-Jun Jiang^b, Jin-Guang Wang^{a,*}, Li-Wei Zou^{b,*}

^a The First Affiliated Hospital, Dalian Medical University, Dalian 116011, China

^b Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China



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ABSTRACT

Carboxylesterases serve as principal Phase I metabolic enzymes that critically regulate the metabolism, efficacy, and safety profiles of ester-based pharmaceuticals in humans. This study investigated 23 clinically relevant cardiovascular agents to assess their inhibitory potential against human carboxylesterase isoforms hCES1A and hCES2A. The findings demonstrated that hypolipidemic agents simvastatin (C-16) and lovastatin (C-17), along with antithrombotic compounds prasugrel (C-22) and clopidogrel (C-23), exhibited potent inhibition against hCES1A, with the lowest IC₅₀ value observed at 0.48 μM. These four therapeutic agents combined with ezetimibe (C-18) demonstrated concurrent inhibition of hCES2A, exhibiting the lowest observed IC₅₀ value of 0.55 μM. Selectivity profiling demonstrated that C-16, C-17, C-22 and C-23 exhibited preferential inhibition against hCES1A/hCES2A compared with three other serine hydrolases, whereas C-18 specifically targeted hCES2A. Further research has found that these five drugs can inhibit hCES2A-mediated CPT-11 hydrolytic metabolism. Kinetic characterization delineated the inhibition modality exerted by the test compounds on hCES1A/hCES2A-mediated hydrolytic activity towards NLM/FD. Molecular docking analysis elucidated the reason why these drugs (C-16/18 and C-22/23) did not exhibit competitive inhibition behavior in hCES2A inhibition experiments. In addition, the five drugs demonstrated significant inhibition of hCES2A activity in Caco2 cells, exhibiting clear concentration dependence. These drugs represent potent inhibitors of carboxylesterases, providing valuable insights into clinical medication.

1. Introduction

Cardiovascular and cerebrovascular diseases have long been a major global concern. Over the past several decades, the global count of hypertension patients has experienced remarkable growth, increasing from 650 million in 1990 to 1.3 billion in 2019 [1]. Additionally, the prevalence of dyslipidemia in adults globally ranges between 30 % and 40 % [2]. Cardiovascular diseases, including hypertension, typically require long-term medication use to manage disease progression [3]. Cardiovascular drugs' consumption has remained consistently high worldwide. For example, statins are widely used in patients with atherosclerotic cardiovascular disease [4]. Angiotensin-converting enzyme inhibitor (ACEI) drugs are also commonly used for hypertension prevention and applied in secondary cardiovascular prevention [5]. Drug-drug interactions (DDIs) resulting from cardiovascular drugs can significantly

impact the absorption, distribution, metabolism, and excretion processes of other medications. For instance, fenofibrate, an inhibitor of CYP2C9, used to treat hyperlipidemia, has been reported to increase bleeding risks in patients when combined with gemfibrozil and warfarin [6]. Therefore, precise risk assessment of DDIs among commonly used cardiovascular and cerebrovascular drugs is of significant clinical importance.

Carboxylesterases, as crucial phase I metabolic enzymes, are indispensable for maintaining human health. They are involved in the metabolism and transport of lipid metabolites, including cholesterol esters and free fatty acids, and mediate the metabolic clearance of exogenous ester drugs and environmental toxicants [7]. Carboxylesterases in humans are categorized into five subtypes, with hCES1A and hCES2A being the most notable. hCES1A and hCES2A display 40–50 % homology in their amino acid sequences. Marked differences

* Corresponding authors.

E-mail addresses: dlwangjg@163.com (J.-G. Wang), chemzlw@163.com (L.-W. Zou).

¹ These authors (Chang-Sheng Lv and Bin Wang) contributed equally.

are observed in their tissue distribution and substrate selectivity characteristics. Such differences significantly impact the metabolic processes of endogenous and exogenous substances, ultimately affecting the pharmacokinetics and pharmacodynamics of drugs interacting with these enzymes [8]. hCES1A is primarily expressed in the liver, where it is commonly known as hepatic carboxylesterase. On the other hand, its expression within the gastrointestinal tract remains relatively low. In contrast, hCES2A, also referred to as intestinal carboxylesterase enzyme, is primarily localized within the gastrointestinal tract. Notably, the enzyme is highly abundant in the small intestine, with its concentration and activity being especially notable there. The distinct tissue-specific distribution of hCES1A and hCES2A plays a significant role. It serves as a key factor in the metabolism of endogenous and exogenous substances, determining the unique functions these enzymes perform. In the long term, these physiological mechanisms have a significant influence on how the body responds to drugs [9]. hCES1A exhibits a propensity to catalyze the hydrolysis of compounds with a large amide moiety and a small alcohol group. Notable examples of compounds exhibiting specific characteristics enumerated by clopidogrel, enalapril, cocaine, and heroin. The substrate selectivity of hCES1A is notable for its significant impact within the fields of pharmacology and toxicology, which significantly impacts the metabolism, efficacy, and safety profiles of pharmacological agents and xenobiotics. The efficiency and specificity of hydrolysis reactions are determined by the combined effects of the active-site structure of hCES1A and the chemical characteristics of the substrates, which jointly regulate their specific interactions [10,11]. Compounds exhibiting small acyl moieties and large alcohol groups, such as irinotecan, capecitabine, and flutamide, exhibit minimal hydrolysis by hCES1A. Conversely, hCES2A can utilize these compounds effectively as substrates [12].

Carboxylesterases influence the disposition of various ester drugs in the human body, thereby affecting their efficacy and safety profiles. hCES1A drug substrates are likely to escape first-pass metabolism in the gastrointestinal tract and enter systemic circulation, but are subject to

metabolic elimination by elevated levels of hCES1A in the liver. hCES1A converts clopidogrel into clopidogrel acid, which is biologically inactive, and 90 % of clopidogrel can be metabolically inactivated through this pathway [13]. hCES1A converts oseltamivir into its active metabolite, oseltamivir carboxylate. When hCES1A enzyme activity is reduced or inhibited, oseltamivir may not demonstrate effective clinical efficacy [14]. For hCES2A drug substrates that escape first-pass hydrolysis in the small intestine and enter systemic circulation, they are less likely to directly contact a significant amount of hCES2A in the small intestine, thereby prolonging their drug efficacy. The local anesthetic procaine and the anticholinergic drug oxybutynin are hydrolyzed and inactivated, and inhibiting hCES2A in the gastrointestinal tract prolongs their drug effects [15]. Irinotecan (CPT-11), an anti-cancer drug with broad-spectrum anti-cancer activity, has certain limitations in clinical application due to its severe and unpredictable delayed diarrhea response. In this pathogenesis, hCES2A plays a key role in hydrolytic metabolism and inhibits the diarrhea resulting from irinotecan when hCES2A inhibitors are co-administered [16]. Evaluating the inhibitory effects of commonly used drugs on carboxylesterases significantly impacts the clinical efficacy, safety, and tolerance of carboxylesterase substrate drugs.

In this research, 23 cardiovascular medications were chosen. A high-throughput screening system developed by our research group was used to assess the inhibitory effects and mechanisms of action of these drugs on hCES1A and hCES2A (Fig. 1). The findings provide valuable insights for the clinical application of these drugs.

2. Experimental

2.1. Chemicals and reagents

Twenty-three cardiovascular drugs, CPT-11 and SN-38 were obtained from Dalian Meilunbio. Co., Ltd. (Dalian, China) for use in this study. Bis-p-nitrophenyl phosphate (BNPP) and loperamide (LPA) were procured from TCI (Tokyo, Japan). According to our previous work

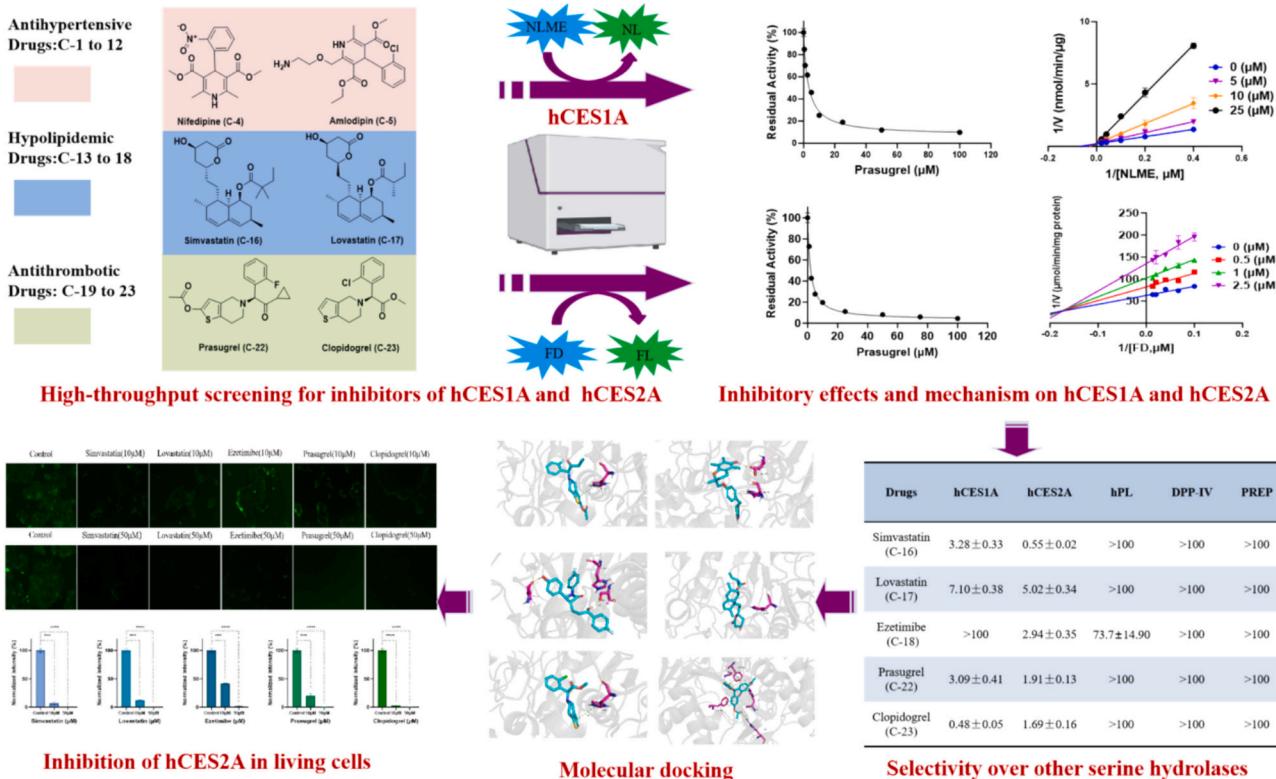


Fig. 1. Schematic diagram of experimental research.

[17], we obtained the specific substrate DDAO-ol and its hydrolyzed product DDAO. Pancrelipase, dipeptidyl peptidase IV (DPP-IV) and hCES2A were derived from recombinant proteins produced in our lab. FD, a fluorogenic substrate for hCES2A, was commercially sourced from TCI Chemical Industry (Tokyo, Japan). The NLME substrate of hCES1A, the GP-BAN substrate of DPP-IV, and the Z-GP-ACM substrate of PREP were successfully synthesized in our laboratory [18]. Every inhibitor and each substrate were dissolved in LC-grade DMSO supplied by Tedia, USA. All inhibitors and substrates were dissolved in LC-grade DMSO provided by Tedia, USA. During all experimental procedures, phosphate-buffered brine at 0.1 M concentration (pH 6.8–7.4), stored at 4 °C, Millipore Water (pH 6.8–7.4), and LC-grade acetonitrile (pH 6.8–7.4) from Tedia, USA were utilized. All the compounds are of purity >98 %, and each solution was stored at 4 °C before use.

2.2. Fluorescence-based enzyme inhibition assays

2.2.1. Fluorescence-based hCES1A inhibition assay

The inhibitory effect of diverse cardiovascular drugs on the hydrolysis of NLME was examined for its potential as an enzyme source [19]. The experimental protocol was carried out as detailed below. Initially, 1 μL of HLM solution, which led to a final concentration of 2 μg/mL, and 1 μL of either drugs or BNPP, achieving a final concentration of 100 μM, were added to 97 μL of 10 mM potassium phosphate buffer (PBS, pH 6.5). The mixture underwent pre-incubation at 37 °C for 10 min. Subsequently, 1 μL of the substrate NLMe, achieving a final concentration of 25 μM, was added to initiate the reaction. Following a 10-min reaction period, 50 μL of luminescence detection reagent (LDR) was added to the reaction mixture. Following an additional 20-min reaction period, luminescence intensity was measured to monitor product formation. A negative control was established, using an equal volume of PBS in place of the enzyme, to measure background fluorescence. Additionally, a blank control using 1 % (v/v) DMSO was established as a reference for 100 % enzyme activity. All data were derived from at least three independent replicates. To determine the IC₅₀ values, human liver microsomes were tested at various concentrations of the selected drugs and bis-p-nitrophenyl phosphate (BNPP). The inhibitory effect of each substance was measured as the percentage decrease in bioluminescent intensity compared to a control group. Specifically, the IC₅₀ is defined as the concentration of an inhibitor that reduces the enzyme activity by 50 %. The IC₅₀ values were determined using nonlinear regression analysis via GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, USA).

2.2.2. Fluorescence-based hCES2A inhibition assay

HLM served as the enzymatic matrix to assess inhibitory effects of clinically used cardiovascular agents on FD hydrolysis [20]. The reaction mixture was prepared by supplementing 196 μL PBS (pH 7.4) with 1 μL HLM (final conc. 2 μg/mL) and 1 μL test compounds/LPA control (final conc. 100 μM). The reaction system was subjected to incubation with agitation (37 °C, 210 rpm) to ensure proper enzyme-substrate interaction. After a 5-min incubation stage, 2 μL of FD, with a final concentration reaching 20 μM, was added to initiate the reaction. Following homogenization, aliquots were dispensed into 96-well opaque microplates for fluorescence quantification using a multimode plate reader. The excitation wavelength was set at 480 nm, the emission wavelength at 525 nm, and fluorescence detection lasted for 20 min. To assess the IC₅₀ values, HLM was exposed to various concentrations of selected drugs and LPA treatment. For the remaining experimental details, refer to the description in 2.2.1.

2.2.3. Fluorescence-based hPL inhibition assay

Using DDAO-ol as the substrate and orlistat as the positive inhibitor for human pancreatic lipase (hPL), we assessed the inhibitory activities of the screened cardiovascular drugs that strongly inhibit hCES1A and hCES2A against hPL. The specific experimental method was described in

our previous publications [17].

2.2.4. Fluorescence-based DPP-IV inhibition assay

Using GP-BAN as the substrate for dipeptidyl peptidase IV (DPP-IV), the inhibitory activities of the screened cardiovascular drugs that exhibit potent inhibitory effects on hCES1A and hCES2A against dipeptidyl peptidase IV were measured. The methodology for assessing DPP-IV inhibition was previously reported [21].

2.2.5. Fluorescence-based PREP inhibition assay

The inhibitory effects of the screened drugs on prolyl endopeptidase (PREP) were determined with Z-GP-ACM as the substrate. The inhibitory effect of PREP on the cardiovascular drugs that were screened out and exhibited significant inhibitory effects on hCES1A and hCES2A was determined. The method for evaluating the inhibitory effect of PREP has been reported previously [18].

2.3. Inhibition kinetic analysis

Inhibition kinetic analysis was conducted on compounds exhibiting strong inhibitory activity against hCES1A and hCES2A (IC₅₀ < 10 μM). To determine the K_i values, varying substrate concentrations (selected based on K_m values) and different inhibitor concentrations were used to measure the corresponding reaction rates. The K_i values were determined by analyzing the secondary slope of Lineweaver-Burk plots in relation to inhibitor concentration. For competitive (a), non-competitive (b), and mixed inhibition (c) modes, all kinetic data were analyzed using the appropriate kinetic equations:

$$\text{Competitive inhibition: } V = (V_{\max}S)/[K_m(1 + I/K_i) + S] \quad (\text{a})$$

$$\text{Non-competitive inhibition: } V = (V_{\max}S)/[(K_m + S) \times (1 + I/K_i)] \quad (\text{b})$$

$$\text{Mixed inhibition: } V = (V_{\max}S)/[(K_m + S) \times (1 + I/\alpha K_i)] \quad (\text{c})$$

Here, V represents the reaction's hydrolytic velocity, V_{max} represents the maximum velocity, and S and I represent the concentrations of the substrate (NLMe, FD) and inhibitor (cardiovascular drugs), respectively. K_i represents the inhibition constant of the tested inhibitor for hCES1A and hCES2A, while K_m represents the Michaelis constant.

2.4. LC-FD based hCES2A inhibition assay

The recombinant hCES2A was used as the enzyme source to evaluate the inhibitory effects of cardiovascular drugs on the hydrolysis of CPT-11. The reaction mixture was prepared as follows: 1 μL of recombinant hCES2A (with a final concentration of 25 μg/mL) and 1 μL of the test drug/LPA (with a series of concentrations) were added to 98 μL of phosphate buffer solution (PBS, pH 7.4). The reaction system was incubated under stirring conditions (37 °C, 210 rpm) to ensure sufficient interaction between the enzyme and the substrate. After 15 min of incubation, 1 μL of CPT-11 (with a final concentration of 5 μM) was added to initiate the reaction. After 60 min of incubation, all samples were treated with an equal volume of acetonitrile. Then, the samples were centrifuged at a speed of 20,000 g, and the supernatants were collected for LC-FD analysis. Both CPT-11 and SN-38 were analyzed using LC-FD (Shimadzu, Japan). A packed VP-ODS C18 column (4.6 mm × 150 mm, 5 μm, Shimadzu) was used. The mobile phase consisted of 0.1 % formic acid in water (A) and acetonitrile (B) with the following gradients: 0.01–1.50 min, 75 % A; from 1.50 to 5.00 min, 75 %–60 % formic acid in water; from 5.00 to 6.00 min, 60 %–10 % formic acid in water; from 6.00 to 8.00 min, 10 %–75 % formic acid in water. The system was operated at a flow rate of 0.4 mL min⁻¹. The peak areas of CPT-11 and SN-38 were detected at Ex = 368 nm and Em = 535 nm.

2.5. Cell culture and cytotoxicity assays

The Caco-2 cell line, derived from colorectal adenocarcinoma, was obtained from the Chinese Academy of Sciences Cell Bank in the Shanghai branch. Cells were cultured in DMEM (Corning) supplemented

with 10 % FBS (Thermo Fisher Scientific) and 100 U/mL penicillin +100 µg/mL streptomycin (Meilun Biotechnology). They were maintained at 37 °C in 5 % CO₂. To evaluate compound effects on Caco-2 cell viability, the Cell Counting Kit-8 (Meilun Biotechnology) was used. Caco-2 cells (5×10^4 cells/mL, 100 µL) were seeded in 96-well plates. After 24 h incubation, cells were treated with test compounds (0–100 µM) for another 24 h. Then, 100 µL of 10 % (v/v) CCK-8 was added, and plates were incubated at 37 °C for 2 h. Absorbance at 450 nm was measured. A control group (cells in medium only) and a blank group (medium +10 % CCK-8) were set up. Additionally, a cell- and drug-free medium was prepared to serve as a reference control. Cell viability was determined by subtracting the mean absorbance of the blank group from those of the control and experimental groups, followed by dividing the adjusted absorbance of the experimental group by that of the control group. Each experiment included at least three independent replicates with multiple wells per condition.

2.6. Fluorescence imaging analysis

Caco-2 cells were cultured in DMEM with 10 % FBS. Once 80 % confluent in a 12-well plate, 1×10^5 cells were seeded per 20-mm-diameter dish and incubated at 37 °C, 5 % CO₂ for 24 h. After incubation, cells were rinsed twice with PBS and treated with simvastatin (C-16), lovastatin (C-17), ezetimibe (C-18), prasugrel (C-22), and clopidogrel (C-23) at 10 µM and 50 µM for 24 h. A 50 mM probe FD stock solution was diluted to 20 µM in MEM without NEAA and FBS, added to cells for 30-min incubation. 15 min later, 5 µL of Hoechst 33,342 (Beyotime Biotechnology) was added for nuclear staining. Cells were washed twice with PBS (pH 7.4), fixed with 4 % paraformaldehyde for 15 min at RT, washed again with PBS. Finally, PBS was added, and fluorescence imaging was done with a CLSM (Leica, Germany), capturing blue (430–480 nm) and green (520–570 nm) emissions.

2.7. Molecular docking

To delve into the binding mechanisms of simvastatin (C-16), lovastatin (C-17), ezetimibe (C-18), prasugrel (C-22), and clopidogrel (C-23) with hCES2A at the molecular level, hCES2A was selected as the receptor and the aforementioned drugs were used as ligands. Molecular docking was then performed using Maestro (Version 13.5), a software developed by Schrödinger [22]. First, the crystal structure of hCES2A was downloaded from the SWISS-MODEL repository (UniProtKB AC: [000748](#)). Following standard operating procedures, hydrogen atoms were incorporated, bond orders and charges were assigned, and energy minimization was carried out using the OPLS4 force field. Subsequently, using the SiteMap module of Maestro, the binding sites of the receptor were identified at the center of the active pocket grid box defined by coordinates x = -69.68, y = -29.67, and z = 9.55. The overall grid box was set to a size of $20 \times 20 \times 20 \text{ Å}^3$. Molecular docking was performed via the Glide module in Maestro, utilizing the extra precision mode and 32 processors for calculations to identify binding poses with the lowest binding energy.

2.8. Statistical analysis

Systematic and reliable measurements were systematically conducted in triplicate mode. The experimental results were expressed in the form of mean ± standard deviation (SD). The IC₅₀ and K_i values of the cardiovascular drugs that strongly inhibited hCES1A and hCES2A were determined using non-linear regression analysis in GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, USA).

3. Results and discussion

3.1. Screening of inhibitors of hCES1A and hCES2A

Previous research studies have been validated as providing unambiguous evidence that NLMe and FD are established as reliable probe substrates for hCES1A and hCES2A [19,20]. The enzymatic activities of hCES1A and hCES2A within HLM can be accurately determined by quantification of the amounts of NL and fluorescein, which represent the hydrolyzed products of NLMe and FD, respectively. In the present study, the inhibitory effects of 23 cardiovascular drugs, as shown in Fig. 2, and SR26334, the hydrolytic product of clopidogrel by human carboxylesterase, on hCES1A and hCES2A were evaluated. The research findings demonstrate that at an inhibitory concentration of 100 µM, five drugs exhibit highly potent and highly significant inhibitory activities against hCES1A, resulting in <50 % residual inhibition Fig. 3. In addition, ten drugs exhibit similar remarkable and potent inhibitory effects on hCES2A, resulting in residual activity levels below 50 %. Simultaneously, the residual activities of BNPP (a positive inhibitor of hCES1A) and LPA (a positive inhibitor of hCES2A), both at a final concentration of 100 µM, were 0.08 % and 13.50 %, respectively, thereby demonstrating the feasibility of this screening system. 23 drugs exhibiting their residual activities after inhibiting NLMe and FD hydrolysis at 100 µM are summarized in Table S1.

3.2. Inhibitory effects of cardiovascular drugs against hCES1A and hCES2A

To more deeply and accurately explore the inhibitory characteristics and potencies of these drugs, we subsequently conducted the calculation of the half-maximal inhibitory concentration (IC₅₀). The concentration-response relationships of the inhibitors were examined. The results showed that simvastatin (C-16), lovastatin (C-17), prasugrel (C-22) and clopidogrel (C-23) had strong inhibitory effects on hCES1A and hCES2A, with IC₅₀ values below 10 µM (Table 1). Meanwhile, ezetimibe (C-18) shows a strong inhibitory effect on hCES2A (IC₅₀ = 2.94 µM) but no inhibitory effect on hCES1A (IC₅₀ > 100 µM). amlodipine (C-5), verapamil (C-6), atorvastatin (C-13) and ticagrelor (C-21) display relatively weak inhibitory efficacies on hCES2A, specifically manifested as IC₅₀ > 10 µM (Fig. S1). It is worth noting that nifedipine (C-4), has a weak inhibitory effect on both hCES1A and hCES2A, with its IC₅₀ value exceeding 10 µM.

Following a detailed analysis of the inhibitory effects of atorvastatin (C-13), rosuvastatin (C-14), pravastatin (C-15), simvastatin (C-16), and lovastatin (C-17) on hCES1A and hCES2A, these findings collectively demonstrate that the lactone ring structure found in simvastatin (C-16) and lovastatin (C-17) plays a significant role in inhibiting carboxylesterases. Conversely, lactone ring-opening to yield free hydroxy acids renders detrimental to carboxylesterase inhibition. A detailed comparison of the inhibitory effects of clopidogrel (C-23) and SR26334 (C-24) on hCES1A and hCES2A reveals that the 5-methyl ester group in clopidogrel (C-23) facilitates carboxylesterase inhibition. In direct contrast, when the 5-methyl ester group is transformed into a 5-carboxylic acid (as seen in SR26334, C-24), the inhibition of carboxylesterases has a detrimental effect, as shown in Tables 1 and S1.

3.3. Inhibition mechanism analyses of identified inhibitors towards hCES1A

The inhibitory mechanisms of the identified inhibitors on hCES1A-mediated NLMe hydrolysis were elucidated using a range of NLMe and selected drug concentrations. These drugs exhibited IC₅₀ values below 10 µM. Based on Fig. 4, Lineweaver-Burk plots indicated that simvastatin (C-16) and lovastatin (C-17) were non-competitive inhibitors, whereas prasugrel (C-22) and clopidogrel (C-23) were competitive inhibitors of hCES1A in HLM. The K_i values in Table 2 clearly demonstrate

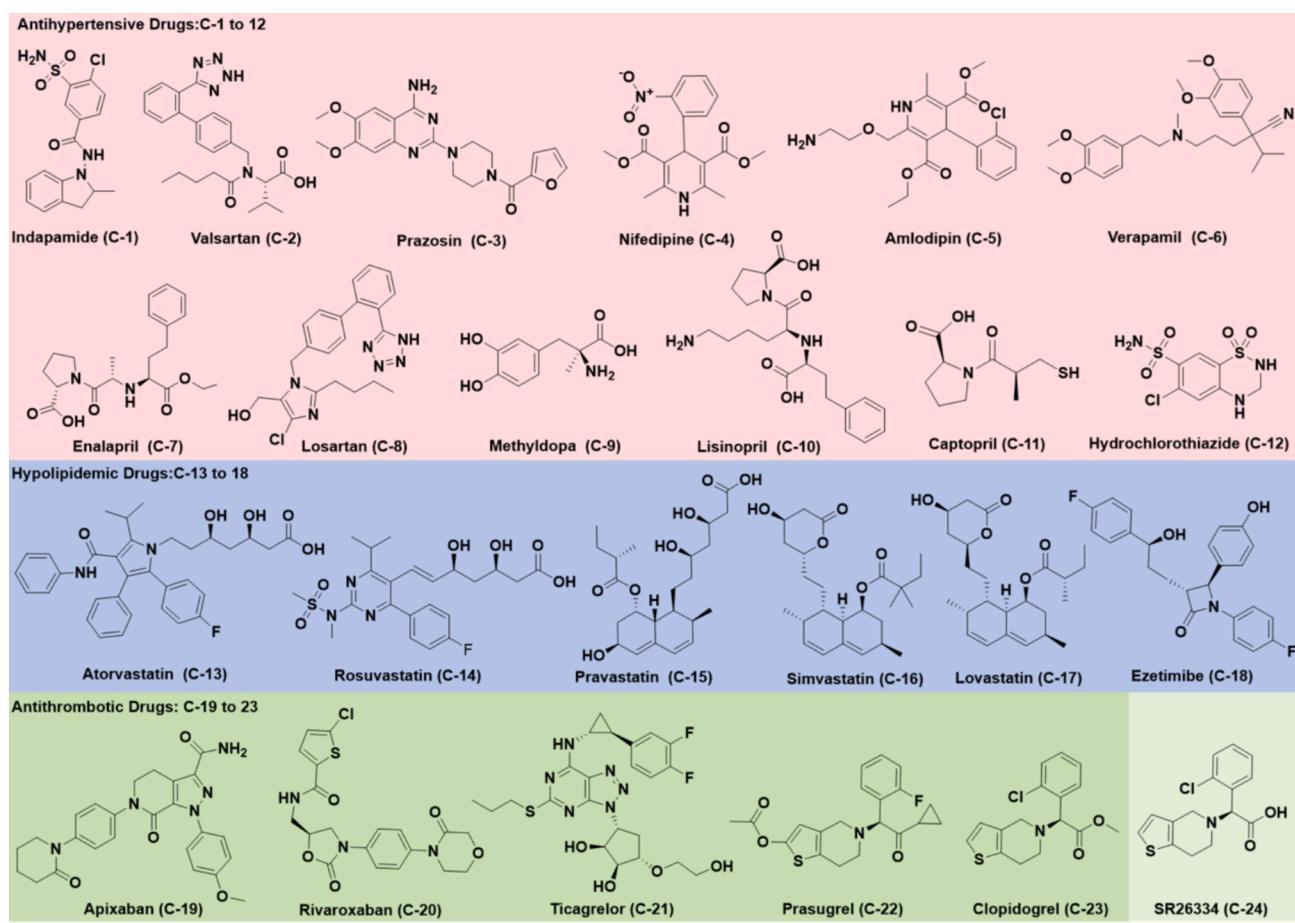


Fig. 2. Chemical structures of cardiovascular drugs.

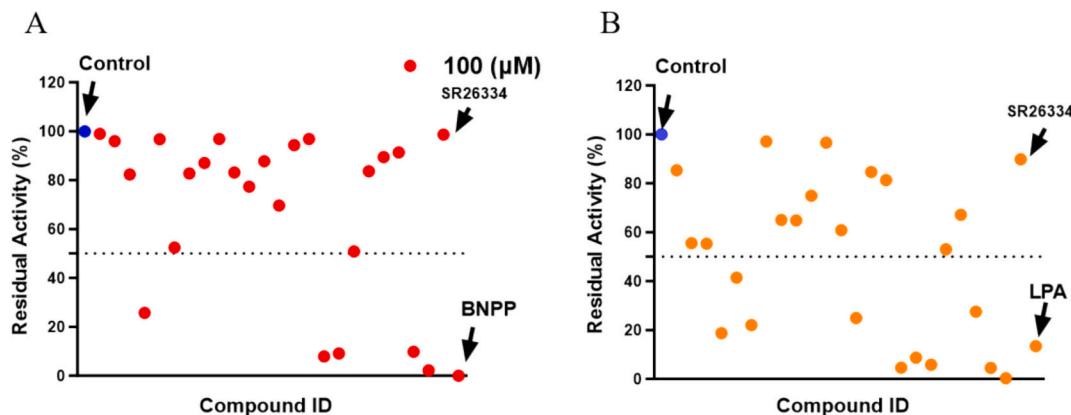


Fig. 3. The inhibitory effects of cardiovascular drugs against hCES1A (A) and hCES2A (B).

Table 1
IC₅₀ values of cardiovascular drugs towards hCES1A and hCES2A in HLM.

Drugs	hCES1A IC ₅₀ (μM)	hCES2A IC ₅₀ (μM)	Drugs	hCES1A IC ₅₀ (μM)	hCES2A IC ₅₀ (μM)
Nifedipine(C-4)	17.47 ± 2.22	28.56 ± 4.85	Lovastatin(C-17)	7.10 ± 0.38	5.02 ± 0.34
Amlodipine(C-5)	>100	16.76 ± 3.15	Ezetimibe(C-18)	>100	2.94 ± 0.35
Verapamil(C-6)	>100	23.28 ± 3.62	Ticagrelor(C-21)	>100	11.86 ± 2.01
Atorvastatin(C-13)	>100	26.73 ± 5.24	Prasugrel(C-22)	3.09 ± 0.41	1.91 ± 0.13
Simvastatin(C-16)	3.28 ± 0.33	0.55 ± 0.02	Clopidogrel(C-23)	0.48 ± 0.05	1.69 ± 0.16

All data were shown as mean ± SD ($n = 3$).

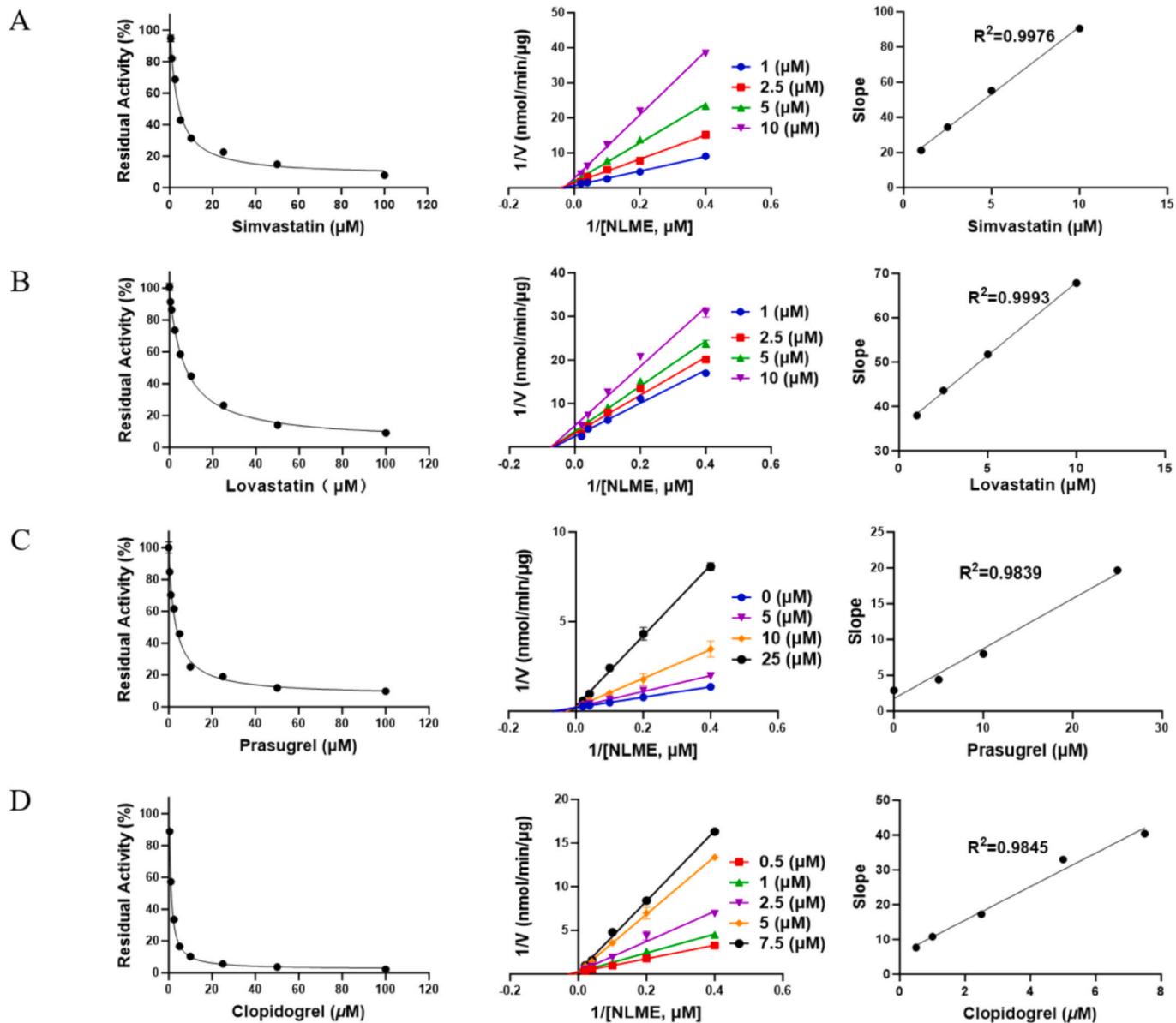


Fig. 4. Inhibition of hCES1A-mediated NLMe hydrolysis by Simvastatin (C-16, A), Lovastatin (C-17, B), Prasugrel (C-22, C), Clopidogrel (C-23, D). Left: Curves of dose-dependent inhibition. Middle: Lineweaver-Burk plots. Right: Second plots of slopes derived from the Lineweaver-Burk plots. HLM (2 μg/mL) was pre-incubated with different concentrations of the drug for 10 min. Then, the reaction was initiated by adding the substrate NLME (25 μM) and allowed to proceed for 10 min. Subsequently, a 20-minute fluorescence reaction with LDR was carried out. The data presented are the average values obtained from three repeated measurements.

Table 2
Inhibitory parameters of hCES1A in HLM.

Drugs	K_i (μM)	Inhibition mode	Goodness of fit (R^2)
Simvastatin(C-16)	1.65 ± 0.25	Non-competitive	0.9839
Lovastatin(C-17)	9.71 ± 1.49	Non-competitive	0.9806
Prasugrel(C-22)	4.71 ± 0.59	Competitive	0.9854
Clopidogrel(C-23)	0.51 ± 0.07	Competitive	0.9823

Data were shown as mean ± SD ($n = 3$).

the strong inhibitory effects of the inhibitors.

3.4. Inhibition mechanism analyses of identified inhibitors towards hCES2A

Various concentrations of FD and drugs with $IC_{50} < 10 \mu M$ were tested to investigate the inhibitory mechanisms of identified inhibitors

on hCES2A-mediated FD hydrolysis. As depicted in Fig. 5, the Lineweaver-Burk plots evidently revealed that simvastatin (C-16), lovastatin (C-17), prasugrel (C-22), and clopidogrel (C-23) functioned as mixed-competitive inhibitors of hCES2A. Notably, ezetimibe (C-18) acts as a non-competitive inhibitor of hCES2A. The K_i values, as presented in Table 3, clearly demonstrate that the drugs previously mentioned exhibit potent inhibitory effects on hCES2A.

3.5. Selectivity of five drugs towards hCES1A and hCES2A over other serine hydrolases

To further assess the selectivity of the resultant hCES1A and hCES2A inhibitors, simvastatin (C-16), lovastatin (C-17), ezetimibe (C-18), prasugrel (C-22), and clopidogrel (C-23), against other serine proteases, the inhibitory potential of dipeptidyl peptidase-4 (DPP-IV), proline endopeptidase (PREP), and human pancreatic lipase (hPL) was evaluated. The investigations utilized the probe substrates GP-BAN, Z-GP-ACM, and

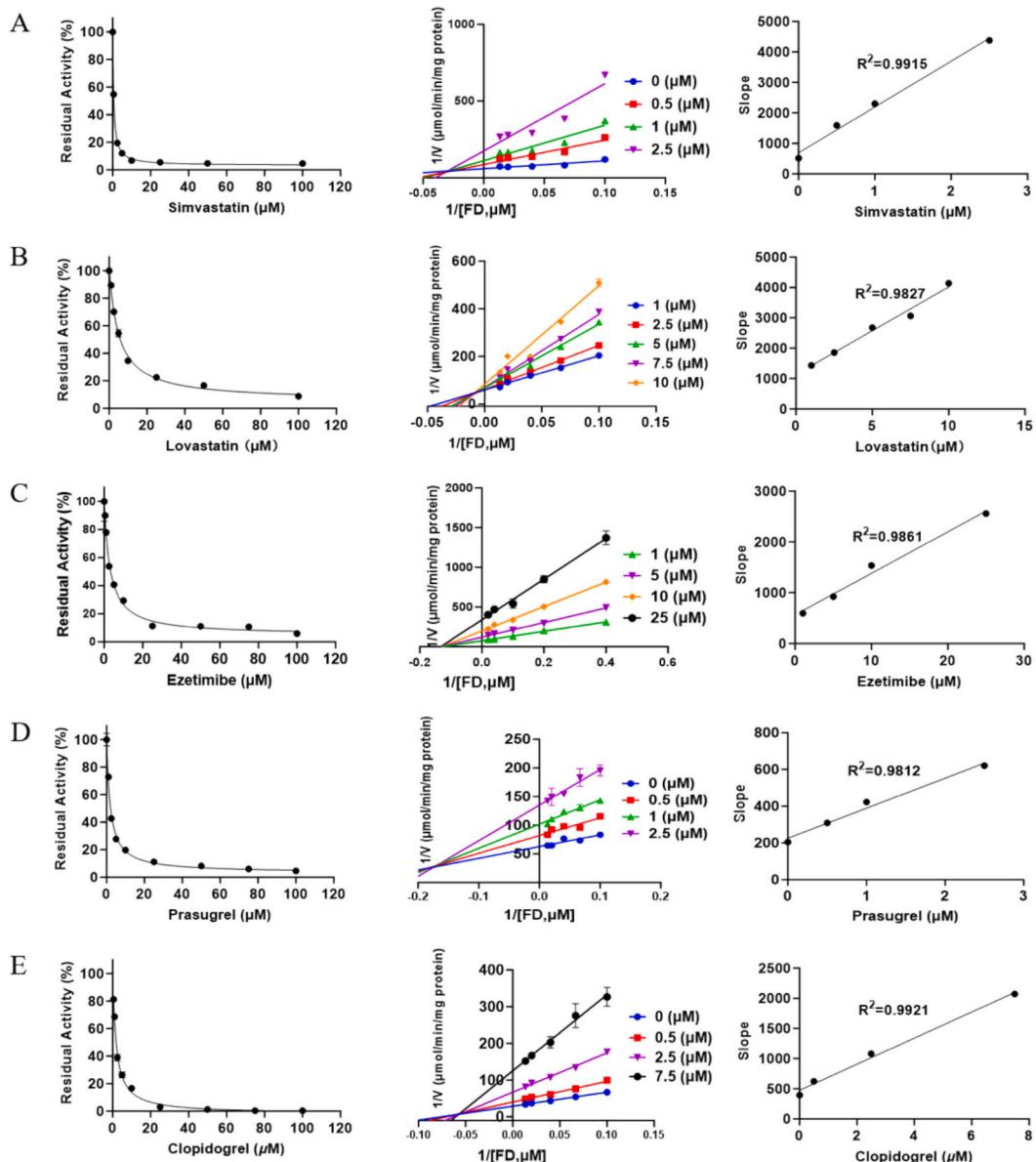


Fig. 5. Inhibition of hCES2A-mediated FD hydrolysis by Simvastatin (C-16, A), Lovastatin (C-17, B), Prasugrel (C-22, C), Clopidogrel (C-23, D). Left: Curves of dose-dependent inhibition. Middle: Lineweaver-Burk plots. Right: Second plots of slopes derived from the Lineweaver-Burk plots. The data presented are the average values obtained from three repeated measurements. HLM (2 $\mu\text{g}/\text{mL}$) was pre-incubated with various concentrations of the drug for 10 min. Subsequently, the reaction was initiated by adding the substrate FD (20 μM) and allowed to proceed for 20 min. The data presented are the average values obtained from three repeated measurements.

Table 3
Inhibitory parameters of hCES2A in HLM.

Drugs	K_i (μM)	Inhibition mode	Goodness of fit (R^2)
Simvastatin(C-16)	0.27 ± 0.09	Mixed inhibition	0.9826
Lovastatin(C-17)	5.65 ± 2.24	Mixed inhibition	0.9814
Ezetimibe(C-18)	5.03 ± 0.44	Non-competitive	0.9864
Prasugrel(C-22)	1.19 ± 0.99	Mixed inhibition	0.9610
Clopidogrel(C-23)	1.45 ± 0.63	Mixed inhibition	0.9836

Data were shown as mean \pm SD ($n = 3$).

DDAO-ol, respectively, which had been developed earlier in the research process [17,18,21]. The experimental results are presented in Table 4. The results demonstrated that these five drugs did not exhibit inhibitory effects on DPP-IV and PREP. hPL is responsible for the metabolic processing of fats in vivo and is currently acknowledged as a key target for

obesity treatment. The experimental results clearly demonstrate that ezetimibe exhibited moderate inhibitory activity, with an IC_{50} value of 73.7 μM , while the other four inhibitors did not demonstrate any inhibitory activity on hPL.

3.6. Verification of the inhibitory effects of the identified inhibitors on CPT-11

CPT-11 is a commonly used first-line anti-cancer drug in clinical practice. Meanwhile, it is metabolically activated by hCES2A in the body and generates SN-38. Verifying the metabolic inhibition of the above-mentioned drugs on CPT-11 can further clarify whether the combined use of drugs affects the efficacy and toxicity of CPT-11. It could provide key evidence for rational clinical drug use, avoiding adverse reactions, and optimizing treatment regimens. Therefore, we used a series of concentrations of simvastatin (C-16), lovastatin (C-17), ezetamibe (C-

Table 4

The inhibitory effects of drugs Simvastatin (C-16), Lovastatin (C-17), Ezetimibe (C-18), Prasugrel (C-22) and Clopidogrel (C-23) towards other serine hydrolases.

Drugs	hCES1A	hCES2A	hPL	DPP-IV	PREP
Simvastatin (C-16)	3.28 ± 0.33	0.55 ± 0.02	>100	>100	>100
Lovastatin (C-17)	7.10 ± 0.38	5.02 ± 0.34	>100	>100	>100
Ezetimibe (C-18)	>100	2.94 ± 0.35	73.7 ± 14.90	>100	>100
Prasugrel (C-22)	3.09 ± 0.41	1.91 ± 0.13	>100	>100	>100
Clopidogrel (C-23)	0.48 ± 0.05	1.69 ± 0.16	>100	>100	>100

Data were shown as mean ± SD ($n = 3$). The values in the table represent the IC₅₀ values (μM) of chosen drugs towards the target enzymes hCES1A, hCES2A, hPL, DPP-IV and PREP.

18), prasugrel (C-22), and clopidogrel (C-23) to verify their metabolic inhibitory effects on CPT-11 at LC-FD. LPA was used as a positive control to verify the authenticity and reliability of this method. As shown in Figs. 6 and S2, the results showed that the above-mentioned drugs had a significant inhibitory effect on hCES2A-mediated CPT-11 hydrolytic metabolism, and their IC₅₀ values were all lower than 10 μM (as shown in Table 5). This result directly verified that the combined use of these drugs with CPT-11 may lead to metabolic drug-drug interactions.

3.7. Docking simulation

The molecular docking simulations investigated the interaction patterns of simvastatin (C-16), lovastatin (C-17), ezetimibe (C-18), prasugrel (C-22), and clopidogrel (C-23) with hCES2A (Figs. 7 and S3). The five compounds were evaluated, and the docking score of simvastatin with the hCES2A structural protein was -5.566 kcal/mol, as shown in Table 6. The docking outcomes indicated that simvastatin exhibited hydrogen bonding with the HIP457 groups of the protein. The docking score of lovastatin with the hCES2A structural protein, as presented in Table 6, was -4.352 kcal/mol. The docking score of ezetimibe with the hCES2A structural protein was -6.846 kcal/mol (Table 6). The docking analysis revealed that the compound formed hydrogen bonds with residues such as Val102 and SER228 in the protein.

After interacting with the hCES2A structural protein, prasugrel achieved a docking score of -6.705 kcal/mol, compared to clopidogrel, which had a docking score of -6.362 kcal/mol (as shown in Table 6). The results of docking analysis indicated that both compounds engaged in π - π interactions with HIP457 in the protein structure.

Furthermore, probe substrate FD was docked with the hCES2A structural protein, achieving a docking score of -5.395 kcal/mol (Table 6). Docking results showed that FD formed hydrogen bonds with PHE153, HIP457, and PHE469 within the protein structure. Besides

Table 5

IC₅₀ values of cardiovascular drugs for the inhibition of hCES2A-mediated CPT-11 hydrolytic metabolism.

Drugs	hCES2A IC ₅₀ (μM)	Goodness of fit (R^2)
Simvastatin(C-16)	0.39 ± 0.06	0.9997
Lovastatin(C-17)	2.19 ± 0.88	0.9614
Ezetimibe(C-18)	0.33 ± 0.04	0.9998
Prasugrel(C-22)	1.24 ± 0.17	0.9990
Clopidogrel(C-23)	0.30 ± 0.04	0.9836
LPA	0.72 ± 0.07	0.9996

All data were shown as mean ± SD ($n = 3$).

hydrogen bonding, there were π - π interactions between FD and residues GLY148 and ARG449 in the protein.

The above-mentioned studies revealed that the amino acid residues with which the probe substrate FD and the drugs (Simvastatin, Lovastatin, Ezetimibe, Prasugrel, and Clopidogrel) interacted within the hCES2A active cavity were significantly different (Table 6). The results indicate that ezetimibe interacts via hydrogen bonds with residues Val102, SER228, and others upon binding to the structural protein of hCES2A. Conversely, when the probe substrate FD binds to hCES2A, it forms hydrogen bonds with residues PHE153, HIP457, PHE469, etc., and engages in π - π interactions with GLY148 and ARG449. The disparity in binding sites between ezetimibe and the probe substrate FD for hCES2A demonstrates significant differences in binding sites, which is consistent with the characteristic of non-competitive inhibition. This inhibition mechanism allows both the inhibitor and the substrate to bind to distinct sites on the enzyme simultaneously. Furthermore, simvastatin interacts with the HIP457-binding group of hCES2A via hydrogen bonds, whereas prasugrel and clopidogrel interact with the same HIP457-binding group via π - π interactions. FD additionally forms hydrogen

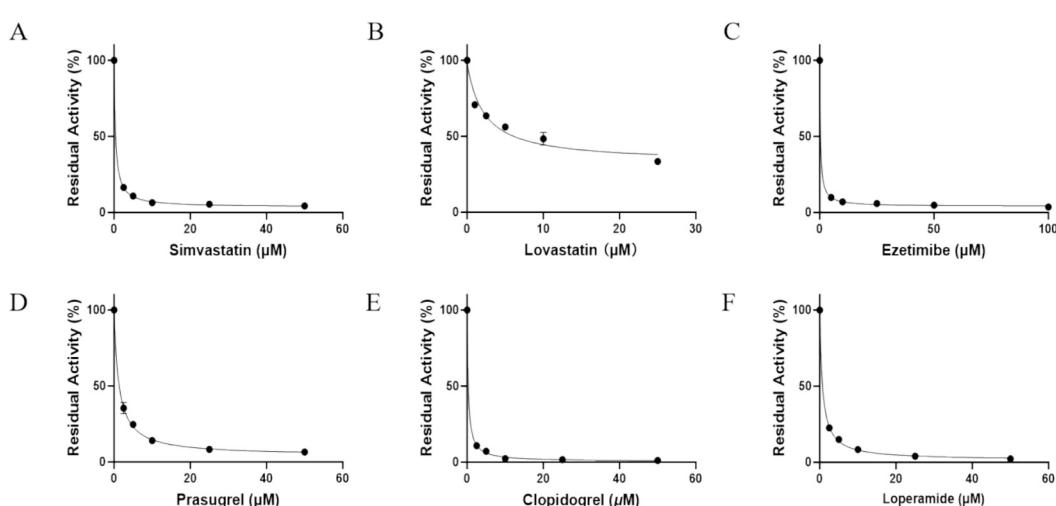


Fig. 6. Inhibition of hCES2A-mediated CPT-11 hydrolysis by Simvastatin (C-16, A), Lovastatin (C-17, B), Ezetimibe (C-18, C), Prasugrel (C-22, D), Clopidogrel (C-23, E), and Loperamide (C-23, F). hCES2A (25 $\mu\text{g}/\text{mL}$) was pre-incubated with various concentrations of the drug for 10 min. Subsequently, the reaction was initiated by adding the substrate CPT-11 (5 μM) and allowed to proceed for 60 min.

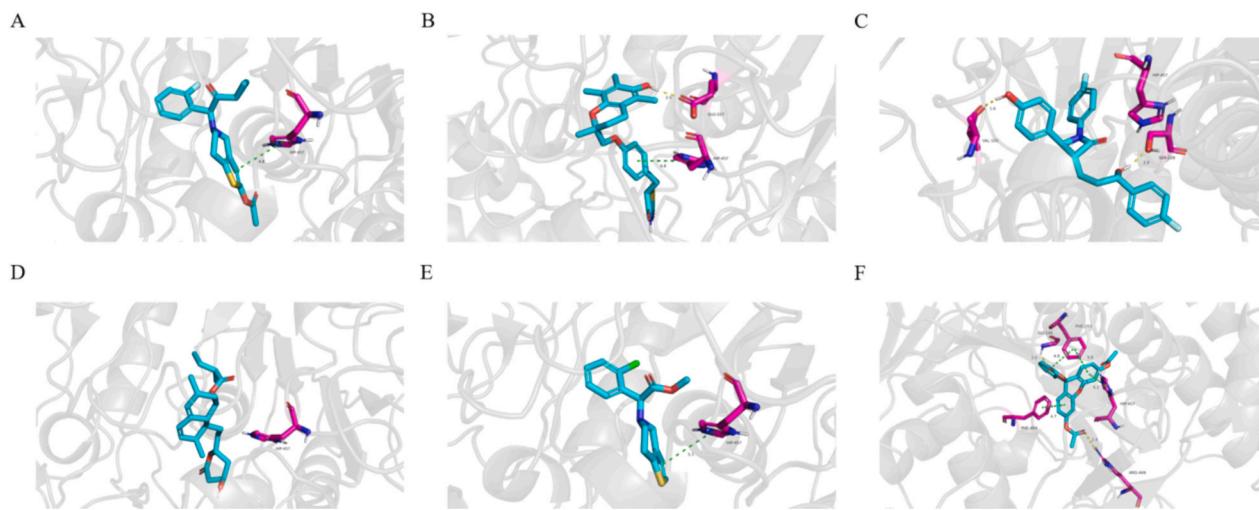


Fig. 7. Docking interactions between tri(indolyl)methanes/FD and hCES2A. Simvastatin (A), Lovastatin (B), Ezetimibe(C), Prasugrel (D), Clopidogrel (E) and FD (F), overall view with hCES2A.

Table 6
Inhibition parameters towards hCES2A.

Ligand_name	Docking_score (kcal/mol)	Pi-Pi stocking	Hydrogen bond
Simvastatin(C-16)	-5.566	–	HIP457
Lovastatin(C-17)	-4.352	–	–
Ezetimibe(C-18)	-6.846	–	Val102, SER228
Prasugrel(C-22)	-6.705	HIP457	–
Clopidogrel(C-23)	-6.362	HIP457	–
FD	-5.395	PHE153,HIP457, PHE469	GLY148, ARG449

bonds with HIP457. This indicates that when these drugs bind to hCES2A, there is a scenario where partial binding site overlap occurs. This represents a key feature of mixed-type inhibition, indicating both partial overlap and partial congruence in the binding sites between the inhibitor and substrate. The studies above demonstrate that these inhibitors (C-16-18 and C-22-23) and substrate FD exhibit significant differences from their interacting amino acid residues in the hCES2A active cavity (**Table 6**), which may explain why these inhibitors do not exhibit competitive inhibition.

3.8. Cell culture and fluorescence imaging analysis

To evaluate the inhibitory effects of simvastatin (C-16), lovastatin (C-17), ezetimibe (C-18), prasugrel (C-22), and clopidogrel (C-23) on hCES2A *in vitro*, their cytotoxicity against Caco-2 cells was first assessed. As shown in Fig. S4, the five drugs exhibited weak cytotoxicity

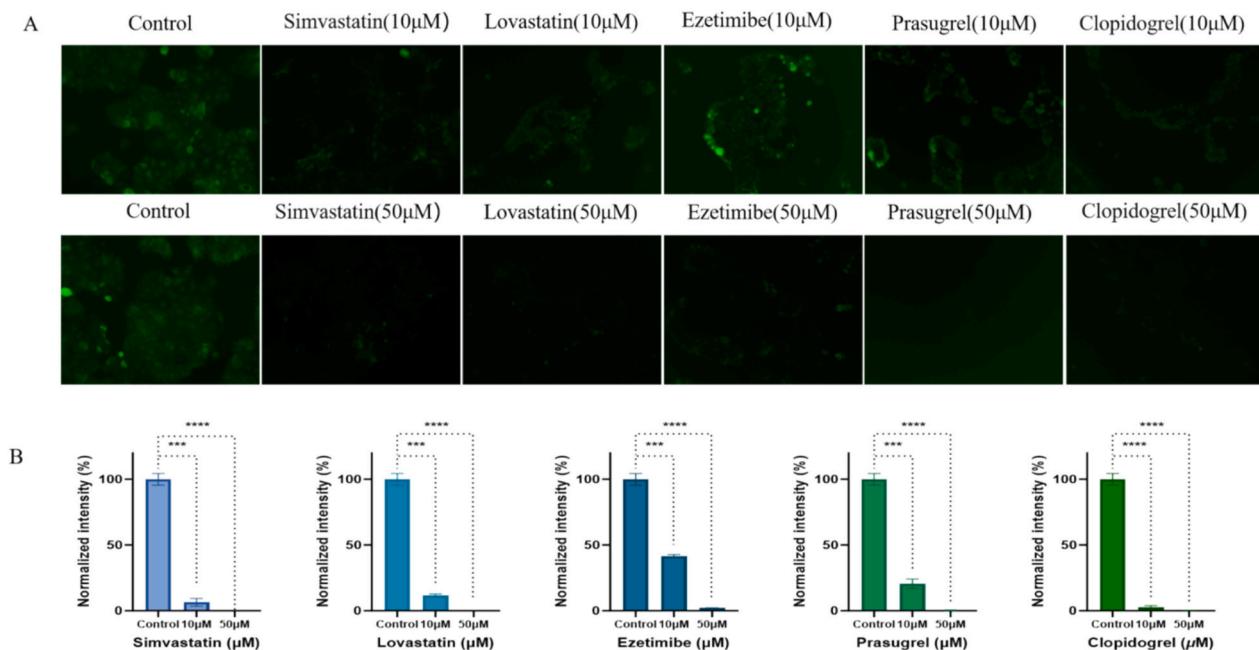


Fig. 8. (A) Fluorescence images of the inhibitory effects of simvastatin, lovastatin, ezetimibe, prasugrel, and clopidogrel on hCES2A in living Caco-2 cells. (B) Inhibition of cellular CES2A activity by different concentrations of Simvastatin (C-16), Lovastatin (C-17), Ezetimibe (C-18), Prasugrel (C-22), and Clopidogrel (C-23). The data were expressed as mean \pm SD, *** p < 0.001 vs. Control group , **** p < 0.0001 vs. Control group.

against Caco-2 cells at concentrations up to 100 μM . To evaluate the cellular-level inhibition effects, 10 and 50 μM concentrations of the tested drugs were used, with fluorescence images presented in Fig. 8A. All five drugs demonstrated concentration-dependent inhibition of intracellular hCES2A-mediated FD hydrolysis, resulting in a reduction in fluorescence intensity within the cells. As shown in Fig. 8B, quantitative analysis of fluorescence intensity was performed at the cellular level using Image J software. Results demonstrate that the five tested drugs are permeable to membranes and exhibit the ability to inhibit endothelial hCES2A within living cells.

4. Conclusion

Carboxylesterases, as phase I enzymes in the human body, play a significant role in the metabolism of diverse drugs. Clinically, numerous cardiovascular drugs might influence their activities. This study systematically characterized the *in vitro* inhibitory profiles of 23 marketed cardiovascular agents against hCES1A and hCES2A. The hypolipidemic agents simvastatin (C-16, $K_i = 1.65 \mu\text{M}$) and lovastatin (C-17, 9.71 μM), along with antithrombotic compounds prasugrel (C-22; 4.71 μM) and clopidogrel (C-23, 0.51 μM), demonstrated potent hCES1A inhibition with distinct inhibitory capacities. At the same time, these drugs also significantly inhibited the activity of hCES2A with K_i values of 0.27 μM , 5.65 μM , 1.19 μM , and 1.45 μM respectively. Notably, the hypolipidemic agent ezetimibe (C-18, $K_i = 5.03 \mu\text{M}$) exhibited selective hCES2A inhibition without cross-activity. Kinetic analyses delineated distinct inhibition mechanisms: C-16/C-17 acted as non-competitive inhibitors of hCES1A, contrasting with C-22/C-23 functioning as competitive inhibitors; while all four compounds demonstrated mixed-type inhibition towards hCES2A, C-18 emerged as a non-competitive inhibitor of this isoform. Molecular docking results showed that the substrate FD interacts with different amino acid residues in the active cavity of hCES2A compared to the aforementioned drugs. This may explain why these inhibitors fail to exhibit competitive inhibition. Detailed research has clearly shown that the cytotoxicity of the five drugs is extremely low and negligible. These drugs can significantly inhibit the activity of hCES2A in Caco-2 cells, with the inhibitory effect becoming increasingly pronounced as drug concentrations increase. This investigation definitively establishes the HMG-CoA reductase inhibitors simvastatin and lovastatin, along with the antiplatelet agents prasugrel and clopidogrel, as potent and selective dual inhibitors of both hCES1A and hCES2A isoforms. Moreover, ezetimibe exhibits strong inhibitory activity against hCES2A. In addition, in order to further strengthen the correlation between the inhibitory potency and the functional metabolic outcomes, the metabolic inhibitory effects of the above-mentioned drugs on CPT-11 were verified. The results showed that all of the above-mentioned drugs could significantly inhibit the conversion of CPT-11 into the active metabolite SN-38. These research results can undoubtedly provide valuable reference for the formulation and optimization of clinical medication regimens, enabling clinical medical workers to make more scientific and rational decisions in drug selection and use. However, these findings still require further investigation and verification through *in vivo* experiments and clinical trials. Furthermore, further mechanistic studies on the specific roles of carboxylesterase in clinical drug metabolism and the potential risks/benefits when co-administered with cardiovascular drugs can be conducted through in-depth research and prediction using omics technologies and network pharmacology [23,24].

CRediT authorship contribution statement

Chang-Sheng Lv: Writing – original draft, Formal analysis. **Bin Wang:** Writing – original draft, Formal analysis. **Yan-Ze Zheng:** Methodology. **Yu-Heng Wang:** Methodology. **Jing Zhang:** Investigation. **Ze-Jun Jiang:** Investigation. **Jin-Guang Wang:** Writing – review & editing, Supervision, Project administration. **Li-Wei Zou:** Writing – review &

editing, Supervision, Project administration.

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Declaration of competing interest

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2025.143967>.

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