Evaluation of the Inhibitory Effects of Antihypertensive Drugs on Human Carboxylesterase In Vitro

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Summary: Human carboxylesterase (CES) 1A and CES2, two major forms of human CES, dominate the pharmacokinetics of most prodrugs such as imidapril and irinotecan (CPT-11). Antihypertensive drugs are often prescribed for clinical therapy concurrently with others. Moreover, two or more antihypertensive drugs are ubiquitously combined. The influences of antihypertensive drugs on the activity of CES remain undefined. In the present study, the inhibitory effects of 17 antihypertensive drugs on the CES1A1 and CES2 activities were evaluated. Imidapril and CPT-11 were used as substrates and cultured with liver microsomes in vitro. The imidapril hydrolase activities by recombinant CES1A1 and human liver microsomes (HLM) were intensely inhibited by telmisartan and nitrendipine (K_i = 0.49 ± 0.09 and 1.12 ± 0.39 µM for CES1A1, 1.69 ± 0.17 µM and 1.24 ± 0.27 µM for HLM, respectively). However, other drugs did not exert strong inhibition. The enzyme hydrolase activity of recombinant CES2 was substantially inhibited by diltiazem and verapamil (K_i = 0.25 ± 0.02 and 3.84 ± 0.99 µM, respectively). Hence, diltiazem, verapamil, nitrendipine and telmisartan may attenuate the drug efficacy of catalyzed prodrugs by changing the activities of CES1A1 and CES2.

Keywords: carboxylesterase; antihypertensive drugs; enzyme kinetics; inhibition; microsomes; toxicology; prodrugs; drug interaction

Introduction

Human carboxylesterase (CES), a member of the serine hydrolase superfamily, metabolizes a variety of ester, carbamate, thioester and amide drug compounds, environmental toxicants and carcinogens. In humans, the CES1A and CES2 families are the two major isoforms of CES and they play important roles in drug metabolism. The two isoforms of human CES1A, CES1A1 and CES1A2, share high homology at the mRNA level (99.3%).1) Since mature proteins produced from both CES1A1 and CES1A2 mRNA are identical, Hosokawa et al. revealed that in the liver, the levels of CES1A1 mRNA transcribed from the CES1A1 gene were substantially higher than those of CES1A2 mRNA transcribed from the CES1A2 gene.2) Therefore, it is plausible that the level of CES1A1 mRNA rather than that of CES1A2 mRNA affects the level of mature protein and enzyme activity.3) CES1A is predominantly expressed in the liver and the lung, whereas CES2 is expressed in the gastrointestinal tract and the liver.4) CES1A mainly catalyzes the hydrolysis of a wide variety of substrates, especially those with acyl moieties sterically larger than alcohol groups, such as imidapril and oseltamivir.5,6) In contrast, CES2 can hydrolyze the substrates with small acyl groups, such as CPT-11 and heroin.7,8)

In multiple drug therapy, drug interactions are important issues that must be taken into consideration. Probably, co-administering several drugs can change the efficacy of each drug per se by inhibiting drug-metabolizing enzymes. Human CES1A and CES2 govern the pharmacokinetic behaviors of most prodrugs, the activities of which are influenced by the direct interactions. Goel et al. conducted a clinical trial to identify potential drug-drug interactions between capecitabine and irinotecan. They exhibited in vitro synergistic anti-cancer activity and both are substrates of CES. The outcome revealed that capecitabine significantly delayed the conversion of irinotecan to SN-38, suggesting the drug-drug interaction at the level of CES.9) Another clinical trial reported that a severe mania episode occurred in a patient treated with

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the combined aripiprazole and dl-three-methylphenidate (MPH), and MPH was mainly metabolized by the human CES1 enzyme. However, the underlying mechanism was unclear.\textsuperscript{[9]} Zhu \textit{et al.} revealed the inhibitory effect of antipsychotics on the metabolism of MPH \textit{in vitro}, and found aripiprazole, thioridazine and fluoxetine were the most potent inhibitors of CES1.\textsuperscript{[10]} Moreover, Fukami \textit{et al.} examined the inhibitory effects of various anti-diabetic and antihyperlipidemic drugs on human CES enzyme activity, and found simvastatin (Ki, 0.11 \(\mu\)M), troglitazone (Ki, 0.62 \(\mu\)M) and fenofoamate (Ki, 0.04 \(\mu\)M) inhibited CES1A1 and CES2 enzyme activities.\textsuperscript{[12]} It is well known that patients suffering from hypertension disease may be prone to heart failure, hyperlipidemia or diabetes, and such patients are concurrently treated from hypertension disease may be prone to heart failure, hyperlipidemia or diabetes, and such patients are concurrently treated with multiple medications including antihypertensive, antihyperlipidemic or anti-diabetic drugs. Furthermore, two or more kinds of antihypertensive drugs are frequently co-prescribed in clinical practice, among which angiotensin-converting enzyme (ACE) inhibitors that are mostly hydrolyzed by CES1A dominate the combined drug regimen. Inhibiting the CES enzyme activity by coadministered drugs may impair the effectiveness of pharmacotherapy or even give rise to toxicity. Nevertheless, there have been hardly any systematic studies assessing the inhibitory potential for CES enzyme activity by antihypertensive drugs in humans performed or published. Therefore, we herein investigate the inhibitory effects of different kinds of antihypertensive drugs on the activities of CES1A and CES2 \textit{in vitro}.

**Materials and Methods**

**Materials:** Imidapril and imidaprilat were purchased from Sigma (St. Louis, MO). CPT-11, SN-38, \textit{p}-nitrophenyl acetate and \textit{p}-nitrophenol were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Cetirizine (purity > 99.7\%) and camptothecine were purchased from National Institute for the Control of Pharmaceutical and Biological Product (Beijing, China). Hydrochlorothiazide, losartan, telmisartan, candesartan, nifedipine, felodipine, nicardipine, diltiazem, verapamil, lacidipine, propanolol, atenolol, metoprolol, timolol, amlodipine, nitrendipine, nicardpine, diltiazem, verapamil, lacidipine, propanolol, atenolol, metoprolol and timolol were purchased from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes (HLM) were purchased from BD Gentest (Woburn, MA). Pooled human jejunal microsomes (HJM) were purchased from Tissue Transformation Technologies (Edison, NJ). Human CES1A1 and human CES2 expressed in Baculovirus-insect cells were obtained from BD Gentest. All other chemicals and solvents were of analytical or HPLC grade.

**Imidaprilat formation:** Imidapril formation from imidapril was determined according to the method described previously with a slight modification.\textsuperscript{[10]} A typical incubation mixture (total volume of 200 \(\mu\)l) contained the microsomes, 100 mM Tris-\(\mathrm{HCl}\) buffer (pH 7.4) and inhibitors. After 2 min of preincubation at 37°C, the reaction was initiated by adding imidapril (100 \(\mu\)M), and then the mixture was incubated at 37°C for 15 min. The reaction was terminated by adding 200 \(\mu\)l of ice-cold methanol. Camptothecine was added as the internal standard. After 10 min of centrifugation at 15,000 \(\times\) g, the supernatant (10 \(\mu\)l) was determined by high performance liquid chromatography. CPT-11 and SN-38 were separated on a Hypersil ODS C\textsubscript{18} (4.6 mm \(\times\) 250 mm, 5 \(\mu\)m) column utilizing the mobile phase of 0.05 mol L\(^{-1}\) phosphate-methanol (50:50, \(v/v\)) containing 0.25% triethylamine (adjusted to pH 3.0 with phosphate),\textsuperscript{[16]} and the chromatograms were recorded by being monitored at the excitation and emission wavelengths of 380 nm and 550 nm, respectively. The linearity of the standard curve of SN-38 was confirmed (\(r = 0.9992\)), within which the concentration in the incubation were determined. The variability and relative recovery rate were suitable for inhibitory study.

**\textit{p}-Nitrophenyl acetate hydrolase activity:** The \textit{p}-nitrophenyl acetate hydrolase activity was determined according to the methods described previously.\textsuperscript{[9,17]}

**Inhibition analysis of CES enzyme activities:** The inhibitory effects of 17 drugs on the imidapril and CPT-11 hydrolase activities were investigated. Hydrochlorothiazide, losartan, telmisartan, candesartan, nifedipine, felodipine, nicardipine, diltiazem and verapamil were dissolved in DMSO. Propranolol, atenolol, metoprolol, timolol, amlodipine, nitrendipine, diltiazem and verapamil were dissolved in distilled water. These drugs were added to the incubation mixtures described above to investigate their inhibitory effects on the imidaprilat and SN-38 formations. The final concentration of DMSO in the incubation mixture was <0.1%. All data were analyzed using the mean ± SD.

To screen of the inhibitory effects, the enzyme activities at 100 \(\mu\)M imidapril and 5 \(\mu\)M CPT-11 were examined in the presence of the 17 drugs (200 \(\mu\)M).\textsuperscript{[18]} For determination of the Ki (inhibition constant) values of hydrolase activity, the concentrations of imidapril ranged between 25 and 200 \(\mu\)M. The concentrations of the inhibitors for the imidapril hydrolase activity test ranged as follows: telmisartan, 0.2 to 2 \(\mu\)M and 1 to 3 \(\mu\)M for recombinant CES1A1 and HLM, respectively; nitrendipine, 0.5 to 5 \(\mu\)M and 0.5 to 10 \(\mu\)M, respectively. The protein concentrations of HLM and CES1A1 were 0.15 and 0.42 mg/ml, respectively.

For determination of the Ki values, the concentrations of CPT-11 ranged from 2 to 20 \(\mu\)M for recombinant CES2 and 5 to 50 \(\mu\)M for HLM and HJM, respectively. The concentrations of the inhibitors for the CPT-11 hydrolase activity test ranged as follows: diltiazem, 0.2 to 2 \(\mu\)M for recombinant CES2, 2 to 15 \(\mu\)M for HLM and 1 to 10 \(\mu\)M for HJM; and verapamil, 0.5 to 10 \(\mu\)M for recombinant CES2, 0.5 to 20 \(\mu\)M for HLM and 5 to 30 \(\mu\)M for HJM. The protein concentrations of recombinant CES2, HLM and HJM were 0.38, 0.06 and 0.14 mg/ml, respectively.

To compare the inhibitory effects, the enzyme activities at 150 \(\mu\)M \textit{p}-nitrophenyl acetate were examined in the presence of the 17 drugs (200 \(\mu\)M).\textsuperscript{[12]} To determine the inhibitor concentration that caused 50% inhibition (IC\textsubscript{50}), the \textit{p}-nitrophenyl acetate hydrolase activities by recombinant CES1A1 and CES2 at 150 \(\mu\)M...
were examined in the presence of the inhibitors. The concentrations of the inhibitors ranged as follows: telmisartan, 0.2–5 µM, nitrendipine, 0.5–30 µM, diltiazem, 0.5–20 µM, and verapamil, 1–50 µM.

The \( K_i \), \( K_m \), and \( V_{max} \) values and inhibition types were determined by fitting the kinetic data to a competitive, noncompetitive, uncompetitive or mixed inhibition model by nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). The \( K_i \), \( K_m \), and \( V_{max} \) values were expressed as the mean ± SD. IC\(_{50}\) values were determined by plotting the percentage of experimental activity to the control one versus log concentration, and fitting the data to a sigmoidal dose-response equation.

Results

Inhibitory effects of 17 drugs on imidapril hydrolase activities of recombinant human CES1A1: The inhibitory effects of the 17 drugs on the imidapril hydrolase activity of human recombinant CES1A1 were investigated (Fig. 1A). DMSO (0.1%) inhibited the imidapril hydrolase activity by CES1A1 by 1.2%. The inhibition rates of the drugs dissolved in DMSO were calculated as the percentages of the tested activity to the control one after eliminating the impact of 0.1% DMSO. The imidapril formation catalyzed by CES1A1 was strongly inhibited by telmisartan (percentage of the control: 0.8%), nitrendipine (1.3%), diltiazem (12%) and felodipine (16%). The activity of recombinant CES1A1 was moderately inhibited by losartan and propranolol (20–50% of the control). The rest inhibited less than 50% of the imidapril hydrolysis activity.

Inhibitory effects of 17 drugs on CPT-11 hydrolase activities of recombinant CES2: To investigate the inhibitory effects of the drugs on human CES2 enzyme activity, the CPT-11 hydrolase activity that was suppressed by 0.1% DMSO by 0.8% was evaluated (Fig. 1B). In case of being dissolved in DMSO, the inhibition rates of the drugs were calculated as the percentages of the experimental activity to the control one. The CPT-11 hydrolase activity of recombinant CES2 was dramatically inhibited by diltiazem (percentage of control: 4.2%) and verapamil (7%), and moderately inhibited by timolol and cilnidipine (20–50% of the control). The others exhibited weak inhibition (>50% of the control).

Inhibition constant and inhibition patterns of imidapril hydrolase activities of recombinant human CES1A1 and HLM: The \( K_i \), values and inhibition patterns of telmisartan and nitrendipine that tremendously inhibited the imidapril hydrolase activities of recombinant CES1A1 and HLM were determined, and the representative Lineweaver-Burk plots are illustrated in Figure 2. The \( K_i \) values of telmisartan and nitrendipine for recombinant CES1A1 were 0.49 ± 0.09 and 1.12 ± 0.39 µM, respectively, with competitive- and mixed-type inhibition. In contrast, the \( K_i \) values of telmisartan and nitrendipine for HLM were 1.69 ± 0.17 and 1.24 ± 0.27 µM with noncompetitive- and competitive-type inhibition, respectively.

Inhibition constant and inhibition patterns of CPT-11 hydrolase activities of recombinant human CES2, HLM, and HJM: The \( K_i \), values and inhibition patterns of diltiazem and verapamil evidently inhibiting the CPT-11 hydrolase activities by recombinant CES2, HLM and HJM were determined, and the representative Lineweaver-Burk plots are shown in Figure 3. The \( K_i \) values of diltiazem and verapamil for recombinant CES2 were 0.25 ± 0.02 and 3.84 ± 0.99 µM with noncompetitive- and competitive-type inhibition, respectively. The \( K_i \) values of diltiazem and verapamil for HLM were 2.89 ± 0.39 and 11.54 ± 1.20 µM with noncompetitive-type inhibition. The \( K_i \) values of diltiazem and verapamil for HJM were 4.67 ± 2.12 and 15.75 ± 2.63 µM with mixed- and noncompetitive-type inhibition, respectively.

Inhibitory effects of 17 drugs on \( p \)-nitrophenyl acetate hydrolase activities of recombinant human CES1A1 and CES2: To compare the inhibitory effects of the drugs on human CES1A1 and CES2 enzyme activities, the activities of the \( p \)-nitrophenyl acetate hydrolase catalyzed by both CES1A1 and CES2 were assessed (Fig. 4). DMSO (0.1%) inhibited the \( p \)-nitrophenyl acetate hydrolase activity by 0.5%. If the drugs were dissolved in DMSO, the inhibition rates were calculated as the percentage of the tested activity to the control one. Telmisartan and nitrendipine remarkably inhibited (percentage of control: 1.8% and 3%, respectively) the \( p \)-nitrophenyl acetate hydrolase activity of CES1A1, but mildly inhibited that of CES2 (higher than 50% of the control). Diltiazem substantially suppressed (21% and 1.6% of the control, respectively) the activities of CES1A1 and CES2.
However, verapamil inhibited their activities differently (5% and 74% of the control, respectively). In addition, nifedipine and felodipine led to modest inhibitions, but the rest performed weakly and diversely.

**IC₅₀ value of p-nitrophenyl acetate hydrolase activities of recombinant human CES1A1 and CES2:*** Telmisartan and nitrendipine intensely inhibited the p-nitrophenyl acetate hydrolase activity of recombinant CES1A1 (Fig. 4A), while diltiazem and verapamil apparently inhibited that of recombinant CES2 (Fig. 4B). To further compare the inhibitory effects of telmisartan, nitrendipine, diltiazem and verapamil on the CES1A1 and CES2 enzyme activities, the IC₅₀ values of p-nitrophenyl acetate hydrolase activity were determined (Fig. 5) based on 150 µM substrate. The IC₅₀ values of telmisartan and nitrendipine for recombinant CES1A1 were 0.89 and 3.7 µM, respectively, while those of diltiazem and verapamil were 18.8 and 20.1 µM. Besides, the IC₅₀ value of telmisartan and nitrendipine for recombinant CES2 were >5 µM and >30 µM, respectively. The IC₅₀ values of diltiazem and verapamil for recombinant CES2 were 3.98 and 7.94 µM, respectively. Thus, the activities of CES1A1 and CES2 were differently inhibited by these drugs.

**Discussion**

Drug interactions may lead to undesirable side effects. Alterations in drug-metabolizing enzymes in particular inhibitions are recognized as a prevalent factor, so it is necessary to understand and clarify the inhibitory effects. Human CES1A and CES2, which account for the biotransformation of both endogenous and exogenous compounds into polar products to be facilely eliminated, determine the drug efficacy eventually. Although antihypertensive drugs and other drugs such as antihyperlipidemic and anti diabetic drugs have been extensively co-prescribed for clinical therapy, the profiles of the drug-drug interaction between antihypertensive drugs and other combined therapeutic drugs have not been fully established. In particular, the influences of the hypertension therapy medications on human CES remain essentially unexplored. In the present study, we investigated the inhibitory effects of antihypertensive drugs on both CES1A1 and CES2 utilizing imidapril and CPT-11 as the representative substrates.

In the imidaprilat formation from imidapril catalyzed by CES1, telmisartan and nitrendipine strongly inhibited both HLM and recombinant CES1A1, and the Kᵣ value of telmisartan for recombinant CES1A1 (0.49 ± 0.09 µM) was lower than that of nitrendipine (1.12 ± 0.39 µM). Furthermore, telmisartan competitively inhibited recombinant CES1A1, suggesting that telmisartan may bind to the active site of CES1A1. The results may partially be attributed to the CES expression pattern and the plastic nature of the active site that accommodates variously structured substrates.¹⁹

SN-38 formation from CPT-11 was inhibited evidently by diltiazem and verapamil, which are calcium channel blocks (CCBs) prescribed simultaneously with ACE inhibitors for hypertension therapy. In this study, we investigated the inhibitory effects of diltiazem and verapamil on HLM, HJM and recombinant CES2. The Kᵣ values of diltiazem for HLM, HJM and recombinant CES2 were all lower than those of verapamil, and the Kᵣ value of verapamil for recombinant CES2 was 15-fold higher than that of diltiazem. Chemically, CCBs are classified into three classes, benzothiazepines (e.g., diltiazem), dihydropyridines (e.g.,...
Fig. 3. Inhibitory effects of diltiazem (A, C and E) and verapamil (B, D and F) on imidapril hydrolase activities of recombinant CES2 (A and B), HLM (C and D) and HJM (E and F)
Each data point represents the mean ± SD. The Kᵢ value represents the mean ± SE.

Fig. 4. Inhibitory effects of 17 antihypertensive drugs on p-nitrophenyl acetate hydrolase activities of recombinant CES1A1 (A) and CES2 (B)
The activities were determined at 150 µM p-nitrophenyl acetate, and the concentrations of the 17 drugs were 200 µM. Each data point represents the mean ± SD. The control activities by recombinant CES1A1 and CES2 were 3.25 nmol/min/mg and 38.9 pmol/min/mg, respectively.

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nifedipine) and phenylalkylamines (e.g., verapamil). Metabolic intermediate (MI) complexing agents, such as diltiazem, commonly contain an amine functional group and allow N-dealkylation. However, the formation mechanisms concerning such complexes on the basis of benzothiazepines and phenylalkylamines have never been reported hitherto. The differently structured diltiazem and verapamil functioned distinctly. In the meantime, the Ki values of diltiazem and verapamil for HLM, HJM and recombinant CES2 also differed. Firstly, more CES1 mRNAs are expressed in human liver than those in the small intestine. In contrast, human CES2 is abundant in the small intestine. Secondly, recombinant CES2 is subject to post-translational modifications specifically in insect cells, which somewhat affects the interactions between the protein and substrates/inhibitors. As a result, the differences between CES2 expressions in human liver and intestine and recombinant factor may contribute to distinct inhibition behaviors against HLM, HJM and recombinant CES2.

To compare the inhibitory effects of telmisartan, nitrendipine, diltiazem and verapamil on human CES1A1 and CES2 enzyme activities, the IC50 values of the p-nitrophenyl acetate hydrolase activity were evaluated (Fig. 5). Telmisartan and nitrendipine inhibited CES1A1 more effectively than CES2. In contrast, diltiazem and verapamil inhibited CES2 more potently than CES1. Moreover, telmisartan and diltiazem showed strongest inhibition on CES1A1 (IC50 = 0.89 µM) and CES2 (IC50 = 3.98 µM), respectively. Collectively, the different inhibitions may be ascribed to the structural differences between drugs and enzymes.

In this study, we found that telmisartan, nitrendipine, diltiazem and verapamil exhibited potent inhibitory effect on human CES in vitro. However, the corresponding results in vivo are still unrevealed because the drugs undergo sophisticated metabolism and may be susceptible to many physiological factors. Fukami et al. reported that simvastatin and lovastatin strongly inhibited the CES1A1 enzyme activity in vitro. However, in contrast to that in vitro study, simvastatin and lovastatin may not affect CES1A1 hydrolase activity in vivo in humans. Thus, the inhibitory effects of drugs on human CES in vivo as well as the relevant mechanisms still need to be evaluated comprehensively.

In conclusion, we evaluated the inhibitory effect of antihypertensive agents on human CES, and found that telmisartan and nitrendipine dramatically inhibited both HLM and recombinant CES1A1. Besides, the CES2 enzyme activity was strongly inhibited by CCBs such as diltiazem and verapamil belonging to the benzothiazepine and phenylalkylamine class, respectively. This study provides theoretical insights for predicting drug-drug interactions.
References


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