DMD9381

Substrate specificity of carboxylesterase isozymes and their contribution to hydrolase activity in human liver and small intestine

Teruko Imai, Megumi Taketani, Mayumi Shii, Masakiyo Hosokawa, Kan Chiba

Graduate School of Pharmaceutical Sciences, Kumamoto University (TI, MT, MS), Faculty of Pharmaceutical Sciences, Chiba Institute of Sciences (MH), Graduate School of Pharmaceutical Sciences, Chiba University (KC), Japan

1

DMD9381

Running title:

Tissue-specific hydrolase activity of human carboxylesterases

Corresponding author:

Teruko Imai, Ph.D.

Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-Honmachi,

Kumamoto 862-0973, Japan

Telephone: +81-96-371-4626, Fax: +81-96-371-4639

E-mail address: iteruko@gpo.kumamoto-u.ac.jp

Number of text pages: 19

Number of tables: 3

Number of figures: 7

Number of references: 31

Number of words in the *Abstract*: 241

Number of words in the *Introduction*: 695

Number of words in the *Discussion*: 1456

Abbreviations

CES, carboxylesterase; hCE-1, human carboxylesterase-1; hCE-2, human carboxylesterase-2; NAG, *N*-acetylglucosamine; FP, flurbiprofen; PL, propranolol; EG, ethylene glycol; TMG, trimethylene glycol

2

DMD9381

Abstract

Hydrolase activity from human liver and small intestine microsomes was compared with that of recombinant human carboxylesterases, hCE-1 and hCE-2. Although both hCE-1 and hCE-2 are present in human liver, the dominant component was found to be hCE-1, while the hydrolase activity of the human small intestine was found to be predominantly hCE-2. hCE-2 has a limited ability to hydrolyze large acyl compound substrates. Interestingly, propranolol derivatives, good substrates for hCE-2, were easily hydrolyzed by substitution of the methyl group on the 2-position of the acyl moiety, but were barely hydrolyzed when the methyl group was substituted on the 3-position. These findings suggest that hCE-2 does not easily form acylated intermediates due to conformational interference in its active site. In contrast, hCE-1 could hydrolyze a variety of substrates. The hydrolytic activity of hCE-2 increased with increasing alcohol chain length in benzoic acid derivative substrates, while hCE-1 preferentially catalyzed the hydrolysis of substrates with short alcohol chains. Kinetic data showed that the determining factor for the rate of hydrolysis of p-amino benzoic acid esters was V_{max} for hCE-1 and K_m for hCE-2. Furthermore, the addition of hydrophobic alcohols to the reaction mixture with p-amino benzoic acid propyl ester induced high and low levels of transesterification by hCE-1 and hCE-2, respectively. When considering the substrate specificities of hCE-1, it is necessary to consider the transesterification ability of hCE-1, in addition to the binding structure of the substrate in the active site of enzyme.

Introduction

The hydrolase activity of various tissues is increasingly used as the basis for drug design, particularly of prodrugs containing functional groups such as carboxylic acid esters (Buchwald and Bordor, 2002; Bordor and Buchwald, 2002). Introduction of an ester linkage generally improves the bioavailability of therapeutic agents, due to increased passive transport following oral administration. The requirements for a better oral prodrug are that it is stable to hydrolytic breakdown in its absorptive stage and that it is easily hydrolyzed to an active drug once it enters the systemic circulation (Beaumont et al., 2003). Carboxylesterases (CESs, EC.3.1.1.1.) play an important role in biotransformation of a variety of ester-containing drugs and prodrugs such as angiotensin-converting enzyme inhibitors (e.g., temocapril, cilazapril, quinapril and imidapril; Takai et al., 1997), anti-tumor drugs (CPT-11 and capecitabin; Humerickhouse et al., 2000; Tabata et al., 2004) and narcotics (cocaine, heroin and meperidine; Pindel et al., 1997; Zhang et al., 1999).

CESs are members of the α/β-hydrolase family and show ubiquitous tissue expression profiles with high levels in the liver, small intestine and lung (Satoh and Hosokawa, 1998; Satoh et al., 2002). CESs use a catalytic triad (Ser-His-Glu) for catalysis, which is located at the base of a deep catalytic gorge (Bencharit et al., 2002; 2003a,b). CES cleaves esters via a two-step process that involves the formation and degradation of an acyl–enzyme intermediate. Firstly, the acyl carbonyl group of the substrate binds to the hydroxyl group of serine to produce an acyl–enzyme complex while the alcohol moiety is released. Then the acid component is released by an attack of histidine-activated water on this acyl–enzyme complex. The micro-environment surrounding the catalytic triad is important, not only for binding of substrate but also for the ease with which alcohol and/or acyl components can be released.

DMD9381

Mammalian CESs comprise a multigene family, in which the isozymes are classified into four main groups and several subgroups according to the homology of the amino acid sequence (Satoh and Hosokawa, 1998; Satoh et al., 2002). The majority of CESs belong to the CES1 and CES2 families, and are differentiated on the basis of substrate specificity, tissue distribution, immunological properties and gene regulation. For example, hCE-1 (CES1A1, HU1), a human CES1 family isozyme, is widely distributed in many tissues but is only found at very low levels in the intestine, while hCE-2 (CES2A1, hiCE), a human CES2 family isozyme, is widely distributed in the intestine, liver and kidney (Xu et al., 2002). While hCE-1 preferentially catalyses the hydrolysis of compounds esterified with a small alcohol group, hCE-2 hydrolyzes compounds with a relatively small acyl group and large alcohol group (Pindel et al., 1997; Takai et al., 1997; Satoh et al., 2002). In the case of cocaine, hCE-1 catalyzes the hydrolysis of the methyl ester of cocaine, producing benzoylecgonine and methanol, while hCE-2 catalyzes the hydrolysis of the benzoyl ester (Pindel et al., 1997). hCE-1 has also been found to catalyze the transesterification of cocaine with ethanol to generate cocaethylene (Brzezinski et al., 1994). Furthermore, hCE-1 has been reported to possess acyl coenzyme A:cholesterol acyltransferase activity, which generates cholesterol esters from fatty-acyl coenzyme A and free cholesterol (Becker et al., 1994). Therefore, bioconversion of orally administered ester-containing drugs is affected by the expression level of hCE-1 and hCE-2 in the liver and small intestine. Some reports have described greater expression of hCE-1 than hCE-2 in human liver and little expression of hCE-1 in human small intestine (Satoh et al., 2002; Xie et al., 2002). However, the extent to which the overall hydrolase activity of the liver and small intestine is dependent upon hCE-1 and hCE-2 activities has not been reported and differences of substrate specificity between hCE-1 and hCE-2 have not been systematically demonstrated using structurally related compounds.

DMD9381

The purpose of the present study is to demonstrate the relative contributions of CES isozymes in the hydrolase activity of microsomes from human liver and small intestine, and to investigate differences in substrate specificity between hCE-1 and hCE-2 expressed in V79 and Sf9 cells using several structurally related compounds. In addition, we examine whether or not the transesterification activity of enzymes via acyl-CES intermediates affects substrate specificity for hCE-1 and hCE-2.

DMD9381

MATERIALS AND METHODS

Materials

O-Acyl-propranolol hydrochloride was synthesized from propranolol hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and an appropriate acyl chloride (Tokyo kasei, Toykyo, Japan) according to previously described methods (Shameem et al., 1993). Flurbiprofen derivatives were synthesized from the appropriate alcohol and 2-(2-fluoro-4-biphenylyl) propionyl chloride which had been synthesized by flurbiprofen and $SOCl_2$, according to a previously reported method (Imai et al., 1993). The identity and purity of the propranolol derivatives and the flurbiprofen derivatives were confirmed by IR, NMR, atomic analysis, and HPLC. Temocapril and temocaprilat were kindly provided by Sankyo Co., Ltd. (Tokyo, Japan). Betamethasone valerate, betamethasone and 1-naphthylbutyrate were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Benzoic acid derivatives were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Fast Red TR was purchased from Sigma (St. Louis, MO, USA). Pooled human liver microsomes from 10 subjects (5 males and 5 females, 21–61 years old, of mixed Caucasian, Hispanic and African American races) and pooled small intestine microsomes from 10 subjects (4 males and 6 females, 24–63 years old, of mixed Caucasian, Hispanic and African American races) were obtained from Gentest Co. (Woburn, MA). All other chemicals and reagents were of analytical grade.

Expression of hCE-1 and hCE-2

Expression of hCE-1 (GI: 34740321) and hCE-2 (GI: 37622885) in V79 cells was carried out as described previously (Hosokawa et al., 2001, Mori et al., 1999). The

7

DMD9381

parental V79 cells (V79-4, American Type Cell Culture Collection CL93, Rockville, MD, USA) were cultured at cell densities of 1×10^6 cells/100-mm plate with D-MEM (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), penicillin/streptomycin (Gibco) and L-glutamine (Gibco) in an atmosphere of 5% CO_2 and 95% air at 37°C. The next day, 10 µg of vector DNA (CES/pTARGET or pTARGET) was transfected into the cells using Lipofectamin Transfection Reagent (Gibco) with OPTI-MEM medium (Gibco). On day 3, the V79 cells were spread over 100-mm plates with media containing the selective agent, G418 (Gibco), at 400–800 µg/ml. The medium was changed every 2–3 days, and the cells were maintained for at least 3 weeks to obtain stable expression colonies. Ten colonies were identified and homogenized in SET buffer (0.25 M sucrose, 1 mM EDTA,100 mM Tris-HCl buffer, pH 7.4), and the expression of hCE-1 or hCE-2 was determined by measurement of hydrolase activity, immunoblot analysis and the molecular weight of a subunit. The background esterase activity in the mock V79 cells (V79 cells transfected by pTARGET) which were used as a control, was extremely low, comparable with the hydrolysis activity in buffer solution. These G418-resistant colonies could be stored in liquid N_2 for several months without loss of expression level.

Recombinant hCE-1 was expressed in Sf9 cells using a BAC-TO-BAC Baculovirus Expression System (Invitrogen, Calsbad, CA, USA) according to a previously described procedure (Furihata et al., 2003). The cDNA in the cloning vector was subcloned into the pFAST BAC1 vector using *EcoRI* and alkaline phosphatase. The pFAST BAC1 vector containing hCE-1 was transformed into DH10Bac cells, and this was followed by transposition of the inserts into bacmid DNA. Likewise, non-recombinant bacmid DNA (mock) was also prepared. The recombinant and mock

8

bacmid DNAs were separately transfected into Sf9 cells with CELL FECTIN Reagent (Invirogen), and the virus was harvested 72 h later. The cells were centrifuged at 1700 × g for 10 minutes to separate cells and virus. The supernatant containing the virus was stored at 4°C in the dark with 5% fetal bovine serum until required for use. Cells were routinely infected with the virus and were harvested 72 h after infection, washed twice with phosphate-buffered saline, and stored –80°C until used. Lysates were prepared by disrupting the cells with a sonicator until the cells were completely lysed as determined by microscopy. Cytosol from Sf9 cells expressing hCE-1 and from Sf9 cells infected with mock virus were prepared by subjecting the cell lysate to centrifugation (105 000 × g for 60 min at 4°C). The expression of hCE-1 was identified by molecular weight of a subunit, immunoblot analysis and hydrolase activity. The background esterase activity in the mock cells was extremely low, comparable to hydrolysis activity in buffer solution.

The recombinant hCE-1 and hCE-2 showed the same substrate specificity as purified human hCE-1 and hCE-2 in the preliminary experiment (data not shown), indicating expression of actual CES isozyme. Although the recombinant hCE-1 expressed in V79 cells showed the same pattern as hydrolysis of propranolol derivatives by hCE-1 expressed in Sf9 cells, the activity of the hCE-1 expressed in V79 cells was very low due to its low expression. Therefore, the activity of recombinant hCE-1 was evaluated using the recombinant hCE-1 in Sf9 cells.

Hydrolysis experiments

Hydrolysis experiments were performed using tissue microsomes and the homogenates or cytosol of cells expressing CES. The initial hydrolytic activity was measured under

DMD9381

reaction conditions which were such that less than 25% of substrate was hydrolyzed. Enzyme solutions were diluted to the appropriate concentration with HEPES buffer (50 mM, pH 7.4). The hydrolysis reaction was initiated by the addition of test compounds dissolved in dimethylsulfoxide (DMSO) after preincubation of each subcellular fraction (200 µL) for 5 min. The final concentration of DMSO was less than 1%, which has no effect on hydrolase activity. The reaction of benzoic acid derivatives was terminated by the addition of acetonitrile. After centrifugation, the supernatant was injected onto an HPLC column. For betamethasone valerate, propranolol derivatives and flurbiprofen derivatives the reaction was terminated by the addition of 5 mL of ethylacetate. Saturated NaCl (0.5 mL), adjusted to pH 1 by phosphoric acid, was added to reaction samples of flurbiprofen derivatives, while 0.5 mL of saturated NaCl, adjusted to pH 4 by phosphate buffer, was added to the reaction samples of propranolol derivatives. After each sample had been shaken for 10 min, the isolated organic phase was evaporated and the residue was redissolved[PP7] in HPLC mobile phase and injected onto HPLC column. The rate of hydrolysis of aspirin and methyl salicylate were determined by the increase in fluorescence intensity of salicylic acid at ex. 296 nm and em. 405 nm over 7 min (Hitachi F-4500, Tokyo, Japan). The rate of hydrolysis of p-nitrophenylacetate was determined by the initial linear increase in absorbance of p-nitrophenol at 405 nm over 3 min (Jasco,V530, Tokyo, Japan). The rate of hydrolysis was determined by subtraction of the hydrolysis rate in buffer from that in tissue microsomes, and by subtraction of the hydrolysis rate in mock/cell preparations from that in CES/cell preparations. Hydrolytic activity was represented as the concentration of hydrolysis product per milligram of protein. Protein content was determined using the method described by Bradford (1976), with bovine serum albumin as standard.

DMD9381

HPLC analysis

Concentrations of betamethasone, flurbiprofen, propranolol and benzoic acid derivatives were determined by HPLC. The HPLC system comprised a JASCO PU-980 pump, a JASCO 980-UV detector, a JASCO AS950 autosampler, a JASCO CO-965 column oven and a JASCO FP-1520S fluorescence detector (JASCO Co., Tokyo, Japan), and a Shimadzu chromatopac C-R7A plus (Shimadzu Co., Ltd., Kyoto, Japan). The column and composition of the mobile phase used for each assay are listed in Table 1. All substrates and hydrolysates were clearly separated. Each hydrolysate was measured in a quantitatively linear range.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed as described by Mentlein et al. (1980). Polyacrylamide gels (7.5% w/w) containing 1% w/v nonidet P-40 for solubilization of proteins were used for the separation of native enzymes. After electrophoresis of the microsomes and S9 samples (5–15 µg protein), the gels were stained for esterase activity with 1-naphthylbutyrate, through coupling of the liberated 1-naphthol with Fast Red TR-salt.

Inhibition experiments for hydrolysis of valeryl-propranolol using anti-hCE-1 polyclonal antibody

Anti-hCE-1 polyclonal IgG (Yamada et al., 1994) was purified from anti-hCE-1 rabbit serum using a Protein D column (Funakoshi, Tokyo, Japan). Human liver microsomes (1 mg protein/mL in pH7.4 HEPES buffer) were incubated with 0.5% of cholic acid for

11

30 min on ice. After centrifugation at 10 000 × g for 30 min at 4˚C, the supernatant was diluted to 25 µg protein/mL by 50 mM HEPES buffer (pH7.4), and then incubated with anti-hCE-1 IgG for 30 min at 37˚C. The mixtures (200 µL) were allowed to stand for 24 h at 4˚C before adding 30 µL of nProtein A Sepharose 4 Fast Flow (Amersham Biosciences Corp., Piscataway, NJ, USA). After centrifugation at 20 000 × g for 5 min, the resulting supernatant was used to assay hydrolase activity for valeryl-propranolol. The control activity was measured using control rabbit IgG (Sigma, St. Louis, MO, USA).

RESULTS

Hydrolase activity of human liver and small intestine microsomes

Fig. 1 shows the hydrolase activity of human liver and small intestine microsomes for several substrates. In a preliminary study, bis(*p*-nitrophenyl)phosphate, a CES-specific inhibitor (Brandt et al., 1980), was found to inhibit the hydrolysis of a variety of compounds tested in this study by more than 90% (data not shown). In comparison with the liver microsomes, the small intestine microsomes showed lower activity for substrates with a large acyl group, for example, temocapril, *p*-nitrobenzoic acid methylester and methyl salicylate. On the other hand, betamethasone valerate and aspirin, substrates with a small acyl group, were hydrolyzed to either the same or a greater extent in the small intestine microsomes than in the liver microsomes. Furthermore, the substrate with the largest alcohol group, betamethasone valerate, was preferentially hydrolyzed in the small intestine microsomes.

Fig. 2 shows the native PAGE gel stained by esterase activity using 1-naphthylbutyrate. The small intestine microsomes showed only one band corresponding to hCE-2, while the liver microsomes showed two bands, a strong upper band and lower

weak band, corresponding to hCE-1 and hCE-2, respectively. Therefore, the hydrolase activity of small intestine microsomes is due principally to hCE-2 activity while that in the liver microsomes is attributable to both hCE-1 and hCE-2.

Hydrolysis of flurbiprofen derivatives in human liver and small intestine microsomes

Flurbiprofen ethylene glycol ester and trimethylene glycol ester were used as model compounds with large acyl groups. As shown in Fig. 3, both flurbiprofen derivatives were barely hydrolyzed in the small intestine microsomes, while being rapidly hydrolyzed in the liver microsomes. Moreover, the R-isomers were preferentially hydrolyzed in the liver microsomes. The recombinant hCE-1 showed a hydrolysis pattern similar to that of the human liver microsomes, although the latter expressed both hCE-1 and hCE-2. In common with the small intestine microsomes, the recombinant hCE-2 showed only low activity. A significant difference in the enantioselectivity of hydrolysis was not observed in recombinant hCE-2 or the small intestine microsomes due to the low activity observed.

Hydrolysis of propranolol derivatives

Fig. 4 gives the structures of the propranolol derivatives used as model compounds with large alcohol groups. Compounds 1 to 6, 7 to 13, and 14 to 16, possess straight acyl chains, branched acyl chains and cyclic acyl chains, respectively. The hydrolase activity of the human liver and small intestine microsomes and recombinant hCE-1 and hCE-2 are shown in Fig. 5. The human liver microsomes hydrolyzed R-isomers preferentially for all propranolol derivatives. The hydrolysis rates of compounds with straight acyl chains increased until butyryl propranolol (No.3) and varelyl propranolol (No.4) for R-

and S-isomers, respectively. Although pivaroyl propranolol (No. 13) and cyclopropanoyl propranolol (No.14) showed low hydrolysis rates, no relation between hydrolysis rate and structure was observed among branched and cyclic acyl derivatives. The recombinant hCE-1 showed a comparable pattern of hydrolysis activity to human liver microsomes for all propranolol derivatives.

The human small intestine and recombinant hCE-2 hydrolyzed both R- and S-form substrates at almost equal rates. The human small intestine and recombinant hCE-2 showed similar patterns for substrate specificity, including markedly lower activities for propranolol derivatives Nos 9 and 11, as well as pivaroyl propranolol (No. 13) and cyclopropanoyl propranolol (No.14). Interestingly, derivatives Nos. 9 and 11, with substituted methyl groups at the 3-position, were only slowly hydrolyzed, in contrast with the derivatives with substituted methyl groups at the 2-position (Nos. 8 and10) which were hydrolyzed as readily as their corresponding straight acyl derivatives (Nos. 4 and 5, respectively). With the exception of compounds Nos. 9 and 11, R-isomers of propranolol derivatives were easily hydrolyzed by both hCE-1 and hCE-2 isozymes, while S-isomers were better substrates for hCE-2 than hCE-1.

The major CES isozyme in human liver microsomes was identified using immuno-inhibition experiments (Fig. 6). Racemic valeryl propranolol was used as the substrate. Inhibition by anti-hCE-1 IgG (Yamada et al., 1994) was dose dependent and hydrolysis of R- and S-isomers was inhibited by 95% and 80% at 500 μg/ml of anti-hCE-1 IgG, respectively. The residual hydrolysis rates were nearly same for R- and S-isomers, indicating that the residual activity was responsible for hCE-2. These data suggest that major component of human liver hydrolase activity is hCE-1.

Hydrolysis of benzoic acid derivatives

14

DMD9381

The hydrolysis of benzoic acid derivatives was determined in human small intestine and liver microsomes and the recombinant CES isozyme (Fig. 7). *p*-Amino- , *o*-amino-, and *p*-hydroxy-benzoic acid derivatives were used as substrates. Interestingly, the hydrolysis activity of recombinant hCE-1 and the liver microsomes decreased with increasing alcohol chain length of all types of benzoic acid derivatives. On the other hand, recombinant hCE-2 and small intestine microsomes preferentially catalyzed those derivatives with longer alcohol chains. The responses toward benzoic acid derivatives showed that the hydrolase activity of the human liver and small intestine microsomes could be explained by hCE-1 and hCE-2, respectively.

In order to examine the different substrate requirements for hCE-1 and hCE-2, the kinetic parameters were determined using *p*-amino benzoic ethyl, propyl and butyl esters. K_m and V_{max} values are listed in Table 2. Interestingly, the K_m value for hydrolysis by recombinant hCE-1 varied little among the three compounds, but the V_{max} value of the butyl ester was much smaller than that of the ethyl ester. On the other hand, V_{max} values for hydrolysis by recombinant hCE-2 were similar for all three compounds and K_m values decreased with increasing alcohol chain length. These data suggest that the determining factor for the hydrolysis rate among the three *p*-amino benzoic acid esters is the V_{max} value for hCE-1 and the K_m value for hCE-2.

Hydrolytic activity as a function of affinity for hCE-2 is a normal property of the enzyme reaction. On the other hand, hCE-1 showed a nearly identical K_m value and increasing V_{max} value with alcohol chain length. Since CES catalyzes ester hydrolysis in two steps, the V_{max} value might depend on the binding velocity of acyl group with the serine residue of CES and the releasing velocity of the acyl group from the acyl-enzyme intermediate upon attack of surrounding water. The acyl group for *p*-amino benzoic acid derivatives is *p*-amino benzoyl in all these substrates. The essential velocity for binding of the *p*-amino benzoyl group with the serine residue of hCE-1 might be similar for the

15

DMD9381

three ester compounds, given the invariance of the K_m values and their similar structures. However, the velocity of the release of the *p*-amino benzoyl group from the acyl-enzyme intermediate might be affected by alcohol released in the first step of the reaction. That is, interference may occur if the released alcohol can attack the acyl-enzyme intermediate. Therefore, *p*-amino benzoic acid propyl ester was selected as a substrate, ethanol and butanol were added to the reaction mixture, and production of *p*-amino benzoic acid and its ethyl or butyl ester was measured. The results are given in Table 3. Ethanol and butanol inhibited the production of *p*-amino-benzoic acid in reactions with both recombinant hCE-1 and hCE-2. The inhibition ratio was greater for hCE-1. The formation of ethyl ester on the addition of ethanol was nearly the same for hCE-1 and hCE-2. Interestingly, when the more hydrophobic alcohol, butanol, was added, *p*-amino-benzoic acid butyl ester was easily formed by hCE-1, but barely by hCE-2. These data suggest that hCE-1 catalyzes transesterification with hydrophobic rather than hydrophilic alcohols, and that hCE-2 possesses low transesterification ability.

Discussion

The present study demonstrated that CES is the main hydrolyzing enzyme for ester compounds; suitable substrates are shown in Fig. 1. hCE-1 and hCE-2 were the predominant isoforms for hydrolysis in the human liver and small intestine, respectively. The predominance of hCE-2 activity in the small intestine was also confirmed by native PAGE, which showed only one band of hydrolase activity for 1-naphthylbutyrate (Fig. 2). In the liver, hydrolase activity was mainly attributed to hCE-1, although native PAGE revealed a relatively high hydrolase activity for hCE-2 in the liver microsomes (Fig. 2). As shown in Fig. 6, anti-hCE-1 antibody inhibited 80–95% of the hepatic hydrolysis of S- and R-valeryl-propranolol; the residual 5–20% of hydrolase activity

was due to hCE-2 which showed non-enantioselective hydrolysis of valeryl-propranolol.

This result closely agreed with a previous report showing the nearly complete inhibition

of *p*-nitrophenylacetate hydrolase activity in human liver microsomes by anti-RH1 IgG,

a cross-reactive antibody with hCE-1 (Hosokawa et al., 1995). These findings indicate

the predominant contribution of hCE-1 to human hepatic hydrolase activity. Individual

levels of both human liver CESs, hCE-1 and hCE-2, are highly variable. Xu et al.

(2002) reported a 3-fold range variance for hCE-2 among 13 human liver microsomes,

while Hosokawa et al. (1995) reported a more than 8-fold range variance of CES1

protein levels among 12 human liver microsomes. Therefore, in the present study, we

used pooled human liver microsomes from 10 subjects whose expression levels of hCE-

1 and hCE-2 showed interindividual differences. Our results nevertheless indicate that

hCE-1 is the main component in human liver.

hCE-1 and hCE-2 exhibit 48% sequence identity and differing substrate

specificities have been proposed. That is, hCE-1 preferentially recognizes a substrate

with large acyl and small alcohol moieties, while hCE-2 prefers substrates with smaller

acyl and bulky alcohol moieties (Satoh et al., 2002; Bosron and Hurley, 2002). Our

results regarding hydrolysis by hCE-2 concurred with this proposal; a bulky acyl moiety,

such as flurbiprofen, was barely recognized by hCE-2 while propranolol derivatives,

with a small acyl moiety and bulky alcohol These findings suggested the presence of

sterically distorted regions, which affect the formation of an acyl-enzyme intermediate,

in the active site of hCE-2. group, were good substrates for hCE-2. Interestingly,

propranolol derivatives Nos. 9 and 11 (see Fig. 5) were scarcely hydrolyzed by hCE-2

or human small intestine microsomes. These compounds have a branched acyl moiety

with a methyl group at the 3-position. In contrast, compounds Nos. 8 and 10 were easily

DMD9381

hydrolyzed at almost the same rate as the corresponding straight acyl derivatives, although they possessed methyl groups at the 2-position. In general, the chemical hydrolysis of ester bonds is affected by substitution of the methyl group at the 2-position but not the 3-position, due to steric hindrance. The reduction in the hydrolysis rate resulting from substitution of the methyl group at the 3-position also suggests the presence of conformational interference in the active site of hCE-2.

In contrast to hCE-2, hCE-1 recognized substrates with either large or small acyl moieties. Flurbiprofen derivatives (a bulky acyl moiety), and R-propranolol derivatives (a small acyl moiety and bulky alcohol group) were recognized by hCE-1, although S-propranolol derivatives were poor substrates for hCE-1. Furthermore, acetyl derivatives such as p-nitrophenyl acetate and aspirin were hydrolyzed by both liver and small intestine microsomes (Fig.1). The crystal structure of hCE-1 has been defined by Bencharit et al. (2003a, b). They reported that the substrate-binding site of hCE-1 was composed of a 'small, rigid' pocket and a 'large, flexible' pocket; the 'small, rigid' pocket being selective, and the 'large, flexible' pocket being promiscuous with regard to substrate specificity. These pockets allow hCE-1 to act on structurally distinct compounds containing either large or small acyl moieties. Therefore, R-propranolol derivatives might be easily recognized by hCE-1. In preliminary studies, we determined the K_m and V_{max} values for R- and S-butyryl-propranolol in human liver microsomes. The K_m value ($22.1 \pm 0.8 \ \mu M$) for the S-isomer was 10-fold smaller than that for the R-isomer ($251 \pm 18.8 \ \mu M$), indicating the higher affinity of the S-enantiomer for hCE-1, in spite of the lower hydrolysis rate of the S-isomer. The V_{max} value for S-butyryl-propranolol (95.7 ± 23.4 nmol/min/mg protein) was much smaller than that obtained for the R-enantiomer (1580 ± 4.0 nmol/min/mg protein). These preliminary findings

18

DMD9381

suggest that both the R- and S-enantiomers are recognized by hCE-1, although their reactivity may be greatly affected by the conformational orientation upon incorporation into the active site pocket of hCE-1. It has been reported that S-cocaine and *cis*-cypermethrin analogues are poor substrates for hCE-1, in contrast to R-cocaine and *trans*-cypermethrin analogues (Brzezinski et al., 1997; Huang et al., 2005). The differences of hydrolysis rate between these enantiomers have been explained by steric clashes with the loop containing Gly142 and Gly143 in the rigid pocket (Bencharit et al., 2003a; Huang et al., 2005), where Gly residues form the oxianion hole (140–144) to stabilize the transition state of substrate via their amide nitrogen. Bencharit et al. (2003a) also determined the Z-site surface ligand binding site for an inactive substrate. This Z-site is formed by inter-digitation of the $\Omega1$ and $\Omega2$ loops adjacent to the active site of hCE-1. The enantioselective hydrolysis of propranolol derivatives is possibly explained by enantiomerically distinct active site orientations in hCE-1 due to structural clashes. Structural analysis will be required to establish the detailed nature of such enantioselectivity.

It was interesting that the substrate requirements for hydrolysis of various benzoate derivatives by hCE-1 and hCE-2 were quite different. Such substrate specificity might be explained by conformational fitting of substrates in the active sites. However, we propose that transesterification via hCE-1 may be due to different hydrolytic mechanisms in hCE-1 and hCE-2. CES-catalyzed hydrolysis proceeds in two steps. For benzoate derivatives, the first step is the formation of a covalent benzoyl-CES intermediate. At same time, the released alcohol moiety is present in the active site gorge. Subsequently, the benzoyl-CES intermediate is attacked by histidine-activated water, and benzoic acid is released from the enzyme. Bencharit et al. (2002) reported the

19

DMD9381

crystal structure of rabbit liver CES (rCE), in which 4-piperidino piperidine, a product of CPT-11 activation, was bound between the first N-acetylglucosamine (NAG) of the Asn389 glycosylation site and the Trp550 side-chain of the C-terminal helix. There are two N-linked glycosylation sites in rCE at Asn residues 79 and 389; Asn 389 is modified via a long carbohydrate chain and plays a role as a novel exit pore for the release of small products from the active site of the enzyme. hCE-2 contains glycosylation sites at two positions (residues 103 and 267). Although there are no crystallographic data for hCE-2, one of these two glycosylation sites might function as an exit pore for the release of alcohol.

In contrast to hCE-2, hCE-1 maintains a glycosylation site at Asn79 but not at residue 389. Asn79 is modified by two NAG groups and appears to be involved in the stabilization of the hCE-1 trimer by packing into adjacent monomer (Bencharit et al., 2003a, b). hCE-1 is also capable of transesterifying cocaine in the presence of ethanol to cocaethylene (Brezinski et al., 1994). During the two-step hydrolysis of cocaine, hCE-1 forms a covalent acyl-enzyme intermediate at the carboxylic methyl ester position of cocaine, which is then attacked by ethanol to create cocaethylene. Bencharit et al., (2003a) proposed the following mechanism based on their X-ray crystalline analysis. Ethanol enters the active site of hCE-1 through the side-door secondary pore adjacent to the 'large, flexible' substrate-binding pocket. The entrance to the side-door secondary pore on the surface of hCE-1 is lined by structurally flexible residues including both β-strands (β14 and β15) and random coils. This flexibility may facilitate the passage of small molecules through the side-door. The alcohol produced may also be released from this side-door. The presence of the side-door secondary pore adjacent to the 'large, flexible' substrate-binding pocket in hCE-1 might prolong the presence of alcohol

molecule so that the alcohol can attack the benzoyl-hCE-1 intermediate to reproduce the original substrate. Furthermore, the transition time of alcohol in hCE-1 might be prolonged with increasing hydrophobicity due to interaction of alcohol with the flexible site of hCE-1. This may be another explanation for the apparently slow hydrolytic rate of substrates with large alcohol moieties by hCE-1. On the other hand, if hCE-2 can release an alcohol from the glycosylation site, its transesterification activity may be extremely low. This would agree with the observation of the low transesterification ability of hCE-2.

Although our proposal is a speculative mechanism of hydrolysis of hCE-1 and hCE-2, the fact that hCE-1 possesses transesterification activity, suggests that, in addition to the binding structure of the substrate, this property should be considered in a complete analysis of the hydrolytic mechanism.

References

Beaumont K, Webster R, Gardner I and Dack K (2003) Design of ester prodrugs to enhance oral absorption of poorly permeable compounds: challenges to the discovery scientist. *Curr Drug Metab* **4**: 461–85.

Becker A, Bottcher A, Lackner KJ, Fehringer P, Notka F, Aslanidis C and Schmitz G (1994) Purification, cloning, and expression of human enzyme with acyl coenzyme a: cholesterol acyl-transferase activity, which is identical to liver carboxylesterase. *Arterioscler Thromb* **14**: 1346–55.

Bencharit S, Morton CL, Howard-Williams EL, Danks MK, Potter PM and Redinbo MR (2002) Structural insights into CPT-11 activation by mammalian carboxylesterases. *Nat Struct Biol* **9**: 337–42.

Bencharit S, Morton CL, Xue Y, Potter PM and Redinbo MR (2003a) Structural basis of heroin and cocaine metabolism by a promiscuous human drug-processing enzyme. *Nat Struct Biol*. 10: 349–56.

Bencharit S, Morton CL, Hyatt JL, Kuhn P, Danks MK, Potter PM and Redinbo MR (2003b) Crystal structure of human carboxylesterase 1 complexed with the alzheimer's drug tacrine: from binding promiscuity to selective inhibition. *Chem Biol* **10**: 341–9.

Bodor N and Buchwald P (2002) Designing safer (soft) drugs by avoiding the formation of toxic and oxidative metabolites. *Methods Mol Biol* **186**:301–12.

DMD9381

Bosron WF and Hurley TD (2002) Lessons from a bacterial cocaine esterase. *Nat Struct Biol* **9**: 4–5.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–54.

Brandt E, Heymann E and Mentlein R (1980) Selective inhibition of rat liver carboxylesterases by various organophosphorus diesters *in vivo* and *in vitro*. *Biochem Pharmacol*. **29:** 1927–31.

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**: 1747–55.

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**: 1089–96.

Buchwald P and Bodor N (2002) Physicochemical aspects of the enzymatic hydrolysis of carboxylic esters. *Pharmazie* **57**: 87–93.

DMD9381

Furihata T, Hosokawa M, Nakata F, Satoh T and Chiba K (2003) Purification, molecular cloning, and functional expression of C57BL/6 inducible mouse liver acylcarnitine hydrolase, belonging to the carboxylesterase multigene family *Arch Biochem Biophys* **416**: 101–9.

Hosokawa M, Endo T, Fujisawa M, Hara S, Iwata N, Sato Y and Satoh T (1995) Interindividual variation in carboxylesterase levels in human liver microsomes. *Drug Metab Dispos* **23:** 1022–7.

Hosokawa M, Suzuki K, Takahashi D, Mori M, Satoh T and Chiba K (2001) Purification, molecular cloning and expression of dog liver microsomal acyl-CoA hydrolase: A member of the carboxylesterase multigene family. *Arch Biochem Biophys* **389**: 245–53.

Huang H, Fleming CD, Nishi K, Redinbo MR and Hammock BD (2005) Stereoselective hydrolysis of pyrethroid-like fluorescent substrates by human and other mammalian liver carboxylesterases. *Chem Res Toxicol* **18**: 1371–7.

Humerickhouse R, Lohrbach K, Li L, Bosron WF and Dolan ME (2000) Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. *Cancer Res* **60:** 1189–92.

Imai T, Fukuhara A, Ueda I and Otagiri M (1993) An evaluation of an anti-

24

inflammatory-histamine H2 antagonist drug complex on gastric erosions in rat. *J Pharmacol Exp Ther* **265**: 328–33.

Mentlein R, Heiland S and Hwymann E (1980) Simultaneous purification and comparative characterization of six serine hydrolases from rat liver microsomes. *Arch Biochem Biophys* **200**: 547–59.

Mori M, Hosokawa M, Ogasawara Y, Tsukada E and Chiba K (1999) cDNA cloning, characterization and stable expression of novel human bran carboxylesterase. FEBS Letters **458**: 17-22.

Pindel EV, Kedishvili NY, Abraham TL, Brzezinski MR, Zhang J, Dean RA and Bosron WF (1997) Purification and cloning of a broad substrate specificity human liver carboxylesterase that catalyzes the hydrolysis of cocaine and heroin. *J Biol Chem* **272:** 14769–75.

Satoh T and Hosokawa M (1998) The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxicol* **38:** 257–88.

Satoh T, Taylor P, Bosron WF, Sanghani P, Hosokawa M and Du BN (2002) Current progress on esterases: from molecular structure to function. *Drug Metab Dispos* **30:**488–93.

DMD9381

Shameem M, Imai T and Otagiri M (1993) An in-vitro and in-vivo correlative approach to the evaluation of ester prodrugs to improve oral delivery of propranolol. *J Pharm Pharmacol* **45**:246–52.

Tabata T, Katoh M, Tokudome S, Nakajima M and Yokoi T (2004) Identification of the cytosolic carboxylesterase catalyzing the 5'-deoxy-5-fluorocytidine formation from capecitabine in human liver. *Drug Metab Dispos* **32:** 1103–10.

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. *Biol Pharm Bull* **20:** 869–73.

Xie M, Yang D, Liu L, Xue B and Yan B (2002) Human and rodent carboxylesterases: immunorelatedness, overlapping substrate specificity, differential sensitivity to serine enzyme inhibitors, and tumor-related expression. *Drug Metab Dispos* **30**: 541-547.

Xu G, Zhang W, Ma MK and McLeod HL (2002) Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. *Clin. Cancer Res*. **8**: 2605–11.

Yamada T, Hosokawa M, Satoh T, Moroo I, Takahashi M, Akatsu H, Yamamoto T. (1994) Immunohistochemistry with an antibody to human liver carboxylesterase in

human brain tissues. *Brain Res* **658**: 163–7.

Yamaoka K, Tanigawara Y, Nakagawa T, Uno T (1981) A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobiodyn* **4:** 879–85.

Zhang J, Burnell JC, Dumaual N and Bosron WF (1999) Binding and hydrolysis of meperidine by human liver carboxylesterase hCE-1. *J Pharmacol Exp Ther* **290:** 314–8.

DMD9381

Footnote

This work was supported in part by a Grant-in-Aid for Scientific Research (16590085)

from the Japan Society for the Promotion of Science (T.I.)

DMD9381

Legends for figures

Figure 1

Hydrolysis of several ester-containing compounds by the human liver and small intestine microsomes.

Concentrations in parenthesis show the initial concentrations of substrate in hydrolysis experiment.

Figure 2

Polyacrylamide gel electrophoresis of human liver (25 µg protein) and small intestine (9.5 µg protein) microsomes followed by staining for esterase activity using 1-naphthylbutyrate.

Figure 3

Structure of flurbiprofen derivatives and their hydrolysis in human liver and small intestine microsomes, and recombinant hCE-1 and hCE-2.

Open and filled columns show hydrolase activity of human liver microsomes for S- and R-flurbiprofen derivatives, respectively. Protein concentration: liver 50 µg/mL, small intestine 100 µg/mL, hCE-1 50 µg/mL , hCE-2 60 µg/mL; racemic concentration of EG-FP and TMG-FP: 250 µM; reaction time: liver and small intestine 10 min, hCE-1 20 min, hCE-2 60 min; temperature: 37°C. Values represent the mean ± S.D. (n=3). *The difference between R- and S-isomers in the liver microsomes and recombinant hCE-1 was statistically significant ($p<0.05$, n=3)

29

DMD9381

Figure 4

Structure of propranolol (PL) derivatives

Figure 5

Hydrolysis of propranolol derivatives in human liver and small intestine microsomes, and recombinant hCE-1 and hCE-2.

Open and filled columns show hydrolase activity of the human liver microsomes for S- and R-propranolol derivatives, respectively. Protein concentration: liver and small intestine 10 μg/mL, recombinant hCE-1 30 μg/mL , hCE-2 20 μg/mL; racemic concentration of substrate: 100 μM; reaction time: liver and small intestine 5 min, hCE-1 and hCE-2 15 min; temperature: 37°C. Values represent the mean ± S.D. (n=3). There was a significant difference between R- and S-isomers for all substrates in the liver microsomes and recombinant hCE-1 (p<0.05)

Figure 6

Effect of polyclonal anti-hCE-1 antibodies on hydrolase activity for racemic valeryl-propranolol in human liver microsomes.

Columns and circles show hydrolase activity of human liver microsomes for valeryl-propranolol at 100 μM racemic concentration in the presence of anti hCE-1 rabbit IgG and rabbit IgG, respectively. Open columns and circles show the hydrolysis for S-valeryl-propranolol, and filled columns and circles indicate the hydrolysis for R-valeryl-propranolol.

Values represent the mean ± S.D. (n=3). * indicates statistically significant differences

DMD9381

compared with hydrolase activity for the S-isomer ($p<0.05$).

Figure 7

Hydrolysis of benzoic acid (BA) derivatives in human liver and small intestine microsomes, and recombinant hCE-1 and hCE-2.

The numbers 1 to 4 indicate the carbon number of the alcohol group (1, methyl; 2, ethyl; 3, propyl; 4, butyl). Protein concentration: liver, 40 µg/mL, small intestine, 100 µg/mL, recombinant hCE-1, 40 µg/mL , hCE-2, 25 µg/mL; concentration of substrate: 500 µM; reaction time: liver and small intestine 15–30 min, hCE-1 and hCE-2 60 min; temperature: 37°C. Values represent the mean ± S.D. (n=3).

DMD9381

TABLE 1

Conditions for HPLC analysis

Compound	Column	Mobile phase	Detection
Betamethasone	Lichrospher RP-18[a)]	Acetonitrile/H_2O (45:55) 1 mL/min	UV 254 nm
Flurbiprofen	Chiralpak AD [b)]	Hexane/2-propranol/TFA (95:5:0.5) 0.5 mL/min	Fluorescence Ex.247 nm, Em.314 nm
Propranolol	Chiralcel OD [b)]	Hexane/2-propranol (70:30) 1 mL/min	Fluorescence Ex.285 nm, Em.340 nm
p-Amino benzoic acid	TSKgel ODS-80T$_M$ [c)]	Acetonitrile/H_2O (20:80) 1 mL/min	Fluorescence Ex.291 nm, Em.330 nm
o-Amino benzoic acid	TSKgel ODS-80T$_M$ [c)]	Acetonitrile/H_2O (30:70) 1 mL/min	Fluorescence Ex.251 nm, Em.394 nm
p-Hydroxy benzoic acid	TSKgel ODS-80T$_M$ [c)]	Acetonitrile/H_2O (20:80) 1 mL/min	UV 252 nm
p-Nitro benzoic acid	TSKgel ODS-80T$_M$ [c)]	Acetonitrile/H_2O (20:80) 1 mL/min	UV 274 nm

a) 4.6 mmφ ×150 mm (Merck KGaA, Darmstadt, Germany)

b) 4.6 mmφ ×250 mm (Daicel Chemical Industries, Ltd., Tokyo, Japan)

c) 4.6 mmφ ×150 mm (TOSOH Co., Tokyo, Japan)

32

DMD9381

TABLE 2.

Kinetic parameters for hydrolysis of *p*-amino benzoic acid ethyl, propyl, butyl ester by hCE-1
and hCE-2

Substrate	hCE-1			hCE-2		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Ethyl ester	356 ±18.1	29.3 ±3.46	0.0822	1198 ±140	1.04 ±0.12	0.000870
Propyl ester	322 ±58.8	7.88 ±0.65*	0.0244	398 ±58.7*	1.01 ±0.39	0.00255
Butyl ester	130 ±0.81	2.30 ±0.51 **	0.0176	110 ±33.7**	2.25 ±0.90	0.0205

*K_m : µM, V_{max} : nmol /min /mg protein, V_{max}/K_m : mL /min /mg protein. K_m and V_{max} were
calculated by least square method using the MULTI program (Yamaoka et al., 1981).*

Protein concentration : hCE-1, 45 µg /mL, hCE-2, 25 µg /mL

Substrate concentration : 10 -500 µM; reaction time: 15 min; temperature: 37°C

*Values represent the mean ±S.D. * and **indicate statistically significant differences compared
with ethyl ester and propyl ester, respectively ($p<0.05$).*

DMD9381

TABLE 3.

Hydrolysis of *p*-amino benzoic acid (BA) propyl ester by hCE-1 and hCE-2 in the presence and absence of alcohol

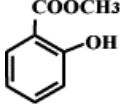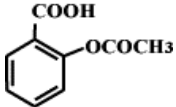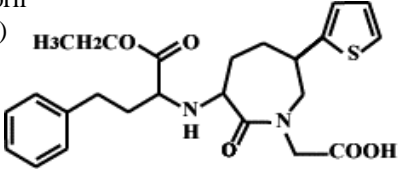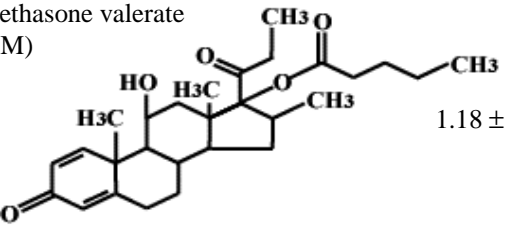|		hCE-1		hCE-2	
	Alcohol (mM)	*p*-Amino BA (µM)	*p*-Amino BA ester (µM)	*p*-Amino BA (µM)	*p*-Amino BA ester (µM)
Control	——	6.29 ±0.31		4.41±0.27	
EtOH	0.5	5.90 ±0.80	N.D.	4.14 ±0.13	N.D.
	5	4.78 ±0.44	1.03 ±0.06	4.10 ±0.37	1.54 ±0.08
	10	4.26 ±0.77	1.59 ±0.55	3.97 ±0.11	2.26 ±0.30
	25	3.05 ±0.23	2.99 ±0.55	3.68 ±0.13	2.78 ±0.13
BuOH	0.5	5.76 ±0.47	N.D.	3.72 ±0.07	N.D.
	5	4.93 ±0.46	1.24 ±0.81	3.49 ±0.10	N.D.
	10	2.83 ±0.88	3.30 ±0.27	2.99 ±0.31	0.33 ±0.19
	25	1.99 ±0.37	4.97 ±0.31	2.22 ±0.84	0.83 ±0.11
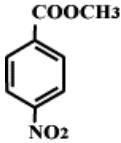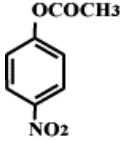
Protein concentration: hCE-1, 45 µg /mL, hCE-2, 25 µg /mL

Substrate concentration: 500 µM; EtOH and BuOH concentration: 0.5–-25 mM; reaction time: 60 min; temperature: 37°C
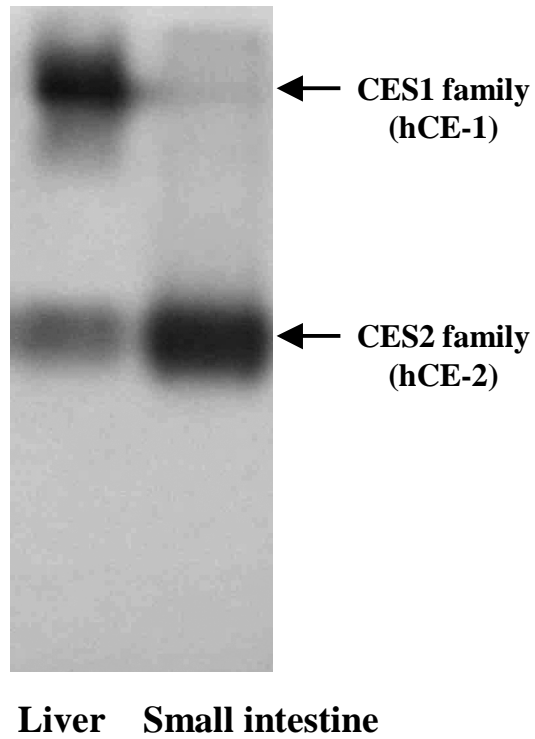
Values represent the mean ±S.D.

Fig.1

Substrate	Structure	Microsomes		Ratio (Liver/Intestine)
		Liver	Intestine	
		nmol/min/mg protein		
p-Nitrobenzoic acid methyester (500μM)	COOCH3 / NO2	253 ± 18.3	45.9 ± 4.92	5.51
p-Nitrophenyl acetate (500μM)	OCOCH3 / NO2	5020 ± 740	2330 ± 170	2.15
Methyl salycylate (500μM)	COOCH3 / OH	147 ± 2.20	9.59 ± 0.458	15.3
Aspirin (500μM)	COOH / OCOCH3	0.511 ± 0.140	0.543 ± 0.075	0.939
Temocapril (250μM)		119 ± 13.4	0.162 ± 0.003	734
Betamethasone valerate (100μM)		1.18 ± 0.32	5.62 ± 0.30	0.210

Fig. 2

←CES1 family
(hCE-1)

←CES2 family
(hCE-2)

Liver   Small intestine

Fig.3

Flurbiprofen(FP) derivatives

Fig. 4

The core structure shows:

$$O - \overset{\overset{O}{\parallel}}{C} - C_nH_m \; :R$$

with the position marked by $*$:

$$OCH_2\overset{*}{C}HCH_2NHCH(CH_3)_2$$

attached to a naphthalene ring.

Compound	R
Propranolol (PL)	H

Straight–chain:

Compound	R
O-Acetyl PL(1)	$COCH_3$
O-Propionyl PL(2)	$COCH_2CH_3$
O-Butyryl PL(3)	$COC_2H_4CH_3$
O-Valeryl PL(4)	$COC_3H_6CH_3$
O-Caproyl PL(5)	$COC_4H_8CH_3$
O-Enantyl PL(6)	$COC_5H_{10}CH_3$

Branched–chain:

Compound	R
O-Isobutyryl PL(7)	$COCH(CH_3)_2$
O-2-methyl-n-butyryl PL(8)	$COCH(CH_3)CH_2CH_3$
O- 3-methyl-n-butyryl PL(9)	$COCH_2CH(CH_3)_2$
O-2-methyl-n-valeryl PL(10)	$COCH(CH_3)C_2H_4CH_3$
O-3-methyl-n-valeryl - PL(11)	$COCH_2CH(CH_3)CH_2CH_3$
O-Isocaproyl PL(12)	$COC_2H_4CH(CH_3)_2$
O-Pivaloyl PL(13)	$COC(CH_3)_3$

Cyclic–chain:

Compound	R
O-Cyclopropanoyl PL(14)	$COcC_3H_5$
O-Cyclopentanoyl PL(15)	$COcC_5H_9$
O-Cyclohexanoyl PL(16)	$COcC_6H_{11}$

Fig. 5

Fig.6

Fig.7