

In Vitro Drug Metabolism by Human Carboxylesterase 1: Focus on Angiotensin-Converting Enzyme Inhibitors

Ragnar Thomsen, Henrik B. Rasmussen, Kristian Linnet, and The INDICES Consortium

Section of Forensic Chemistry, Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark (R.T., K.L.); and Institute of Biological Psychiatry, Mental Health Centre Sct. Hans, Copenhagen University Hospital, Roskilde, Denmark (H.B.R.)

Received June 27, 2013; accepted October 18, 2013

ABSTRACT

Carboxylesterase 1 (CES1) is the major hydrolase in human liver. The enzyme is involved in the metabolism of several important therapeutic agents, drugs of abuse, and endogenous compounds. However, no studies have described the role of human CES1 in the activation of two commonly prescribed angiotensin-converting enzyme inhibitors: enalapril and ramipril. Here, we studied recombinant human CES1- and CES2-mediated hydrolytic activation of the prodrug esters enalapril and ramipril, compared with the activation of the known substrate trandolapril. Enalapril, ramipril, and trandolapril were readily hydrolyzed by CES1, but not by CES2. Ramipril and trandolapril exhibited Michaelis-Menten kinetics, while enalapril demonstrated substrate inhibition kinetics. Intrinsic clearances were 1.061, 0.360, and 0.02 ml/min/mg protein for ramipril, trandolapril, and enalapril,

respectively. Additionally, we screened a panel of therapeutic drugs and drugs of abuse to assess their inhibition of the hydrolysis of *p*-nitrophenyl acetate by recombinant CES1 and human liver microsomes. The screening assay confirmed several known inhibitors of CES1 and identified two previously unreported inhibitors: the dihydropyridine calcium antagonist, isradipine, and the immunosuppressive agent, tacrolimus. CES1 plays a role in the metabolism of several drugs used in the treatment of common conditions, including hypertension, congestive heart failure, and diabetes mellitus; thus, there is a potential for clinically relevant drug-drug interactions. The findings in the present study may contribute to the prediction of such interactions in humans, thus opening up possibilities for safer drug treatments.

Introduction

Carboxylesterase 1 (CES1) is the predominant hydrolase in the human liver (Imai, 2006). The enzyme is involved in the metabolism of several therapeutic agents, drugs of abuse, and endogenous compounds. CES1 activates several ester-containing prodrugs, including oseltamivir, dabigatran etexilate, mycophenolate mofetil, and a number of angiotensin-converting enzyme (ACE) inhibitors, including trandolapril, imidapril, benazepril, and quinapril (Takai et al., 1997; Shi et al., 2006; Zhu et al., 2009; Fujiyama et al., 2010; Hu et al., 2013). The enzyme also inactivates a multitude of drugs, including methylphenidate, pethidine, clopidogrel, rufinamide, and oxybutynin (Zhang et al., 1999; Sun et al., 2004; Tang et al., 2006; Williams et al., 2011; Sato et al., 2012).

Polypharmacy is common in the treatment of hypertension, diabetes mellitus, congestive heart failure, and other multifactor diseases.

This work is part of the INDICES research collaboration (*Individualized drug therapy based on pharmacogenomics: Focus on carboxylesterase 1, CES1*) and financed by the Danish Council for Strategic Research, Programme Commission on Individuals, Disease, and Society [Grant 10-092792/DSF].

Primary laboratory of origin: Section of Forensic Chemistry, Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, Frederik V's vej 11, DK-2100 Copenhagen, Denmark

See Appendix for a list of all partners in the INDICES Consortium.

dx.doi.org/10.1124/dmd.113.053512.

Several of the drugs prescribed for these diseases are substrates of CES1. Likewise, drug addicts may abuse several drugs that can be metabolized by CES1 concomitantly. These groups are particularly prone to the potential for clinically relevant drug interactions that involve CES1.

ACE-inhibitors are commonly prescribed antihypertensive agents. Of importance, clinical studies have failed to show consistent antihypertensive action with these agents in individuals that received kidney transplants (Hiremath et al., 2007; Cross et al., 2009). Because these agents are activated by CES1, a possible mechanism for this lack of effect could be the simultaneous administration of immunosuppressive drugs that inhibit CES1. A previous study demonstrated that the immunosuppressive agent, mycophenolate mofetil, was a substrate for CES1 (Fujiyama et al., 2010). Other immunosuppressive agents have not been tested for their activity as esterase inhibitors. Although several ACE inhibitors have been shown to be substrates of CES1, no study has investigated the human enzyme responsible for the activation of two of the most commonly prescribed compounds in this family: enalapril (EPL) and ramipril (RPL). Thus, in the present study, we explored the metabolism of these compounds by CES1 and CES2, in comparison with the known CES1 substrate trandolapril (TPL). Additionally, we screened a panel of cardiovascular, antiplatelet, and anticoagulant drugs; drugs of abuse; and immunosuppressive agents for their inhibitory potential against CES1. We further examined the inhibition kinetics of some identified inhibitors.

ABBREVIATIONS: ACE, angiotensin-converting enzyme; CES1, carboxylesterase 1; CES2, carboxylesterase 2; DMSO, dimethyl sulfoxide; EPL, enalapril; EPLA, enalaprilat; HLM, human liver microsomes; PNPA, *p*-nitrophenyl acetate; RPL, ramipril; RPLA, ramiprilat; TPL, trandolapril; TPLA, trandolaprilat.

Materials and Methods

Chemicals, Reagents, and Reference Compounds. *p*-Nitrophenyl acetate, morphine, and fentanyl were from Sigma-Aldrich (St. Louis, MO); *p*-nitrophenol was from Fluka (Buchs, Switzerland). Amphetamine, cocaine, methamphetamine, 3,4-methylenedioxy-*N*-methylamphetamine, methadone, 6-monoacetyl-morphine, and heroin were from Lipomed AG (Arlesheim, Switzerland). Carvedilol was from GlaxoSmithKline (London, United Kingdom). Dabigatran, dabigatran etexilate, and trandolaprilat-d5 were from Toronto Research Chemicals (Toronto, Canada). Metoprolol, pethidine, diazepam, and nitrazepam were from Nycomed Denmark (Roskilde, Denmark). Spironolactone was from Ercopharm A/S (Kvistgaard, Denmark). Eplerenone, amlodipine, and sirolimus were from Pfizer (New York City, NY). Verapamil was from GEA (Copenhagen, Denmark). Diltiazem was from Norpharma (Hørsholm, Denmark). Furosemide was from Hoechst AG (Frankfurt, Germany). Bendroflumethiazide was from LEO Pharma (Ballerup, Denmark). Ketamine was from Parke-Davis (Pontypool, United Kingdom). Clopidogrel, ramipril, ramiprilat, and irbesartan were from Sanofi-Aventis (Paris, France). Zopiclone was from Actavis (Zug, Switzerland). Simvastatin, bisoprolol, and enalapril were from Merck & Co. (Whitehouse Station, NJ). Tacrolimus was from Fujisawa Pharmaceutical (Osaka, Japan). Azathioprine and labetalol were from Glaxo Wellcome (London, United Kingdom). Propranolol was from Zeneca (London, United Kingdom). Atorvastatin was from Bie & Berntsen (Herlev, Denmark). Sotalol was from Bristol-Myers Squibb (New York City, NY). Enalaprilat-d5 was from CDN isotopes (Quebec, Canada). Trandolapril and trandolaprilat were from Roussel (Paris, France). Pindolol was from DuraScan (Odense, Denmark). Enalaprilat was from USP (Rockville, MD). Nimodipine was from Bayer Denmark (Frederiksberg, Denmark). Isradipine was from Sandoz (Basel, Switzerland). Lercanidipine was from Recordati (Milano, Italy). Valsartan was from Novartis (Basel, Switzerland). Pooled human liver microsomes (HLMs), recombinant human CES1 (CES1b/CES1A1), and CES2 (prepared from baculovirus-infected High-Five insect cells) were obtained from BD Gentest (Woburn, MA). Phosphate buffer (100 mM, pH 7.4) was produced from potassium phosphate and dipotassium phosphate (both from Merck, Darmstadt, Germany). Other solvents and chemicals were commercially available and of liquid chromatography–mass spectrometry grade.

Detection of CES1 and CES2 Metabolism. The hydrolysis of TPL, RPL, and EPL by recombinant human CES1 and CES2 was investigated. Substrate (100 μ M) was incubated for 20 minutes at 37°C with a final protein concentration of 100 μ g/ml. Negative controls were included in which substrate was incubated in buffer without enzyme. The hydrolytic products were quantified as described in the analytical methods section below. Hydrolysis rates were corrected for spontaneous hydrolysis (i.e., nonenzymatic hydrolysis) by subtracting any product formed in the negative controls.

Kinetic Studies. Michaelis-Menten kinetic parameters were determined for TPL, RPL, and EPL in recombinant human CES1. An initial study was performed to establish time and protein linearity of the reactions by measuring product formed at several time points (5, 10, 15, 20, and 30 minutes) using three protein concentrations (50, 100, and 150 μ g/ml). Final protein and substrate concentrations, as well as incubation times, were selected such that a quantifiable amount of hydrolysis product was formed and so that the sampling was done in the linear range of the reactions. No more than 20% of substrate was hydrolyzed at the time of sampling. Substrate concentrations were 0.2–4 mM for TPL, 0.1–4 mM for RPL, and 0.05–4 mM for EPL. Final recombinant CES1 protein concentrations were 50 μ g/ml for TPL and RPL, and 100 μ g/ml for EPL. No organic solvent was present in the incubations. The drug and enzyme combinations were incubated in 96-well, twin.tec polymerase chain reaction plates (Eppendorf, Hamburg, Germany) in 100 mM phosphate buffer at 37°C, in a final reaction volume of 50 μ l. Control incubations with no enzyme were performed for each compound. Reactions (8 minutes for RPL, 10 minutes for TPL, and 20 minutes for EPL) were terminated by transferring 20 μ l to an equal volume of cold acetonitrile with 0.5% formic acid and the corresponding internal standard—500 nM trandolaprilat (TPLA)-d5 for TPL or RPL, and 1,000 nM enalaprilat (EPLA)-d5 for EPL. Next, 110 μ l of mobile phase was added, and the samples were centrifuged at 2,000g, 5°C, for 10 minutes. The supernatant was analyzed by liquid chromatography–tandem mass spectrometry as described in the analytical methods section below. The hydrolysis rate versus the substrate concentration was plotted and fit with

a standard Michaelis-Menten equation or a substrate inhibition equation, where a second substrate molecule was treated as an uncompetitive inhibitor (Cornish-Bowden, 2012). The two fits were compared with an extra-sum-of-squares F-test. The test compares the improvement of sum-of-squares with the more complicated model (the substrate inhibition model) versus the loss of degrees of freedom. The null-hypothesis was that the data were adequately described by the standard Michaelis-Menten equation. *P* values < 0.05 were considered significant. Nonlinear regression fitting and statistical analysis was performed with Prism, version 6.02 (GraphPad Software, Inc., San Diego, CA). Intrinsic clearance (CL_{int}) was calculated by dividing V_{max} by K_m .

Screening Assay. We screened the inhibitory potential of a variety of therapeutic drugs and drugs of abuse with the well-known esterase substrate, *p*-nitrophenyl acetate (PNPA). The incubations were performed in 96-well, pureGrade BRANDplates (BRAND, Wertheim, Germany) in 100 mM phosphate buffer at 37°C in a final volume of 200 μ l. Human CES1 and HLM were used for the enzyme source. Dimethyl sulfoxide (DMSO) was used for the dissolution of all compounds. The final concentration of DMSO was 2% v/v in all incubations, including the controls. An initial study was performed to analyze the effect of this concentration of DMSO on enzyme activity. The substrate and inhibitor were premixed to allow simultaneous addition of both. The final concentrations of substrate and inhibitor were 50 μ M and 100 μ M, respectively; the final protein concentrations were 10 μ g/ml. The hydrolytic product, *p*-nitrophenol, was determined by measuring the absorbance at 405 nm after 3 minutes with a Sunrise microplate reader (Tecan, Grödig, Austria). The sampling time was tested to be in the linear range of the reaction. The assay was performed in triplicate. Substrate in buffer without enzyme was included as a negative control. The results were corrected for spontaneous hydrolysis by subtracting the absorbance of the negative control and comparing with a positive control containing enzyme, but no inhibitor.

Inhibition Kinetics and Pattern. The inhibition constant K_i was determined for selected inhibitors of CES1, with TPL as substrate. The analytical method for TPLA is described in the analytical methods section below. Substrate concentrations were 0.1, 0.2, 0.5, and 1 mM. Five concentrations of inhibitors were used, including zero. The final protein concentration was 50 μ g/ml. K_i constants were determined by fitting the data with a competitive, non-competitive, uncompetitive, or mixed-mode inhibition model with Prism software.

Analytical Methods. Formations of TPLA, RPLA, and EPLA were determined with liquid chromatography–tandem mass spectrometry. Chromatographic separation was performed with an Agilent 1100 series high-performance liquid chromatography system from Agilent Technologies (Santa Clara, CA). The system consisted of a binary pump, a degasser, a column compartment with thermostat, and a 1200 series autosampler. We used a Zorbax SB-C18 column (3.5 μ m, 2.1 \times 50 mm) for TPLA and ramiprilat (RPLA), and a Chromsep SS100 Pursuit3 column (3 μ m, 3 \times 100 mm) for EPLA (both from Agilent Technologies). Isocratic chromatographic methods were developed to separate the prodrugs from their respective metabolites. The mobile phase was 0.1% formic acid and isopropanol (65:35, v/v) for TPLA, 0.1% formic acid and isopropanol (72:25, v/v) for EPLA, and 10 mM ammonium formate and methanol (40:60, v/v) for RPLA. Flow rates were 0.3 ml/min for TPLA, 0.5 ml/min for EPLA, and 0.2 ml/min for RPLA. A column temperature of 50°C was used for all methods. Injection volume was 5 μ l for TPLA, 3 μ l for EPLA, and 10 μ l for RPLA. Run times were 2 minutes for TPLA and 3 minutes for EPLA and RPLA.

A QuattroMicro triple quadrupole tandem mass spectrometer (Waters Corporation, Milford, MA) was coupled to the high-performance liquid chromatography system. Data were acquired in positive electrospray ionization mode with Masslynx 4.1 software (Waters). Mass spectrometry conditions were optimized by infusing a standard solution through a T-piece and engaging the autotune function in the software. Multiple reaction monitoring was used for data collection. Monitored mass transitions were m/z 403.2 $>$ 170.1, m/z 389.3 $>$ 206.2, and m/z 349.1 $>$ 206.2 for TPLA, RPLA, and EPLA, respectively, and m/z 408.2 $>$ 170.1 and m/z 354.3 $>$ 211.2 for TPLA-d5 and EPLA-d5, respectively. Cone voltage and collision energy of 30 V and 20 eV, respectively, were applied for all analytes except for EPLA-d5, where a cone voltage of 25 V was applied. Other mass spectrometry parameters were capillary voltage 1 kV, source temperature 120°C, desolvation temperature

350°C, cone gas flow 30 L/h, and desolvation gas flow 800 L/h. Argon was used as the collision gas.

The measuring range was 1–400 μ M for all analytes. The methods were validated for precision and accuracy by analyzing blank samples spiked at four concentration levels; quadruplicate determinations were performed on three different days. The precision (CV%) was <7%, and the accuracy was 91% to 111% for all methods.

Results

Hydrolytic Activities of CES1 and CES2 for TPL, RPL, and EPL. The hydrolytic activities of recombinant human CES1 and CES2 were measured for TPL, RPL, and EPL. CES2 showed no hydrolytic activity above the spontaneous hydrolysis toward any of the three ACE inhibitors. In contrast, all three compounds were readily hydrolyzed by CES1 (Fig. 1). The hydrolytic activity of CES1 was roughly 10 times higher for RPL and TPL than for EPL.

Kinetics of CES1-Mediated Hydrolysis of EPL, TPL, and RPL. We investigated the kinetics of recombinant CES1-mediated hydrolysis of EPL, TPL, and RPL. All three reactions were linear up to at least 30 minutes at the protein concentrations used in the final assay. Hydrolysis rates were plotted versus substrate concentrations (Fig. 2). EPL displayed obvious substrate inhibition kinetics, illustrated by a lack of linearity at low $v/[S]$ on the Eadie-Hofstee plot (inset in Fig. 2C). For this compound, an extra-sum-of-squares F-test was used to compare fitting to the standard Michaelis-Menten equation and the substrate inhibition equation. The F-test revealed the inclusion of an extra parameter (K_{si} , the inhibition constant for a second substrate molecule) to be justified (P value < 0.0001). For EPL, the standard Michaelis-Menten equation was therefore rejected, and the substrate inhibition equation applied. The kinetic parameters are summarized in Table 1. Intrinsic clearance (CL_{int}) was approximately 3-fold higher for RPL than for TPL and roughly 18-fold higher for TPL than for EPL.

Inhibitory Effects of Therapeutic Drugs and Drugs of Abuse on the Hydrolase Activity of Recombinant Human CES1 and HLM. We investigated a panel of therapeutic drugs and drugs of abuse by measuring their inhibition of human CES1 and HLM hydrolysis of

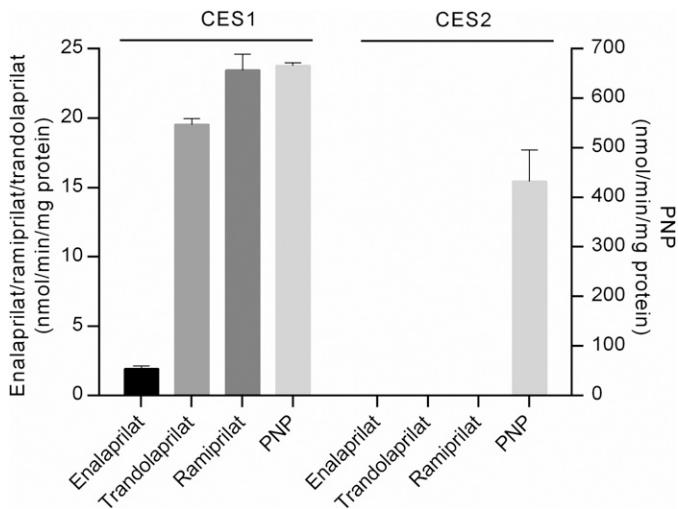


Fig. 1. Enzymatic activity of CES1 and CES2 on the hydrolysis of three ACE inhibitors. Substrates EPL, TPL, and RPL (100 μ M) were incubated with recombinant human CES1 and CES2 at 37°C for 20 minutes. PNPA (100 μ M) was included as a positive control. The corresponding hydrolytic products were determined and normalized by time and protein contents. Values represent mean \pm S.E.M. ($n = 4$).

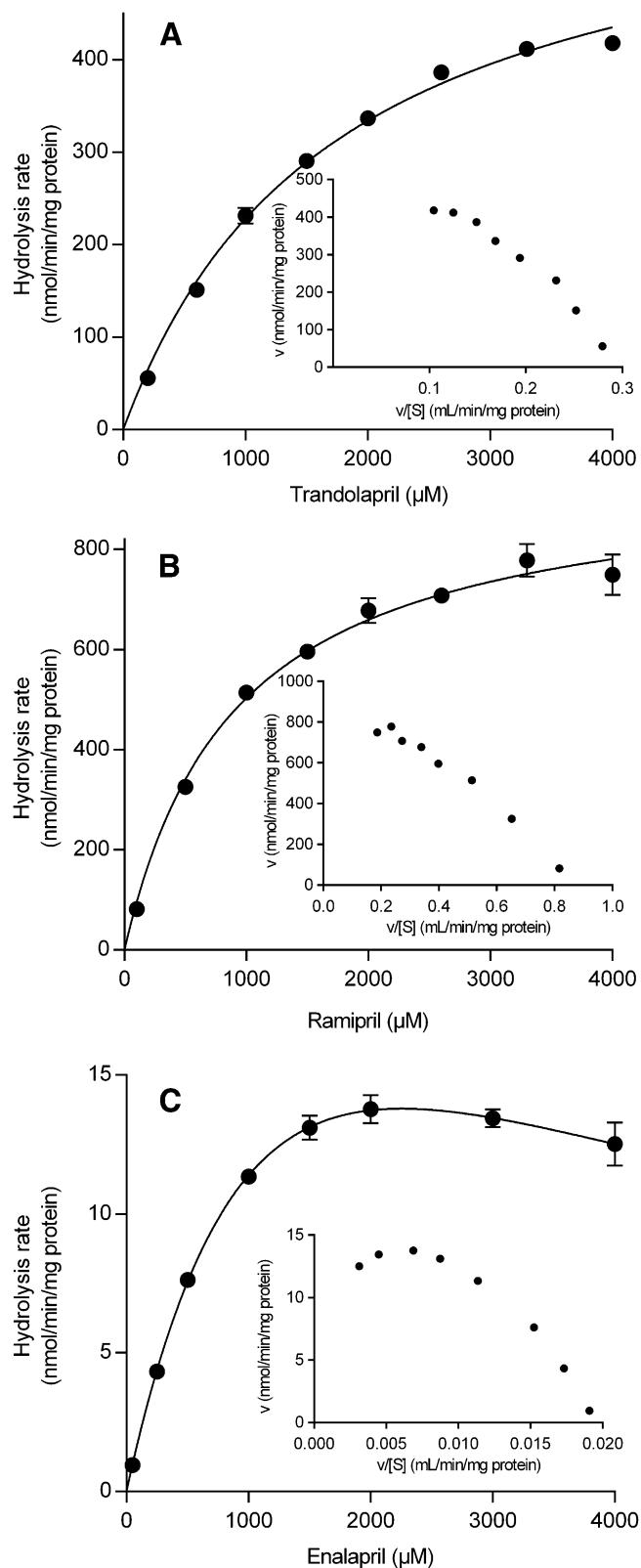


Fig. 2. Determination of kinetic constants of the hydrolysis of three ACE inhibitors by CES1. The hydrolysis of TPL (A), RPL (B), and EPL (C) were determined at substrate concentrations of 50–4,000 μ M. K_m and V_{max} constants were estimated by nonlinear regression using Prism. Eadie-Hofstee plots are provided in the insets. Data represent mean \pm S.E.M. ($n = 4$).

TABLE 1

Kinetic parameters for hydrolysis of three ACE inhibitors by recombinant human CES1

Substrate	K_m	V_{max}	K_{si}	CL_{int}
	μM	nmol/min/mg protein	μM	ml/min/mg protein
Trandolapril	1734	624	—	0.360
Ramipril	901	956	—	1.061
Enalapril	1721	34	2956	0.020

PNPA. The concentration of DMSO at 2% v/v was found to have only a minor impact on PNPA hydrolysis in both recombinant CES1 ($92 \pm 0.2\%$ residual activity compared with a control without DMSO, mean \pm S.E.M., $n = 3$) and HLM ($98 \pm 1.6\%$, mean \pm S.E.M., $n = 3$).

The results for 26 therapeutic drugs and dabigatran (the active metabolite of the prodrug dabigatran etexilate) are shown in Fig. 3. In general, the tested drugs showed comparable inhibition patterns for the recombinant enzyme and HLM, although some compounds showed less inhibition of HLM than of CES1.

No inhibition was detected for dabigatran etexilate and its active metabolite. The two tested statin drugs, simvastatin and atorvastatin, both inhibited the enzyme with 0.2% and 44.1% residual activity, respectively, of the recombinant enzyme. The known substrate and inhibitor of CES1, clopidogrel, showed potent inhibition of recombinant CES1, with only 12.0% residual activity. The β -blocking agent, carvedilol, suppressed all but 13.9% of recombinant CES1 activity; the other β -blocking agents tested showed no major inhibition of CES1. The two nondihydropyridine calcium channel blockers, diltiazem and verapamil, demonstrated potent inhibition of recombinant CES1, with 20.6% and 30.0% residual activity, respectively. All four dihydropyridine calcium channel blockers tested inhibited the enzyme with variable potency. Of these, isradipine and amlodipine showed the most potent inhibition of recombinant CES1, with 17.6% and 40.6% residual activity, respectively. The diuretic agents and angiotensin II receptor antagonists tested did not exhibit any noteworthy inhibition. Tacrolimus showed moderate inhibition with 28.4% remaining activity of recombinant CES1, while no major inhibition was observed for sirolimus or azathioprine.

The results for 10 common drugs of abuse, 6-monoacetylmorphine, two benzodiazepine drugs, and zopiclone are shown in Fig. 4. Of these compounds, only cocaine showed potent inhibition, with 8.8% and 67.8% residual activity in the recombinant enzyme and HLM, respectively.

Kinetics of Inhibition by Diltiazem and Verapamil on CES1 Hydrolase Activity. Both diltiazem and verapamil inhibited the recombinant human CES1 hydrolysis of TPL (Fig. 5). The kinetics of the inhibition was determined in two independent experiments and the results summarized in Table 2.

Discussion

In this study, we identified two novel substrates of human CES1, EPL, and RPL. These drugs are among the most commonly prescribed drugs in the ACE-inhibitor family. Thus, they are included in the group of drugs metabolized by CES1 that are typically prescribed for cardiovascular diseases, such as congestive heart failure and hypertension, but also for diabetes mellitus. The kinetics of CES1 hydrolysis of RPL and TPL were fitted with the standard Michaelis-Menten equation, while EPL displayed obvious substrate inhibition kinetics, and a modified equation that took into account the uncompetitive inhibition by a second substrate molecule was therefore employed for this compound. The kinetics of TPL also hinted at a substrate

inhibition character. Higher substrate concentrations would likely reveal a more pronounced substrate inhibition character for this compound, which, unfortunately, was not possible due to limitations in solubility. Of the three tested compounds, RPL was most efficiently hydrolyzed by CES1, with a CL_{int} of 1.061 ml/min/mg protein. The catalytic efficiency of CES1 for EPL was much lower (18-fold and 53-fold lower, respectively) than for TPL and RPL. Both RPL and TPL are more lipophilic compounds than EPL, due to the additional cyclopentane and cyclohexane rings, respectively, fused to the pyrrolidine ring. The increased lipophilic character of RPL and TPL relative to EPL is likely to improve hydrophobic interactions with CES1, which would lead to increased CES1 catalytic efficiency. The fact that no CES2-mediated hydrolysis was detected for any of the three compounds suggested that the primary site for activation of these prodrugs is in the liver, rather than the intestinal wall, where CES2 is most highly expressed (Schwer et al., 1997). This finding was consistent with those of Pang et al., who found no significant EPLA in portal venous plasma after delivery of EPL into the superior mesenteric artery with an in situ perfused rat intestine-liver preparation (Pang et al., 1985). Zhu and coworkers previously determined the kinetic parameters of TPL hydrolase activity by recombinant CES1 and found a K_m of 639.9 μM and a V_{max} of 103.6 nmole/min/mg protein (Zhu et al., 2009). These results are somewhat different from the values determined in the present study. However, Zhu et al. did not use the commercially available enzyme, but instead constructed and purified their own recombinant version. Differences in construction methods could lead to variations in post-translational modifications and hence enzyme activity and affinity. Additionally, they used a maximum substrate concentration of 2 mM, while a maximum of 4 mM was used in the present study. The higher maximal concentration leads to more accurate determination of V_{max} and hence K_m .

Several known inhibitors were confirmed in the screening assay. These included clopidogrel, diltiazem, and verapamil. Clopidogrel is a prodrug that is oxidized to a 2-oxo intermediate, followed by conversion to the pharmacologically active thiol metabolite. Clopidogrel is a known substrate for CES1; in fact, CES1 is the primary esterase that inactivates clopidogrel and its 2-oxo- and thiol-metabolites (Bouman et al., 2011). This is consistent with our finding that clopidogrel was also a potent inhibitor of CES1. Diltiazem and verapamil were recently identified as inhibitors of human CES1 and CES2 (Yanjiao et al., 2013). In addition, both drugs are known inhibitors of CYP3A4 (Sutton et al., 1997; Wang et al., 2004). Thus, the inhibition of CES1 and CES2 provides an additional mechanism for drug-drug interactions caused by diltiazem and verapamil, and it widens the range of potentially affected drugs. Carvedilol was previously determined as an inhibitor of imidapril hydrolase activity in human liver microsomes and cytosol (Takahashi et al., 2009). The present study provides evidence that the effect on imidapril hydrolase in these preparations is most likely caused by inhibition of CES1. Carvedilol was the only β -blocking agent out of the seven tested that demonstrated inhibition of CES1. Thus, inhibition of CES1 did not appear to be a class effect for this family of drugs. Cocaine was previously shown to inhibit heroin and 4-methylumbelliferyl acetate hydrolysis in enzyme purified from human liver (Kamendulis et al., 1996; Brzezinski et al., 1997). Studies have shown conflicting data on whether the drug was a substrate for CES1. Early studies, using CES1 enzyme purified from human liver, determined that cocaine was a substrate (Dean et al., 1991; Brzezinski et al., 1994), but a later study, using recombinant CES1 enzyme, failed to detect any CES1-mediated hydrolysis of cocaine (Hatfield et al., 2010). The present study showed cocaine to be a potent inhibitor of recombinant CES1 (8.8% residual activity); however, there was only a minor inhibition of

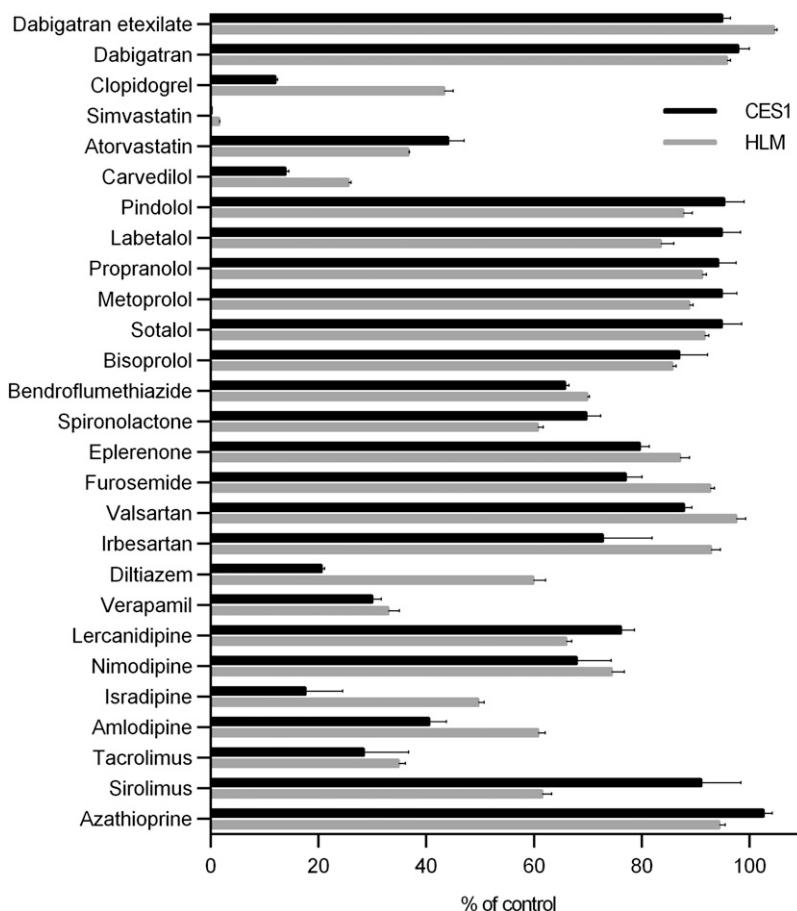


Fig. 3. Inhibition of CES1 by therapeutic drugs. Inhibitory effects of 26 cardiovascular, antiplatelet, anticoagulant, and immunosuppressant drugs and dabigatran on the hydrolysis of 50 μ M PNPA in recombinant human CES1 or HLM. Concentrations of tested compounds were 100 μ M. Values represent mean \pm S.E.M. ($n = 3$). Control activity was 382 nmol/min/mg protein for CES1 and 838 nmol/min/mg protein for HLM.

HLM (67.8% residual activity). This discrepancy could be a result of cocaine being metabolized by other esterases present in HLM.

The four tested dihydropyridine calcium channel blockers all inhibited CES1 to varying degrees, with isradipine and amlodipine having the greatest inhibition potential. Isradipine is previously unreported as an inhibitor of human CES1. Yanjiao and coworkers identified nitrendipine and felodipine as strong, and amlodipine as

a weaker inhibitor of CES1 (Yanjiao et al., 2013). Thus, it appears that most calcium channel blockers of the dihydropyridine class inhibit CES1 to varying degrees. Dihydropyridine calcium antagonists possess two ester groups at positions 3 and 5 of the 1,4-dihydropyridine ring. Most of these drugs are transformed in vivo, where the dihydropyridine ring is oxidized to pyridine, and one or both of the ester groups may be cleaved (Weidolf et al., 1984; Stopher et al., 1988; Böcker et al., 1990).

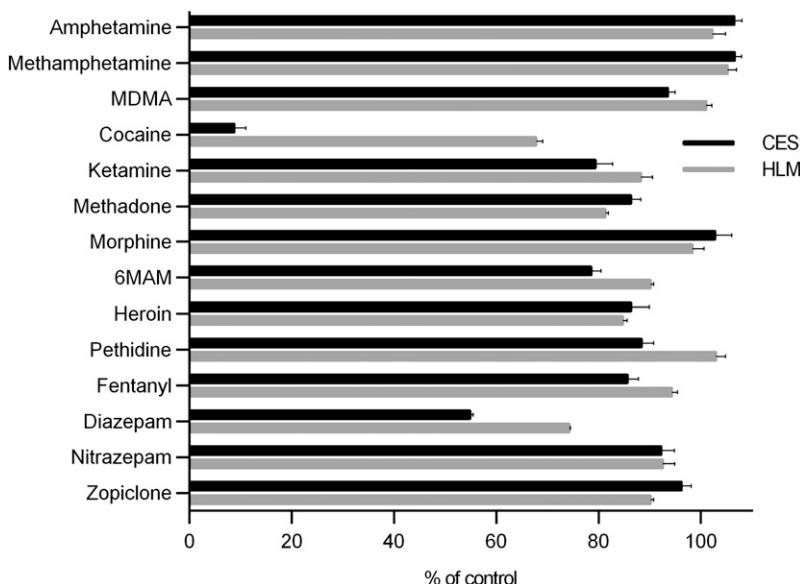


Fig. 4. Inhibition of CES1 by common drugs of abuse and related compounds. Inhibitory effects of common drugs of abuse, including two benzodiazepines, zopiclone, pethidine, fentanyl, and 6-monoacetylmorphine (6MAM) on the hydrolysis of 50 μ M PNPA in recombinant human CES1 or HLM. Concentrations of tested compounds were 100 μ M. Values represent mean \pm S.E.M. ($n = 3$). Control activity was 382 nmol/min/mg protein for CES1 and 838 nmol/min/mg protein for HLM. MDMA, 3,4-methylenedioxy-N-methylamphetamine.

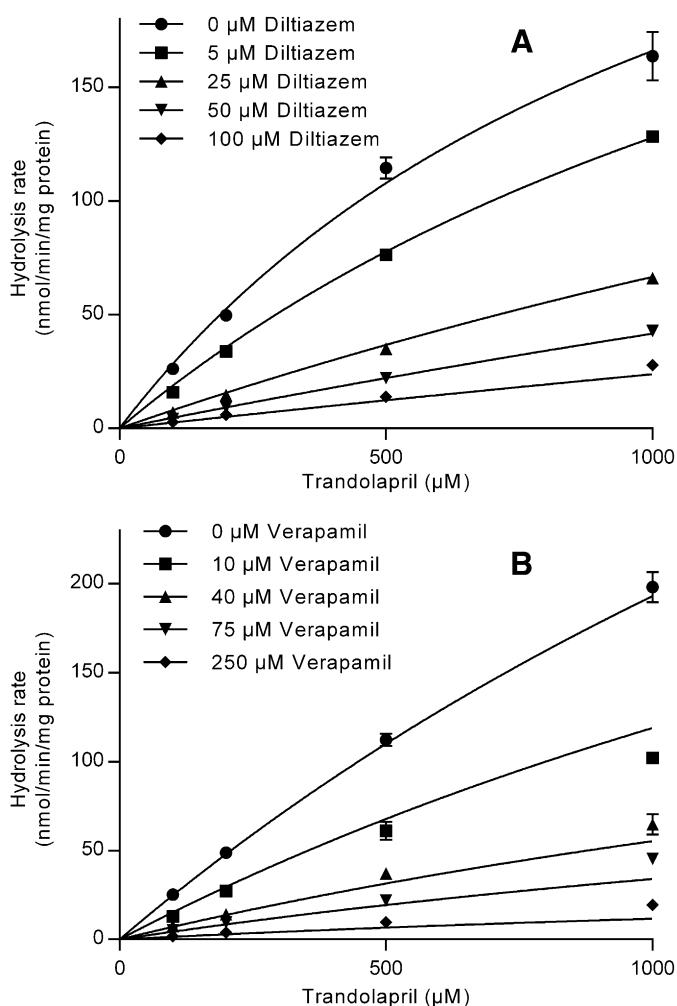


Fig. 5. Determination of inhibition constants for inhibition by diltiazem (A) and verapamil (B) on the trandolapril hydrolase activities of recombinant CES1. Plotted values represent mean \pm S.E.M. ($n = 2$).

The oxidation seems to be mostly catalyzed by the 3A4 isoform of the cytochrome P450 enzymes (Katoh et al., 2000). It was also demonstrated that CYP3A4 was capable of oxidative cleavage of the ester bond in nifedipine (Funaki et al., 1989). To our knowledge, no study has investigated a potential role of esterases in hydrolytic cleavage of ester bonds at the 3 or 5 position in dihydropyridine calcium antagonists. Considering that several of the dihydropyridine calcium channel blockers are inhibitors of CES1, carboxylesterases may at least contribute to the metabolism of these drugs.

One of the immunosuppressive agents, tacrolimus, moderately inhibited CES1. Thus, this inhibition could play a role in the lack of effect of ACE inhibitors seen in renal-transplant recipients who are receiving concomitant immunosuppressive therapy with this agent.

TABLE 2

Inhibition constants for inhibition of trandolapril hydrolase activity in recombinant CES1 by diltiazem and verapamil

Inhibitor	K_i^a	Inhibition type
Diltiazem	9.0 ± 0.5	Competitive
Verapamil	16.0 ± 1.1	Noncompetitive

^a Mean \pm S.E.M. for two independent determinations.

However, this finding needs to be further examined in clinical studies.

Simvastatin was confirmed as a potent inhibitor of CES1. Additionally, atorvastatin moderately inhibited the enzyme. In the study by Fukami et al., several statin drugs were tested as inhibitors of CES1 (Fukami et al., 2010). Their study found the lactone-containing statins, simvastatin and lovastatin, to be more potent inhibitors of CES1 than the statins existing as hydroxy acids. The results in the present study confirm this observation, with simvastatin being a more potent inhibitor than atorvastatin; the latter compound not containing a lactone.

For some compounds there were discrepancies between the degree of inhibition in recombinant CES1 and HLM, with some compounds having a greater inhibition potential of the recombinant enzyme. Cocaine, clopidogrel, diltiazem, and isradipine were among the compounds that inhibited recombinant CES1 more potently than HLM. There are several possible explanations for this finding, including other PNPA-hydrolyzing enzymes found in the microsomal preparation, inhibitors being metabolized by other enzymes in HLM, differences in protein binding and thus of free drug concentrations, and possible differences in the structure of the recombinant form of the enzyme and the natural enzyme found in HLM preparations, caused by, for example, post-translational modifications.

Most of the identified inhibitors of CES1 are compounds containing an ester or amide bond, and are thus possible substrates of the enzyme. However, two of the identified inhibitors, carvedilol and verapamil, do not contain any such bonds. There are no particularly reactive groups, such as aldehydes, alkenes, or phenols, in the structure of these compounds, so an irreversible inhibition mode is unlikely. Also, verapamil was found to inhibit the TPL hydrolase activity of recombinant CES1 in a noncompetitive manner (Table 2). This means that the compound binds equally well to the free enzyme and the enzyme-substrate complex (Cornish-Bowden, 2012). It is thus likely that verapamil binds to an allosteric site on the enzyme. However, discerning the binding mechanism for these compounds is difficult without crystal structures of CES1 cocrystallized with these compounds, but molecular docking studies may help elucidate the binding region.

Fukami and coworkers identified simvastatin as an inhibitor of recombinant human CES1 hydrolysis of imidapril; they determined a K_i of $0.11 \mu\text{M}$ (Fukami et al., 2010). The present study confirmed the strong inhibition of CES1 by simvastatin. We also found that diltiazem and verapamil ($K_i 9.0 \pm 0.5 \mu\text{M}$ and $16.0 \pm 1.1 \mu\text{M}$, respectively) were weaker than simvastatin at inhibiting CES1 hydrolysis of TPL. Little or no clinical interactions have been demonstrated between EPL/RPL and simvastatin (Shionoiri, 1993; Meyer et al., 1994); therefore, these drugs might also possess a low potential for affecting the pharmacokinetics of ACE inhibitors. However, with the lengthening list of CES1 substrates and inhibitors, the possibility of clinically significant interactions has increased for scenarios in which several of these drugs are administered concomitantly. Additionally, genetic variations that cause reduced expression and/or activity of CES1 may further increase this possibility. Polymorphisms in the gene or promoter region for CES1 have been shown to cause significant changes in the pharmacokinetics of and/or the clinical response to methylphenidate, oseltamivir, clopidogrel, and imidapril (Geshi et al., 2005; Zhu et al., 2008; Tarkiainen et al., 2012; Lewis et al., 2013). Individuals with CES1 polymorphisms that produce a poor CES1 phenotype in relation to metabolism of substrates, may be at increased risk of experiencing drug interactions that involve CES1 substrates and inhibitors. Considering the wide usage of many of these drugs, the potential for clinically relevant drug interactions should not be ignored.

In conclusion, our results showed that the widely prescribed ACE inhibitors, EPL and RPL, were substrates of human CES1 but not of CES2. Additionally, we confirmed several known inhibitors of CES1 and discovered isradipine and tacrolimus to be new inhibitors of CES1. This knowledge may assist in the prediction of drug-drug interactions and thus improve the safety of future drug therapies.

Appendix

Henrik Berg Rasmussen, Institute of Biological Psychiatry, Mental Health Centre Sct. Hans, Copenhagen University Hospital, Roskilde, Denmark.

Ditte Bjerre, Institute of Biological Psychiatry, Mental Health Centre Sct. Hans, Copenhagen University Hospital, Roskilde, Denmark.

Kristian Linnet, Section of Forensic Chemistry, Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, Denmark.

Ragnar Thomsen, Section of Forensic Chemistry, Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, Denmark.

Gesche Jürgens, Department of Clinical Pharmacology, Bispebjerg University Hospital, Copenhagen, Denmark.

Kim Dalhoff, Department of Clinical Pharmacology, Bispebjerg University Hospital, Copenhagen, Denmark.

Claus Stage, Department of Clinical Pharmacology, Bispebjerg University Hospital, Copenhagen, Denmark.

Hreinn Stefansson, CNS Division, deCODE Genetics, Reykjavik, Iceland.

Thomas Hankemeier, The Leiden/Amsterdam Center for Drug Research LACDR, Leiden University, Gorlaeus Laboratories, Leiden, The Netherlands.

Rima Kaddurah-Daouk, Department of Psychiatry and Behavioral Sciences, Duke University, Durham, NC, USA.

Søren Brunak, Center for Biological Sequence Analysis, Technical University of Denmark, Kgs. Lyngby, Denmark.

Olivier Taboureau, Center for Biological Sequence Analysis, Technical University of Denmark, Kgs. Lyngby, Denmark.

Grace Shema Nzabonimpa, Center for Biological Sequence Analysis, Technical University of Denmark, Kgs. Lyngby, Denmark.

Tine Houmann, Child and Adolescent Psychiatric Center, Mental Health Services, Capital Region of Denmark, Copenhagen, Denmark.

Pia Jeppesen, Child and Adolescent Psychiatric Center, Mental Health Services, Capital Region of Denmark, Copenhagen, Denmark.

Kristine Kaalund-Jørgesen, Child and Adolescent Psychiatric Center, Mental Health Services, Capital Region of Denmark, Copenhagen, Denmark.

Peter Riis Hansen, Department of Cardiology, Copenhagen University Hospital, Hellerup, Denmark.

Karl Emil Kristensen, Department of Cardiology, Copenhagen University Hospital, Hellerup, Denmark.

Anne Katrine Pagsberg, Child and Adolescent Psychiatric Center, Mental Health Services, Capital Region of Denmark, Copenhagen, Denmark.

Kerstin Plessen, Child and Adolescent Psychiatric Center, Mental Health Services, Capital Region of Denmark, Copenhagen, Denmark.

Poul-Erik Hansen, Department of Science, Systems and Models, Roskilde University, Roskilde, Denmark.

Wei Zhang, Department of Science, Systems and Models, Roskilde University, Roskilde, Denmark.

Thomas Werge, Institute of Biological Psychiatry, Mental Health Centre Sct. Hans, Copenhagen University Hospital, Roskilde, Denmark.

Jørgen Dyrborg, Child and Adolescent Psychiatric Center, Mental Health Services, Capital Region of Denmark, Hillerød, Denmark.

Maj-Britt Lauritzen, Child and Adolescent Psychiatric Center, Mental Health Services, Capital Region of Denmark, Hillerød, Denmark.

Authorship Contributions

Participated in research design: Thomsen, Linnet.

Conducted experiments: Thomsen.

Performed data analysis: Thomsen.

Wrote or contributed to writing of the manuscript: Thomsen, Rasmussen, Linnet.

References

- Bouman HJ, Schömöig E, van Werkum JW, Velder J, Hackeng CM, Hirschhäuser C, Waldmann C, Schmalz H-G, ten Berg JM, and Taubert D (2011) Paraoxonase-1 is a major determinant of clopidogrel efficacy. *Nat Med* **17**:110–116.
- Brzezinski MR, Abraham TL, Stone CL, Dean RA, and Bosron WF (1994) Purification and characterization of a human liver cocaine carboxylesterase that catalyzes the production of benzoylecgonine and the formation of cocaethylene from alcohol and cocaine. *Biochem Pharmacol* **48**:1747–1755.
- Brzezinski MR, Spink BJ, Dean RA, Berkman CE, Cashman JR, and Bosron WF (1997) Human liver carboxylesterase hCE-1: binding specificity for cocaine, heroin, and their metabolites and analogs. *Drug Metab Dispos* **25**:1089–1096.
- Böcker RH, Preuss E, and Peter R (1990) High-performance liquid chromatography of the metabolites of nitrendipine and investigation into the metabolic pathways of this dihydropyridine. *J Chromatogr A* **530**:206–211.
- Cornish-Bowden A (2012) *Fundamentals of Enzyme Kinetics*, 4th ed. Wiley-Blackwell, Weinheim, Germany.
- Cross NB, Webster AC, Masson P, O'Connell PJ, and Craig JC (2009) Antihypertensive treatment for kidney transplant recipients. *Cochrane Database Syst Rev* (3):CD003598.
- Dean RA, Christian CD, Sample RH, and Bosron WF (1991) Human liver cocaine esterases: ethanol-mediated formation of ethylcocaine. *FASEB J* **5**:2735–2739.
- Fujiyama N, Miura M, Kato S, Sone T, Isobe M, and Satoh S (2010) Involvement of carboxylesterase 1 and 2 in the hydrolysis of mycophenolate mofetil. *Drug Metab Dispos* **38**:2210–2217.
- Fukami T, Takahashi S, Nakagawa N, Maruichi T, Nakajima M, and Yokoi T (2010) In vitro evaluation of inhibitory effects of antidiabetic and antihyperlipidemic drugs on human carboxylesterase activities. *Drug Metab Dispos* **38**:2173–2178.
- Funaki T, Soons PA, Guengerich FP, and Breimer DD (1989) In vivo oxidative cleavage of a pyridine-carboxylic acid ester metabolite of nifedipine. *Biochem Pharmacol* **38**:4213–4216.
- Geshi E, Kimura T, Yoshimura M, Suzuki H, Koba S, Sakai T, Saito T, Koga A, Muramatsu M, and Katagiri T (2005) A single nucleotide polymorphism in the carboxylesterase gene is associated with the responsiveness to imidapril medication and the promoter activity. *Hypertens Res* **28**:719–725.
- Hatfield MJ, Tsurkan L, Hyatt JL, Yu X, Edwards CC, Hicks LD, Wadkins RM, and Potter PM (2010) Biochemical and molecular analysis of carboxylesterase-mediated hydrolysis of cocaine and heroin. *Br J Pharmacol* **160**:1916–1928.
- Hiremath S, Fergusson D, Doucette S, Mulay AV, and Knoll GA (2007) Renin angiotensin system blockade in kidney transplantation: a systematic review of the evidence. *Am J Transplant* **7**:2350–2360.
- Hu Z-Y, Parker RB, Herring VL, and Laizure SC (2013) Conventional liquid chromatography/triple quadrupole mass spectrometry based metabolite identification and semi-quantitative estimation approach in the investigation of in vitro dabigatran etexilate metabolism. *Anal Bioanal Chem* **405**:1695–1704.
- Imai T (2006) Human carboxylesterase isozymes: catalytic properties and rational drug design. *Drug Metab Pharmacokinet* **21**:173–185.
- Kamendulis LM, Brzezinski MR, Pindel EV, Bosron WF, and Dean RA (1996) Metabolism of cocaine and heroin is catalyzed by the same human liver carboxylesterases. *J Pharmacol Exp Ther* **279**:713–717.
- Katoh M, Nakajima M, Shimada N, Yamazaki H, and Yokoi T (2000) Inhibition of human cytochrome P450 enzymes by 1,4-dihydropyridine calcium antagonists: prediction of in vivo drug-drug interactions. *Eur J Clin Pharmacol* **55**:843–852.
- Lewis JP, Horenstein RB, Ryan K, O'Connell JR, Gibson Q, Mitchell BD, Tanner K, Chai S, Bliden KP, and Tantry US, et al. (2013) The functional G143E variant of carboxylesterase 1 is associated with increased clopidogrel active metabolite levels and greater clopidogrel response. *Pharmacogenet Genomics* **23**:1–8.
- Meyer BH, Scholtz HE, Müller FO, Luus HG, de la Rey N, Seibert-Grafe M, Eckert HG, and Metzger H (1994) Lack of interaction between ramipril and simvastatin. *Eur J Clin Pharmacol* **47**:373–375.
- Pang KS, Cherry WF, and Ulm EH (1985) Disposition of enalapril in the perfused rat intestine-liver preparation: absorption, metabolism and first-pass effect. *J Pharmacol Exp Ther* **233**:788–795.
- Sato Y, Miyashita A, Iwatsubo T, and Usui T (2012) Conclusive identification of the oxybutynin-hydrolyzing enzyme in human liver. *Drug Metab Dispos* **40**:902–906.
- Schwer H, Langmann T, Daig R, Becker A, Aslanidis C, and Schmitz G (1997) Molecular cloning and characterization of a novel putative carboxylesterase, present in human intestine and liver. *Biochem Biophys Res Commun* **233**:117–120.
- Shi D, Yang J, Yang D, LeCluyse EL, Black C, You L, Akhlaghi F, and Yan B (2006) Anti-influenza prodrug oseltamivir is activated by carboxylesterase human carboxylesterase 1, and the activation is inhibited by antiplatelet agent clopidogrel. *J Pharmacol Exp Ther* **319**:1477–1484.
- Shionoiri H (1993) Pharmacokinetic drug interactions with ACE inhibitors. *Clin Pharmacokinet* **25**:20–58.
- Stopher DA, Beresford AP, Macrae PV, and Humphrey MJ (1988) The metabolism and pharmacokinetics of amlodipine in humans and animals. *J Cardiovasc Pharmacol* **12** (Suppl 7):S55–S59.
- Sun Z, Murry DJ, Sanghani SP, Davis WI, Kedishvili NY, Zou Q, Hurley TD, and Bosron WF (2004) Methylnaphenide is stereoselectively hydrolyzed by human carboxylesterase CES1A1. *J Pharmacol Exp Ther* **310**:469–476.
- Sutton D, Butler AM, Nadin L, and Murray M (1997) Role of CYP3A4 in human hepatic diltiazem N-demethylation: inhibition of CYP3A4 activity by oxidized diltiazem metabolites. *J Pharmacol Exp Ther* **282**:294–300.
- Takahashi S, Katoh M, Saitoh T, Nakajima M, and Yokoi T (2009) Different inhibitory effects in rat and human carboxylesterases. *Drug Metab Dispos* **37**:956–961.
- Takai S, Matsuda A, Usami Y, Adachi T, Sugiyama T, Katagiri Y, Tatematsu M, and Hirano K (1997) Hydrolytic profile for ester- or amide-linkage by carboxylesterases pI 5.3 and 4.5 from human liver. *Biof Sci Bull* **20**:869–873.
- Tang M, Mukundan M, Yang J, Charpentier N, LeCluyse EL, Black C, Yang D, Shi D, and Yan B (2006) Antiplatelet agents aspirin and clopidogrel are hydrolyzed by distinct carboxylesterases, and clopidogrel is transesterified in the presence of ethyl alcohol. *J Pharmacol Exp Ther* **319**:1467–1476.

- Tarkiainen EK, Backman JT, Neuvonen M, Neuvonen PJ, Schwab M, and Niemi M (2012) Carboxylesterase 1 polymorphism impairs oseltamivir bioactivation in humans. *Clin Pharmacol Ther* **92**:68–71.
- Wang YH, Jones DR, and Hall SD (2004) Prediction of cytochrome P450 3A inhibition by verapamil enantiomers and their metabolites. *Drug Metab Dispos* **32**:259–266.
- Weidolf L, Borg KO, and Hoffmann KJ (1984) Urinary metabolites of felodipine, a new vasodilator drug, in man, dog, rat and mouse. *Xenobiotica* **14**:657–666.
- Williams ET, Carlson JE, Lai WG, Wong YN, Yoshimura T, Critchley DJ, and Narurkar M (2011) Investigation of the metabolism of rufinamide and its interaction with valproate. *Drug Metab Lett* **5**:280–289.
- Yanjiao X, Chengliang Z, Xiping L, Tao W, Xiuhua R, and Dong L (2013) Evaluation of the inhibitory effects of antihypertensive drugs on human carboxylesterase in vitro. *Drug Metab Pharmacokinet* [published ahead of print].
- Zhang J, Burnell JC, Dumaul N, and Bosron WF (1999) Binding and hydrolysis of meperidine by human liver carboxylesterase hCE-1. *J Pharmacol Exp Ther* **290**:314–318.
- Zhu H-J, Appel DI, Johnson JA, Chavin KD, and Markowitz JS (2009) Role of carboxylesterase 1 and impact of natural genetic variants on the hydrolysis of trandolapril. *Biochem Pharmacol* **77**:1266–1272.
- Zhu H-J, Patrick KS, Yuan H-J, Wang J-S, Donovan JL, DeVane CL, Malcolm R, Johnson JA, Youngblood GL, and Sweet DH, et al. (2008) Two CES1 gene mutations lead to dysfunctional carboxylesterase 1 activity in man: clinical significance and molecular basis. *Am J Hum Genet* **82**:1241–1248.

Address correspondence to: Ragnar Thomsen, Section of Forensic Chemistry, Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, Frederik V's vej 11, DK-2100 Copenhagen, Denmark. E-mail: ragnar.thomsen@sund.ku.dk