

RESEARCH PAPER

Biochemical and molecular analysis of
carboxylesterase-mediated hydrolysis of cocaine
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Background and purpose: Carboxylesterases (CEs) metabolize a wide range of xenobiotic substrates including heroin, cocaine, meperidine and the anticancer agent CPT-11. In this study, we have purified to homogeneity human liver and intestinal CEs and compared their ability with hydrolyse heroin, cocaine and CPT-11.

Experimental approach: The hydrolysis of heroin and cocaine by recombinant human CEs was evaluated and the kinetic parameters determined. In addition, microsomal samples prepared from these tissues were subjected to chromatographic separation, and substrate hydrolysis and amounts of different CEs were determined.

Key results: In contrast to previous reports, cocaine was not hydrolysed by the human liver CE, hCE1 (CES1), either as highly active recombinant protein or as CEs isolated from human liver or intestinal extracts. These results correlated well with computer-assisted molecular modelling studies that suggested that hydrolysis of cocaine by hCE1 (CES1), would be unlikely to occur. However, cocaine, heroin and CPT-11 were all substrates for the intestinal CE, hiCE (CES2), as determined using both the recombinant protein and the tissue fractions. Again, these data were in agreement with the modelling results.

Conclusions and implications: These results indicate that the human liver CE is unlikely to play a role in the metabolism of cocaine and that hydrolysis of this substrate by this class of enzymes is via the human intestinal protein hiCE (CES2). In addition, because no enzyme inhibition is observed at high cocaine concentrations, potentially this route of hydrolysis is important in individuals who overdose on this agent.

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Keywords: carboxylesterase; cocaine; heroin; CPT-11; inhibitor; molecular modelling

Abbreviations: BChE, butyrylcholinesterase; CE, carboxylesterase; clogP, calculated logP; CPT-11, irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; DMSO, dimethyl sulfoxide; hBChE, human BChE; hBr3, human brain CE, CES3; hCE1 (CES1), human carboxylesterase 1, CES1; hiCE (CES2), human intestinal carboxylesterase, CES2, hCE2; *i*, fractional inhibition; IEC, ion-exchange chromatography; *k*_{cat}, turnover constant; *K*_i, inhibition constant; *K*_m, Michaelis constant; o-NPA, o-nitrophenyl acetate; TBAP, tetrabutyl ammonium phosphate; *V*_{max}, maximal reaction velocity

Introduction

Carboxylesterases [carboxylic ester hydrolase (CE), EC 3.1.1.1] are responsible for the detoxification of numerous xenobiotics including heroin, cocaine, meperidine and lidocaine, as well as the activation of the anticancer agents, capecitabine

and CPT-11 (Pindel *et al.*, 1997; Danks *et al.*, 1999; Zhang *et al.*, 1999; Tabata *et al.*, 2004). CEs tend to be expressed in tissues likely to be exposed to such agents, for example, the liver, gut, kidney, lung. Although numerous CEs have been identified in rodents and other mammals, currently only three such enzymes have been characterized from humans. Human liver CE (hCE1; CES1) is predominantly expressed in the liver and is efficient at metabolizing small esterified substrates (Wadkins *et al.*, 2001). Human intestinal CE (hiCE, hCE2, CES2) is expressed in the epithelial lining of the gut and the liver, and has been demonstrated to be efficient in the hydrolysis of the anticancer prodrug CPT-11 (Humerickhouse

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et al., 2000; Khanna *et al.*, 2000). A third human CE, human brain CE (hBr3; CES3) has been recently identified, but as yet little is known about its pattern of expression and/or substrate specificity (Mori *et al.*, 1999).

Cocaine and heroin abuse is a major clinical problem in the 25 to 49 year-old age group, with drug overdose being the fourth leading cause of death, on a par with motor vehicle accidents (Anderson, 1999). Hence devising alternative treatment options for patients who have overdosed on these agents is desirable. Although naloxone has significantly reduced the incidence of heroin-induced fatalities, there are currently few effective drugs for cocaine overdose. We have proposed that in patients who have overdosed on cocaine, administration of a recombinant enzyme (e.g. a CE) that could metabolize the drug to inactive metabolites, may be effective in ameliorating the toxicity associated with this agent (Potter and Wadkins, 2006; Redinbo and Potter, 2005). However, for any approach to be successful, a detailed analysis of the metabolism of cocaine by different enzymes will be essential.

Although the metabolism of heroin and cocaine has been presumed to occur via CE-mediated hydrolysis of the ester functions, it is clear that butyrylcholinesterase (BChE) can also hydrolyse these drugs (Gatley, 1990; Lockridge and LaDu, 1980). However, the relative contribution of CEs and BChE towards metabolism of each of these substrates is unclear, in part due to the lack of availability of the purified proteins, and also due to the lack of specific reagents that could inhibit the different enzyme isoforms.

As hCE1 (CES1), hiCE (CES2) and BChE are all expressed in the liver, the determination of which enzyme can metabolize particular substrates has been difficult. In this article, we have purified recombinant hiCE (CES2) to homogeneity, assessed its hydrolytic activity towards several different substrates including heroin and cocaine, and determined the ability of specific inhibitors to modulate enzyme activity. Furthermore, we have correlated these results using human liver and intestinal specimens and corroborated our experiments using computer-assisted molecular modelling. These studies confirm that cocaine is exclusively metabolized by hiCE (CES2) and not by hCE1 (CES1).

Methods

CE activity assay

CE activity was measured by following *o*-nitrophenyl acetate (*o*-NPA) hydrolysis at 420 nm as previously described

(Beaufay *et al.*, 1974; Danks *et al.*, 1998). Protein concentrations were determined using the Bradford method (using Bio-Rad reagent, Hercules, CA, USA). Data were expressed as nmoles of *o*-nitrophenol produced per minute per milligram of protein (nmol/min/mg) as calculated using the extinction coefficient for *o*-nitrophenol ($13.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

Kinetics of nitrophenyl ester hydrolysis

The kinetic parameters K_m , k_{cat} and V_{max} for a panel of nitrophenyl esters (Table 1) were determined as previously described (Wadkins *et al.*, 2001). Briefly, assays were performed in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 and formation of the products was determined spectrophotometrically. Product yields were then calculated using the extinction coefficients for nitrophenol, and kinetic parameters were determined from these values

Kinetics of CPT-11 metabolism

The kinetic parameters for CPT-11 hydrolysis were determined as previously described (Wadkins *et al.*, 2001).

Analysis of cocaine hydrolysis

To assess the hydrolysis of cocaine to benzoylecgonine and ecgonine methyl ester, the drug (1 mM) was incubated with either media harvested from Sf21 cells (150 μL) or purified CEs (20 μg) in 50 mM HEPES pH 7.4 at 37°C. After 60 min, samples were analysed by high-performance liquid chromatography (HPLC) for metabolite formation. For kinetic studies, 0–5 mM of cocaine was incubated with hiCE (CES2) (250 U/reaction, 10 min) at 37°C. One unit of enzyme activity is equivalent to 1 nmol of nitrophenol produced per min. Reactions were terminated by addition of cold acidified methanol and drug concentrations were determined using isocratic HPLC analysis. Samples (20 μL) were separated using a $3.9 \times 300 \text{ mm } 4 \mu\text{m}$ Nova-Pak C_{18} reverse-phase column (Waters Corporation, Milford, MA, USA) and a mobile phase containing 25% (v/v) acetonitrile in 50 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 150 μM tetrabutyl ammonium phosphate (TBAP), pH 2.2. Metabolites were eluted at a flow rate of 1.0 ml/min, and identified by ultraviolet detection (235 nm). As ecgonine methyl ester absorbs poorly at this wavelength, hydrolysis of cocaine to this product was monitored by the formation of benzoic acid (the acid released from enzymatic hydrolysis). Under these conditions, cocaine, benzoylecgonine and

Table 1 Purification of hiCE (CES2) to homogeneity

Sample	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Recovery (%)
Crude media	1100	472 200	300	1 550	100
After ultrafiltration	520	472 700	260	1 840	100
After IEC	146	116 400	4.00	29 300	25
After ultrafiltration	0.3	96 900	3.20	30 700	21
After Sephacryl S200	5	41 700	0.80	52 100	9

A typical purification profile for hiCE (CES2), isolated from serum-free media, harvested from *Spodoptera frugiperda* Sf21 cells expressing a secreted form of the enzyme. Carboxylesterase (CE) activity was determined using *o*-NPA as a substrate and 1 U is equivalent to 1 nmol of *o*-nitrophenol produced per minute.

benzoic acid eluted at 4.3, 2.8 and 7.4 min, respectively. Drug concentrations were calculated using System Gold software (Beckmann, Brea, CA, USA) by comparison with known amounts of standard compounds. The sensitivity of detection for this system was 2, 2 and 1 ng/ μ L for cocaine, benzoylecgonine and benzoic acid, respectively. All reactions were corrected for non-enzymatic hydrolysis by subtraction of the levels of products when cocaine was incubated with buffer alone.

Analysis of heroin hydrolysis

Enzymatic hydrolysis of heroin to 6-monoacetylmorphine was examined under the following conditions. Drug was added to a pre-warmed (37°C) mixture containing purified enzyme and 50 mM HEPES buffer pH 7.4. Typically, 0–5 mM heroin was incubated for 2 min with hCE1 (CES1) (25 U/reaction) or hiCE (CES2) (10 U/reaction), at 37°C. All reactions were terminated by addition of equal volume of ice-cold acidified methanol (containing 0.5% 1 M HCl), and proteins were pelleted by centrifugation for 5 min at 14 500 \times g at 4°C.

The supernatants from the above reactions were then analysed by reverse phase HPLC using a similar system to that for cocaine. However, heroin and its metabolites were separated and quantitated using a gradient profile as follows: 100% buffer A for 1.5 min changing to 100% buffer B over 0.5 min for 5.5 min [where buffer A is 5% (v/v) acetonitrile in 50 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 150 μ M TBAP, pH 2.2 and buffer B is 25% (v/v) acetonitrile in 50 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 150 μ M TBAP, pH 2.2]. Using a flow rate of 1 ml/min, heroin, 6-acetylmorphine and morphine eluted at 7.0, 5.6 and 4.1 min, respectively, and were detected at 235 nm. Concentrations were determined in an identical fashion to that described for cocaine earlier, and the sensitivity of this system was 3 ng/ μ L⁻¹, 2 ng/ μ L⁻¹ and 4 ng/ μ L⁻¹ for heroin, 6-acetylmorphine and morphine, respectively. All reactions were corrected for non-enzymatic hydrolysis by subtraction of the levels of products when cocaine was incubated with buffer alone.

Prediction of logP values

Calculated logP (clogP) values were obtained using ChemSilico Predict v2.0 software (ChemSilico LLC, Tewksbury, MA, USA).

Experimental design

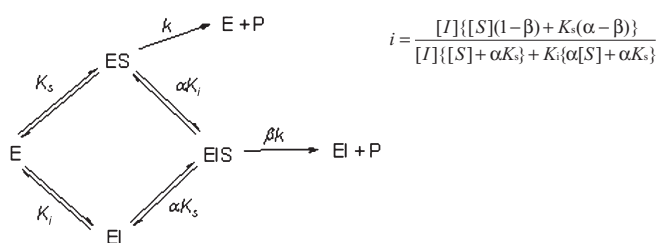
Expression and purification of recombinant hiCE (CES2). The purification of hiCE (CES2) was achieved by overexpression of a secreted form of the protein from *Spodoptera frugiperda* Sf21 cells using baculoviral expression vectors (Morton and Potter, 2000). All purification steps were carried out at 4°C. Media harvested from infected Sf21 cells was buffer exchanged with 50 mM HEPES pH 7.4 buffer using ultrafiltration with Ultracel PL-30 membranes (Millipore, Bedford, MA) and the sample was then applied to a 2.5 \times 6 cm macro-prep diethylaminoethyl (DEAE) column equilibrated in the same buffer. Following washing with 1 column volume (~30 mL) of 50 mM HEPES pH 7.4, the sample was then washed with 5 column

volumes of 50 mM HEPES pH 7.4 buffer containing 125 mM NaCl. Elution of hiCE (CES2) was accomplished with 50 mM HEPES pH 5.5 buffer containing 130 mM NaCl. The hiCE (CES2)-containing fractions were pooled, concentrated by ultrafiltration and applied to a 1.5 \times 170 cm Sephacryl S-200 HR column (column volume ~300 mL; Amersham Biosciences Inc., Piscataway, NJ, USA), pre-equilibrated with 50 mM HEPES pH 7.4. hiCE (CES2) was eluted from the column using a flow rate of 6 mL \cdot h⁻¹ and was estimated to be greater than 98% pure by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Chromatography of human intestinal microsomal extracts. Extracts, typically containing ~10 mg of total protein (equivalent to ~10 000 U of CE activity), were applied to a 1 \times 170 cm Sephacryl S-200 HR column equilibrated in 150 mM NaCl, 50 mM HEPES pH 7.4, 0.5% Triton X-100 and samples were eluted using the same buffer. Routinely 3 ml fractions were collected, concentrated and then assayed for the desired enzyme activity. Western analysis for hCE1 (CES1) and hiCE (CES2) was undertaken using specific antibodies for these proteins using chemiluminescent detection.

Data analysis and statistical procedures

Enzyme inhibition assays. CE enzyme inhibition was determined using the previously described, selective CE inhibitors (Wadkins *et al.*, 2004; Wadkins *et al.*, 2005). Compounds analysed were benzil (1), 1,4-bisbenzil (2), 1-(3,4-dimethylphenyl)-2-phenyl-ethane-1,2-dione (3), *N,N'*-(2,3,5,6-tetramethyl-1,4-phenylene)bis(4-fluorobenzene sulfonamide) (4) or *N,N'*-1,4-phenylenebis(3-bromobenzene sulfonamide) (5) (see the chemical structures of these compounds in Table 4) using cocaine, heroin, o-NPA or CPT-11 as substrates. Briefly, the formation of product in the presence of inhibitor at concentrations ranging from 1 nM to 100 μ M was determined (Wadkins *et al.*, 2004; Wadkins *et al.*, 2005). Routinely at least eight concentrations of inhibitor were used. To determine the K_i values (inhibition constants), data were fitted to the following equation (Webb, 1963):



where i = fractional inhibition, $[I]$ = inhibitor concentration, $[S]$ = substrate concentration, α = change in affinity of the substrate for the enzyme in the presence of the inhibitor (where $\alpha > 0$), b = change in the rate of enzyme substrate complex decomposition in the presence of the inhibitor (where $1 > b > 0$), K_s is the dissociation constant for the enzyme substrate complex (assuming negligible commitment to catalysis) and K_i is the inhibitor constant. The data sets were analysed using Prism 4.0 software (GraphPad, La Jolla, CA, USA) and Perl Data Language, and the mode of enzyme

inhibition was determined by evaluating the r^2 values for the curve fits using Akaike's information criteria (Wadkins *et al.*, 2005). K_i values were then calculated using the best fit model described from these analyses.

In most cases, enzyme inhibition was partially competitive (i.e. where the inhibitor does not affect rate of enzyme/substrate complex dissociation and only partially hinders substrate binding).

Molecular modelling studies. Molecular modelling analyses were performed using ICM Pro software v3.6 (Molsoft LLC, La Jolla, CA, USA) running on a dual Xeon computer in a Windows XP environment. Recent studies indicate that docking experiments using ICM Pro compare favorably with FlexX, GOLD and GLIDE (Chen *et al.*, 2006). For hCE1 (CES1), the coordinates derived from the CE/homatropine crystal structure were used (1MX5; Bencharit *et al.*, 2003b). For hiCE (CES2), a homology model was developed using the 1MX5 coordinates With Modeller 8 (Sali and Blundell, 1993) software.

For these analyses, substrate and inhibitor structures were constructed in ChemDraw (CambridgeSoft, Cambridge, MA, USA) and subjected to energy minimization using ICM-Pro. The receptor site, consisting of the residues that encompassed the enzyme active site gorge, was then selected as a pocket into which the minimized ligand could be docked. For cocaine and heroin, binding of both the protonated and the native form of the molecule was assessed. Docking studies were performed using essentially the default parameters for the ICM Pro software. A rigid receptor/flexible ligand approach was adopted that used five potential energy maps combining hydrophobicity, electrostatics, hydrogen bond formation and two van der Waals parameters. However, minor modifications included constructing receptor electrostatic charge maps at a resolution of 0.1 Å and increasing the 'Thoroughness' parameter to 10. The latter increased the time and number of poses that were evaluated during the docking simulations.

Materials

Cocaine ((-)-cocaine) and heroin were purchased from Cerilant Corporation (Round Rock, TX) and were dissolved in acetonitrile. Routinely, solvent concentrations in biochemical reactions did not exceed 2%, although no effect was seen on CE activity at 5% acetonitrile. CPT-11 was kindly provided by Dr J. P. McGovren (Pharmacia, Peapack, NJ, USA) and was dissolved in methanol. The Macro-Prep® DEAE support was from Bio-Rad and Sephacryl S-200 high resolution resin was obtained from Amersham Biosciences Inc. All other chemicals were purchased from Fisher (Fair Lawn, NJ, USA), Organic Research (Cincinnati, OH, USA), Sigma-Aldrich (St. Louis, MO, USA) or VWR Scientific Products, Inc. (Suwanee, GA, USA).

hCE1 (CES1) was purified from the media of baculovirus-infected *Spodoptera frugiperda* Sf21 cells as previously described (Bencharit *et al.*, 2003a; Bencharit *et al.*, 2003b; Morton and Potter, 2000). The GenBank accession numbers for the cDNAs encoding the CEs used in these studies were as follows; hiCE (CES2) - Y09616 (Schwer *et al.*, 1997); and

hCE1 (CES1) - M73499 (Munger *et al.*, 1991). Rabbit polyclonal antibodies directed towards hiCE (CES2) and hCE1 (CES1) were generated by repeated immunization of rabbits with recombinant proteins. Antibodies were purified from sera using IgA affinity chromatography and sensitivity and specificity were verified by enzyme-linked immunosorbent assay and Western analyses. Human liver and intestinal microsomal extracts were purchased from BD Biosciences (Woburn, MA, USA) and Celsis In Vitro (Baltimore, MD, USA), respectively.

Results

Expression and purification of recombinant hiCE (CES2)

Media was obtained from *S. frugiperda* Sf21 cells that had been infected with baculovirus expressing a secreted form of the hiCE (CES2) protein (Potter *et al.*, 1998; Khanna *et al.*, 2000). Initial attempts at purification using isoelectric focusing (Morton and Potter, 2000) proved unsuccessful and ion-exchange chromatography was chosen as the preferred method. A step-wise wash and elution was used to allow the separation of hiCE (CES2) from two problematic contaminants, bovine serum albumin (present in minor amounts in the culture media) and baculoviral chitinase, both of which have molecular masses similar to hiCE (CES2) (~65 kDa). The anion-exchange column allowed for a 15-fold purification followed by an additional 2-fold purification by gel filtration, based on yields of CE activity (Table 1). Typically, we obtained 1 mg of hiCE (CES2) per liter of insect cell culture media. Multiple batches of hiCE (CES2) have been made by this protocol with final specific activities ranging from 40 to 55 000 U·mg⁻¹, with a typical average of 50 000 U·mg⁻¹; however, selected fractions typically showed a specific activity as high as 60 000 U·mg⁻¹. Purity of the samples was assessed by SDS-PAGE analysis (Figure 1) and protein identification by Matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

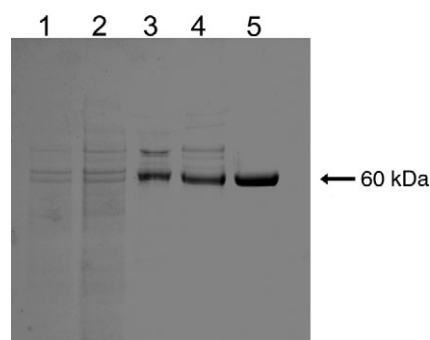


Figure 1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of different fractions following purification of hiCE (CES2). Approximately 3 µg of protein was loaded into each lane and following electrophoresis, the gel was stained with Coomassie Blue. Lane 1, Crude culture media; Lane 2, After ultrafiltration; Lane 3, After ion-exchange chromatography using DEAE; Lane 4, After ultrafiltration; Lane 5, After chromatography on Sephacryl S200 HR.

Table 2 A comparison of kinetic parameters of hiCE (CES2) and hCE1 (CES1) with various nitrophenyl esters

Substrate	clogP	Enzyme	$K_m \pm \text{s.e.m.}$ (μM)	$V_{\max} \pm \text{s.e.m.}$ (nmol min^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)	Ratio of k_{cat}/K_m [hiCE (CES2)/ hCE1 CES1]
o-Nitrophenyl acetate	1.12	hiCE (CES2)	79 ± 7.6	0.94 ± 0.02	67 ± 6	8.5
		hCE1 (CES1)	73 ± 7.4	0.15 ± 0.00	7.9 ± 0.7	
p-Nitrophenyl acetate	1.16	hiCE (CES2)	976 ± 60	4.17 ± 0.14	24 ± 1	6.3
		hCE1 (CES1)	822 ± 73	0.81 ± 0.02	3.8 ± 0.3	
p-Nitrophenyl propionate	1.86	hiCE (CES2)	201 ± 18	9.3 ± 0.6	261 ± 7	8.2
		hCE1 (CES1)	249 ± 9	2.05 ± 0.03	32 ± 0.6	
p-Nitrophenyl butyrate	2.46	hiCE (CES2)	117 ± 5	12 ± 0.4	580 ± 5	15.7
		hCE1 (CES1)	169 ± 8	1.65 ± 0.04	37 ± 1	
p-Nitrophenyl trimethylacetate	2.75	hiCE (CES2)	19.8 ± 1.1	0.77 ± 0.02	220 ± 9	14.7
		hCE1 (CES1)	29 ± 5	0.8 ± 0.1	15 ± 0.6	
p-Nitrophenyl valerate	3.01	hiCE (CES2)	71 ± 11	10.5 ± 1.2	421 ± 27	8.1
		hCE1 (CES1)	27 ± 2.7	0.73 ± 0.04	52 ± 2.5	

Assessment of substrate hydrolysis was determined spectrophotometrically by monitoring the formation of nitrophenol at 420 nm. Kinetic parameters were then calculated from these data sets.

s.e.m., standard error of the mean; CE, carboxylesterase.

To determine the oligomerization state of hiCE (CES2), we calibrated a Sephacryl S200 column using low molecular mass protein standards and applied a sample of the purified CE. These studies indicated that hiCE (CES2) had a predicted molecular mass of ~66 kDa. Furthermore, in co-chromatographic studies, it was observed that hiCE (CES2) and bovine serum albumin (66 kDa) eluted together off this Sephacryl column. As the hiCE (CES2) cDNA contains an open reading frame that would give rise to a protein of predicted molecular mass of 61 807 Da (Schwer *et al.*, 1997), we conclude that under the conditions employed here, the hiCE (CES2) protein purifies as a monomer.

Kinetic parameters of recombinant hiCE (CES2) with nitrophenyl ester substrates

Having isolated recombinant hiCE (CES2) and having homogenous hCE1 (CES1) at hand (Bencharit *et al.*, 2003b; Morton and Potter, 2000), we evaluated the kinetic parameters of these proteins with a variety of different esterase substrates. The K_m , V_{\max} and k_{cat}/K_m were therefore determined for various nitrophenyl esters using hiCE (CES2), and were compared with those for hCE1 (CES1) (Table 2). As can be seen, the K_m values demonstrated considerable variation with no obvious correlation with either enzyme. However, the V_{\max} values for hiCE (CES2) ranged from 0.77 to 12.0 $\text{nmol}\cdot\text{min}^{-1}$ for the different substrates, and these values were up to 6-fold higher than that observed for hCE1 (CES1). Peak velocities were seen with p-nitrophenyl butyrate and p-nitrophenyl propionate for hiCE (CES2) and hCE1 (CES1), respectively. However, maximal catalytic efficiency for hCE1 (CES1) was demonstrated with the valerate ester. This is a consequence of the much lower K_m constants that are observed with this substrate, resulting in higher k_{cat}/K_m values.

It was also noted that with the larger, more bulky substrates, such as p-nitrophenyl butyrate or p-nitrophenyl trimethylacetate, hiCE (CES2) was much more proficient at substrate hydrolysis. This is exemplified by the significantly larger k_{cat}/K_m values for these esters (Table 2). For example,

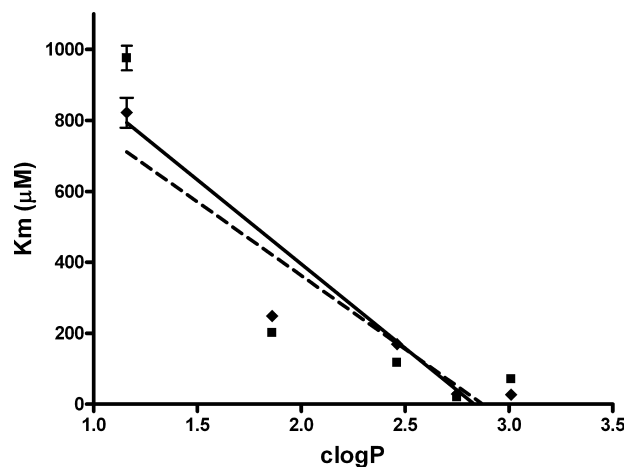


Figure 2 A graph demonstrating the linear relationships between the clogP values of the *para*-substituted nitrophenyl esters, and their observed K_m constants for hiCE (CES2) (■), and hCE1 (CES1) (◆). The correlation coefficients (r^2) for the data sets are indicated on the graph.

hiCE (CES2) was ~16-fold more efficient at p-nitrophenyl butyrate hydrolysis than hCE1 (CES1). As indicated in Table 2, for all substrates analysed, hiCE (CES2) was more efficient at substrate turnover as compared with hCE1 (CES1).

As the active sites of the CEs are lined with aromatic amino acids (Bencharit *et al.*, 2003b; Potter and Wadkins, 2006), we hypothesized that more hydrophobic molecules would preferentially localize within these domains. Therefore, we compared the affinity constants (K_m) for the *para*- series of substituted nitrophenyl esters for the two CEs, with their clogP values (calculated water/octanol partition coefficients). As shown in Figure 2, excellent correlations were seen with data obtained from both enzymes, with r^2 for the curve fits of 0.79 and 0.89 for hiCE (CES2) and hCE1 (CES1), respectively. These results confirm that the affinity of the compounds for the CEs is directly related to their clogP (or hydrophobicity).

Table 3 Kinetic parameters for the metabolism of heroin, cocaine, and CPT-11 for hiCE (CES2) and hCE1 (CES1)

Substrate	Enzyme	$K_m \pm \text{s.e.m. } (\mu\text{M})$	$V_{\max} \pm \text{s.e.m. } (\text{pmol min}^{-1})$	$k_{\text{cat}}/K_m \text{ (s}^{-1} \text{ mM}^{-1}\text{)}$	Ratio of k_{cat}/K_m hiCE (CES2)/hCE1 (CES1)
Heroin	hiCE (CES2)	1440 ± 630	8130 ± 910	5.9	0.65
	hCE1 (CES1)	445 ± 73	$10\,660 \pm 450$	9.7	
Cocaine	hiCE (CES2)	202 ± 45	589 ± 33	0.44	–
	hCE1 (CES1)	ND ^a	ND ^a	–	
CPT-11	hiCE (CES2)	3.9 ± 0.3	1.6 ± 0.12	0.41	93
	hCE1 (CES1)	82.8 ± 9.6	0.36 ± 0.02	0.0044	

The hydrolysis of the respective substrates was determined using HPLC separation and detection of the products, from which the kinetic parameters were calculated.

^aND, not determined. No hydrolysis was apparent for this substrate.

s.e.m., standard error of the mean; CE, carboxylesterase.

This is consistent with the notion that the longer alkyl chain esters localize within the hydrophobic active site gorges present within the protein.

Metabolism of cocaine by CEs

Having demonstrated that hiCE (CES2) could hydrolyse large, bulky esters, we evaluated the ability of this enzyme to metabolize cocaine and heroin. As before, we also determined the ability of recombinant hCE1 (CES1) to hydrolyse these compounds. As indicated in Table 3 and displayed in Figure 3, hiCE (CES2) readily hydrolysed cocaine to yield benzoic acid (and ecgonine methyl ester) with K_m and V_{\max} values of 202 μM and 589 $\text{pmol}\cdot\text{min}^{-1}$, respectively. In contrast, no hydrolysis of the drug to either benzoylecgonine, or benzoic acid, was observed with hCE1 (CES1) (Figure 3A). Indeed, no enzyme-mediated metabolism of cocaine was seen, even after prolonged incubation (~20 h) with hCE1 (CES1) (data not shown). Under these same conditions using hiCE (CES2), all of the cocaine was hydrolysed to benzoic acid (data not shown). We conclude therefore, that hiCE (CES2) is the primary CE responsible for cocaine metabolism *in vivo*.

Analysis of the curve fits for benzoic acid formation versus substrate concentration (Figure 3B), indicated that no inhibition of hiCE (CES2) activity was observed with high concentrations of cocaine (up to 5 mM). This is in contrast to that seen for BChE where cocaine concentrations greater than 300 μM resulted in reduced product formation (Stewart *et al.*, 1977). This suggests that in situations where individuals overdose with very high levels of drug, hiCE (CES2) may play a significant role in detoxification of cocaine.

The metabolism of the anticancer agent CPT-11 was included in these studies since previous reports have indicated that this substrate is hydrolysed much more effectively by hiCE (CES2) than by hCE1 (CES1) (Humerickhouse *et al.*, 2000). Our studies are in agreement with these results (Table 3), confirming the specificity and hydrolytic activity of the recombinant purified hiCE (CES2) protein.

Metabolism of heroin by CEs

In contrast to the results obtained when using cocaine as a substrate, both human CEs did hydrolyse heroin. The efficiencies of hydrolysis of this drug to 6-acetylmorphine were similar, as exemplified by the k_{cat}/K_m values (Table 3). It should

be noted however, that the k_{cat}/K_m values for heroin catalysis [5.9 and 9.7 $\text{s}^{-1} \text{ mM}^{-1}$ for hiCE (CES2) and hCE1 (CES1), respectively] were at least 10-fold higher than that observed for cocaine with hiCE (CES2) (0.44 $\text{s}^{-1} \text{ mM}^{-1}$). Indeed, based upon these kinetic analyses, the catalytic efficiency of heroin hydrolysis by CEs was ~12- to 40-fold more efficient than cocaine metabolism by the latter enzyme. Again, no inhibition of hiCE (CES2) enzyme activity was observed in the presence of high concentrations of heroin (Figure 3C).

Inhibition of hiCE (CES2) and hCE1 (CES1) with CE-specific inhibitors

As the inhibition of CE-mediated hydrolysis of drugs may be used to modulate the levels of toxic metabolites (such as morphine from heroin), we determined the ability of a series of recently identified CE inhibitors to alter heroin, cocaine or CPT-11 hydrolysis. The benzil based compounds (1-3) are generic CE inhibitors, whereas the bis-benzene sulfonamides, 4 and 5, demonstrate specificity for hiCE (CES2). As indicated in Table 4, inhibitors 1-3 displayed very low K_i values towards hiCE (CES2) and hCE1 (CES1) for all substrates. With compounds 4 and 5, which are hiCE (CES2)-specific inhibitors (Wadkins *et al.*, 2004), no inhibition of metabolism of o-NPA or heroin was observed with hCE1 (CES1). However, potent inhibition of hiCE (CES2)-mediated metabolism was seen for all substrates, with K_i values as low as 35 nM with heroin. These studies confirm the applicability of using inhibitors to modulate these reactions and indicate that the selectivity of the sulfonamides towards hiCE (CES2) may have utility in modulating either CPT-11 or heroin hydrolysis *in vivo*.

Metabolism of heroin and cocaine by human intestinal and liver extracts

To confirm the results observed with the recombinant protein, we evaluated the ability of human intestinal and liver microsomal extracts to hydrolyse the drugs. We chose these tissues because they are rich in hiCE (CES2) and hCE1 (CES1), respectively, and demonstrate significantly different ratios of the two human CEs. This is exemplified by the Western analysis of total microsomal extracts shown in Figure 4A and the hydrolytic activities of these samples (Figure 4B). As can be seen, the intestinal microsomes contained significantly more hiCE (CES2) than hCE1 (CES1); the converse being true for the liver sample.

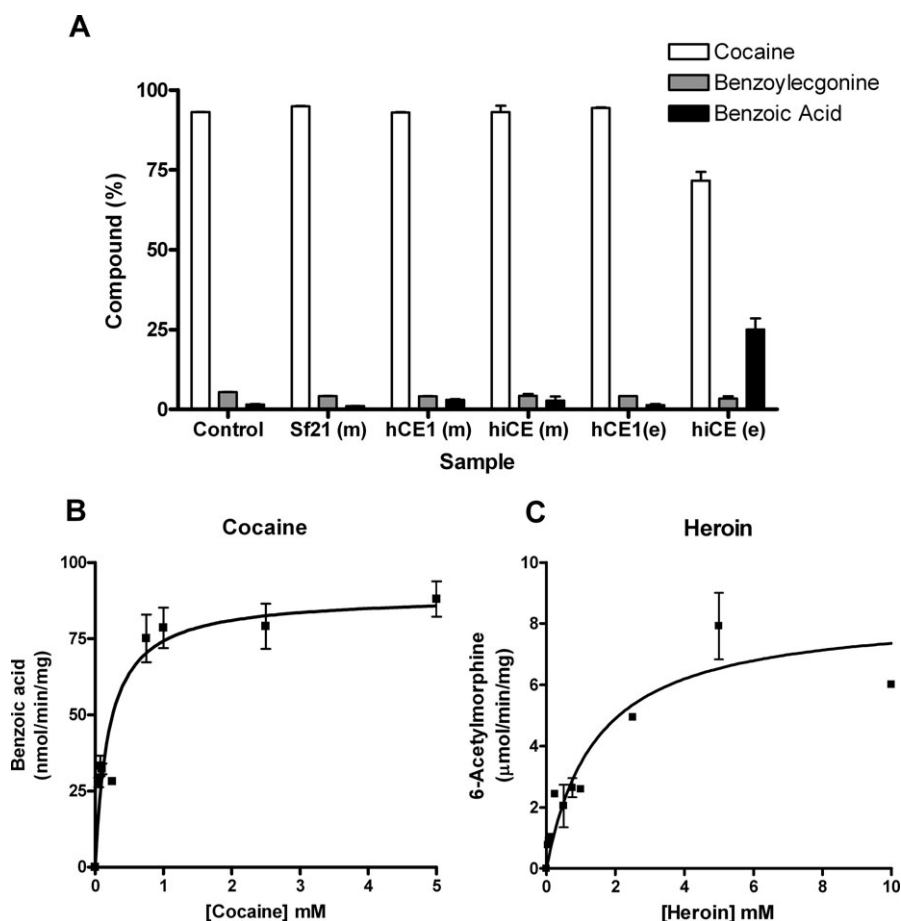


Figure 3 (A) Metabolism of cocaine by different carboxylesterases (CEs). Cocaine (1 mM) was incubated with the different samples for 1 h. The yields of benzoylecgonine and benzoic acid were determined by HPLC. Essentially, only hiCE (CES2) was able to hydrolyse cocaine. Control – 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7.4 only; Sf21 (m) – media harvested from uninfected Sf21 cells; hCE1 (m) – media harvested from Sf21 cells infected with baculovirus expressing hCE1 (CES1) (~250 units of CE); hiCE (m) – media harvested from Sf21 cells infected with baculovirus expressing hiCE (CES2) (~75 units of CE); hCE1 (e) – reaction containing pure hCE1 (CES1) (20 μg = ~2000 units of CE); hiCE (e) – reaction containing pure hiCE (CES2) (20 μg = ~500 units of CE). (B) The concentration versus velocity curve for hiCE (CES2) with cocaine. No evidence of enzyme inhibition by high concentrations of substrate was observed in these assays. (C) The concentration versus velocity curve for hiCE (CES2) with heroin. Similar to that seen for cocaine, no substrate-mediated enzyme inhibition was observed.

Chromatography of extracts was performed on Sephacryl S-200 HR and the CE activity of individual fractions was determined. In addition, the presence of hiCE (CES2) or hCE1 (CES1) in each fraction was assessed by Western analysis and hydrolysis of cocaine and heroin was monitored by HPLC. Figure 4C, D demonstrate the activity profile of the fractions obtained from this chromatographic technique.

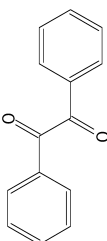
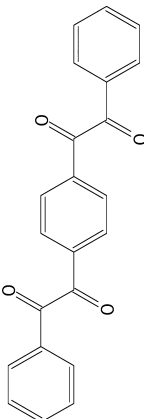
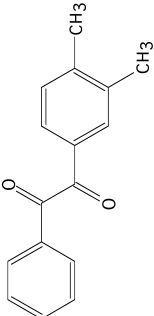
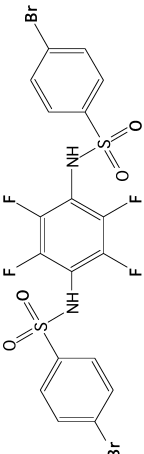
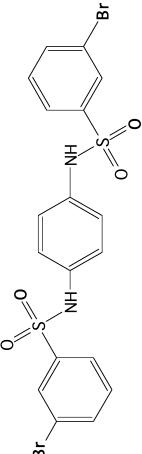
As can be seen with the intestinal microsomal sample, two peaks of esterase activity were observed (fractions 8–11 and 14–19) with the latter correlating with the presence of hiCE (CES2) (see the Western analyses under the graph – Figure 4D). Interestingly, the earlier peak did not correspond to hCE1 (CES1) or BChE (data not shown), suggesting that this represents another unknown esterase in this sample. Assessment of cocaine and heroin metabolism indicated that although the latter was effectively converted to 6-acetylmorphine by both fractions, only hiCE (CES2) was capable of hydrolyzing the former drug (Figure 4C, bottom graph). Although the identity of this enzyme that eluted

between fractions 8–11 is not known, the data presented in Figure 4 indicate that this protein did not contribute to cocaine hydrolysis.

As a comparison, we performed similar studies using a human liver microsomal extracts that demonstrated high expression of hCE1 (CES1) and low levels of hiCE (CES2) (see Figure 4A). Little or no cocaine hydrolysis was observed with either the initial extract (Table in Figure 4B) or from any of the fractions following chromatography (Figure 4C, bottom graph, red line), even though large amounts of hCE1 (CES1) were present in these samples (Figure 4D). Although hiCE (CES2) was present in the liver sample, the actual level of this enzyme was very low (see Figure 4A) and the cocaine hydrolysis products were probably below the limit of detection for these assays.

Overall, the results from these studies are consistent with the *in vitro* experiments using recombinant protein, that demonstrate that hiCE (CES2) is responsible for conversion of cocaine to benzoic acid and ecgonine methyl ester.

Table 4 K_i values for the inhibition of hiCE (CES2) and hCE1 (CES1) by a variety of CE inhibitors

Inhibitor	Structure	hiCE (CES2) $K_i \pm \text{s.e.m. (nM)}$			hCE1 (CES1) $K_i \pm \text{s.e.m. (nM)}$				
		o-NPA	Cocaine	Heroin	CPT-11	o-NPA	Cocaine	Heroin	CPT-11
1		14.7 ± 1.9 ^a	85.5 ± 19.0	92.4 ± 12.5	40.0 ± 10.0	45.1 ± 3.2 ^a	NA ^b	45.7 ± 8.8	NA ^b
2		5.6 ± 0.4 ^a	88.9 ± 22.0	42.2 ± 7.7	13.9 ± 2.3	8.0 ± 0.9 ^a	NA	29.2 ± 9.5	NA
3		4.1 ± 0.4 ^a	53.3 ± 11.5	20.1 ± 5.1	13.9 ± 2.3	99.1 ± 10.0 ^a	NA	77.7 ± 16.9	NA
4		23.7 ± 2.7 ^c	69.5 ± 21.9	111 ± 17	35.8 ± 8.3	>100 000	NA	>100 000	NA
5		67.0 ± 9.6 ^c	38.7 ± 10.6	404 ± 78	144 ± 27	>100 000	NA	>100 000	NA

The inhibition of the hydrolysis of o-NPA, cocaine, heroin or CPT-11 by hiCE (CES2) or hCE1 (CES1), with various CE inhibitors, was determined as described in the text.

^aData has been reported previously (Hyatt *et al.*, 2005) and is included for comparative purposes.

^bNA, not applicable. The K_i values were not determined for these substrates due to their very poor hydrolysis by hCE1 (CES1).

^cData has been recently reported (Hicks *et al.*, 2009) and is included for comparative purposes.
s.e.m., standard error of the mean; CE, carboxylesterase.

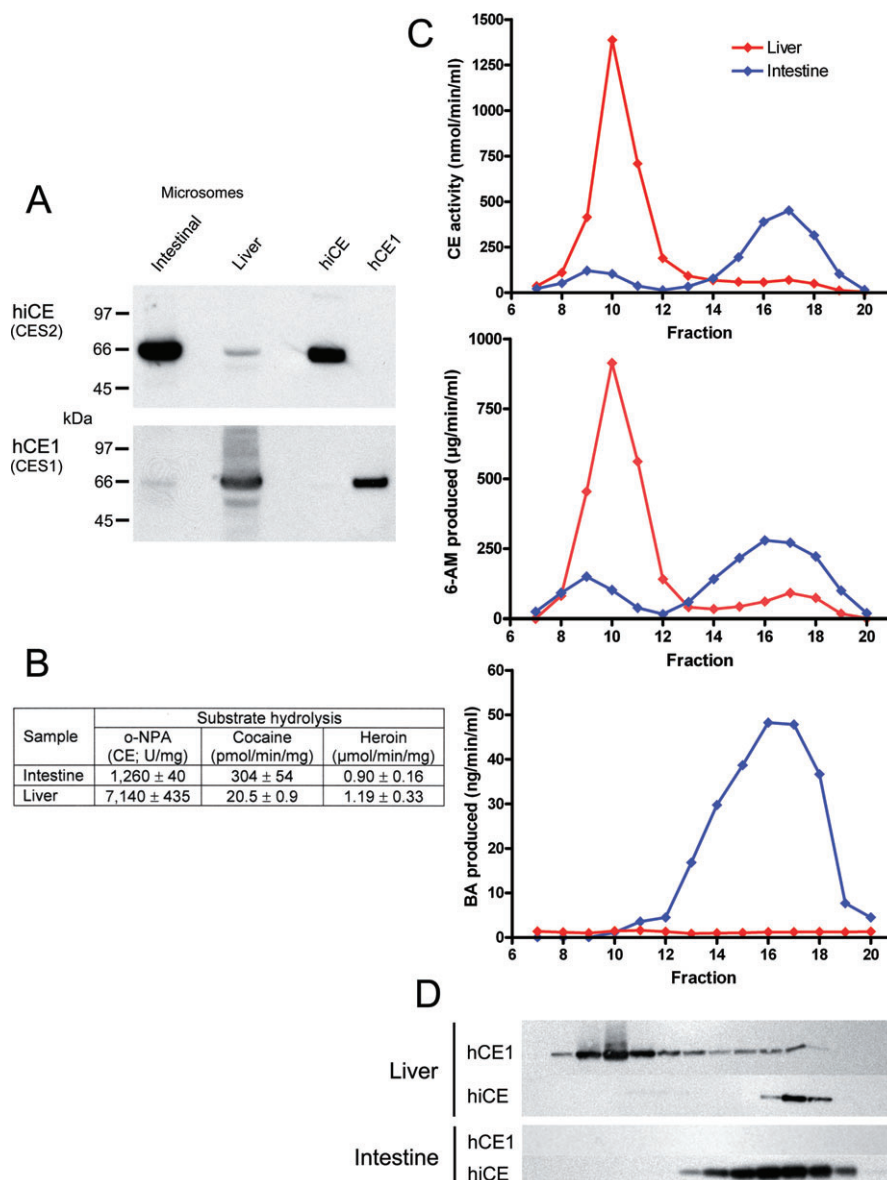


Figure 4 Chromatographic separation of carboxylesterases (CEs) in human intestinal and liver microsomal extracts using Sephacryl S-200 HR resin. (A) Western analysis demonstrating the amounts of hiCE (CES2) and hCE1 (CES1) present in the samples prior to chromatography. Standards present on the right hand side of the image represent 50 ng of each pure protein. (B) Table demonstrating the levels of CE activity, as well as the cocaine and heroin hydrolysis of the intestine and liver microsomal extracts prior to chromatography. (C) Elution profile for intestinal (blue line) and liver (red line) microsomal extracts demonstrating levels of CE activity (top graph), 6-acetylmorphine (6-AM) formation (heroin hydrolysis; middle graph) and benzoic acid (BA) production (cocaine hydrolysis; bottom graph). (D) Western analysis of these same fractions analysed in panel C, using antibodies specific for hiCE (CES2) or hCE1 (CES1). The images are aligned such that the signals correspond to the fraction number indicated on the abscissa axis.

Molecular analysis of cocaine and heroin binding to CEs

Having demonstrated that hiCE (CES2) was markedly more efficient at cocaine hydrolysis than hCE1 (CES1), we sought to determine whether molecular modelling studies could provide insight into the alignment of the substrates within the enzyme active sites. Therefore, we docked the drug (and a variety of other compounds) into the active sites of both enzymes. As the crystal structure for hiCE (CES2) is currently not available, we generated a homology model using the coordinates for hCE1 (CES1) when bound to homatropine.

Interestingly, when the hCE1 (CES1) and derived hiCE (CES2) structures were overlaid, the catalytic residues (Ser, His

and Glu) were essentially in the same position and orientation in both proteins (Figure 5B). However, as can be seen in Figure 5A, one loop of amino acids (residues 303–320 in hCE1) was displaced as compared with hiCE (CES2). It is unclear whether movement of this domain affects either the biological activity and/or docking studies, but due to its proximity to the active site, it is likely to play a role in substrate hydrolysis.

Following docking of different molecules the distances between the catalytic serine residue and the carbonyl carbon atoms were measured. As can be seen in Table 5 and Figure 5, the carbonyl carbon atom of the benzyl ester in cocaine localized 2.8 Å from the serine O_γ atom for hiCE (CES2)

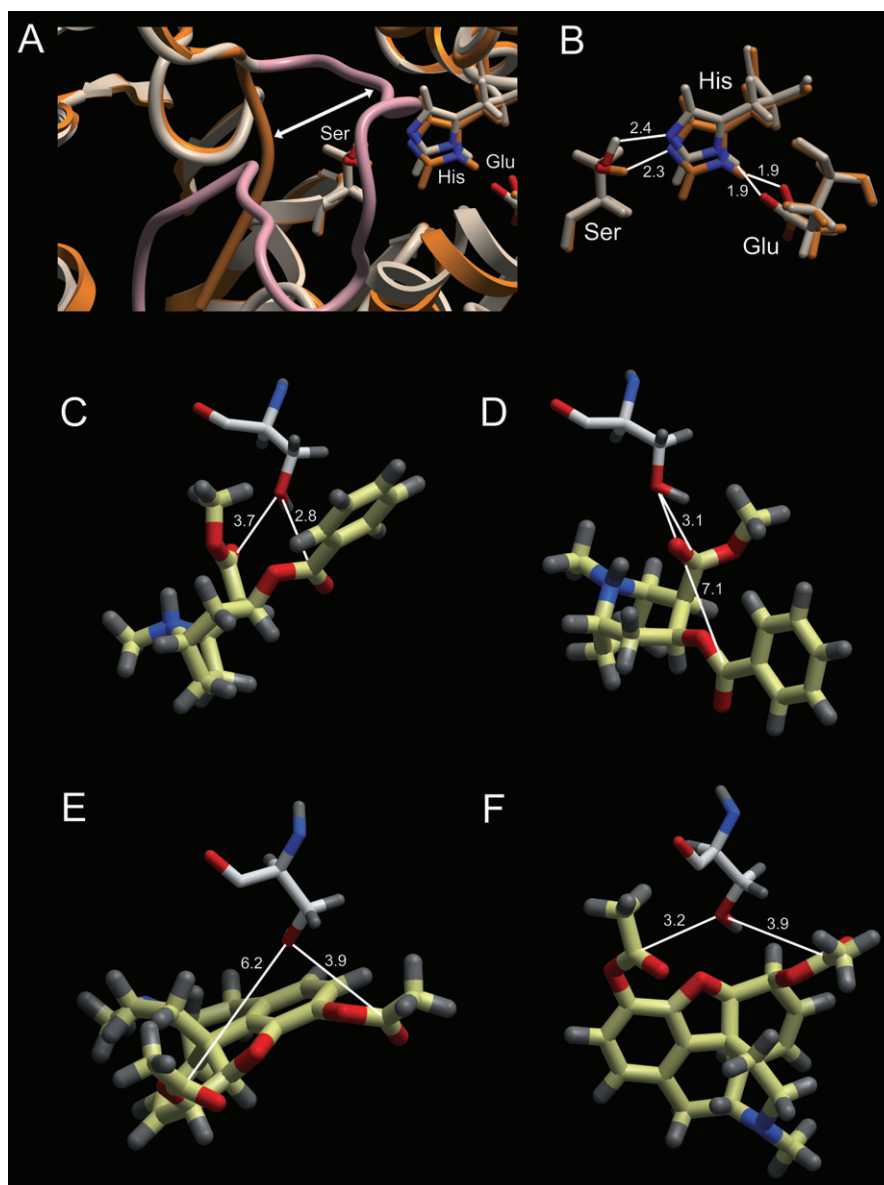


Figure 5 Computer-assisted docking of cocaine or heroin into the active sites of hCE1 (CES1) or hiCE (CES2). (A) An overlay of the ribbon representations of the hCE1 (CES1) (taupe) and hiCE (CES2) (orange/brown) structures used for the docking studies. As can be seen one loop of hCE1 (CES1) (highlighted in pink) is displaced (marked by the white arrow) and projects towards the active site catalytic amino acids (Ser, His, Glu). (B) An overlay of the catalytic amino acids in hCE1 (CES1) (taupe) and hiCE (CES2) (orange/brown) with the distances between the relevant atoms indicated. (C, D) – Docking of cocaine into the active sites of hiCE (CES2) and hCE1 (CES1), respectively. The active site serine is depicted and the distance from the O γ atom to the carbonyl carbon atoms in the drug are displayed. (E, F) – Docking of heroin into the active sites of hiCE (CES2) and hCE1 (CES1), respectively. The active site serine is depicted and the distance from the O γ atom to the carbonyl carbon atoms in the drug are displayed. In all panels, the nitrogen and oxygen atoms displayed in blue and red, respectively, and distances are marked in Å.

(Figure 5C), but was localized greater than 7 Å away in hCE1 (CES1) (Figure 5D). As the latter distance is too large for nucleophilic attack, this suggests that hCE1 (CES1) would be unable to hydrolyse this ester function. This was true for both the protonated and un-protonated forms of the drug.

However, following docking of heroin, the carbonyl carbon atoms for the methyl ester in the 3-position localized within 3.9 Å for both CEs (Figure 5E and F).

Indeed, for hCE1 (CES1) the distance to the ester was smaller than that for hiCE (CES2). This is in agreement with our experimental results that demonstrate that the k_{cat}/K_m

value for heroin with hCE1 (CES1) is ~threefold greater than that seen for hiCE (CES2) (Table 3).

As controls for the docking studies, we also assessed the binding of a series of related substrates and inhibitors. As noted in Table 5, the carbonyl carbon atom was present within 3.7 Å of the serine O γ atom for o-NPA, p-nitrophenyl valerate, CPT-11 and benzil, for both hCE1 (CES1) and hiCE (CES2). Finally as a negative control, we docked camphorquinone into the enzyme active sites. This molecule contains the ethane-1,2-dione chemotype (similar to benzil), but is not an inhibitor of either of the human CEs (Hyatt *et al.*,

Table 5 Distance between the catalytic serine O γ atom and the carbonyl carbon atoms for a series carboxylesterase (CE) substrates and inhibitors

Compound	Distance from Ser O γ H to C = O (Å)	
	hCE1 (CES1)	hiCE (CES2)
Cocaine ^a	4.9 (Bz) 3.9 (Me)	2.6 (Bz) 5.1 (Me)
Cocaine ^a (protonated)	7.1 (Bz) 3.1 (Me)	2.8 (Bz) 3.7 (Me)
Heroin ^b	3.2 (3-) 3.9 (6-)	3.9 (3-) 6.2 (6-)
Heroin ^b (protonated)	2.8 (3-) 5.7 (6-)	3.6 (3-) 5.2 (6-)
o-NPA	3.3	3.7
o-Nitrophenyl valerate	3.7	2.8
CPT-11	NA ^c	3.1
Benzil ^d	3.9, 4.8	3.0, 3.3
Camphorquinone ^e	5.8 (2-) 5.0 (3-)	6.7 (2-) 6.8 (3-)

The distance between the catalytic serine O γ atom and the carbonyl carbon atoms for the listed compounds were determined following docking into the active sites of either hCE1 (CES1) or hiCE (CES2). Values were calculated using ICM Pro software.

^aDistances are indicated to the carbonyl carbon atom of either the benzyl ester (Bz) or the methyl ester (Me).

^bDistances are indicated to the carbonyl carbon atom of the methyl ester at either the 3- or 6- position of the molecule.

^cDocking of CPT-11 into the active site of hCE1 (CES1) was not undertaken as steric constraints present at the entrance to the active site gorge significantly impede access of the drug to the catalytic amino acids (Wadkins *et al.*, 2001; Wierdl *et al.*, 2008).

^dTwo distance values are given for benzil as it contains two carbonyl groups, however, because this molecule is symmetrical, no assignment of these atoms is included.

^eDistances are indicated to the carbonyl carbon atom present at the 2- or 3-position of the molecule.

2007). As indicated, the measured distances [\sim 6.8 Å for hiCE (CES2) and 5.0–5.8 Å for hCE1 (CES1)] would be too large for nucleophilic attack by the O γ atom towards this molecule. In summary, the results of these docking studies are in complete agreement with the obtained experimental data.

Discussion and conclusions

We have purified recombinant human intestinal CE, hiCE (CES2), to homogeneity and determined the kinetic parameters for this enzyme with a variety of clinically relevant substrates. This has demonstrated that hiCE (CES2) efficiently metabolized CPT-11, cocaine and heroin. In contrast, the human liver CE, hCE1 (CES1), only hydrolysed heroin. Until recently, the enzymes involved in the metabolism of all of these agents have not been unambiguously identified. For example, although hiCE (CES2) has been proposed as the principal CE responsible for CPT-11 activation *in vivo* (Humrickhouse *et al.*, 2000; Khanna *et al.*, 2000), other esterases, for example, BChE can also activate this drug (Dodds and Rivory, 1999; Morton *et al.*, 1999). Similarly, previous reports have indicated that cocaine can be metabolized by both hCE1 (CES1) and hiCE (CES2) (Kamendulis *et al.*, 1996; Brzezinski *et al.*, 1997; Pindel *et al.*, 1997). However, these studies were

performed using enzymes purified from extracts derived from human liver. As hCE1 (CES1) and hiCE (CES2) (as well as BChE and other esterases) are expressed in this organ, potentially, contamination of 'pure' CEs with other liver esterases could confuse the results obtained from these biochemical experiments. We are unable to assess the level of purity of the enzymes used in these previous studies and hence we cannot comment on which protein would be definitively responsible for cocaine hydrolysis; however, it should be noted that no specific assays or techniques were used by these authors to assess the levels of other esterases present in their enzyme preparations.

We relied on the use of recombinant hCE1 (CES1) or hiCE (CES2), purified from the culture media of the insect cell line, *S. frugiperda*. We have used this approach, rather than purifying the proteins from cell extracts, to avoid any possible cross contamination with intracellular insect CEs, and to minimize proteolytic degradation of the mammalian enzymes. As the only protein contaminants we have observed during the hiCE (CES2) purification have been bovine serum albumin and *Autographa californica* chitinase, and neither of these proteins has esterase activity, it is likely that the enzymes used in our studies were homogenous. We did not observe hydrolysis of cocaine by the recombinant hCE1 (CES1) that we used in these studies, and as this protein is catalytically active towards a variety of substrates, we cannot confirm the results generated in previous reports that indicate that the drug is metabolized by this enzyme (Kamendulis *et al.*, 1996; Brzezinski *et al.*, 1997).

Potentially, there may be different isoforms of hCE1 (CES1) that result from nucleotide polymorphisms (Zhu *et al.*, 2008) that could account for the discrepancy between our results and those of Brzezinski *et al.* (1997). However, as we have sequenced the cDNA that encodes the hCE1 (CES1) protein used in these studies (Danks *et al.*, 1999), determined that this enzyme is pure by both physical and biochemical methods (Bencharit *et al.*, 2003b; Morton and Potter, 2000; Wadkins *et al.*, 2001), and obtained the crystal structure of this protein in complex with a variety of different small molecules (Bencharit *et al.*, 2003b; Fleming *et al.*, 2005; Bencharit *et al.*, 2006), our results clearly indicate that the protein encoded by this cDNA cannot hydrolyse cocaine. Additionally, our studies implicate hiCE (CES2) as the principal CE involved in drug hydrolysis. As hiCE (CES2) is also expressed at relatively high levels in liver, skeletal muscle, heart, colon and kidney (Wu *et al.*, 2003), it is likely that this enzyme will be important in the hydrolysis of this molecule, regardless of the route of cocaine administration.

To validate the *in vitro* studies, we evaluated the esterase levels and drug hydrolyzing properties of intestinal and liver microsomal extracts after chromatographic separation. Consistent with our previous results, we determined that hiCE (CES2) was responsible for cocaine metabolism and that hCE1 (CES1) was inactive towards this substrate (Figure 4). This was achieved using samples that were either, rich in hiCE (CES2) and low in hCE1 (CES1) (intestine), or where the converse was true (liver). Confirmation of the identity of the enzymes involved was documented using antibodies specific for these proteins. Hence, our results using these human microsomal

samples corroborate our previous studies using the recombinant proteins.

In an attempt to understand the differences in cocaine hydrolysis by these two CEs, we undertook molecular docking studies using the hCE1 (CES1) crystal structure and a homology model for hiCE (CES2) based upon these same coordinates. Initially, we were concerned that as the former enzyme cannot hydrolyse cocaine, the use of the hCE1 (CES1) coordinates might result in a conformation in hiCE (CES2) that would preclude informative results from these analyses. However, upon docking of this drug (and others small molecules displayed in Table 5), it was apparent that the homology model demonstrated sufficient variation in the active site gorge, such that different conformations of the CE/drug complexes were observed. Gratifyingly, results from these docking studies placed the active site serine O γ atom within 3.9 Å of the carbonyl carbon atoms for all substrates and inhibitors with all enzymes, the exceptions being hCE1 (CES1) with cocaine and camphorquinone with both enzymes. The latter 1,2-dione is not an inhibitor of these CEs (Hyatt *et al.*, 2007), even though it demonstrates properties suitable for enzyme inhibition. The lack of CE inhibition and the failure of camphorquinone to dock adjacent to the catalytic residues is likely to be due to steric hindrance enforced by the methyl bridge present in this bicyclic structure (Hyatt *et al.*, 2007).

In previous modelling studies, based upon the crystal structure of homatropine bound in the hCE1 (CES1) active site (Bencharit *et al.*, 2003b), the tropine ring of cocaine was overlaid onto the coordinates for the corresponding moiety of the former molecule. This demonstrated that (–)-cocaine would be preferentially hydrolysed by hCE1 (CES1). However, in these experiments, no computational docking studies were performed and hence the electrostatics of drug binding were not taken into account. Here, we demonstrate that when these forces are considered in the context of cocaine docking into the active sites of the CEs, significant differences in the interaction of the drug with the respective catalytic residues were observed. This resulted in models that confirmed our experimental data, that is, that the carbonyl group present within the benzyl ester chemotype of cocaine was juxtaposed to the catalytic serine O γ atom in hiCE (CES2) (2.8 Å), but was up to 4 Å further away in hCE1 (CES1). This would make drug hydrolysis by the latter enzyme unlikely, again consistent with the biochemical results presented earlier.

Recent analysis of the pharmacokinetics of oral heroin administration demonstrated that the free drug and 6-acetylmorphine could not be detected in the arterial blood following dosing (Girardin *et al.*, 2003). However, appreciable levels of morphine were detected, suggesting that an esterase present within the alimentary canal mediated the hydrolysis of heroin to this metabolite. Because high levels of hiCE (CES2) are present within the epithelial lining of the human gut, this suggests that this enzyme may be responsible for metabolism of heroin when this drug is taken orally. Our results, presented here, confirm that this enzyme is proficient in hydrolysing this drug, suggesting that in previous studies (Girardin *et al.*, 2003), hiCE (CES2) present in the gut metabolized heroin immediately after absorption. This would account for the lack of the parent molecule present within the circulation.

As hiCE (CES2) has been identified as being proficient in the hydrolysis of heroin and the activation of CPT-11, potentially, selective CE inhibitors that prevent these hydrolytic reactions may prove useful in ameliorating the toxicity observed with these drugs. Therefore, we assessed the ability of a panel of benzil-based compounds and bis benzene-sulfonamides to modulate drug hydrolysis. As we have previously reported that these types of inhibitors act in a partially competitive fashion (Wadkins *et al.*, 2004; Wadkins *et al.*, 2005), the potency of enzyme inhibition will be dependent upon the substrate used for the analyses. Our results indicate however, that the phenylethane-1,2-dione based compounds that were chosen for analysis were potent inhibitors of all mammalian CEs, with all of the substrates tested (o-NPA, cocaine, heroin and CPT-11), whereas the sulfonamides only demonstrated selectivity for hiCE (CES2). The K_i values for enzyme inhibition were typically in the nM range, although the benzil analogues demonstrated greatest potency towards hiCE (CES2) when using CPT-11 as a substrate. We believe that selective administration of CE inhibitors after CPT-11 dosing, may alleviate the delayed diarrhea that results from intestinal drug activation. Because this toxicity is delayed up to 96 h following chemotherapy, this may provide a suitable window in which to inhibit hiCE (CES2). We are currently evaluating this hypothesis in animal models.

The studies described here demonstrate that hiCE (CES2) is efficient in hydrolysing cocaine and that inhibition of this enzyme by benzil-based compounds can be achieved in the presence of a variety of different clinically relevant substrates. These results should allow the development of approaches that may have utility in modulating the toxicity for such agents. For example, i.v. administration of hiCE (CES2) to rapidly convert cocaine to its inactive metabolites may be possible. Alternatively, selective hiCE (CES2) inhibitors may reduce drug-induced toxicity associated with CPT-11 hydrolysis *in vivo*.

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Conflicts of interest

None.

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