0090-9556/11/3908-1329–1333$25.00
DRUG METABOLISM AND DISPOSITION
Vol. 39, No. 8
39628/3701443
DMD 39:1329–1333, 2011
Printed in U.S.A.

Short Communication

Characterization of Recombinant Human Carboxylesterases: Fluorescein Diacetate as a Probe Substrate for Human Carboxylesterase 2

Received March 17, 2011; accepted May 3, 2011

ABSTRACT:

Human carboxylesterase (CES) 1 and CES2 are members of the serine hydrolase superfamily, and both exhibit broad substrate specificity and are involved in xenobiotic and endobiotic metabolism. Although expression of CES1 and CES2 occurs in several organs, their expression in liver and small intestine is predominantly attributed to CES1 and CES2, respectively. We successfully expressed CES1 form b (CES1-b) and form c (CES1-c) as well as CES2 in baculovirus-infected High Five insect cells. With 4-nitrophenyl acetate (4-NPA) as the probe substrate, the K_m values of recombinant CES1-b and CES2 matched those of human liver microsomes (HLM) and human intestinal microsomes (HIM) with approximately 200 and 180 μM, respectively. Bis(4-nitrophenyl) phosphate potently inhibited 4-NPA hydrolysis by HLM, CES1-b, CES1-c, HIM, and CES2 with IC_{50} values less than 1 μM. With fluorescein diacetate (FD) as the substrate, the K_m values were similar for all enzyme systems, with the exception of CES1-b, which was slightly lower; however, the V_{max} values for HIM and CES2 were 39.5 and 14.6 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, respectively, which were at least 50-fold higher than those of CES1-b or CES1-c. Loperamide potently inhibited HLM, HIM, and CES2 with similar IC_{50} values of approximately 1 μM. Substrate specificity was compared between human tissues and recombinant enzymes. The data suggest the following: 1) FD is a probe substrate for CES2; 2) CES1-b is the predominant form in human liver; and 3) recombinant CES1-b and CES2 expressed in insect cells are functionally consistent with native carboxylesterases expressed in human liver and intestine, respectively.

Introduction

Human carboxylesterases (CES) are members of the serine hydrolase superfamily. They are categorized as phase I drug-metabolizing enzymes that can hydrolyze a variety of ester-containing drugs and prodrugs, such as angiotensin-converting enzyme inhibitors (temocapril, cilazapril, quinapril, and imidapril), anticancer drugs (irinotecan and capecitabine), and narcotics (cocaine, heroin, and meperidine) (Hosokawa, 2008). In humans, most carboxylesterases that have been identified belong to the CES1 and CES2 family, and they are differentiated on the basis of tissue distribution, substrate specificity, immunological properties, and gene regulation. CES1 protein is expressed in many organs, especially in the liver, but its expression in the gastrointestinal tract is markedly low (Imai, 2006). CES2 protein is also expressed in many extrahepatic tissues, especially in the gastrointestinal tract and at lower levels in the liver (Xu et al., 2002). CES1 preferentially hydrolyzes substrates that, upon hydrolysis, yield a smaller alcohol group and larger acyl group, whereas CES2 prefers substrates that yield a larger alcohol group and smaller acyl group (Imai et al., 2006).

This work was funded by BD Biosciences.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.111.039628.

There are three CES1 forms present in the National Center for Biotechnology Information database, CES1 form a (CES1-a; accession number NM_001025195), form b (CES1-b; accession number AB119997), and form c (CES1-c; accession number BC012418). Sequence alignment indicated that CES1-b and CES1-c lack Ala18 near the N terminus (Fig. 1A), whereas CES1-c lacks Gln362 in the proposed active site (Fleming et al., 2005) and several amino acids near the N terminus are different. Many recent studies have identified CES1-b in human livers by mRNA reverse transcription, purified enzyme analysis, and mRNA expression level (Danks et al., 1999; Tabata et al., 2004b; Hosokawa, 2008). To understand the differences in the enzymatic activities between the CES1-b and CES1-c forms, and to identify the predominant CES1 form in human liver, we successfully expressed CES1-b, CES1-c, and CES2 in baculovirus-infected High Five insect cells. The data presented in this study suggest that fluorescein diacetate (FD) is a probe substrate for CES2, and CES1-b is the dominant form in human liver.

Materials and Methods

Enzyme Preparations. cDNAs of hCE1-b, hCE1-c, and hCE2 were cloned into an engineered baculovirus expression vector using EcoRI site and confirmed with DNA sequencing. The constructs were cotransfected into Sf9 insect cells using the BD BaculoGold Transfection Kit (BD Pharmingen, San Diego, CA). For each construct, 30 plaques were screened for activity. Two

ABBREVIATIONS: CES1, human carboxylesterase 1; CES1-a, CES1 form a; CES1-b, CES1 form b; CES1-c, CES1 form c; FD, fluorescein diacetate; HLM, human liver microsomes; 4-NPA, 4-nitrophenyl acetate; HIM, human intestinal microsomes; BNPP, bis(4-nitrophenyl) phosphate; M4NB, methyl 4-nitrobenzoate.

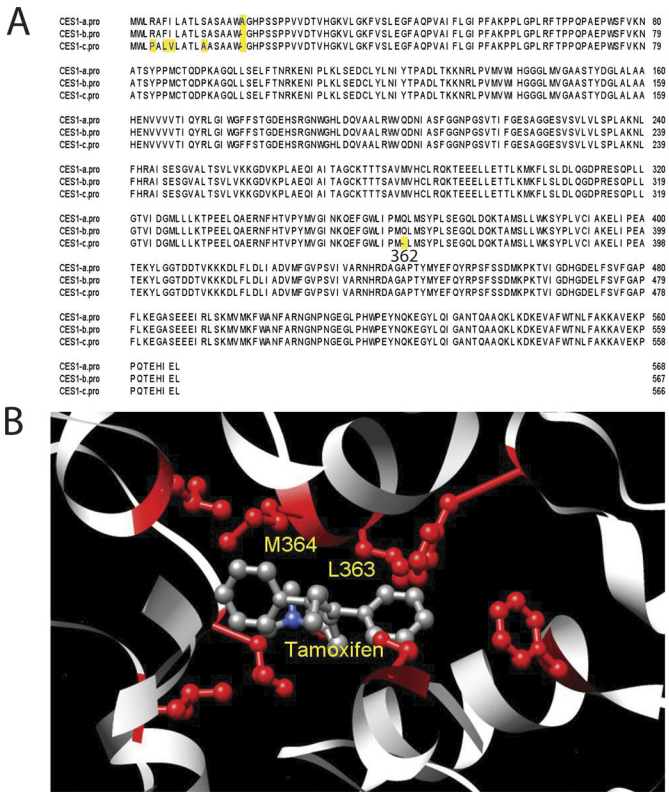


FIG. 1. The sequences of CES1-b and CES1-c are identical, with the exception of one residue, Gln362 (active site residue), and N terminus. A, sequence alignment between three CES1 forms. B, the active site of CES1-c in complex with tamoxifen (Protein Data Bank code 1YA4). Eight amino acid residues making hydrophobic contacts with tamoxifen are colored red (Fleming et al., 2005). Figure 1B was created with Chimera (Pettersen et al., 2004).

liters of High Five cells were infected and then incubated for 48 to 66 h at 27°C. Membrane fractions were collected to make recombinant enzyme microsomes. CES1-b, CES1-c, and CES2 were from BD Gentest (Woburn, MA). HLM was obtained from BD Gentest HLM 50 Donor Pool, HIM was obtained from BD Gentest pooled HIM, and control membrane protein was obtained from BD Gentest Insect Control.

Kinetic Curve Assays. Assays were conducted in 96-well plates at 37°C. The assay buffer was 0.1 M potassium phosphate, pH 7.4. Incubation time and protein concentration were chosen within a linear condition response. The final acetonitrile concentration was not greater than 1.5% for all assays. Assays were initiated by adding the substrate/buffer mix to the enzyme/buffer mix. For the 4-NPA assay, the protein concentration was 10 μg/ml for HLM and 20 μg/ml for HIM, CES1-b, CES1-c, and CES2. Formation of 4-nitrophenol from 4-NPA hydrolysis was monitored at 410 nm using $A = \varepsilon bc$ ($\varepsilon b = 11\ mM^{-1}$). For the FD assay, protein concentration was 0.5 μg/ml for HIM, 2.5 μg/ml for HLM and CES2, and 50 μg/ml for CES1-b and CES1-c. Product (fluorescein) was detected using excitation 483 nm, emission 525 nm, and $\varepsilon b = 554000\ \mu M^{-1}$ at a gain setting of 70 on a Safire^2 instrument (Tecan, Männedorf, Switzerland). The studies were conducted in triplicate. Kinetic parameters (K_m and V_{max}) were determined by nonlinear regression analysis using the Michaelis-Menten equation.

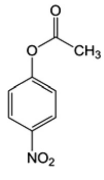
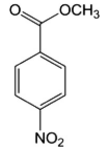
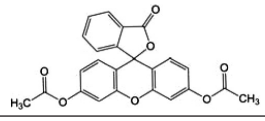
Inhibition Assays. Assays were conducted in similar methods as stated above with 1 mM 4-NPA for the colorimetric assay and 5 μM FD for the fluorescent assay. The maximal final concentration was 100 μM for bis(4-nitrophenyl) phosphate (BNPP) and loperamide with a 3-fold serial dilution to obtain the lower inhibitor concentrations. Reactions were initiated by the addition of the substrate/buffer mix to the plate. Metabolite formation was less than 20% in all conditions. The studies were conducted in duplicate. The IC_{50} value for each assay was determined by nonlinear regression using the 4-parameter Hill equation.

Specific Activity Assays. For the specific activity assays, the data from the top 4-NPA and FD assays were used. To determine the specific activity for methyl 4-nitrobenzoate (M4NB), enzyme solutions were diluted to 0.005 mg/ml (HLM, CES1-b, CES1-c) or 0.05 mg/ml (HIM, CES2) with 0.1 M potassium phosphate buffer, pH 7.4. The final substrate concentration was 500 μM. After a 10-min incubation at 37°C, the reaction was terminated by the addition of an equal volume of ice-cold acetonitrile. After centrifugation, supernatant was injected onto a high-performance liquid chromatography column (Zorbax SB-C18, 250 × 4.6 mm, 5 μm; Agilent Technologies, Santa Clara, CA). Mobile phases were 0.1% trifluoroacetic acid in H_2O (A) and 0.1% trifluoroacetic acid in acetonitrile (B). Separation was achieved with a gradient elution from 10% B to 90% B in 25 min. Column temperature was 45°C and the flow rate was 1 ml/min. The eluent was monitored at 274 nm.

TABLE 1

Hydrolysis of three representative ester-containing compounds by HLM, HIM, and recombinant enzymes

High Five control showed little activity compared with recombinant enzyme microsomes (data not shown).

Substrate	Structure	Specificity	Activity				
			HLM	CES1-b	CES1-c	HIM	CES2
			$\mu mol \cdot mg^{-1} \cdot min^{-1}$				
4-Nitrophenyl acetate		CES1≈ CES2	2.86	1.43	1.40	1.18	0.682
Methyl 4-nitrobenzoate		CES1	2.39	0.957	0.680	0.223	0.0990
Fluorescein diacetate		CES2	16.9	0.357	0.281	37.2	13.8

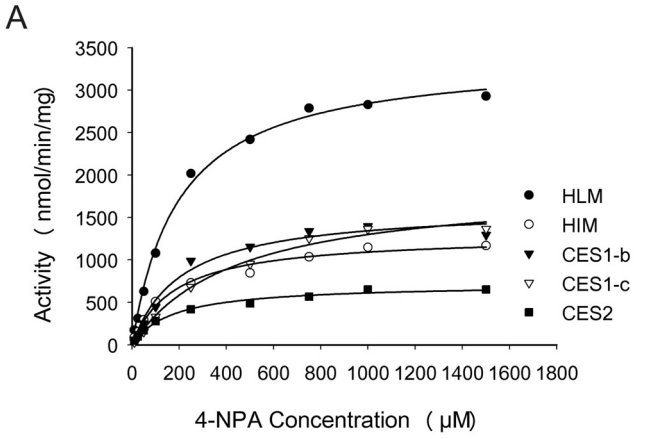
Results

Specific Activity. Recombinant CES1-b, CES1-c, and CES2 were successfully expressed in High Five insect cells, and their substrate specificities were compared with HLM and HIM. With 4-NPA as the substrate, CES1-b and CES2 showed specific activities of 1.43 and 0.682 μmol · mg^{-1} · min^{-1}, respectively (Table 1). With M4NB as the substrate, CES1-b and CES2 had specific activities of 0.957 and 0.0990 μmol · mg^{-1} · min^{-1}, respectively. With FD as the substrate, CES1-b and CES2 had specific activities of 0.357 and 13.8 μmol · mg^{-1} · min^{-1}, respectively.

Kinetics. The kinetics of CES1-b, CES1-c, and CES2 were compared with HLM and HIM using a common carboxylesterase substrate, 4-NPA (Li et al., 2005) (Fig. 2A), and a fluorogenic esterase substrate, FD (Battin, 1997) (Fig. 2B). All enzymes exhibited a typical Michaelis-Menten saturation curve. The kinetic parameters are summarized in Fig. 2C. With 4-NPA as the substrate, CES1-b, CES2, HIM, and HLM had similar K_m values between 170 and 210 μM, but CES1-c showed a 2-fold higher K_m value (441 μM). Both CES1-b and CES1-c had similar V_{max} values near 1.7 μmol · mg^{-1} · min^{-1}, which was approximately half of the V_{max} value for HLM (3.41 μmol · mg^{-1} · min^{-1}). In addition, the V_{max} values between CES2 and HIM were approximately 2-fold, 0.718 and 1.29 μmol · mg^{-1} · min^{-1}, respectively.

When FD was used as the substrate, all enzyme systems except CES1-b had similar K_m values, near 4 μM. CES1-c had a 2-fold higher K_m value than CES1-b (1.94 μM). The V_{max} values for CES2, HIM, and HLM were 14.6, 39.5, and 18.5 μmol · mg^{-1} · min^{-1}, respectively, which are greater than 50-fold higher than CES1-b (0.307 μmol · mg^{-1} · min^{-1}) and CES1-c (0.213 μmol · mg^{-1} · min^{-1}).

Inhibition. The IC_{50} values of the generic carboxylesterase inhibitor BNPP (Tabata et al., 2004a,b) and the CES2-specific inhibitor loperamide (Quinney et al., 2005) were compared with recombinant CESs and human tissues (Fig. 3). When 4-NPA was used as the substrate, BNPP exhibited potent inhibition toward HLM, CES1-b, CES1-c, HIM, and CES2 with IC_{50} values less than 1 μM. With the same substrate 4-NPA, loperamide inhibited HIM and CES2 with similar IC_{50} values near 1 μM; however, it showed minimal inhibition against HLM, CES1-b, and CES1-c. When FD was used as the substrate, loperamide inhibited HLM (0.664 μM) with similar IC_{50} values as HIM (1.07 μM) and CES2 (0.287 μM) (Fig. 3).



C

	4-NPA as substrate		FD as substrate	
Enzyme	K_m(μM)	V_{max}(μmol/mg/min)	K_m(μM)	V_{max}(μmol/mg/min)
HLM	198 ± 17	3.41 ± 0.08	4.87 ± 0.51	18.5 ± 0.5
CES1-b	208 ± 41	1.62 ± 0.09	1.94 ± 0.27	0.307 ± 0.011
CES1-c	441 ± 67	1.87 ± 0.11	4.31 ± 0.70	0.213 ± 0.011
HIM	182 ± 25	1.29 ± 0.05	4.04 ± 0.96	39.5 ± 2.5
CES2	173 ± 22	0.718 ± 0.024	4.82 ± 1.11	14.6 ± 0.9

FIG. 2. Michaelis-Menten plots with 4-NPA (A) or FD (B) as the substrate. The Michaelis-Menten kinetic parameters were summarized in C.

Discussion

Recombinant Enzymes Have Similar Characteristics as Native Enzymes. Sequence alignment indicated that the difference between CES1-a and CES1-b was due to the insertion of Ala18 near the N terminus (Fig. 1A), whereas the differences between CES1-b and CES1-c were the deletion of Gln362 as well as several amino acid changes near the N terminus. The crystal structure of CES1-c in complex with the breast cancer drug tamoxifen (Fleming et al., 2005) (Protein Data Bank code 1YA4) indicated that the N-terminal residues were flexible and far from the active site, whereas Gln362 is adjacent to two substrate binding residues (Leu363 and Met364) in the active site (Fig. 1B). Because Leu363 and Met364 are both involved in substrate binding, it is possible that CES1-a and CES1-b have similar substrate binding characteristics due to the presence of Gln362; however, the absence of Gln362 may alter the substrate binding for CES1-c.

The observed difference of K_m values between CES1-b and CES1-c with 4-NPA and FD seems to be consistent with the crystal structure (Fig. 1B). Leu363 and Met364 are two of the eight residues involved in tamoxifen binding, and the absence of Gln362 may change the geometry of the binding site and directly affect the affinity of the substrate and impact the K_m value. Furthermore, Gln362 is away from the catalytic triad Ser221-His468-Glu354, which is on the other side of the active site, and the absence of Gln362 may have less of an effect on the maximal rate of enzyme catalysis. Although the k_{cat} values for CES1-b and CES1-c are unknown, there seems to be comparable expression levels of CES1-b and CES1-c based on Western Blot analysis (data not shown) and similar V_{max} values.

Because CES1 was more abundant than CES2 in liver, when 4-NPA was used, HLM activity is mainly attributed to CES1. Because the IC_{50} values with BNPP and K_m values with CES1-b and HLM are similar, the data suggest that CES1-b, or CES1-a, is the primary CES1 form in the liver. This conclusion seems to be supported by the results of other laboratories finding CES1-b but not CES1-a in human liver (Danks et al., 1999; Tabata et al., 2004b; Hosokawa et al., 2008). Based upon these results, it is reasonable to conclude that CES1-b is the primary form in human liver.

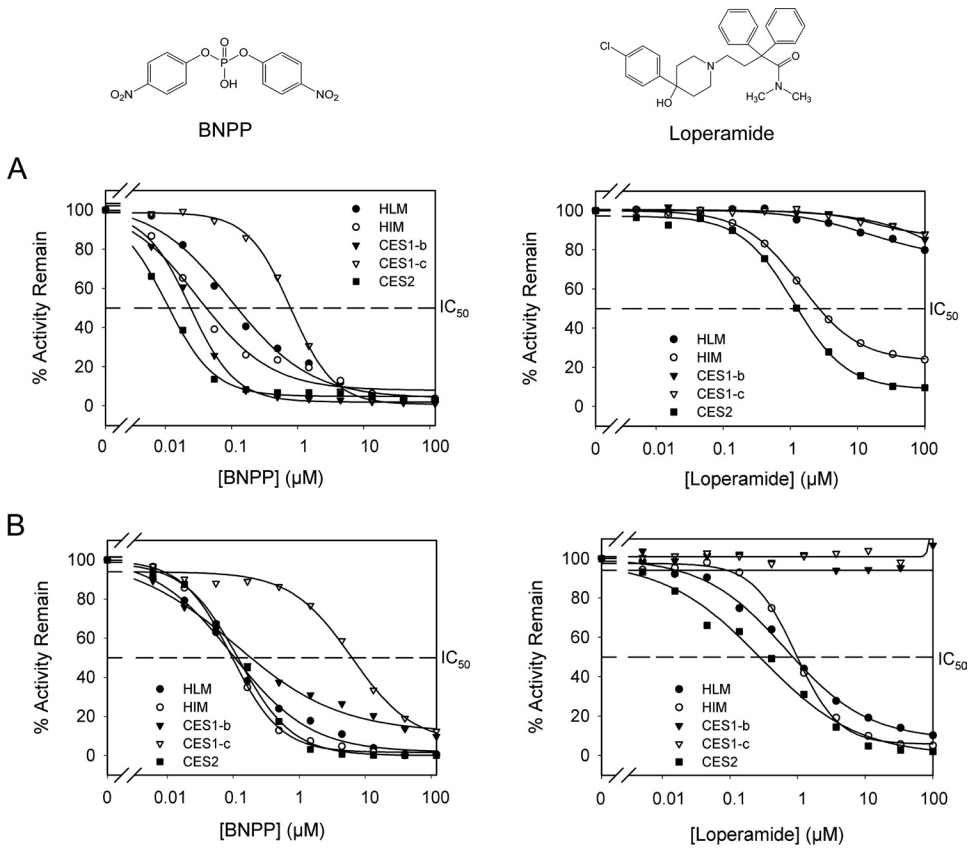


FIG. 3. Effects of inhibitors BNPP and loperamide with either 4-NPA (A) or FD (B) as the substrate. The structures of BNPP and loperamide are shown at the top. The IC_{50} values were summarized in C.

C

IC_{50} (μM)

	4-NPA as Substrate		FD as Substrate	
Enzyme	BNPP	Loperamide	BNPP	Loperamide
HLM	0.0990	>100	0.0969	0.664
CES1-b	0.0236	>100	0.107	>100
CES1-c	0.788	>100	6.31	>100
HIM	0.0316	1.45	0.0944	1.07
CES2	0.0104	1.12	0.120	0.287

The specific activity, K_m values, and IC_{50} values with CES1-b and CES2 match the values obtained in HLM or HIM, respectively, indicating that the insect cell-expressed recombinant enzymes have similar characteristics with the native enzymes. In spite of its structure, 4-NPA does not display the same substrate preference for CES1 or CES2 as other substrates. M4NB and FD prefer CES1 and CES2, respectively, favoring a larger acyl group or a larger alcohol group, respectively (Imai, 2006; Imai et al., 2006). The specific activity comparison was in agreement with the previously reported trend.

FD Is a Probe Substrate for CES2. A previous study showed that purified recombinant CES1 and CES2 had similar k_{cat} values with 4-NPA as the substrate (E. T. Williams, personal communication). In this study, the V_{max} ratio with 4-NPA for recombinant CES1 over CES2 is 2:1, which indicates that the expression levels of CES1 over CES2 is roughly 2-fold. When FD was used as the substrate, CES2 had a 50-fold greater V_{max} value than CES1, suggesting a roughly 100-fold greater k_{cat} value for CES2 than CES1. Therefore, FD was a probe substrate for CES2. This was further confirmed by the observation that loperamide inhibited HLM with similar IC_{50} values as HIM and CES2, when FD was used as the substrate. Although Western blot analysis (data not shown) and previous studies (Imai et al., 2006) indicated that HLM contains both CES1 and CES2, the high FD hydrolysis by HLM was most likely due to the presence of CES2 because the CES1 Supersomes showed substantially lower V_{max} values. The structure of FD is also consistent with the proposed CE substrate selectivity (Imai et al., 2006).

The V_{max} value of HIM was higher than HLM with FD as the substrate, which suggested that CES2 is less abundant in liver than in intestine, consistent with Western blot analysis (data not shown) and hydrolysis in a nondenaturing protein gel (Imai et al., 2006). High concentrations of loperamide inhibited HIM hydrolysis of 4-NPA by nearly 100%, suggesting that CES2 is the primary carboxylesterase in intestine. Both findings were consistent with a previous report of esterase activity in a nondenaturing protein gel (Imai et al., 2006).

Conclusions

In conclusion, FD was demonstrated as a fluorogenic in vitro CES2-selective probe substrate. In addition, the results further support that CES1-b is the major expressed form in human liver, and the K_m difference of the CES1 forms may be explained by its structure. The insect cell-expressed recombinant enzymes have similar characteristics with native enzymes.

Authorship Contributions

Participated in research design: Wang, Patten, and Williams.
Conducted experiments: Wang and Bourgea.

Contributed new reagents or analytic tools: Wang.

Performed data analysis: Wang.

Wrote or contributed to the writing of the manuscript: Wang, Williams, Wong, and Patten.

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