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Characterization of pyrethroid hydrolysis by the human liver carboxylesterases hCE-1 and hCE-2

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Abstract

Carboxylesterases hydrolyze a large array of endogenous and exogenous ester-containing compounds, including pyrethroid insecticides. Herein, we report the specific activities and kinetic parameters of human carboxylesterase (hCE)-1 and hCE-2 using authentic pyrethroids and pyrethroid-like, fluorescent surrogates. Both hCE-1 and hCE-2 hydrolyzed type I and II pyrethroids with strong stereoselectivity. For example, the *trans*-isomers of permethrin and cypermethrin were hydrolyzed much faster than corresponding *cis*-counterparts by both enzymes. Kinetic values of hCE-1 and hCE-2 were determined using cypermethrin and 11 stereoisomers of the pyrethroid-like, fluorescent surrogates. K_m values for the authentic pyrethroids and fluorescent surrogates were in general lower than those for other ester-containing substrates of hCEs. The pyrethroid-like, fluorescent surrogates were hydrolyzed at rates similar to the authentic pyrethroids by both enzymes, suggesting the potential of these compounds as tools for high throughput screening of esterases that hydrolyze pyrethroids.

Keywords

Carboxylesterase; Pyrethroid; Fluorescent substrate

Synthetic pyrethroid insecticides represented about 17% of the global pesticide market in 2002 [1]. In the coming years, the use of pyrethroids is predicted to further increase with the phase out of the use of organophosphorus insecticides. Additionally, due to the emergence and re-emergence of mosquito-vectored diseases such as West Nile fever, Japanese encephalitis, and dengue fever, pyrethroids are being used with increased frequency against adult mosquitoes

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[1]*Abbreviations used:*

GC-MS gas chromatography-mass spectrometry

IEF isoelectric focusing

hCE human carboxylesterase

LD_{50} lethal dose 50%*p*

NPA *p*-nitrophenyl acetate.

[2]. Because of the increased agricultural and residential usage of pyrethroids, human exposure to these compounds is also expected to increase.

Pyrethroids are ester-containing compounds consisting of various acid and alcohol moieties. Type I pyrethroids are esters of primary alcohols, whereas type II pyrethroids are esters of secondary alcohols that contain a cyano group at the α-carbon of the alcohol moiety. Chiral centers can exist in both the acid and alcohol moieties, leading to the existence of several stereoisomers for each pyrethroid. Ester hydrolysis by carboxylesterases and oxidation by cytochrome P450s are the main detoxification routes of pyrethroids in animals [3]. Differences in the activities of carboxylesterases and P450s between mammals and insects contribute to the low mammalian toxicity of pyrethroids (i.e., pyrethroids are metabolized faster in mammals than in insects) [3]. In general, whole animals or liver microsomes have been used to study pyrethroid metabolism in mammals, and little has been done to identify the specific carboxylesterase isozymes that hydrolyze pyrethroids [4]. Butte and Kemper [5]have shown ester-hydrolytic activities in human serum using pyrethroid-like colorimetric substrates that are not hydrolyzed by acylesterase, arylesterase, acetylcholinesterase, or butyrylcholinesterase. However, to our knowledge, the human carboxylesterase isozymes that hydrolyze pyrethroids have not been identified.

Recently, two pyrethroid-hydrolyzing carboxylesterases have been identified from mouse liver in our laboratory [6]. These mouse carboxylesterases are highly similarity to two major human carboxylesterase isozymes, human carboxylesterase (hCE[1])-1 and hCE-2 at the amino acid sequence level. hCE-1 and hCE-2 are known as α/β-hydrolase fold proteins that play major roles in the metabolism of a wide variety of exogenous ester-containing compounds, including numerous pharmaceuticals and pesticides [7]. From these facts, we hypothesize that hCE-1 and hCE-2 are involved in pyrethroid hydrolysis. To test this hypothesis, recombinant hCE-1 and hCE-2 were produced using the baculovirus expression vector system. In addition, fluorescent surrogates that structurally resemble pyrethroids [8] were synthesized to facilitate the measurement of esterase-mediated ester hydrolysis. The hydrolysis activities of recombinant hCE-1 and hCE-2 for authentic pyrethroids and the pyrethroid-like fluorescent surrogates were determined. Furthermore, kinetic data of hCE-1 and hCE-2 for cypermethrin and 11 stereoisomers of the pyrethroid-like, fluorescent surrogates were also determined to examine the efficiency of the enzymes. The characterization of hCE-1 and hCE-2 with authentic and fluorescent pyrethroids will help to more accurately assess the risks posed by exposure to this class of insecticides on human health.

Materials and methods

Chemicals

The following compounds are numbered according to the system outlined in Fig.1. (*R*)-**A1**, (*S*)-**A1**, (*R*)-**A2**, (*S*)-**A2**, **A3**, (2*R*)-**A4**, (2*S*)-**A4**, **A5**, **A6**, *cis*-**A7**, *trans*-**A7**, **A8**, and four fenvalerate isomers (α*R*)(2*R*)-**B5**, (α*R*)(2*S*)-**B5**, (α*S*)(2*R*)-**B5**, and (α*S*)(2*S*)-**B5**) were all previously synthesized in this laboratory [6,8-11]. The term α refers to the α-carbon of the alcohol moiety and 2 refers to the 2-position carbon of the acid moiety. The terms *cis* and *trans* refer to the fixed geometry across the cyclopropane ring. All of the fenvalerate isomers have an enantiomeric excess ~96%. The pyrethroids deltamethrin ((α*S*)(1*R*)-*cis*-**B5**), bifenthrin ((*Z*)-*cis*-**E7**), cyfluthrin (**D3**), and λ-cyhalothrin ((α*S*)(*Z*)(1*R*)-*cis*-**B7**; (α*R*)(*Z*)(1*S*)-*cis*-**B7**) were obtained from Chem Service (West Chester, PA). Both *cis*- and *trans*-permethrin (**C3**) were gifts from ICI Agrochemicals (Bracknell, UK). Both *cis*- and *trans*-cypermethrin (**B3**), α-cypermethrin ((α*R*)(1*S*)-*cis*- and (α*S*)(1*R*)-*cis*-**B3**), and ζ-cypermethrin ((α*S*)-**B3**) were gifts from FMC. (Princeton, NJ). All isomers unless otherwise stated were ~99% of the isomer mixture indicated. 6-Methoxy-2-naphthaldehyde was purchased from Avocado Research

Chemicals (Lancashire, UK). All of the other chemicals used in this study were either purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hampton, NH).

cDNA library screening

To isolate full-length cDNA clones encoding hCE-1 and hCE-2, a probe was generated by PCR using degenerate primers (5′-AACTGGGGYYACYTGGACCARKTGGC TGC-3′ and 5′-GCRAAGTTGGCCCAKWRYTTCAT CA-3′) that were designed on the basis of two highly homologous regions between hCE-1 and hCE-2. These primers were predicted to amplify a fragment of approximately 950 bp. A human liver cDNA library (Invitrogen, Carlsbad, CA) was used as the template source for PCR. PCR was performed as follows: 94 °C for 2 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and 72 °C for 7 min. The PCR-generated fragment was gel-purified, labeled using an ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences, Piscataway, NJ), and used to screen the human liver cDNA library. Positive colonies were verified by DNA sequencing (DBS DNA Sequencing Facility, University of California at Davis).

Construction of recombinant baculoviruses expressing hCE-1 and hCE-2

To generate recombinant baculoviruses expressing hCE-1 and hCE-2, the coding sequences of hCE-1 and hCE-2 were PCR-amplified using primer pairs that incorporated*Bgl*II and *Eco*RI endonuclease sites at the 5′- and 3′-ends, respectively, of the coding sequences. The primer pair for hCE-1 was 5′-AGATCTATGTGGCTCCGTGCCTTTA TCCTGGC-3′ and 5′-GAATTCTCACAGCTCTATGTG TTCTGTCTGGGGTG-3′; and the primer pair for hCE-2 was 5′-AGATCTATGCGGCTGCACAGACTTCGTG CG-3′ and 5′-GAATTCCTACAGCTCTGTGTGTCTCT CTTCAGGC-3′. PCR was performed with Ex *Taq* DNA polymerase (Takara Bio, Shiga, Japan) using the cDNA clone encoding full-length hCE-1 or hCE-2 as the template. PCR was performed as follows: 94 °C for 2 min; 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and 72 °C for 7 min. The resulting products were inserted into a pSTBlue-1 vector (EMD Biosciences, Madison, WI) and the nucleotide sequences of the coding sequences were verified. The cDNA fragments were then excised and directionally ligated to the *Bgl*II and *Eco*RI sites of the baculovirus transfer vector pAcUW21 (BD Biosciences Pharmingen, San Diego, CA).

Production and purification of hCE-1 and hCE-2

Recombinant baculoviruses harboring the *hCE-1* or *hCE-2* gene, Ac-hCE1 and Ac-hCE2, respectively, were generated by co-transfection of *Spodoptera frugiperda*-derived Sf21 cells with the recombinant transfer vector plasmid and *Bsu*36I-cleaved BacPAK6 viral DNAs (BD Biosciences Pharmingen) as previously described [12]. The recombinant baculoviruses were plaque-purified and propagated on monolayers of Sf21 cells grown in Ex-Cell 401 medium (JRH Biosciences, Lenexa, KS) supplemented with 2.5% fetal bovine serum. To express the recombinant protein, *Trichoplusia ni*-derived High Five cells (1×10^6 cells/ml) were inoculated with Ac-hCE1 or Ac-hCE2 at a multiplicity of infection of 0.1 virus per cell. High Five cells were cultured in ESF921 medium (Expression Systems LLC, Woodland, CA). At 72 h post-infection, the infected cells were harvested by centrifugation (2000*g*, 20 min, 4 °C) and suspended in 50 mM Tris-HCl (pH 8.0) containing 1 mM DDT, 1 mM EDTA, and 1 mM benzamidine. The cell suspension was then homogenized using a Polytron homogenizer and octyβ-D-glucopyranoside was added to final concentration of 1% (w/v) and mixed on a rotating wheel at 4 °C for 60 min. An octyl-β-D-glucopyranoside-soluble fraction was collected by ultracentrifugation (100,000*g*, 60 min, 4 °C). The fraction was then loaded onto a DEAE anion-exchange chromatography column (3 × 7 cm) and washed with 50 mM NaCl in 20 mM Tris-HCl (pH 8.0) for hCE-1 or 100 mM NaCl in 20 mM Tris-HCl (pH 8.0) for hCE-2. Elution of hCE-1 and hCE-2 was done with 75 mM NaCl or 130 mM NaCl, respectively, in 20 mM Tris-

HCl (pH 8.0). The hCE-containing fractions were detected by measuring *p*-nitrophenyl acetate (*p*NPA) hydrolysis at 405 nm. Following desalting, the protein was further purified by two rounds of preparative isoelectric focusing (IEF) using a Rotofor apparatus (Bio-Rad Laboratories, Hercules, CA) with pH 5-8 ampholytes for hCE-1 or pH 4-6 ampholytes for hCE-2. The hCE fractions were then combined and loaded onto a Superose-12 column (Amersham Biosciences) which had been equilibrated in 20 mM Tris-HCl (pH 8.0). The hCE-containing fractions were eluted by running 20 mM Tris-HCl (pH 8.0) and combined. Protein concentration was determined according to Bradford [13] with bovine serum albumin as a standard. SDS-, native-, and IEF-PAGE were performed using a 12% Tris-glycine, 6% Tris-glycine, and IEF gels pH 3-7 (Invitrogen), respectively.

Enzyme activity assays

Enzymatic activities of the recombinant hCE-1, recombinant hCE-2, and pooled human liver microsomes (Gentest, Woburn, MA) were determined using *p*NPA, pyrethroid-like fluorescent surrogates, and individual pyrethroid isomers or racemic mixtures. All of the isomers or isomer mixtures tested were at least 96% of the stereochemistry indicated. All assays were performed in 20 mM Tris-HCl (pH 8.0) at 37 °C. No more than 10% of the substrate was hydrolyzed during the assay, and solvent content never exceeded 1% of the total assay volume. Three replicates were conducted for each compound with the same enzyme preparations. Hydrolytic activity for *p*NPA was monitored for 2 min at 405 nm in 96-well, flat-bottomed, polystyrene microtiter plates (Dynex Technologies, Chantilly, VA) and analyzed on a Spectramax 200 plate reader (Molecular Devices, Sunnyvale, CA) according to the methods of Ljungquist and Augustinsson [14] as modified in Wheelock et al. [15].

Fluorescent assays were conducted in black 96-well, flat-bottomed polystyrene microtiter plates (Corning, New York, NY) by measuring the production of 6-methoxy-2-naphthaldehyde with a Spectrafluor Plus spectrophotometer (Tecan, Research Triangle Park, NC) using three flash cycles according to the method of Wheelock et al. [10]. Briefly, 1 μl of stock solution (final concentration; **A1** and **2**: 50 μM in ethanol; **A3** to **9**: 25 μM in ethanol) was added to 200 μl of the enzyme (0.025-20 μg/ml) and fluorescence was measured using an excitation wavelength of 330 nm (35 nm bandwidth) and an emission wavelength of 465 nm (35 nm bandwidth). When enzymatic assays were conducted with microsomes, a standard curve was generated using a fluorescent aldehyde (6-methoxy-2-naphthaldehyde) containing an equivalent amount of protein as the standard solution to compensate for protein-induced quenching [10]. In general, protein quenching should be corrected for but this effect is relatively minor with these substrates when using purified recombinant esterases.

Hydrolytic activity for pyrethroids was determined by gas chromatography-mass spectrometry (GC-MS) according to the method of Stok et al. [6]. Briefly, 1 μl of stock solution (final concentration; **B3**, **B4**, **B7**, **C3**, **D3**, and **E7**: 50 μM in ethanol; **B5**: 40 μM in ethanol) was added to 500 μl of the enzyme (10-30 μg/ml). The enzyme mixture was incubated for 1-30 min depending on the compound. The reactions were stopped by adding ethyl acetate (250 μl) and brine (250 μl) to each sample and vortexing. Eight nanomoles of an internal standard (3-(4-methoxyphenoxy)benzaldehyde in ethanol) was added to 100 μl of the ethyl acetate solution. The assay was based upon the detection of the esterase hydrolytic products: 3-phenoxybenzylalcohol for permethrin, 2-methyl-3-biphenylmethanol for bifenthrin, 4-fluoro-3-phenoxybenzylalcohol for cyfluthrin, and 3-phenoxybenzaldehyde for the other pyrethroids. Samples were analyzed on a HP 6890 GC equipped with a HP 5973 MS (Agilent Technologies, Palo Alto, CA). The analytical column was a 0.25 mm i.d. × 30 m, 0.25 μm DB-XLBms column (J&W Scientific, Folsom, CA). The GC-MS operation condition was according to the method of Wheelock et al. [10].

For kinetic studies, stock solutions for each compound were appropriately diluted in ethanol into at least five different concentrations around the K_m values. Kinetic values were obtained from Hanes-Woolf plots against various substrate concentrations. Data were obtained by repetitive assays ($N = 3$).

Results and discussion

Preparation of recombinant human carboxylesterases

Full-length cDNA clones encoding hCE-1 and hCE-2 were isolated from a human liver cDNA library. The nucleotide sequences of the isolated *hCE-1* and *hCE-2* cDNA clones coincided with those of *hCE-1* and *hCE-2* genes previously reported (NCBI Accession Nos. NM_001025194 and NM_003869, respectively). Recently, numerous single nucleotide polymorphisms have been identified in hCE-1 and hCE-2 [16-18]. So far, none of these polymorphisms have been shown to directly affect esterase activity or expression, and the variability in carboxylesterase activity is thought to be more likely of environmental rather than genetic origin [18]. Although the existence of these types of polymorphisms in hCE-1 and hCE-2 are not unexpected, the relative roles that these polymorphisms play in the metabolism of xenobiotics such as pyrethroids and prodrugs are still unclear.

Recombinant baculoviruses expressing the *hCE-1* and *hCE-2* genes in insect cells were prepared to have a reproducible source of recombinant enzymes for this study. Recombinant hCE-1 and hCE-2 produced in High Five cells were solubilized by octyl-β-D-glucopyranoside. After anion-exchange chromatography, each enzyme was subjected to preparative IEF, a suitable tool to purify carboxylesterases [6,19]. The apparent masses of the recombinant hCE-1 and hCE-2 were around 60 kDa by SDS-PAGE as expected, and purity of at least 98%, as determined by gel scanning, was obtained for both enzymes (data not shown). The average yield of each of the two recombinant enzymes in this study was around 2.5 mg from one liter of insect cell culture (Table 1). Purification coefficients between 40 and 50 were regularly obtained, indicating that the recombinant enzymes represent around 2% of the total cell protein. These results are similar to previous expression studies of human carboxylesterase genes in insect cells [20].

Hydrolysis of authentic pyrethroids

The hydrolytic activities of hCE-1 and hCE-2 for authentic pyrethroids were examined by a GC-MS assay. Both type I (e.g., permethrin and bifenthrin) and type II (e.g., cypermethrin, cyfluthrin, fenvalerate, and γ-cyhalothrin) pyrethroids were chosen as target compounds in this study based on their structural variation (Fig. 1) and common use in California [21]. Both hCE-1 and hCE-2 were found to hydrolyze most of the type I and type II pyrethroids tested (Table 2). As shown on Fig. 2, both hCE-1 and hCE-2 displayed very similar selectivity profiles. hCE-1 and hCE-2 hydrolyzed the *trans*-isomers of cypermethrin (**B3**) 14-fold and 29-fold faster, respectively, than the corresponding *cis*-counterparts. Additionally, hCE-1 and hCE-2 hydrolyzed *trans*-permethrin (**C3**) 12-fold and 5-fold faster, respectively, than the corresponding *cis*-isomers. The results were consistent with previous animal studies [22,23] showing that *trans*-permethrin ester is hydrolyzed more rapidly than its *cis*-isomer. Furthermore, no significant *cis*-permethrin metabolism is observed in either human liver cytosolic or microsomal fractions [24]. Therefore, hCE-1 and hCE-2 were assumed to play major roles in the stereoselective metabolism of cypermethrin and permethrin. Our laboratory has previously characterized two recombinant mouse carboxylesterases that are involved in pyrethroid metabolism [6]. In comparison to hCE-1 and hCE-2, the murine carboxylesterase BAC36707 hydrolyzes cypermethrin and permethrin at a faster rate [6]. Additionally, the murine carboxylesterase BAC36707 hydrolyzes *trans*-isomers of cypermethrin and permethrin at least 4-fold faster than the *cis*-counterparts, suggesting that pyrethroid-hydrolyzing

carboxylesterases from mammals in general have similar stereopreferences for *cis*/*trans*-configurations of cypermethrin and permethrin.

hCE-1 and hCE-2 hydrolyzed all of the stereoisomers of fenvalerate (**B4**) except for the (2*S*)(α*R*)-isomer. However, different enantiomeric preferences were found. hCE-1 hydrolyzed the (α*S*)-enantiomers of fenvalerate at least 3-fold faster than the (α*R*)-enantiomers regardless of the configuration at the 2-position carbon. At this latter position hCE-1 preferred the (*R*)-configuration by 3-fold, indicating that the absolute configurations at both chiral centers were important. On the other hand for hCE-2, only the spatial configuration at the 2-position carbon was important with a strong preference for the (*R*)-configuration independent of the configuration at the α-position carbon. It is interesting to note that only the hCE-1 isomer hydrolyzed the active ingredient in the commercially used insecticide esfenvalerate, i.e., the (2*S*, α*S*)-isomer. This suggested that a person with low hCE-1 levels could be more sensitive to toxic effects induced by this particular insecticide, and perhaps this could be linked to some isomer-selective toxicity. Interestingly, the stereopreference of the human carboxylesterases were different from that of murine carboxylesterase BAC36707. BAC36707 hydrolyzed (2*R*)(α*R*)-fenvalerate at least 10-fold faster than the other stereoisomers of fenvalerate and did not hydrolyze (2*S*)(α*S*)-isomer[6]. This indicated that the stereopreference for fenvalerate is different among the pyrethroid-hydrolyzing carboxylesterases in mammals.

One could expect that permethrin and cypermethrin would be less easily hydrolyzed by esterases than fenvalerate because the aromatic-like cyclopropane conjugated through a vinyl group and carbonyl of the ester (**3**) destabilizes the transition state of the compounds in the catalytic pocket. However, hCE-1 and hCE-2 and murine carboxylesterases [6] were found to hydrolyze cypermethrin and permethrin faster than fenvalerate, implying the chlorophenyl group and/or isopropyl group (**4**) sterically affected the enzymes. hCE-2 hydrolyzed the other pyrethroids tested in this study approximately 2-fold faster than hCE-1. Compared to most of the substrates tested, both esterases displayed low activity against bifenthrin. We hypothesize that this is due to its unique chemical structure, a rigid biphenyl group and a methyl group, and/or its absolute configuration. These findings indicate that diffrences in the structure of pyrethroids may greatly alter the hydrolysis activities of hCE-1 and hCE-2.

Characterization of human carboxylesterases with fluorescent substrates

To evaluate the toxicity risk associated with pyrethroid exposure, it is important to determine the hydrolytic activity of metabolic enzymes against pyrethroids. However, the use of authentic pyrethroids as substrates requires an end point GC-MS assay which needs an extraction step and complicated instrumental analysis. Thus, the use of pyrethroid-like fluorescent surrogates could be highly useful for the identification and monitoring of enzymes involved in the hydrolysis of pyrethroids. The use of α-cyanoalcoholsas a reporter system offers substantial advantages in the analysis of several enzymes including cytochrome P450s [25], epoxide hydrolases [26] and, as illustrated here, esterases such as hCE-1 and hCE-2. These advantages include high quantum yield, very large Stokes' shifts, low background, hydrolytic stability, and flexibility of structure.

To evaluate the effectiveness of the use of pyrethroid-like fluorescent surrogates as predictive tools for the quantification of pyrethroid hydrolysis capacity, recombinant and authentic human carboxylesterases were evaluated against a series of fluorescent compounds that have been synthesized in our laboratory [6,8,10]. Fluorescent substrates containing pyrethroid acids (**3** to **8**) were hydrolyzed by hCE-1 and hCE-2 at rates that were 100- to 1000-fold slower than those with simple acids such as acetic acid and butanoic acid (**1** and **2**) (Table 3), suggesting that the ability of the pyrethroid acids to access the active pocket is reduced. Both hCE-1 and hCE-2 had a preference in terms of the length of the alkyl chain of the acid and the configuration at the α-position carbon of **A1** and **A2**. The *S*-isomer and butyrate were several times more rapidly

hydrolyzed than the corresponding *R*-isomer and acetate, respectively. The hydrolytic activities of both enzymes for colorimetric substrate *p*NPA were similar to the values obtained by another group [27] (Table 3). Compared to *p*NPA, the corresponding fluorescent substrate (**A1**) was hydrolyzed 10-fold more slowly, as one would expect for a secondary (**A1**) versus phenolic (*p*NPA) ester. One could expect that the nitrophenol group on *p*NPA is a good leaving group and the cyano group on **A1** and **A2** may somewhat interfere the enzyme attack, and these contribute to higher and lower background signal in assays with *p*NPA and the fluorescent substrates, respectively.

The hydrolysis profile by hCE-1 and hCE-2 for the pyrethroid-like fluorescent surrogates (**A3** to **8**) was similar except for (2*R*)-**A4**. The (2*R*)-**A4** surrogate was hydrolyzed around 60-fold faster by hCE-2. This could be due to the higher preference of hCE-2 for (*R*)-enantiomers at the 2-position as observed above for the fenvalerate isomers (Table 2). The activity of hCE-1 for **A8** was over 6-fold higher than that of hCE-2, implying that hCE-2 recognized the tetramethylcyclopropane carboxylate structure (**8**) better than the chrysanthemate structure (**6**). The replacement of chlorine atoms on **A3** with other halogens (bromine atoms, **A5**; trifluoromethyl group, **A7**) reduced the hydrolytic activities of both enzymes in a similar fashion, indicating that both enzymes have a specific preference for the size and electronegativity of the chlorine atoms. The replacement with methyl groups (**A6**) did not affect the hydrolytic activity of hCE-1. Murine carboxylesterases also prefer chlorine atoms and methyl groups to other halogens [6]. The hydrolytic activities of hCE-1 and hCE-2 for **A3**, **A5**, **A6**, or **A7** showed similar changes in response to substitution of the halogen atoms, indicating that the structure of the substrate-binding pocket was globally similar for both enzymes. However, the hydrolytic activities of hCE-1 and hCE-2 were diffrent for some structures (e.g., a tetramethylcyclopropane (**A8**), a chlorophenyl group ((2*S*)-**A4**)).

Following preparative IEF separation of octyl-β-D-glucopyranoside-soluble proteins from human liver microsomes, the esterase activities of the various fractions against the pyrethroid-like fluorescent surrogates were examined. When the fluorescent cypermethrin-like surrogate (**A3**) was used as a substrate, only fractions that putatively contained hCE-1 and hCE-2 showed weak hydrolytic activity (data not shown). The relative ratio of putative hCE-1 and hCE-2 were estimated to be 2:1 in the human liver microsomes. This was based on the relative specific activities of these enzymes for *p*NPA and **A3**. However, there remained the possibility that other pyrethroid-hydrolyzing esterases were lost during the IEF preparations. The microsomes showed similar trends to those of purified hCE-1 and hCE-2 (Table 3). Additionally, the esterase activities of human butyrylcholinesterase (catalog number C-9971, Sigma-Aldrich) and acetylcholinesterase (catalog number C-1682, Sigma-Aldrich) for our pyrethroid-like fluorescent surrogates were tested because butyrylcholinesterase is also involved in the metabolism of xenobiotic compounds. These cholinesterases showed no activity for these compounds. Our findings suggested that hCE-1 and hCE-2 represent major pyrethroid-hydrolyzing esterases of human liver microsomes.

Characterization of human carboxylesterases with stereoisomers of fluorescent substrates

Pyrethroid stereoisomers are known to diffr widely in their biological activities. For example, (1*R*)-*cis*-permethrin is both insecticidal and toxic to mammals, whereas (1*R*)-*trans*-permethrin lacks measurable acute toxicity to mammals, although having similar insecticidal potency [28]. Cypermethrin (**B3**) has three asymmetric carbons at C-1, C-3, and C-α and fenvalerate (**B4**) has two asymmetric carbon atoms at C-2 and C-α. Thus, there are eight stereoisomers for cypermethrin and four stereoisomers for fenvalerate. To examine the hydrolytic activity of esterases for these optical isomers, eight and four fluorescent stereoisomer surrogates resembling cypermethrin and fenvalerate, respectively, were synthesized in our laboratory [11] and tested against hCE-1 and hCE-2 (Table 4). Both hCE-1 and hCE-2 hydrolyzed the

trans-isomers of **A3** faster than the corresponding *cis*-counterparts as was observed with the authentic pyrethroids. hCE-1 hydrolyzed the *trans*-isomers of **A3** at least 30-fold faster than the corresponding *cis*-isomers, suggesting that the *cis*- or *trans*-configuration at the C-3 carbon on a cyclopropane ring is a major determinant for the hydrolysis activity of hCE-1. On the other hand, hCE-2 showed a preference for (1*S*)-*trans*-**A3**, indicating that the *cis*- or *trans*-configuration at the C-1 and C-3 carbons are much more important than the C-α carbon. In mice, pyrethroidhydrolyzing carboxylesterases also prefer *trans*-isomers of **A3** [11]; and *trans*-permethrin is over 78-fold less toxic than their *cis*-counterparts [29]. This suggests that hCE-1 and hCE-2 partly participate in the detoxification of pyrethroids with a preference for *trans*-isomers, possibly causing diffrent toxicity with certain optical isomers of pyrethroids in humans. In fact, after human ingestion of permethrin, the *trans*-isomers disappear much more rapidly in serum than the *cis*-isomers [30]. The role of P450s and other metabolizing enzymes, however, should also be considered in the degradation of these compounds.

The preference of hCE-1 and hCE-2 for the four stereoisomers of the fluorescent fenvalerate-like surrogates (**A4**) was similar to that of the authentic fenvalerate (**B4**) (Table 2). hCE-2 showed much higher activity for the **A4** isomers than hCE-1. hCE-1 was not able to hydrolyze (2*S*)(α*R*)-**A4** putatively because of steric hindrance. From these results, the fluorescent pyrethroid-like substrates are very useful for the estimation of hydrolytic activity and stereoselectivity of esterases. A crystal structure of hCE-1 has recently been determined in the laboratory of Redinbo [31,32]. Our laboratory in collaboration with the Redinbo laboratory has examined the stereoselectivity of hCE-1 for the optical isomers of the pyrethroid-like substrates **A3** and **A4** by computer modeling [33]. In this study, the mechanism of lower hydrolytic activity of hCE-1 for *cis*-**A3** was caused by protrusion of the dichlorovinyl group of *cis*-**A3** into oxyanion hole, resulting in the inability of the carbonyl carbon to access the active pocket of the enzyme. This mechanism may be applicable to other pyrethroid-hydrolyzing carboxylesterases.

The preferences of hCE-2 for the optical isomers of **A3** and **A4** were similar to those found for two murine pyrethroid-hydrolyzing carboxylesterases [11]. The (1*S*)-*trans*-isomers of **A3** were hydrolyzed much faster by hCE-2 in a similar manner as the mouse pyrethroid-hydrolyzing carboxylesterases. In addition, like hCE-2 the mouse carboxylesterases showed little or no activity for the **A4** stereoisomers with a 2*S* configuration. As mentioned above, the murine carboxylesterase genes NM_133960 and BAC36707 show higher identity with *hCE-2* than with *hCE-1* at the amino acid sequence level. Thus, the structure-activity relationships between hCE-2 and the two mouse carboxylesterases were consistent in terms of the hydrolytic activity and stereoselectivity for optical isomers of **A3** and **A4**. This also implied that the structure of the substrate-binding pocket of hCE-2 and the two murine carboxylesterases are conserved and unique from that of hCE-1. According to the NCBI database, NM_133960 and BAC36707 belong to carboxylesterase classes CES6 and CES5, respectively. In addition to CES5 and CES6, mouse has its own CES1 [34] and CES2 [35] class carboxylesterases that are predicted to also be involved in pyrethroid detoxification.

Kinetic analysis of human carboxylesterases

The kinetic constants of hCE-1 and hCE-2 were determined using *p*NPA, cypermethrin, and fluorescent pyrethroid surrogates as substrates. The results are summarized in Table 5. In comparison to *p*NPA, the k_{cat} values of both enzymes for cypermethrin and the fluorescent compounds were very low, however, the K_m values were even lower. The K_m values for **A3** isomers with hCE-1 and hCE-2 were smaller than those for cypermethrin (**B3**), suggesting that both enzymes prefer a 6-methoxy-2-naphthyl group (**A**) to a phenoxybenzyl group (**B**). The K_m values of hCE-1 for (1*R*)-*cis*-isomers of **A3** were lower than the other isomers of **A3**, while hCE-1 showed similar K_m values for **A4** stereoisomers. The k_{cat} values of hCE-1 for *trans*-

isomers of **A3** were higher than *cis*-counterparts. Interestingly, hCE-1 showed a strong stereopreference for the (α*S*)(1*R*)-*trans*-**A3** isomer in terms of catalytic efficiency (k_{cat}/K_m). On the other hand, hCE-2 had similar affinity for the stereoisomers of **A3** and **A4** in terms of K_m values. The k_{cat} values for (1*S*)-*trans*-isomers of **A3** and (2*R*)(α*S*)-**A4** were higher than the other stereoisomers, resulting in higher catalytic efficiencies for these stereoisomers. hCE-1 showed similar catalytic efficiency among **A3**, **A4**, **A6**, and **A8**. hCE-1 and hCE-2 had similar K_m and k_{cat} values for **A6**. In comparison to general esterase substrates and drugs such as cocaine and heroin (Table 5), both enzymes showed lower K_m values for cypermethrin and the pyrethroid-like fluorescent substrates, except for CPT-11. The catalytic efficiency of hCE-1 and hCE-2 for heroin was comparable to that for cypermethrin and fluorescent pyrethroid-like substrates. The K_m values of hCE-1 and hCE-2 for cypermethrin (molecular weight=416.3) were 20 and 9.1 μM (Table 5); these values are equivalent to 8.3 and 3.8 mg/L, respectively. Institóris et al. [36] reported that acute oral median dose required for 50% lethality (LD_{50}) of cypermethrin in eight-week-old male Wistar rats was 554 (411-746) mg/kg. Thus, assuming that acute toxicity of cypermethrin to rats may be directly extrapolated to that in humans, it seems that both enzymes may hydrolyze cypermethrin with higher hydrolytic activity when exposed to the pyrethroid at LD_{50} or much lower levels to detoxify quickly, possibly contributing lower toxicity of pyrethroid insecticides to humans.

The degree of stereoselectivity between hCE-1 and hCE-2 for the optical isomers of the pyrethroid-like fluorescent substrates were quantified in terms of the following preference index:

Preference index= log((catalytic efficiency of hCE-1)/(catalytic efficiency of hCE-2)). From our results, hCE-1 and hCE-2 seemed to possess strong and opposite selectivities for (α*S*)(1*R*)-*trans*-**A3** and (2*R*)(α*S*)-**A4**, respectively. Because hCE-1 and hCE-2 are involved in the activation of prodrugs, inactivation of ester-containing pharmaceuticals, and metabolism of xenobiotics [7], it is of great importance to predict a prodrug/xenobiotics response in individuals. These fluorescent substrates are thus exceptionally useful for the evaluation of the relative amounts of specific carboxylesterase isozymes. Leng et al. [37] found that carboxylesterases in human lymphocytes are potential markers of pyrethroid susceptibility. From our results, *hCE-1* and/or *hCE-2* may be expressed in lymphocytes. Therefore, the determination of esterase activities in lymphocytes using our pyrethroid-like fluorescent substrates could be a useful tool to assess the ability of individuals to metabolize pyrethroids. Furthermore, this assessment would also be useful to assess the efficiency of some prodrugs containing ester bond(s) in individuals.

In summary, we identified and characterized hCE-1 and hCE-2 as human pyrethroid-hydrolyzing esterases. These hCEs showed diffrent enantio/diastereoselectivities for pyrethroids, suggesting that diffrences in the hydrolysis activities of these enzymes toward stereoisomers of pyrethroids may lead to diffrences in human toxicity. Furthermore, individual-to-individual diffrences in the abundance of these esterases may result in diffrences in sensitivity to pyrethroid toxicity. Finally, the purified recombinant enzymes hydrolyzed a variety of pyrethroid-like fluorescent surrogates. These surrogates proved to be useful tools to evaluate hydrolysis activity for pyrethroids and may be useful for the evaluation of hCE-1 and hCE-2 activities in individuals.

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References

[1]. Khambay, BPS.; Jewess, PJ. Comprehensive Molecular Insect Science. Gilbert, LI.; Iatrou, K.; Gill, SS., editors. 6. Elsevier; Oxford: 2005. p. 1-29.

[2]. Their A. J. Urban Health 2001;72:372–381.

[3]. Abernathy CO, Casida JE. Science 1973;179:1235–1236. [PubMed: 4689016]

[4]. Sogorb MA, Vilanova E. Toxicol. Lett 2002;128:215–228. [PubMed: 11869832]

[5]. Butte W, Kemper K. Toxicol. Lett 1999;107:49–53. [PubMed: 10414780]

[6]. Stok JE, Huang H, Jones PD, Wheelock CE, Morisseau C, Hammock BD. J. Biol. Chem 2004;279:29863–29869. [PubMed: 15123619]

[7]. Satoh T, Hosokawa M. Annu. Rev. Pharmacol 1998;38:257–288.

[8]. Shan G, Hammock BD. Anal. Biochem 2001;299:54–62. [PubMed: 11726184]

[9]. Shan G, Stoutamire DW, Wengatz I, Gee SJ, Hammock BD. J. Agric. Food Chem 1999;47:2145–2155. [PubMed: 10552511]

[10]. Wheelock CE, Wheelock AM, Zhang R, Stok JE, Morisseau C, Le Valley SE, Green CE, Hammock BD. Anal. Biochem 2003;315:208–222. [PubMed: 12689831]

[11]. Huang H, Stok JE, Stoutamire DW, Gee SJ, Hammock BD. Chem. Res. Toxicol 2005;18:516–527. [PubMed: 15777092]

[12]. O'Reilly, DR.; Miller, LK.; Luckow, VA. Baculovirus Expression Vectors: A Laboratory Manual. Oxford University Press; New York: 1992.

[13]. Bradford MM. Anal. Biochem 1976;72:248–254. [PubMed: 942051]

[14]. Ljungquist A, Augustinsson KB. Eur. J. Biochem 1971;23:303–313. [PubMed: 5156375]

[15]. Wheelock CE, Severson TF, Hammock BD. Chem. Res. Toxicol 2001;14:1563–1572. [PubMed: 11743738]

[16]. Wu MH, Chen PX, Wu KL, Liu WQ, Strom S, Das S, Cook EH, Rosner GL, Dolan ME. Pharmacogenetics 2004;14:595–605. [PubMed: 15475733]

[17]. Marsh S, Xiao M, Yu JS, Ahluwalia R, Minton M, Freimuth RR, Kwok PY, McLeod HL. Genomics 2004;84:661–668. [PubMed: 15475243]

[18]. Charasson V, Bellott R, Meynard D, Longy M, Gorry P, Robert J. Clin. Pharmacol. Ther 2004;76:528–535. [PubMed: 15592324]

[19]. Morton CL, Potter PM. Mol. Biotechnol 2000;16:193–202. [PubMed: 11252804]

[20]. Sanghani SP, Quinney SK, Fredenburg TB, Davis WI, Murry DJ, Bosron WF. Drug Metab. Dispos 2004;32:505–511. [PubMed: 15100172]

[21]. Wheelock CE, Miller JL, Miller MJ, Gee SJ, Shan G, Hammock BD. Environ. Toxicol. Chem 2004;23:2699–2708. [PubMed: 15559286]

[22]. Elliott M, Janes NF, Pulman DA, Gaughan LC, Unai T, Casida JE. J. Agric. Food Chem 1976;24:270–276. [PubMed: 1254806]

[23]. Gaughan LC, Unai T, Casida JE. J. Agric. Food Chem 1977;25:9–17. [PubMed: 1002942]

[24]. Choi J, Rose RL, Hodgson E. Pestic. Biochem. Physiol 2002;74:117–128.

[25]. Kang K-D, Jones PD, Huang H, Zhang R, Mostovich LA, Wheelock CE, Watanabe T, Gulyaeva LF, Hammock BD. Anal. Biochem 2005;344:183–192. [PubMed: 16083846]

[26]. Jones PD, Wolf NM, Morisseau C, Whetstone P, Hock B, Hammock BD. Anal. Biochem 2005;343:66–75. [PubMed: 15963942]

[27]. Senter PD, Beam KS, Mixan B, Wahl AF. Bioconjug. Chem 2001;12:1074–1080. [PubMed: 11716702]

[28]. Gray, AJ.; Soderlund, DM. Insecticides. Hutson, DH.; Roberts, TR., editors. Wiley; New York: 1985. p. 193-248.

[29]. Lawrence LJ, Casida JE. Pestic. Biochem. Physiol 1982;18:9–14.

[30]. Gotoh Y, Kawakami M, Matsumoto N, Okada Y. J. Toxicol. Clin. Toxicol 1998;36:57–61. [PubMed: 9541045]

[31]. Bencharit S, Morton CL, Xue Y, Potter PM, Redinbo MR. Nat. Struct. Biol 2003;10:349–356. [PubMed: 12679808]

[32]. Bencharit S, Morton CL, Hyatt JL, Kuhn P, Danks MK, Potter PM, Redinbo MR. Chem. Biol 2003;10:341–349. [PubMed: 12725862]

[33]. Huang H, Fleming CD, Nishi K, Redinbo MR, Hammock BD. Chem. Res. Toxicol 2005;18:1371–1377. [PubMed: 16167828]

[34]. Ellinghaus P, Seedorf U, Assmann G. Biochim. Biophys. Acta 1998;1397:175–179. [PubMed: 9565681]

[35]. Furihata T, Hosokawa M, Nakata F, Satoh T, Chiba K. Arch. Biochem. Biophys 2003;416:101–109. [PubMed: 12859986]

[36]. Institoris L, Undeger U, Siroki O, Nehez M, Desi I. Toxicology 1999;137:47–55. [PubMed: 10513999]

[37]. Leng G, Lewalter J, Rohrig B, Idel H. Toxicol. Lett 1999;107:123–130. [PubMed: 10414789]

[38]. Pindel EV, Kedishvili NY, Abraham TL, Brzezinski MR, Zhang J, Dean RA, Bosron WF. J. Biol. Chem 1997;272:14769–14775. [PubMed: 9169443]

[39]. Brzezinski MR, Abraham TL, Stone CL, Dean RA, Bosron WF. Biochem. Pharmacol 1994;48:1747–1755. [PubMed: 7980644]

[40]. Kamendulis LM, Brzezinski MR, Pindel EV, Bosron WF, Dean RA. J. Pharmacol. Exp. Ther 1996;279:713–717. [PubMed: 8930175]

[41]. Zhang J, Burnell JC, Dumaual N, Bosron WF. J. Pharmacol. Exp. Ther 1999;290:314–318. [PubMed: 10381793]

[42]. Humerickhouse R, Lohrbach K, Li L, Bosron WF, Dolan ME. Cancer Res 2000;60:1189–1192. [PubMed: 10728672]

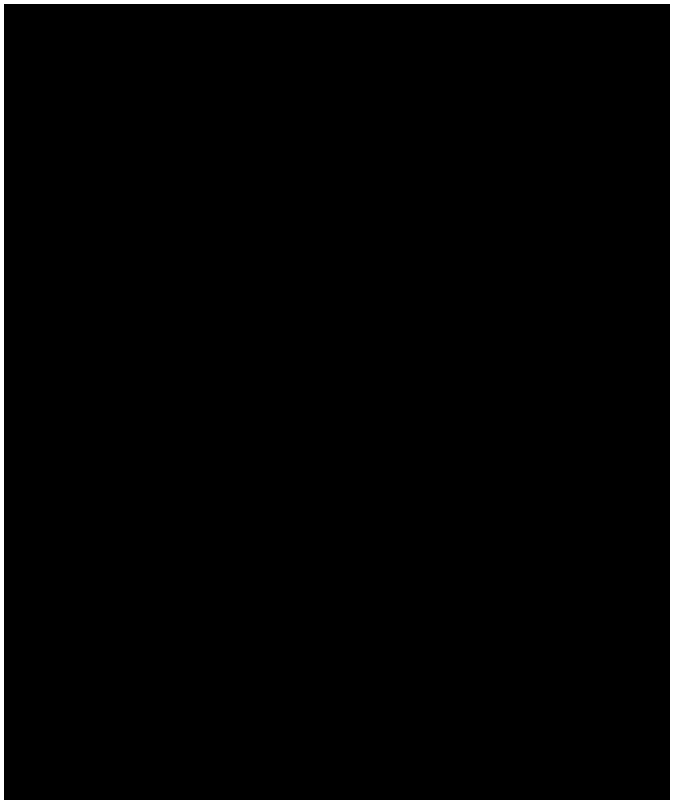

Fig. 1.
Nomenclature scheme for the various pyrethroids and pyrethroid surrogates used in this study. The alcohol and acid moieties are indicated by a *letter* or *number*, respectively. Chiral centers are indicated by an *asterisk*

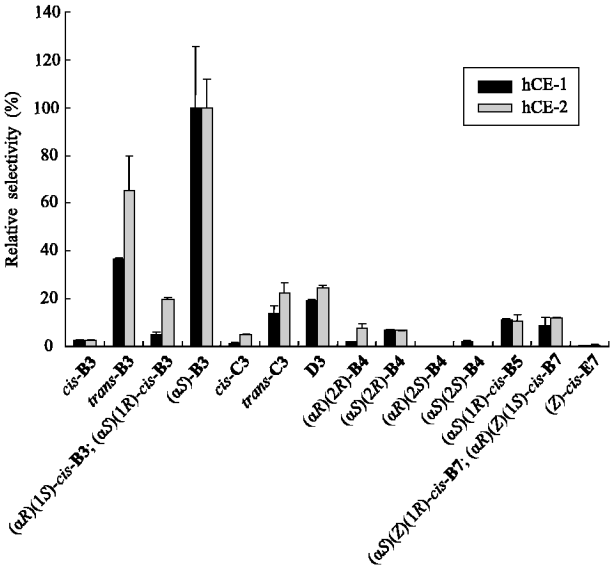

Fig. 2.

Relative selectivities of hCE-1 and hCE-2 for a series of authentic pyrethroids. The relative selectivities were calculated based on the data from Table 2 using (α*S*)-**B3** as a reference.

Table 1

Purification of a recombinant hCE-1 and hCE-2 produced in insect cells

Step	Total activity (nmol/min)[a]	Total protein (mg)	Activity (%)	Specific activity (nmol/min/mg)	Purification fold
hCE-1					
Supernatant 10,000g	224	721	100	0.31	1
Supernatant 100,000g	213	607	95	0.35	1.1
DEAE	57	24	25	2.4	7.7
Preparative IEF	17	1.2	7.6	14	44
hCE-2					
Supernatant 10,000g	218	588	100	0.37	1
Supernatant 100,000g	195	423	89	0.46	1.2
DEAE	110	35	50	3.2	8.6
Preparative IEF	20	1.1	9.2	18	49

[a] Enzyme activity was measured using A3 as described under “Materials and methods.”

Table 2

Specific activity of the recombinant hCE-1 and hCE-2 for various authentic pyrethroids

Substrate	hCE-1 (nmol/min/mg)	hCE-2 (nmol/min/mg)
cis-Cypermethrin (cis-B3)	3.2±0.1	4.8±0.6
trans-Cypermethrin (trans-B3)	47 ± 0.8	137 ± 31
α-Cypermethrin ((αR)(1S)-cis-B3; (αS)(1R)-cis-B3)	6.3 ± 1.1	41 ± 2
ζ-Cypermethrin ((αS)-B3)	129 ± 33	210 ± 25
cis-Permethrin (cis-C3)	5.0 ± 1.3	33 ± 3
trans-Permethrin (trans-C3)	61 ± 14	160 ± 31
CyXuthrin (D3)	25 ± 2	51 ± 3
(2R)(αR)-Fenvalerate ((2R)(αR)-B4)	2.6 ± 0.1	16 ± 4
(2R)(αS)-Fenvalerate ((2R)(αS)-B4)	8.4 ± 0.9	14 ± 0.4
(2S)(αR)-Fenvalerate ((2S)(αR)-B4)	ND[a]	ND
(2S)(αS)-Fenvalerate ((2S)(αS)-B4)	2.6 ± 0.6	ND
Deltamethrin ((αS)(1R)-cis-B5)	14 ± 0.8	22 ± 6
λ-Cyhalothrin ((αS)(Z)(1R)-cis-B7; (αR)(Z)(1S)-cis-B7)	11 ± 4.8	25 ± 1
Bifenthrin ((Z)-cis-E7)	0.3 ± 0.03	1.2 ± 0.1

[a]ND, not measurable. The quantitation limit for 3-phenoxybenzalde-hyde was 2.0 μM.

Table 3

Specific activity of the recombinant hCE-1, hCE-2, and human microsomes for pNPA and various Xuorescent substrates

Substrate	hCE-1	hCE-2 (nmol/min/mg)	Microsomes
(αR)-A1	750 ± 70	3000 ± 300	65 ± 5
(αS)-A1	2500 ± 200	19,000 ± 100	220 ± 10
(αR)-A2	3300 ± 300	14,000 ± 900	100 ± 10
(αS)-A2	16,000 ± 800	38,000 ± 1000	330 ± 20
A3	9.5 ± 0.6	20 ± 0.3	0.24 ± 0.003
(2R)-A4	0.94 ± 0.03	55 ± 1	0.087 ± 0.008
(2S)-A4	ND[a]	ND	ND
A5	0.51 ± 0.01	0.56 ± 0.04	0.093 ± 0.005
A6	6.5 ± 0.2	4.1 ± 0.1	0.10 ± 0.01
cis-A7	0.51 ± 0.01	0.48 ± 0.03	ND
trans-A7	0.65 ± 0.07	1.2 ± 0.02	ND
A8	4.7 ± 0.3	0.71 ± 0.04	0.033 ± 0.003
pNPA	18,000 ± 1000	43,000 ± 2000	1400 ± 100
pNPA[b]	10,000	60,000	

[a] ND, not detected.

[b] Data are from Senter et al. [27].

Table 4

Specific activities of recombinant hCE-1 and hCE-2 for stereoisomers of pyrethroid-like Xuorescent substrates

Substrate	hCE-1		hCE-2	
	nmol/min/mg	Relative activity[a]	nmol/min/mg	Relative activity
(αR)(1R)-cis-A3	0.80 ± 0.04	1	2.4 ± 0.1	1
(αR)(1R)-trans-A3	15 ± 1	19	4.6 ± 0.2	1.9
(αR)(1S)-cis-A3	0.40 ± 0.03	0.50	2.1 ± 0.1	0.88
(αR)(1S)-trans-A3	17 ± 2	21	63 ± 3	26
(αS)(1R)-cis-A3	0.53 ± 0.01	0.66	5.5 ± 0.3	2.3
(αS)(1R)-trans-A3	61 ± 10	76	10 ± 0.2	4.2
(α S)(1S)-cis-A3	0.65 ± 0.08	0.81	16 ± 0.1	6.7
(αS)(1S)-trans-A3	21 ± 1	26	160 ± 3	67
(2R)(αR)-A4	0.67 ± 0.04	0.84	32 ± 0.4	13
(2R)(αS)-A4	0.99 ± 0.05	1.2	71 ± 2	30
(2S)(αR)-A4	ND[b]	0	ND	0
(2S)(αS)-A4	0.24 ± 0.02	0.30	ND	0

[a] Relative activity is a ratio of specific activities between (α R)(1R)-cis-A3 and each compound.

[b] ND, not detected.

Table 5

Kinetic constants of the recombinant hCE-1 and hCE-2 for various substrates

Substrate	hCE-1			hCE-2			Preference index[a]
	K_m(μM)	k_{cat}(min^{-1})	k_{cat}/K_m(μM^{-1} min^{1})	K_m(μM)	k_{cat}(min^{-1})	k_{cat}/K_m(μM^{-1} min^{-1})	
pNPA	27 ± 1	1020 ± 30	38 ± 1.4	31 ± 2	4200 ± 240	140 ± 8	
(αR)(1R)-cis-A3	0.3 ± 0.1	0.19 ± 0.06	0.71 ± 0.29	0.9 ± 0.2	0.11 ± 0.03	0.14 ± 0.04	0.71
(αR)(1R)-trans-A3	1.7 ± 0.5	1.3 ± 0.3	0.74 ± 0.03	2.5 ± 0.1	0.32 ± 0.07	0.13 ± 0.03	0.76
(αR)(1S)-cis-A3	1.5 ± 0.5	0.09 ± 0.03	0.06 ± 0.03	1.7 ± 0.3	0.10 ± 0.02	0.06 ± 0.01	-0.01
(αR)(1S)-trans-A3	1.9 ± 0.8	1.7 ± 0.4	0.87 ± 0.03	1.9 ± 0.7	2.2 ± 0.3	1.2 ± 0.4	-0.14
(αS)(1R)-cis-A3	0.3 ± 0.1	0.19 ± 0.06	0.57 ± 0.03	2.5 ± 0.7	0.28 ± 0.04	0.11 ± 0.03	0.71
(αS)(1R)-trans-A3	0.7 ± 0.4	4.4 ± 0.6	6.6 ± 0.03	2.8 ± 0.1	0.48 ± 0.03	0.17 ± 0.01	1.59
(αS)(1S)-cis-A3	1.4 ± 0.4	0.07 ± 0.02	0.05 ± 0.03	4.0 ± 0.9	0.71 ± 0.21	0.18 ± 0.05	-0.56
(αS)(1S)-trans-A3	2.4 ± 0.8	1.7 ± 0.4	0.71 ± 0.03	3.8 ± 0.4	4.6 ± 0.3	1.2 ± 0.1	-0.23
(2R)(αR)-A4	0.7 ± 0.2	0.10 ± 0.02	0.15 ± 0.03	0.9 ± 0.3	0.51 ± 0.10	0.56 ± 0.16	-0.57
(2R)(αS)-A4	0.8 ± 0.3	0.13 ± 0.02	0.16 ± 0.03	2.0 ± 0.3	3.5 ± 0.7	1.8 ± 0.4	-1.05
(2S)(αR)-A4	—[b]	—	—	—	—	—	
(2S)(αS)-A4	0.8 ± 0.3	0.03 ± 0.01	0.04 ± 0.03	—	—	—	
A6	1.8 ± 0.3	0.25 ± 0.06	0.14 ± 0.03	2.1 ± 0.3	0.34 ± 0.05	0.16 ± 0.02	
A8	1.5 ± 0.2	0.30 ± 0.05	0.20 ± 0.03	5.4 ± 1.2	0.31 ± 0.05	0.06 ± 0.01	
Cypermethrin (B3)	20 ± 2	1.3 ± 0.1	0.07 ± 0.01	9.1 ± 2.0	4.0 ± 0.9	0.44 ± 0.10	
4-Methylumbelliferyl acetate[c]	800		2.0	150		60.0	
Cocaine[d]	120			390		0.0184	
Heroin[e]	6300		0.069	6800		0.314	
6-Monoacetylmorphine[e]	8300		0.000024	130		0.022	
Meperidine[f]	1900		0.00035				
CPT-11[g]	42.7	0.031	0.00073	3.4	0.160	0.047	

[a] Preference indexD log((catalytic eYciency of hCE-1)/(catalytic eYciency of hCE-2)).

[b] Hydrolytic activity was not detected.

[c] Data are from Pindel et al. [38].

[d] Data for hCE-1 are from Brzezinski et al. [39] and for hCE-2 are from Pindel et al. [38].

[e] Data are from Kamendulis et al. [40].

[f] Data are from Zhang et al. [41].

[g] Data are from Humerickhouse et al. [42].