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Review

Structure and Catalytic Properties of Carboxylesterase Isozymes Involved in Metabolic Activation of Prodrugs

Masakiyo Hosokawa

Laboratory of Drug Metabolism and Biopharmaceutics, Faculty of Pharmaceutical Sciences, Chiba Institute of Science, Shiomi-Cho, Choshi-City, Chiba 288-0025, Japan;
E-mail: Masakiyo@cis.ac.jp

Abstract: Mammalian carboxylesterases (CESs) comprise a multigene family whose gene products play important roles in biotransformation of ester- or amide-type prodrugs. They are members of an α,β-hydrolase-fold family and are found in various mammals. It has been suggested that CESs can be classified into five major groups denominated CES1-CES5, according to the homology of the amino acid sequence, and the majority of CESs that have been identified belong to the CES1 or CES2 family. The substrate specificities of CES1 and CES2 are significantly different. The CES1 isozyme mainly hydrolyzes a substrate with a small alcohol group and large acyl group, but its wide active pocket sometimes allows it to act on structurally distinct compounds of either a large or small alcohol moiety. In contrast, the CES2 isozyme recognizes a substrate with a large alcohol group and small acyl group, and its substrate specificity may be restricted by the capability of acyl-enzyme conjugate formation due to the presence of conformational interference in the active pocket. Since pharmacokinetic and pharmacological data for prodrugs obtained from preclinical experiments using various animals are generally used as references for human studies, it is important to clarify the biochemical properties of CES isozymes. Further experimentation for an understanding of detailed substrate specificity of prodrugs for CES isozymes and its hydrolysates will help us to design the ideal prodrugs.

Keywords: carboxylesterase, CES1, CES2, prodrug metabolism, structure

Introduction

Mammalian carboxylesterases (CESs, EC 3.1.1.1) comprise a multi-gene family whose gene products are localized in the endoplasmic reticulum (ER) of many tissues. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals as well as drugs (including prodrugs) to the respective free acids. They are involved in detoxification or metabolic activation of various drugs, environmental toxicants and carcinogens. CESs also catalyze the hydrolysis of endogenous compounds such as short- and long-chain acyl-glycerols, long-chain acyl-carnitine, and long-chain acyl-CoA esters [1-9]. We have reviewed the characteristics of CESs in relation to the metabolism of xenobiotics [10-12]. Multiple isozymes of hepatic microsomal CES exist in various animal species [3, 13, 14], and some of these isozymes are involved in the metabolic activation of certain carcinogens as well as being associated with hepato-carcinogenesis [6].

Mammalian CES are members of an α,β-hydrolase-fold family and are found in various mammal spp. [3, 15-25]. It has been suggested that CESs can be classified into five major groups denominated CES1-CES5, according to the homology of the amino acid sequence [10-12], and the majority of CESs that have been identified belong to the CES1 or CES2 family. It has also been shown that striking species differences exist [3, 13, 26]. For example, Inoue *et al*. [27] showed that esterase activity in the dog intestine is very weak and produced no appreciable active band in a disc electrophoresis coupled with staining of esterase activity. On the other hand, esterase activities were observed in the intestines of other species (human, rat, mouse, guinea pig and rabbit) [12, 26, 28-30] and found to produce a few active bands in an electrophoretic assay. Since pharmacokinetic and pharmacological data for ester-prodrugs obtained from preclinical experiments using various animals are generally used as references for human studies, it is important to clarify the biochemical properties of each CES isozyme such as substrate specificity, tissue distribution and transcriptional regulation.

CESs show ubiquitous tissue expression profiles with the highest levels of CES activity present in liver microsomes in many mammals [1, 6, 8, 31-38]. Drug-metabolizing enzymes that are present predominantly in the liver are involved in biotransformation of both endogenous and exogenous compounds to polar products to facilitate their elimination. These reactions are categorized into phase I and phase II reactions. CESs 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) [1, 39-41], anti-tumor drugs (CPT-11 and Capecitabin) [42-49] and narcotics (cocaine, heroin and meperidine) [16, 50, 51]. In this regard, it is thought that CESs are one of the major determinants for pharmacokinetics and pharmacodynamics of ester-drugs or ester prodrugs (Figure 1). Actually, it has been shown that the dog CES1 isozyme was involved in a pulmonary first-pass effect in the disposition of a propranolol ester prodrug [28, 52]. It has also been shown that the expression level of the human CES isozyme was correlated with the conversion ratio of CPT-11 to SN-38, the active metabolite, which is thought to be a key step for the chemotherapeutic action of this anti-tumor drug [47, 53-55].

This review focuses on the molecular characteristics of CES isozymes, the different structure-activity relationship of substrates with each CES families and genomic structure and regulation of CES genes. This information is important in the design and the development of the prodrugs.

Important role of CES in drug metabolism with other related enzymse or transporters

Since many drug metabolizing enzymes, such as cytochrome P450(CYP), CES, UDP-glucuronosyl-ntransferase (UGT) and sulfotransferase, and transporters, such as P-glycoprotein (P-GP), multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP), were co-expressed in liver and small inteisine, the hydrolase activity in the liver and small intestine is contribute to drug metabolism and drug transport with phase II drug metabolizing enzyme or drug transporter. The CESs and the UGT family, the catalytic domains of which are localized in the luminal sides of the endoplasmic reticulum (ER) membrane, are two major enzyme groups responsible for phase I and II reactions (Figure 2). Products hydrolyzed by CESs, such as SN-38 from CPT-11, are also good substrates for UGT. Thus, we speculated that CES-UGT interaction in the luminal side of the ER membrane is important for drug metabolism. As shown in Figure 1, two hydrolyzed products from ester-substrate are formed by CES: alcohol or phenol, which are substrates for UGT, and organic anions, which are substrates for organic anion transporter such as MRP2 or BCRP. In this regard, we thought that CES is major drug-metabolizing enzymes for enzyme-enzyme interaction or enzyme-transporter interaction.

Figure 1. CES-UGT interaction in the luminal side of the ER membrane and CES-transporter interaction in the cell. Two hydrolyzed products from ester-substrate are formed by CES; alcohol or phenol, which are substrates for UGT, and organic anions, which are substrates for organic anion transporter such as multidrug resistance-associated protein 2 (MRP2) or breast cancer resistance protein (BCRP).
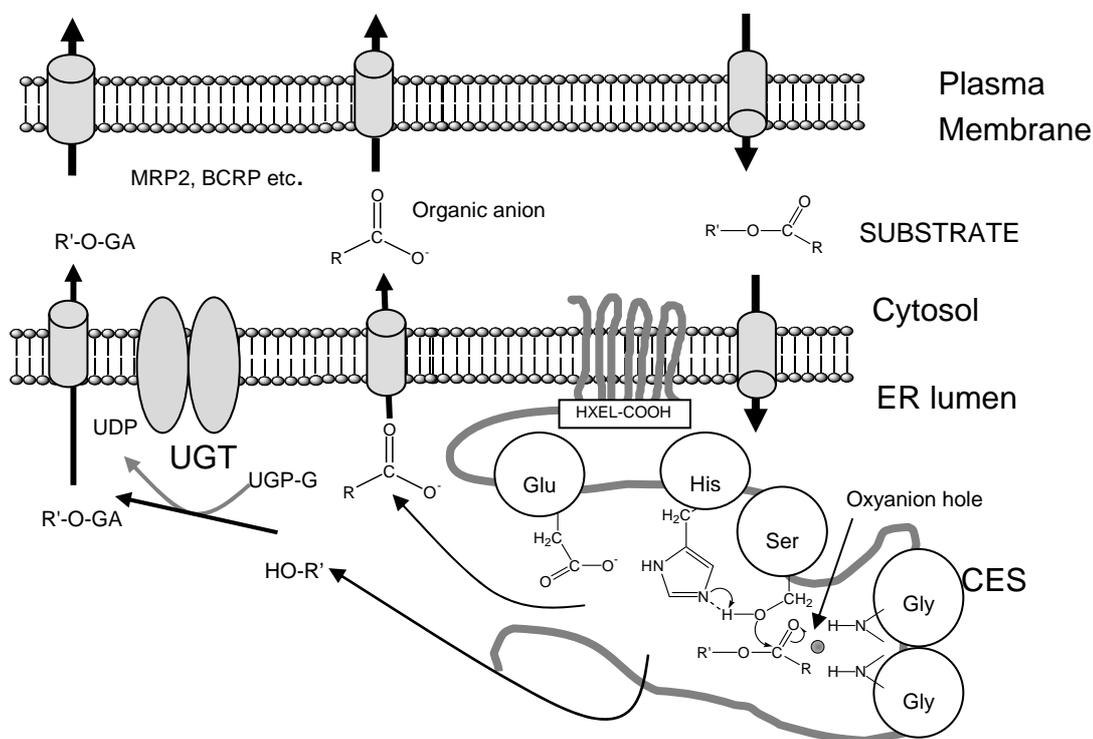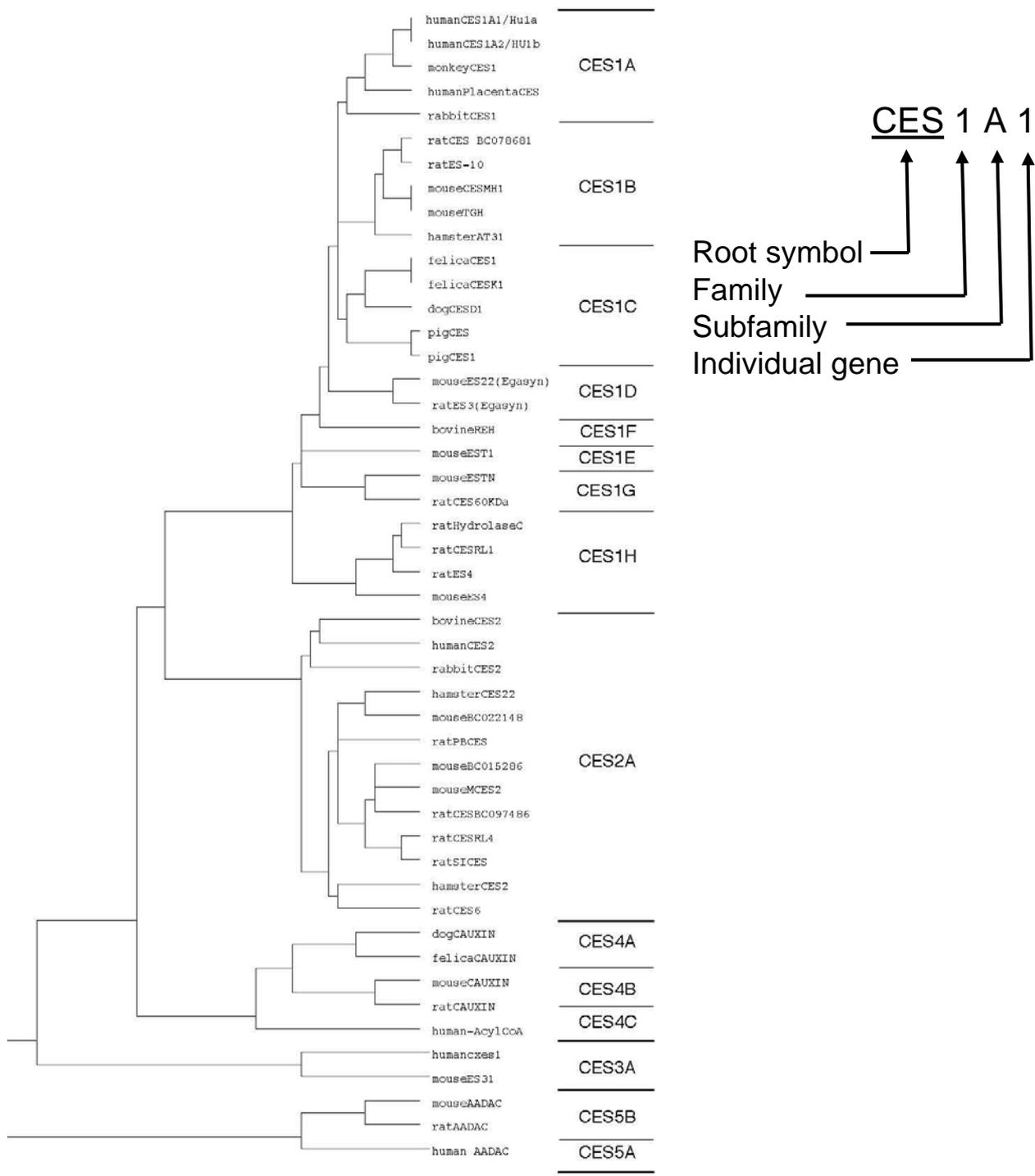
Figure 2. Phylogenic tree and nomenclature of CES families. CES isozymes are classified into five families, CES1, CES2, CES3, CES4 and CES5. Each families also divided into subfamilies.

Classification and Nomenclature of CES Superfamily

According to the classification of esterase by Aldridge [56], the serine superfamily of esterase, i.e., acetylcholine-esterase, butyrylcholine-esterase and CES, fall into the B-esterase group. It is becoming increasingly clear that esterases tend to have a broad and overlapping substrate specificity toward

amides and esters. A single esterolytic reaction is frequently mediated by several kinds of enzyme. Recent studies on esterases as well as other enzymes, such as acetylcholine esterase, butyrylcholine esterase, cholesterol esterase, triacylglycerol lipase and CES, involved in xenobiotic metabolism have provided evidence of multiple forms. It seems almost impossible to classify these CES isozymes based on their substrate specificity along the lines of the International Union of Biochemistry (I.U.B.) Classification because the individual hydrolases exhibit properties of CES, lipase or both. Mentlein *et al.* [37] proposed classification of these hydrolases as "unidentified CESs" (EC 3.1.99.1 to 3.1.99.x). Based on amino acid sequence alignment of the encoding genes, we tried to classify CES isozymes into five families, CES 1, CES 2, CES 3, CES4 and CES 5 (Figure 2) [11, 12]. The CES 1 family includes the major forms of CES isozymes (more than 60% homology of human CES1A1). Thus, they could divide into eight subfamilies. Most of all CES1 family, except CES1G, are mainly expressed in liver. The CES 1A subfamily includes the major forms of human, monkey and rabbit CES, and the CES1B includes the major isoforms of rat, mouse and hamster CES, and CES1C includes the major isoforms of dog, cat and pig CES [1, 4, 5, 23, 40, 46, 57-60]. The CES 1H subfamily includes CES RL1 (CES1H4), mouse ES 4(Ces1H1) and Hydrolase B (CES1H3) and C (CES1H2), which catalyze long-chain acyl-CoA hydrolysis [3, 4, 10, 22, 25, 61]. Members of the CES 1G family are not retain in ER, which CES isozymes are secrete to blood from liver [24, 58]. It is interested that CES1G family isozymes only found in rats and mice, not found in human. Although high level of hydrolase activity can be detected in the blood of the rats and mice, no such activity is detected in the blood of humans. On the other hand, the CES2 family includes human intestinal CES (CES2A1) [28, 30, 44, 62, 63], rCES2 (CES2A10) [64], rat intestinal CES, mCES2 (Ces2A6) [2], rabbit form2 [65] and hamster AT51 (CES2A11) [66], which are mainly expressed in small intestines. The CES3 family includes ES-male (CES3A2) and human CES3 (CES3A1) [67, 68]. Human CES3 (CES3A1) has about 40% amino acid sequence identity with both CEA1A1 and CES2A1, and is expressed in the liver and gastrointestinal tract at an extremely low level in comparison with CES1A1 and CES2A1 [68]. The CES4 family includes carboxylesterase-like urinary excreted protein (CAUXIN) (CES4A2), which is excrete as a major urinary protein in cat urine [69, 70]. The CES 5 family includes 46.5-kDa CES isozymes [71, 72], which have a different structure from the structures of isozyme in other CES families. ES 46.5-kDa from mouse liver [38] and amide hydrolase of monkey liver [19] probably belong to this family. These groupings are similar to the results of phylogenic analysis (Figure 2).

Structure and Catalytic Mechanism of CES Isozymes

It has been shown that several proteins of the ER lumen have a common carboxy-terminal sequence, KDEL-COOH, and that the structural motif is essential for retention of the protein in the luminal side of the ER through the KDEL receptor bound to the ER membrane [73-75]. Korza and Ozols [76] and Ozols [65] have established the primary structures of two microsomal esterases purified from rabbit liver and designated them 60-kDa esterase forms 1 and form 2, respectively. These two forms of CES have the consensus sequence for the ER retention tetra-peptide (HTEL or HIEL in the one-letter code). The HXEL-COOH motif is also essential for retention of the protein in the luminal side of the ER through the KDEL receptor bound to ER membrane [73-75]. Robbi *et al*. [60] reported cDNA cloning of rat liver CES1B4 (ES-10). That was the first report to show that cDNA of liver CES has the

consensus sequence of the ER retention tetrapeptide (HVEL-COOH). Later, Robbi and Beaufay [77] isolated a cDNA clone of another rat liver CES1D2 (ES-3) which encode the consensus sequence of the ER retention tetrapeptide (HTEL-COOH). The other clone encoded egasyn, an accessory protein of β-glucuronidase in the liver microsomes [78]. Egasyn is identical to CES, and it binds β-glucuronidase *via* its CES active site. In the case of mouse rat and mouse, the carboxyl terminal amino acid sequence of clone rat CES60KDa(CES1G1) and mouse Es-N (Ces1G2) is HTEHK-COOH, which could not bound to KDEL-receptor, and these isozymes are secreted to blood [58].

CES have a signal peptide of 17 to 22 amino acid residues of N-terminal amino acid, including hydrophobic amino acid. In CES1 family, exon1 encode a signal peptide [79, 80]. In a case of CES1 family, a bulky aromatic residue (Trp) followed by a small neutral residue (Gly) directly precedes the cleavage site [81].

CES have four Cys residues that may be involved in specific disulfide bonds. Among them, Cys98 is the most highly conserved residue in many CES isozymes. Cygler *et al*. [82] reported an important alignment of a collection of related amino acid sequences of esterase, lipase and related proteins based on X-ray structures of *Torpedo californica* acetylcholinesterase and *Geotrichum candidum* lipase. According to these authors, Ser_{203}, Glu_{336} and His_{450} form a catalytic triad, and Gly_{124}-Gly_{125} may be part of an oxyanion hole. These residues are also highly conserved among CES isozymes. As a result we have started mutation analysis [11]. Site-specific mutation of Ser_{203} to Thr_{203}, Glu_{336} to Ala_{336}, or His_{450} to Ala_{450} greatly reduced the CES activity towards substrates. Therefore, the mutagenesis confirmed a role of Glu_{336} and His_{450} in forming a putative charge relay system with active site Ser_{203} [11].

Frey *et al*. [83] reported that the formation of low barrier hydrogen bonds between His and Asp (Glu for CES) facilitates nucleophilic attack by the β-OH group of Ser on the acyl carbonyl group of peptide in chymotrypsin. The catalytic triad in the tetrahedral addition intermediate is stabilized by the low barrier hydrogen bonds. According to their theory, we speculated that the low barrier hydrogen bond between Glu_{336} and His_{450} facilitates nucleophilic attack by the β-OH group of Ser_{203} on the carbonyl group of the substrate in CES (Figure 3). The mechanism of CES could thus be divided into next steps. 1) The enzyme substrate complex form, positioning the substrate in the correct orientation for reaction. 2) Hydrolysis of the ester bond starts with an attack by the oxygen atom of the hydroxy group of Ser_{203} on the carbonyl carbon atom of the ester bond. 3) The hydrogen bonds between the negatively charged oxygen of the tetrahedral intermediate and the N-H group of Gly_{123} and Gly_{124} stabilize the negatively charged oxygen (O⁻). This configuration, in which negatively charged carboxyl oxygen is hydrogen bonded to two N-H group, is called oxyanion hole. In the general acid-catalyzed step, the ester bond breaks, and the leaving group picks up a proton from the imidazolium ion of His_{450}. The acyl portion of the original ester bond remains bound to the enzyme as an acyl-enzyme intermediate. The alcohol component (R'-OH) diffused away, completing the acylation stage of the hydrolytic reaction. 4) A water molecule attacks the acyl-enzyme intermediate to give a second tetrahedral intermediate. 5) His_{450} then donates the proton to the oxygen atom of Ser_{203}, which then releases the acid component of the substrate. The acid component diffused away and the enzyme is ready for catalysis. The tetrahedral transition state is stabilized by the formation of low barrier hydrogen bonds between His_{450} and Glu_{336}. The low barrier hydrogen bonds facilitated mechanism includes weak hydrogen bonds between the oxyanion (O⁻) and peptide N-H bonds contributed by Gly_{123} and Gly_{124}, which stabilize the tetrahedral adduct on the substrate side of the transition state (Figure 3). Formation of the acyl-enzyme complex in the next step

requires removal of a proton from His_{450}, so that the tetrahedral intermediate is disrupted in the acyl-enzyme intermediate. When the unbound portion of the alcohol group of the first product of the substrate has diffused away, a second step which the deacylation step is essentially the reverse of the acylation step occurs, with a water molecule substituting for the alcohol group of the original substrate (Figure 3).

Figure 3. Proposed mechanism for the action of CES. Conformation of the Ser-His-Glu catalytic triad in CES.
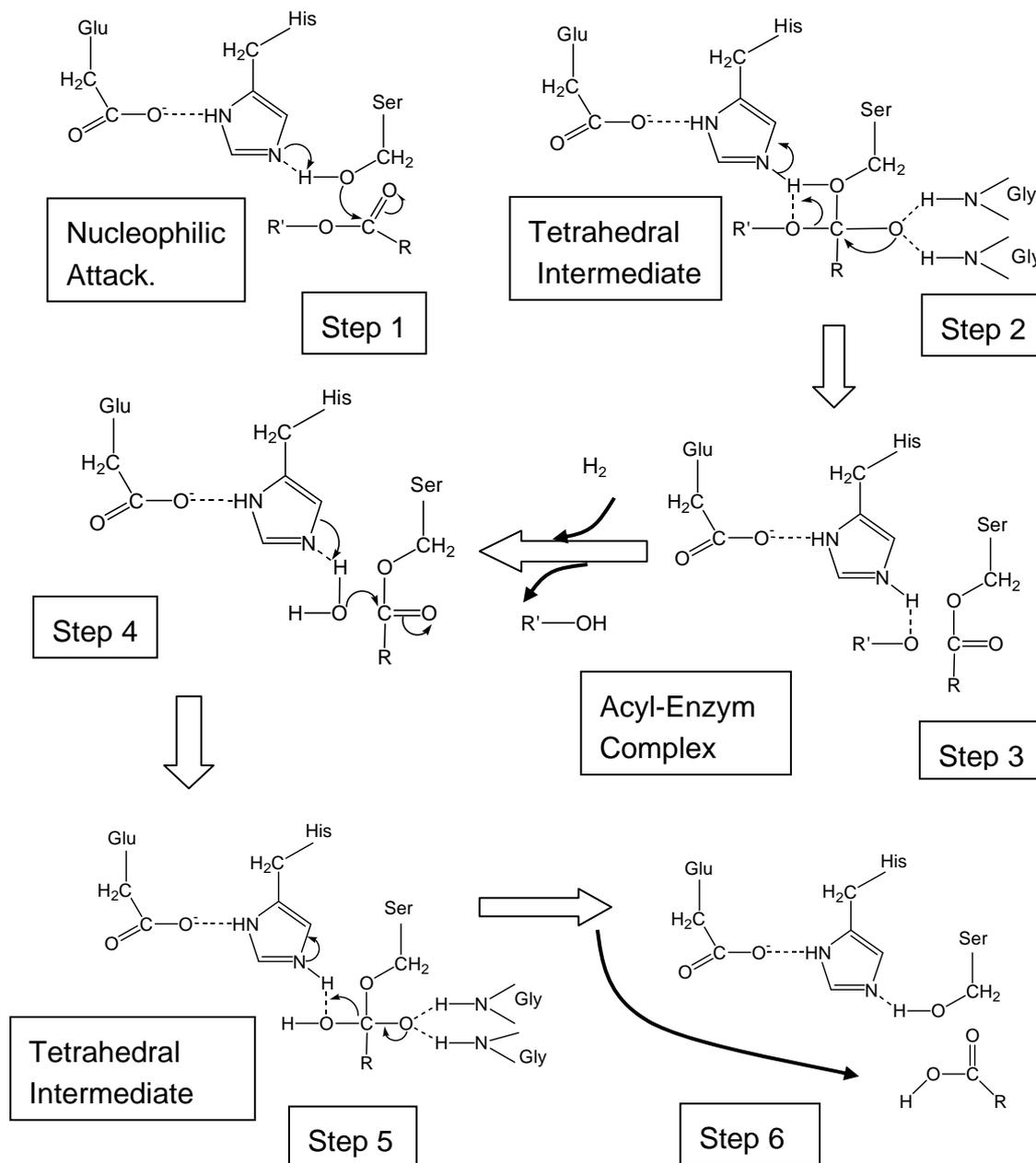
Ser, Ser_{203}; His, His_{450}; Glu, Glu_{336}; Gly, Gly_{123} or Gly_{124}

It is of interest that the sequences required for the hydrolytic capability at the catalytic triad (Glu, His, Ser) of CES, acetylcholine esterase, butyrylcholin esterase, and cholesterol esterase are highly

conserved. This is a common structure of α,β-hydrolase-fold families, which are responsible for the hydrolysis of endogenous and exogenous compounds.

Furthermore, these elements are strongly conserved among orthologous CESs of the mouse, rat, rabbit, monkey and human. A three-dimensional model for human CES has been proposed on the basis of crystal structure coordinates of acetylcholine esterase and overlapping active sites with pancreatic lipase and CES [84]. The modeled structure shares the overall folding and topology of the proteins identified in the recently published crystal structures of the rabbit [85] and human CES [86, 87]. CES has a three-dimensional α,β-hydrolase-fold structure, which is a structural feature of all lipases [88]. In general, the structure of CES may be viewed as comprising a central catalytic domain surrounded by $\alpha-\beta$ and regulatory domains [85-87]. In essence, the α,β-hydrolase-fold consists of a central β-sheet surrounded by a variable number of α-helices and accommodates a catalytic triad composed of Ser, His and a carboxylic acid. This suggests that the catalytic function of these proteins is conserved across species. The catalytic triad is located at the bottom of about 25 Å deep active site cleft, approximately in the center of the molecule and comprises a large flexible pocket on one side of Ser_{203} and a small rigid pocket on the opposite side [86]. The orientation and location of the active site provides an ideal hydrophobic environment for the hydrolysis of a wide variety of hydrophobic substrates [86]. The small rigid active site pocket is adjacent to the oxanion hole formed by $Gly_{123-124}$ and is lined by several hydrophobic residues [86]. Short acyl chains would be easily accommodated within the small rigid pocket. The larger flexible active site pocket is lined by several non-polar residues and could accommodate larger or polycyclic molecules such as cholesterol. The large pocket is adjacent to a side door secondary pore that would permit small molecules (substrates and reaction products) to enter and exit the active site [86]. Longer acyl chains may be oriented for catalysis in such a way that they extend through the side door. Indeed, the presence of a hydrophobic residue at position 423 in mice Ces1B2 and 425 in humans CES1A1 is necessary for efficient hydrolysis of hydrophobic substrates, as mutation of Met present in position 423 of the related rat lung CES (CES1B4) to Ile increased the CES activity towards a more hydrophobic substrate without affecting activity towards short-chain esters [89].

Most CES isozymes are glycoproteins, and the carbohydrate chain is required for the enzyme activity of CESs [10, 18, 86, 90, 91]. Human CES2A1 contains a glycosylation site at two different positions (Asn_{103} and Asn_{267}), while CES1A1 contains only one glycosylation site at Asn_{79}. This glycosylation site is modified by a carbohydrate chain with first N-acetylglucosamine and terminal sialic acid and appears to be involved in the stabilization of the CES1A1 trimer by packing into the adjacent monomer in its crystal structure [86]. According to the X-ray crystal structure of human CES1, this residue lines the flexible pocket adjacent to the side door [86]. Given the wide range of substrates that CESs are known to hydrolyze, the large flexible pocket confers the ability to hydrolyze many structurally distinct compounds, whereas the rigid pocket is much more selective with regard to the substrates that may be accommodated.

Table 1. Structure-activity relationship of substrates with CES1 and CES2 families.
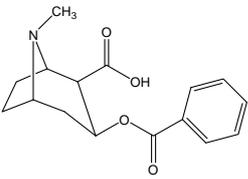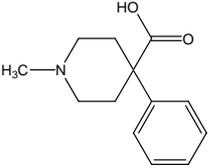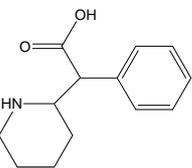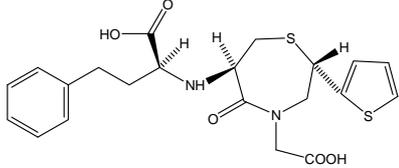
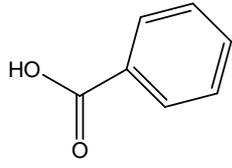| Substrate	Alcohol Substituent	Acyl Substituent	Substrate Specficity
Cocaine (methyl ester)	CH_3OH		CES1
Meperidine	CH_3CH_2OH		CES1
Methylphenidate	CH_3OH		CES1
Temocapril	C_2H_5OH		CES1
Oseltamivir	C_2H_5OH		CES1
Cocaine (benzoyl ester)			CES2>>CES1
CPT-11			CES2>>CES1
Heroin		CH_3COOH	CES2>>CES1
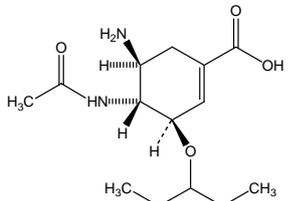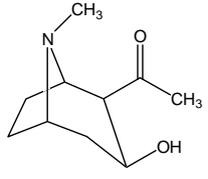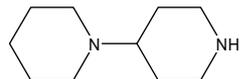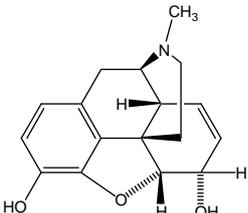
Table 1. Cont.

Substrate	Alcohol Substituent	Acyl Substituent	Substrate Specficity
Methylprednisolone 21-Hemisuccinate		HOOC-CH$_2$-CH$_2$-COONa	CES2>>CES1
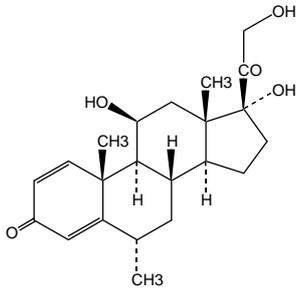
Structure-activity Relationships of Substrates with CES1 and CES2 Families

Recent studies have shown that there are some differences between these families in terms of substrate specificity, tissue distribution, immunological properties, and gene regulation [10]. For example, the preferential substrates for CES1A1, a human CES1 family isozyme, are thought to be compounds esterified by small alcohols, while those for CES2A1, a human CES2 family isozyme, are thought to be compounds esterified by relatively large alcohols (Table 1). CES1A1, but not CES2A1, hydrolyzed the methyl ester of cocaine and the ethyl esters of temocapril, meperidine and delapril [1, 40, 41, 54, 92]. More recently, Sun *et al.* [93] reported that CES1A1 has a high catalytic efficiency for both *d-* and *l*-enantiomers of methylphenidate. No catalytic activity was detected with CES2 and CES3 for either enantiomer. The catalytic efficiency of CES1A1 for l-methylphenidate (kcat/Km= 7.7 mM^{-1}min^{-1}) is greater than that for *d*-methylphenidate (kcat/Km= 1.3-2.1 mM^{-1}min^{-1}). Hence, the catalytic efficiency of CES1A1 for methylphenidate enantiomers agrees with stereoselective clearance of methylphenidate reported in human subjects. Both enantiomers of methylphenidate can fit into the three-dimensional model of CES1A1 to form productive complexes in the active site [93]. Oseltamivir as an anti-influenza viral agent is an ester prodrug, and its antiviral activity is achieved by its hydrolytic metabolite: oseltamivir carboxylate. Shi *et al.* [94] reported that human liver microsomes rapidly hydrolyzed oseltamivir, but no hydrolysis was detected with intestinal microsomes or plasma. Recombinant CES1A1, but not CES2A1, hydrolyzed oseltamivir and produced similar kinetic parameters as the liver microsomes [94]. We also reported that mouse mCES1(CES MH1), a mouse CES1 family isozyme, hydrolyzed temocapril, which esterified a small alcohol, similar to human CES1 isozyme [1].

In contrast to the specificity of CES1 for the methyl ester of cocaine, only CES2 hydrolyzed the benzoyl ester of cocaine [54, 92]. For the remaining substrates that could be hydrolyzed by both enzymes, CES2 exhibited higher catalytic efficiency than did CES1 for heroin; enzymatic conversion of 6-acetylmorphine to morphine was not known before the isolation and characterization of CES2 [50]. CPT-11 and heroin bearing a small acyl moiety and a bulky alcohol group are good substrates for the CES2 isozyme (Table 1). On the other hand, we also reported that rat rCES2 (also called CES RL4), a rat CES2 family isozyme, hydrolyzed methylprednisolone hemisuccinate, which esterified a large alcohol as did the human CES2 family [64]. It has been suggested that although these two CES families exhibit

broad substrate specificity for ester, carbamate, or amide hydrolysis, these CES isozymes do exhibit distinct catalytic efficiencies that correlate with the relative size of the substrate substituents versus that of the enzyme active sites.

Tissue specific expression of CES1 and CES2 was examined by northern blots, RT-PCR and real time PCR analysis. As shown in Table 2, human CES1A was highly expressed in liver and lung, human CES2A was highly expressed in small intestine and kidney. Knowledge of these substrate structure-activity relationships and the tissue distribution of CES isozymes is critical for predicting the metabolism and the pharmacokinetics and pharamcodynamics of ester drugs or prodrugs.

Table 2. Tissue-specific expression profile of CES1 and CES2 isozymes in mammals and humans.

Species	Isozyme	Liver	Small intestine	Kidney	Lung
Mouse	CES1	+++	-	+++	+++
	CES2	+++	+++	+++	-
Rat	CES1	+++	-	+++	+++
	CES2	-	+++	-	-
Hamster	CES1	+++	-	+++	NT
	CES2	+++	+++	-	NT
Guinea Pig	CES1	+++	+++	++	NT
	CES2	-	+	-	NT
Beagle Dog	CES1	+++	-	NT	+++
	CES2	++	-	NT	+
Monkey	CES1	+++	++	-	NT
	CES2	+	+++	+	NT
Human	CES1	+++	-	+	+++
	CES2	+	+++	+++	-

-, undetectable, +, weakly expressed, ++, moderately expressed, +++, strongly expressed, NT, not tested

## Gene Structure and Regulation of CES Isozymes

Both the murine [10] and human [80, 95] CES1 genes span about 30 kb and contain 14 small exons. Recently, sequencing of the mouse and human genomes have been completed, enabling detailed sequence comparisons. Previously published sequences of individual exons, splice junctions, size of the introns and restriction sites within the murine and human CES genes are consistent with their respective genes sequenced by the mouse and human genome projects. Therefore, the organization of the CES gene is evolutionarily conserved in mice and humans. In previous studies, the human CES gene have been mapped to chromosome 16 at 16q13–q22.1 [18, 57]. This region is syntenic to a region of mouse chromosome 8 at 8C5 [57]. The murine CES Es22 [79] and Es-N [58] have been previously mapped to chromosome 8. The completion of the mouse genome sequencing project unambiguously demonstrated that the murine CES gene was located on the minus strand of chromosome 8 at 8C5 in a cluster of six CES genes that spans 260.6 kb in total. These six CES genes are presumed to have originated from repeated gene duplications of a common ancestral gene that encoded a CES [80], and subsequent evolutionary divergence occurred.

Recently, we have identified a mouse liver microsomal acylcarnitine hydrolase, mCES2, as a member of the CES 2 family [2]. It has been revealed that this enzyme is significantly induced by di(2-ethylhexyl)phthalate and shows medium- and long-chain acylcarnitine hydrolase activity [2]. In addition, we have found that mCES2 is expressed in various tissues with higher levels of expression in the liver, kidney and small intestine. It was shown that three transcription factors, specificity protein (Sp) 1, Sp3 and upstream stimulatory factor 1, could bind to the promoter region of the mCES2 gene, leading to a synergistic transactivation of the promoter [96]. Although this mechanism may explain the ubiquitous tissue expression profiles of mCES2, it is unlikely to contribute to the higher levels of mCES2 expression in the liver, kidney and small intestine. Therefore, it is thought that there exists another mechanism controlling this tissue-specific transcription of the mCES2 gene [96]. More recently, we have shown that hepatocyte nuclear factor-4 alpha (HNF-4α) can strongly enhance mCES2 gene transcription and that the involvement of HNF-4α accounts for the high expression level of mCES2 in the liver [97]. These findings are notable when physiological roles of mCES2 are studied, since HNF-4α is involved in various hepatic functions, such as glucose and cholesterol metabolism and drug metabolism. In addition, we found that bile acid can repress mCES2 gene transcription by repressing HNF-4α-mediated transactivation [97].

We have also isolated and characterized two genes encoding the human CES1A1 (AB119997) and CES 1A2 (AB119998), and we also cloned and sequenced the 5' flanking region of each gene in order to elucidate the structure of the promoter [98]. It is noteworthy that both the CES1A1 and CES1A2 genes are located on chromosome 16q13-q22 with a tail-to-tail structure. A comparison of the nucleotide sequences of CES1A1 and CES 1A2 genes revealed about 98% homology in 30 Kbp. There are only six nucleotide differences resulting in four amino acid differences in the open reading frame, and all of the differences existed in exon 1. Gene duplication has generally been viewed as a necessary source of material for the origin of evolutionary novelties, and duplicate genes evolve new functions. The majority of gene duplicates are silenced within a few million years, with the small number of survivors subsequently being subjected to strong purifying selection. Although duplicate genes may only rarely evolve new functions, the stochastic silencing of such genes may play a significant role in the passive origin of new species. Since exon 1 of the CES1 gene encodes a signal peptide region, intracellular localization of the CES1 gene product was preliminary investigated using a signal peptide/EYFP-ER chimera protein-expressing system. It was interesting that the CES1A1 signal peptide/EYFP-ER chimera protein was localized to the endoplasmic reticulum, whereas the CES1A2 signal peptide/EYFP-ER chimera protein was distributed in the endoplasmic reticulum and cytosol. On the other hand, CES1A2 mRNA was found to be expressed only in human adult liver, although CES1A1 is expressed in human adult liver and fetal liver (Hosokawa *et al.* submitted for publication). These results suggested that CES1A1 and CES1A2 have different intracellular localizations and different expression profiles in liver differentiation. We investigated the transcriptional regulation of these two CES genes. Reporter gene assays and electrophoretic mobility shift assays demonstrated that Sp1 and C/EBPα could bind to each responsive element of the CES1A1 promoter but that Sp1 and C/EBP could not bind to the responsive element of the CES1A2 promoter (Figure 4) [98].

The expression level of CES1A1 mRNA is much higher than that of CES1A2 mRNA in the liver [98]. Since CES1A1 is highly variable in the individual liver [33], it was thought that these result provide information on individual variation of human CES1.

Figure 4. Structure of the 5' flanking region of CES 1A1 and CES 1A2 genes. Sp1 and C/EBPα could bind to each responsive element of the CES1A1 promoter but that Sp1 and C/EBP could not bind to the 5' flanking region of the CES1A2 promoter. NF-Y, nuclear factor Y, CBF, CCAAT-binding factor
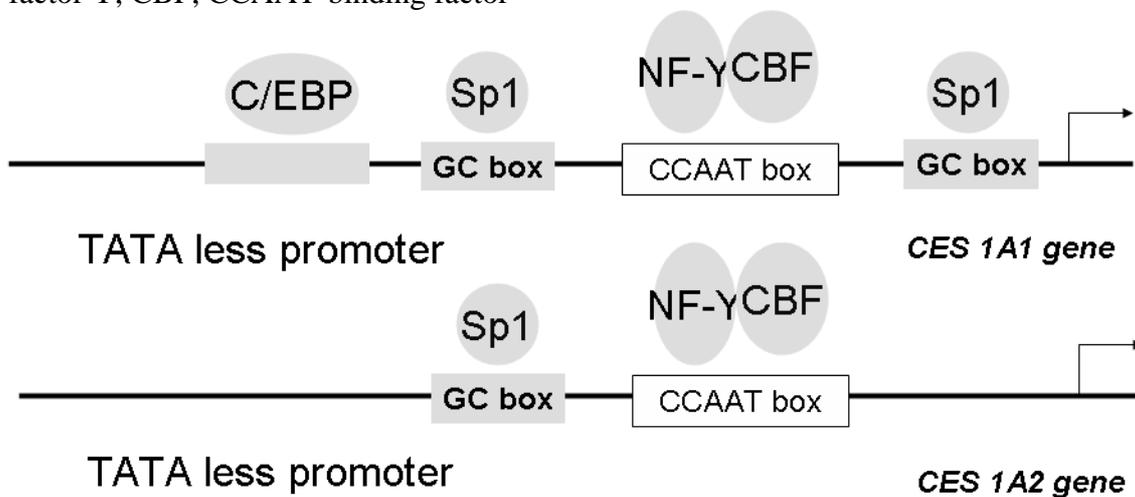
Conclusions

Multiple CES play an important role in the hydrolytic biotransformation of a vast number of structurally diverse drugs. These enzymes are major determinants of the pharmacokinetic behavior of most therapeutic agents containing an ester or amide bond. There are several factors that influence CES activity, either directly or at the level of enzyme regulation. In the clinical field, drug elimination is decreased and the incidence of drug-drug interactions increases when two or more drugs compete for hydrolysis by the same CES isozyme. Exposure to environmental pollutants or to lipophilic drugs can result in induction of CES activity. Several drug-metabolizing enzymes, such as CYP, UGT and SULT, have been extensively studied to clarify the substrate specificity using molecular cloning and cell expression systems. In the present review also described the structure and substrate specificity of CES isozymes, and tissue-specific expression profile of CES isozymes. Therefore, successful design of ester-containing drugs will be greatly improved by further detailed analysis for the mechanism of action and substrate recognition site of human CES isozymes. Further experimentation for an understanding of detailed substrate specificity of prodrugs for CES isozymes and its hydrolysates will help us to design ideal prodrugs.

Acknowledgements

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