HUMAN LIVER CARBOXYLESTERASE hCE-1: BINDING SPECIFICITY FOR COCAINE, HEROIN, AND THEIR METABOLITES AND ANALOGS

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ABSTRACT:

Purified human liver carboxylesterase (hCE-1) catalyzes the hydrolysis of cocaine to form benzoylecgonine, the deacetylation of heroin to form 6-acetylmorphine, and the ethanol-dependent transesterification of cocaine to form cocaethylene. In this study, the binding affinities of cocaine, cocaine metabolites and analogs, heroin, morphine, and 6-acetylmorphine for hCE-1 were evaluated by measuring their kinetic inhibition constants with 4-methylumbelliferyl acetate in a rapid spectrophotometric assay. The naturally occurring (R)-(-)-cocaine isomer displayed the highest affinity of all cocaine and heroin analogs or metabolites. The pseudo- or allopseudococaine isomers of cocaine exhibited lower affinity indicating that binding to the enzyme is stereoselective. The methyl ester, benzoyl, and N-methyl groups of cocaine play important roles in binding because removal of these groups increased K_i values substantially. Compounds containing a variety of hydrophobic substitutions at the benzoyl group of cocaine bound to the enzyme with high affinity. The high K_i value obtained for cocaethylene relative to cocaine is consistent with weaker binding to the esterase and a longer elimination half-life reported for the metabolite. The spectrophotometric competitive inhibition assay used here represents an effective method to identify drug or environmental esters metabolized by carboxylesterases like hCE-1.

Carboxylesterases are hydrolytic enzymes involved in the metabolism of drug and environmental esters in a variety of mammalian tissues. They catalyze the conversion of mostly lipophilic ester substrates to more water-soluble carboxylic acids plus alcohol products, thereby facilitating their elimination (1). The broad substrate specificity exhibited by these enzymes enables the cell to metabolize a wide variety of esters. However, the lack of specificity generally results in relatively high K_m values and low catalytic efficiencies (k_cat/K_m) for hydrolysis of drug or environmental esters. The low catalytic efficiency is usually compensated by the presence of relatively large amounts of these enzymes in tissues, especially in the liver.

A carboxylesterase with broad substrate specificity was recently purified from human liver and partially characterized (2). The enzyme is named hCE-1 for human carboxylesterase-1 according to the suggestion of Kroetz et al. (3), although it also has been referred to as esterase 1 (4). The hCE-1 is involved in the metabolism of cocaine by hydrolyzing cocaine to benzoylecgonine (fig. 1) (4), a major urinary metabolite of cocaine (5). In the presence of ethanol, hCE-1 also catalyzes the ethyl transesterification of cocaine forming the pharmacologically active metabolite, cocaethylene (4). The same enzymatic step may be responsible for the conversion of norcocaine to norcocaethylene (fig. 1) and both compounds may be hydrolyzed by this enzyme to benzoylecgonine. A second carboxylesterase named hCE-2 in human liver catalyzes the hydrolysis of the benzoyl group of cocaine (4, 6) and the 3- or 6-acetyl groups of heroin (7).

The hCE-1 enzyme exhibits broad substrate-specificity for ester hydrolysis. 4-methylumbelliferyl acetate (4) and p-nitrophenyl valerate (8) are used in simple spectrophotometric assays. Both hCE-1 and a highly homologous enzyme from rat called hydrolase A hydrolyze p-nitrophenyl butyrate (3, 9). They also exhibit fatty acid ethyl ester synthase activity by catalyzing the formation of ethyleate from oleic acid and ethanol (2, 9). Additionally, the rat (10) and human enzymes (2) catalyze the ethanol-dependent transesterification of cocaine to cocaethylene.

The substrate specificity of hCE-1 with cocaine metabolites, toxins, and other drug esters has not been studied. Recently Kamendulis et al. (7) demonstrated that the enzyme plays a role in the metabolism of heroin by catalyzing its hydrolysis to 6-acetylmorphine. The binding affinities of heroin, cocaine, and structurally related compounds to hCE-1 were evaluated in this study using the steady-state kinetic analysis of alternative substrates or competitive inhibitors in a spectrophotometric assay employing 4-methylumbelliferyl acetate as substrate. The assay provides an efficient method for identifying compounds that bind with high affinity to the enzyme and for predicting ester drugs hydrolyzed by this carboxylesterase.

Materials and Methods

Materials. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), except ultra pure DTT which was obtained from the United States Biochemical Corp. (Cleveland, OH). The Q Sepharose and Con A Sepharose chromatography resins used for enzyme purification were purchased from Pharmacia (Uppsala, Sweden) and DEAE-cellulose was from Whatman Bio-systems (Maidstone, UK). Solvents were prepared with H_2O treated with a...
Three types of reactions occur in the metabolism of cocaine: 1) hydrolysis, 2) transesterification, and 3) N-demethylation. The competitive inhibition constants ($K_i$) calculated from steady-state kinetic analyses (eqs. 1 and 2) are listed under each of the six compounds.

Milli-Q filtration system (Millipore, Bedford, MA). Enzyme samples were obtained with Amicon ultrafiltration stirred cells (Beverly, MA) using YM30 membranes.

**Drugs and Related Compounds.** Atropine sulfate, tropocaine HCl, and $R$-(-)-coca HCl were obtained from Sigma Chemical. Heroin HCl monohydrate and benzoylecgonine propyl ester HCl were purchased from Alttech-Applied Science Laboratories (State College, PA). Benzoylecgonine and cocaine were prepared in our laboratory by a previously published procedure (11). Norcocaine and norcocaethane were also prepared in our laboratory by the $N$-demethylation of cocaine and cocacethylene, respectively (12). Purity of the synthesized compounds was determined by HPLC and GC/MS (13). Compounds #1 through #7 were synthesized as described below. The structures of these compounds are shown in table 1. All other compounds were obtained from the National Institute on Drug Abuse.

**Synthetic Procedures.** 3β-([Methylphenylphosphonodithionyloxy]-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester (#1). Lawesson’s reagent (42 mg, 104 μmol) and compound #4 (70 mg, 189 μmol) were dissolved in toluene (5 ml) at 4°C under an Ar gas atmosphere. DEA (90 μl, 503 μmol) was added via syringe followed by the addition of diphenylphosphinic chloride (96 μl, 503 μmol). The reaction mixture was stirred 2.5 hr warming slowly to room temperature. The solvent was removed in vacuo and the product was purified by flash chromatography (silica gel; MeOH:CH$_2$Cl$_2$ 10:90, $R_f$ = 0.4) to give a colorless oil (116 mg, 58% yield). $^1$H NMR (CDCl$_3$): δ 1.61–1.71 (m, 1H), 1.88–2.10 (m, 2H), 2.14 (s, 1H), 2.45–2.66 (m, 1H), 3.02 and 3.12 (dt, 1H, $J_{5,3} = 11.6$ Hz), 3.78 (s, 1H), 3.87 (s, 1H), 3.97–4.11 (m, 3H), 4.78–4.92 (m, 1H). 31P NMR (CDCl$_3$): δ 89.47, 94.59.

3β-([Diphenylphosphonyloxy]-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester (#3). (R)-Ecgoline methyl ester (100 mg, 503 μmol) and tetrazole (4 mg, 51 μmol) were dissolved in benzene (10 ml) at 4°C under an Ar gas atmosphere. DEA (90 μl, 503 μmol) was added via syringe followed by the addition of diphenylphosphinic chloride (96 μl, 503 μmol). The reaction mixture was stirred 2.5 hr warming slowly to room temperature. The solvent was removed in vacuo and the product was purified by flash chromatography (silica gel; MeOH:CH$_2$Cl$_2$:NH$_4$OH (30%) 10:90:0.15, $R_f$ = 0.4) to give a pale yellow oil (352 mg, 38% yield). $^1$H NMR (CDCl$_3$): δ 1.61–1.71 (m, 1H), 1.88–2.10 (m, 2H), 2.14 (s, 1H), 2.45–2.66 (m, 1H), 3.02 and 3.12 (dt, 1H, $J_{5,3} = 11.6$ Hz), 3.78 (s, 1H), 3.87 (s, 1H), 3.97–4.11 (m, 3H), 4.78–4.92 (m, 1H). 31P NMR (CDCl$_3$): δ 88.21, 88.95.

3β-([Methylphenylphosphonothionyloxy]-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester (#4). Potassium ethyl xanthate (560 mg, 345 μmol) and compound #2 (116 mg, 314 μmol) were dissolved in dry acetonitrile and refluxed 10 hr after which dimethyl sulfate (33 μl, 345 μmol) was added and the reaction mixture was stirred an additional 0.5 hr. The solvent was removed in vacuo and the product was purified by flash chromatography (silica gel; MeOH:CH$_2$Cl$_2$:NH$_4$OH (30%) 10:90:0.15, $R_f$ = 0.4) to give a pale yellow oil (70 mg, 61% yield). $^1$H NMR (CDCl$_3$): δ 1.61–1.71 (m, 1H), 1.88–2.10 (m, 2H), 2.14 (s, 1H), 2.45–2.66 (m, 1H), 3.02 and 3.12 (dt, 1H, $J_{5,3} = 11.6$ Hz), 3.78 (s, 1H), 3.87 (s, 1H), 3.97–4.11 (m, 3H), 4.78–4.92 (m, 1H). 31P NMR (CDCl$_3$): δ 89.47, 94.59.

3β-([Methyl phenylphosphonothionyloxy]-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester (#5). (R)-Ecgoline methyl ester (300 mg, 1.51 mmol) and tetrazole (11 mg, 0.15 mmol) were dissolved in benzene (10 ml) and chilled to 4°C under an Ar gas atmosphere. DEA (90 μl, 503 μmol) was added via syringe followed by the addition of phenylphosphonic dichloride (224 μl, 1.59 mmol), and the reaction mixture was stirred 15 hr, slowly warming to room temperature. Methanol (100 μl, 2.27 mmol) was added and the reaction stirred an additional 1 hr. The solvent was removed in vacuo and the product was purified by flash chromatography (silica gel; MeOH:CH$_2$Cl$_2$:NH$_4$OH (30%) 10:90:0.15, $R_f$ = 0.4) to give a pale yellow oil (70 mg, 61% yield). $^1$H NMR (CDCl$_3$): δ 1.61–1.71 (m, 1H), 1.88–2.10 (m, 2H), 2.14 (s, 1H), 2.45–2.66 (m, 1H), 3.02 and 3.12 (dt, 1H, $J_{5,3} = 11.6$ Hz), 3.78–4.11 (m, 3H), 4.78–4.92 (m, 1H). 31P NMR (CDCl$_3$): δ 88.21, 88.95.
the product was purified by flash chromatography (silica gel; MeOH:CH$_2$Cl$_2$ 10:90, $R_f = 0.25$) to give a colorless oil (231 mg, 43% yield). $^1$H NMR (CDCl$_3$): $\delta$ 1.45–1.70 (m, 2H), 1.78 –2.14 (m, 3H), 2.17 (s, 3H), 2.37 and 2.53 (dt, 1H, $J$ 5.2, 11.8 Hz), 2.75 and 3.06 (m, 1H), 3.16 and 3.26 (s, 1H), 3.41 and 3.50 (s, 1H), 3.60 and 3.75 (s, 3H), 3.66 and 3.70 (d, 3H, $J$ 11.3 Hz), 4.58 – 4.76 (m, 1H), 7.40 –7.50 (m, 2H), 7.50 –7.58 (m, 1H), 7.72–7.88 (m, 2H). $^{31}$P NMR (CDCl$_3$): $\delta$ 19.83, 20.38. MS (FAB) $m/z$ 354 (M$^+$H$^+$).

$\beta$-(Phenylphosphonic acid)-8-azabicyclo[3.2.1]octane-2$\beta$-carboxylic acid methyl ester (#6). To a solution of compound #5 (20 mg, 57 $\mu$mol) in acetonitrile (100 $\mu$l) under an Ar gas atmosphere was added trimethylsilyl
bromide (12.2 μl, 92 μmol). The reaction mixture was stirred 1.5 hr after which the reaction was quenched with water (50 μl). The solvent was removed in vacuo and the product purified as the ammonium salt by eluting the mixture from a C18 solid phase extraction column (0.5 g) with methanol containing 1% NH4OH (30%) to give a white solid (20 mg, 99% yield). 1H NMR (CDCl3): δ 1.91–2.09 (m, 3H), 2.16–2.40 (m, 3H), 2.75 (s, 3H), 3.08 (dd, 1H, J = 7.3 Hz), 3.70 (s, 3H), 3.80 (s, 1H), 4.02 (d, 1H, J = 4.7 Hz), 4.08–4.72 (m, 1H), 7.31–7.44 (m, 3H), 7.74 (dd, 2H, J = 7.7, 12.6 Hz). 31P NMR (CDCl3): δ 16.77.

3β-(Methanesulfonyloxy)-8-azabicyclo[3.2.1]octane-2β-carboxylic acid methyl ester (#7). Methanesulfonfonyl chloride (69 μl, 0.89 mmol) was added dropwise to a stirring mixture of (R)-ecgonine methyl ester hydrochloride (0.20 g, 0.85 mmol) and triethylamine (0.24 ml, 1.7 mmol) in CH2Cl2 (5 ml) at 0°C. After several hours at room temperature, the reaction mixture was washed with 10% Na2CO3, dried over Na2SO4, and evaporated in vacuo. The yellow residue was purified by silica gel chromatography (10% MeOH/CH2Cl2) to afford the mesylate as a light yellow solid (0.12 g, 51% yield). 1H NMR (CDCl3): 85.04 (m, 1H), 3.92 (s, 3H), 3.71 (m, 1H), 3.46 (m, 1H), 3.21 (m, 1H), 3.19 (s, 3H), 2.70 (m, 1H), 2.37 (s, 3H), 2.20–2.35 (m, 2H), 2.01–2.11 (m, 1H), 1.72–1.88 (m, 2H), MS (EI) m/z 277 (M+).

Carboxylesterase Assays. Acetylesterase activity was measured by incubating approximately 0.7 μg of purified enzyme with 0.2 to 0.5 mM 4-methylumbelliferyl acetate in 90 mM KH2PO4, 40 mM KCl, pH 7.3 (1-ml total volume) at 37°C. The 4-methylumbelliferyl acetate stock solutions (25 mM) were prepared in dimethyl sulfoxide. The formation of 4-methylumbelliferone was monitored for 1.5 min in a Perkin-Elmer Lambda 6 double-beam spectrophotometer at 350 nm. Rates of ester hydrolysis were calculated by linear regression of absorbance versus time using the extinction coefficient 12.2 cm−1 mM−1 for 4-methylumbelliferyl acetate (4). The time course of product formation was linear throughout the time of assay (correlation coefficient for linear regression 98%). Specific activity is expressed as μmol product formed per mg protein. Protein concentration was determined with the Bio-Rad protein assay using bovine serum albumin as a standard.

Purification of Human Liver Carboxylesterase (hCE-1). The DEAE-cellulose and Q Sepharose chromatography steps in the purification of enzyme from 60 g of human liver are identical to those described by Brzezinski et al. (2). However, the last three steps that included Superose 6, Polybuffer Exchanger, and Phenyl Superose columns were substituted by affinity chromatography on Con A Sepharose (7). Fractions of hCE-1 obtained from the Q Sepharose column were pooled and the buffer exchanged for 20 mM Tris-Cl, 0.5 M NaCl, 1 mM CaCl2, 1 mM MnSO4, 1 mM benzamidine, 1 mM EDTA, and 1 mM DTT at pH 7.4 (Buffer A). The sample was loaded onto a Con A Sepharose 4B column (2.5 x 6 cm) and bound protein was eluted with a 260 ml linear gradient of Buffer A to Buffer A + 0.5 M methyl-α-D-mannopyranoside, pH 7.4. Active fractions were pooled and the buffer exchanged for 50 mM Na2HPO4, 1 mM benzamidine, 1 mM EDTA, pH 7.0. The purified enzyme was concentrated to 1.3 mg/ml, sterile-filtered, and stored at 4°C to maintain stability. The purity of hCE-1 was examined by SDS-PAGE (14) and enzyme was concentrated to 1.3 mg/ml, sterile-filtered, and stored at 4°C to maintain stability. The purity of hCE-1 was examined by SDS-PAGE (14) and enzyme was concentrated to 1.3 mg/ml, sterile-filtered, and stored at 4°C to maintain stability. The purity of hCE-1 was examined by SDS-PAGE (14) and enzyme was concentrated to 1.3 mg/ml, sterile-filtered, and stored at 4°C to maintain stability. The purity of hCE-1 was examined by SDS-PAGE (14) and enzyme was concentrated to 1.3 mg/ml, sterile-filtered, and stored at 4°C to maintain stability. The purity of hCE-1 was examined by SDS-PAGE (14) and enzyme was concentrated to 1.3 mg/ml, sterile-filtered, and stored at 4°C to maintain stability. The purity of hCE-1 was examined by SDS-PAGE (14) and enzyme was concentrated to 1.3 mg/ml, sterile-filtered, and stored at 4°C to maintain stability.

\[ K_v = \frac{V_{max}}{K_m + [S]} \]

where \( V_{max} \) is the maximum velocity for 4-methylumbelliferyl acetate hydrolysis, \( K_m \) is the Michaelis constant for 4-methylumbelliferyl acetate, and \( K_v \) is the inhibition or dissociation constant for the dead-end inhibitor. The steady-state kinetic expression for alternative substrates is:

\[ 1/v = (K_d/V_{max})(1 + [B]/K_b)(1/[S]) + 1/V_{max} \]

where \([B]\) is the alternate substrate and \(K_b\) is its Michaelis constant. Note that eqs. 1 and 2 are identical in form.

The enzyme concentration was adjusted so that the activity was approximately 3.6 U/ml with no inhibitor added. The buffer-substrate-inhibitor mixture was equilibrated in the cuvette at 37°C for 1.5 min prior to the addition of inhibitor. Assay buffer was used to make the inhibitor stock solutions. (−)-Cocaine was dissolved in DMSO and compound #4 contained 5% DMSO. For cocaine, heroin, and the metabolites in table 2 and fig. 1, competitive inhibition constants (\(K_i\)) and alternative substrate Michaelis constants (\(K_a\)) were determined from data sets consisting of 56 points generated with four different substrates and concentrations. Duplicate activity readings were obtained for each set of conditions. At the lowest substrate and highest inhibitor concentrations, it was necessary to reduce the assay time to avoid nonlinearity owing to substrate depletion. The data collected for each compound were evaluated for fit to competitive, noncompetitive, and uncompetitive inhibition models (15) by nonlinear regression of inhibition equations using programs written in SAS (SAS Institute, Inc., Cary, NC). Additional data sets were collected for each compound until the \(K_i\) values, defined by the 95% confidence interval, overlapped.

To determine inhibition constants (\(K_i\)) of compounds in tables 1 and 3, a series of inhibitor concentrations were used ranging from 0.125 mM to 10 mM. At least 12 data points were generated from duplicate assays. The 4-methylumbelliferyl acetate concentration, [S] = 0.5 mM, was used to calculate \(V_{max} \) using the following equation:

\[ v = \frac{V_{max}[S]}{K_M + [S]} \]

where \( K_M \) for 4-methylumbelliferyl acetate was 660 μM. Competitive inhibition constants (\(K_i\)) were fit to eq. 1 with no less than six inhibitor concentrations.

The log octanol-water partition coefficient (log \(P\)) of selected compounds was estimated by the atom/fragment contribution method (16). Starting with the experimentally determined log \(P\) of cocaine, coefficients assigned to atoms and fragments were added or subtracted to obtain log \(P\) values for the cocaine derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(R)</th>
<th>(R')</th>
<th>(K_i) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heroin</td>
<td>→COCH3</td>
<td>→COCH3</td>
<td>0.3</td>
</tr>
<tr>
<td>6-Acetylmorphine</td>
<td>H</td>
<td>→COCH3</td>
<td>0.3</td>
</tr>
<tr>
<td>Morphine</td>
<td>H</td>
<td>H</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

\(K_i\) values of heroin, 6-monoacetylmorphine, and morphine were determined through competitive inhibition of the hydrolysis of 4-methylumbelliferylacetate with purified hCE-1 as described in Methods.

Results

The purification procedure for hCE-1 was modified and simplified from that previously described (2). After the Q Sepharose column, an affinity chromatography step was incorporated which gave a higher yield and a more stable enzyme suitable for steady-state kinetic studies (7). Acetylesterase activity was measured at each step of the purification using 4-methylumbelliferyl acetate as the substrate. The
specific activity was 5.5 U/mg for the pure enzyme based on the 4-methylumbelliferyl acetate assay compared with 6.8 U/mg obtained with the original procedure (2). The yield of acetyesterase activity was 31%, whereas the yield by the previous procedure was only 3%. The purified enzyme exhibited a single, dominant band at 59 kD by SDS-PAGE analysis (fig. 2). The carboxylesterase was considerably more stable after this purification scheme since no loss of activity was observed with storage at 4°C over a period of several months.

Inhibition constants were determined for a series of compounds using a single concentration of the substrate 4-methylumbelliferyl acetate.

### TABLE 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>Kᵢ(mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(±)-allopseudococaine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>H</td>
<td>-COOCH₃</td>
<td>H</td>
<td>-OC</td>
<td>0.68</td>
</tr>
<tr>
<td>R-(+)-pseudococaine</td>
<td>H</td>
<td>-COOCH₃</td>
<td></td>
<td>-OC</td>
<td>0.35</td>
</tr>
<tr>
<td>S-(−) pseudococaine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>H</td>
<td>-COOCH₃</td>
<td></td>
<td>-OC</td>
<td>0.19</td>
</tr>
<tr>
<td>S-(+) cocaine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-COOCH₃</td>
<td>H</td>
<td>-OC</td>
<td>H</td>
<td>&gt;10</td>
</tr>
<tr>
<td>R-(−)-cocaine</td>
<td>-COOCH₃</td>
<td>H</td>
<td></td>
<td>-OC</td>
<td>0.01</td>
</tr>
<tr>
<td>cocaethyline</td>
<td>-COOCH₂CH₃</td>
<td>H</td>
<td></td>
<td>-OC</td>
<td>0.1</td>
</tr>
<tr>
<td>benzoylecgonine propyl ester</td>
<td>-COOCH₂CH₂CH₃</td>
<td>H</td>
<td></td>
<td>-OC</td>
<td>0.14</td>
</tr>
<tr>
<td>benzoylecgonine</td>
<td>-COOH</td>
<td>H</td>
<td></td>
<td>-OC</td>
<td>1.5</td>
</tr>
<tr>
<td>ecgonine methyl ester</td>
<td>-COOCH₃</td>
<td>H</td>
<td>-OH</td>
<td>H</td>
<td>&gt;10</td>
</tr>
<tr>
<td>ecgonine</td>
<td>-COOH</td>
<td>H</td>
<td>-OH</td>
<td>H</td>
<td>&gt;10</td>
</tr>
<tr>
<td>tropacocaine</td>
<td>H</td>
<td>H</td>
<td></td>
<td>-OC</td>
<td>2.4</td>
</tr>
<tr>
<td>atropine</td>
<td>H</td>
<td>H</td>
<td></td>
<td>-OC-CH₂OH</td>
<td>H</td>
</tr>
</tbody>
</table>

*Kᵢ values of compounds were determined by their competitive inhibition of the hydrolysis of 4-methyl-umbelliferylacetate with purified hCE-1.

<sup>a</sup> Racemic mixture, R₁ and R₂ groups are attached at the C2 position (shown) or C4 position.

<sup>b</sup> S isomers of cocaine, R₁ and R₂ groups are attached at the C4 position.
The compounds can be divided into four groups according to the substituent groups present at positions R1 through R4. The compounds can be divided into four groups: cocaine, tropacocaine, atropine, plus cocaine isomers and metabolites are listed in table 3 along with the substituent groups present at positions R1 through R4 of the tropane ring. The compounds can be divided into four groups according to the substituent values as follows: cocaine and caocadeine (0.01 to 0.1 mM), benzyloecgonine propyl ester, pseudococaine isomers, and allopseudococaine (0.1 to 1.0 mM), benzoylcoacaine, tropacocaine, and atropine (1.0 to 10.0 mM); and (S)(+)-cocaine, egonine methyl ester, and egonine (>10 mM).

In table 1, the inhibition constants and estimated log octanol-water partition coefficients for cocaine and a series of C3-substituted derivatives are listed. There is a direct relationship between hydrophobicity and binding affinity, i.e., the compounds with a more hydrophobic substituent at the C3 position bind hCE-1 with greater affinity. The compounds can be divided into four groups according to the substituent values as follows: cocaine and caocadeine (0.01 to 0.1 mM), benzyloecgonine propyl ester, pseudococaine isomers, and allopseudococaine (0.1 to 1.0 mM), benzoylcoacaine, tropacocaine, and atropine (1.0 to 10.0 mM); and (S)(+)-cocaine, egonine methyl ester, and egonine (>10 mM).

In the present study, we evaluated the affinities of various compounds, and cocaine plus compounds #1 thru #7. Compound #6 was not included because the $K_i$ was >10. This was probably caused by a negatively charged oxygen atom at the neutral pH of the assay. Compound #5 was not included because the $K_i$ was higher than expected, probably because of nonenzymatic hydrolysis of the compound in stock solution.

The mechanism of inhibition was examined for cocaine, five cocaine metabolites (fig. 1), heroin, and 6-acetylmorphine by varying both 4-methylumbelliferyl acetate and the inhibitor or alternative substrate concentrations. As shown by the example of the Dixon plot for 6-acetylmorphine inhibition of 4-methylumbelliferyl acetate hydrolysis (fig. 4), the inhibition was competitive with respect to the substrate. This was verified by comparing the fit of data to competitive, noncompetitive, and uncompetitive inhibition models. The competitive inhibition fit yielded the highest $F$-statistic. The SE of $K_i$ was <6% of the value obtained in each case. Cocaine, heroin, and their metabolites can be divided into four groups according to $K_i$ values as follows: cocaine (0.01 mM); caocadeine, norcaocaine, heroin, and 6-acetylmorphine (0.1 to 1 mM); norcocaine, benzyloecgonine, and benzoylqnorcocgonine (1.0 to 10 mM); and morphine (>10 mM). Modification at the methyl ester, benzoyl or N-methyl sites of cocaine causes substantial increases in the inhibition constant.

**Discussion**

A family of carboxylesterases has been isolated and characterized in human (8, 17), rat (10, 18), and rabbit tissues (19, 20). The numerous isozymes share several common structural characteristics including about 60 kD subunit weight, microsomal subcellular localization, and an acidic isoelectric point (21). Two human liver esterases, hCE-1 and hCE-2, have been purified and partially characterized (2, 6). These two esterases can be distinguished by their molecular properties: hCE-1 is a 180 kDa trimer with an isoelectric point of 5.8 while hCE-2 is a 60 kDa monomer with an isoelectric point of 4.9. These esterases exhibit different substrate specificities. For example, hCE-1 hydrolyzes the methyl ester group of cocaine while hCE-2 hydrolyzes the benzoyl ester (4). Important information about structure-activity relationships has been obtained by examination of the amino acid sequence alignment of these human esterases with other esterases and lipases (6, 22). Regions around the catalytic triad, including the active-site serine, are conserved. However, there is high variability in residues suspected to be in the substrate binding site that could account for differences in substrate specificity (22).

In the present study, we evaluated the affinities of various compounds, and cocaine plus compounds #1 thru #7. Compound #6 was not included because the $K_i$ was >10. This was probably caused by a negatively charged oxygen atom at the neutral pH of the assay. Compound #5 was not included because the $K_i$ was higher than expected, probably because of nonenzymatic hydrolysis of the compound in stock solution.

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2. (R)(+)-pseudococaine is the C2 epimer of (R)(-)-cocaine, which is found in the leaves of *Erythroxylon* coca.

3. (S)(+)-cocaine is the unnatural enantiomer of (R)(-)-cocaine, and (S)(-)-pseudococaine is the C2 epimer of (S)(-)-cocaine.
A representative Dixon plot generated with data from a single experiment is shown. In the enzyme assay, the rate of 4-methylumbelliferone formation was monitored in the presence of varying substrate and inhibitor concentrations, as described in Methods. Symbols correspond to 1/observed rates and lines correspond to 1/predicted rates. The predicted rates are from the nonlinear regression fit of data to the equation for competitive inhibition. At the point of intersection, the y coordinate is equivalent to 1/V_{max} and the absolute value of the x coordinate is equivalent to K_i.

For compounds hCE-1 by examining their competition with hydrolysis of the substrate 4-methylumbelliferonyl acetate in a spectrophotometric assay. The compounds in fig. 1 and table 2 were examined by steady-state kinetic analysis, and they obeyed a simple competitive inhibition model since the fit of data to this mechanism was significantly better than to noncompetitive or uncompetitive models. Two types of compounds were examined in the kinetic analysis: alternate ester substrates and dead-end, competitive inhibitors. As shown by eqs. 1 and 2, the only mathematical difference between these two modes of inhibition is the definition of the inhibition constant, K_i = inhibitor dissociation constant and K_M = Michaelis constant for the alternate substrate.

The K_i values listed in table 3 for the cocaine isomers, metabolites, and derivatives range from 0.01 mM for the naturally occurring (R)-(−)-cocaine to >10 mM for the unnatural enantiomer (S)-(−)-cocaine. Binding to hCE-1 appears to be stereoselective, since the K_i for (R)-(−)-cocaine was at least 20 times lower than values obtained for (+)- and (−)-pseudococaine and (±)-allopseudococaine (table 3). Interestingly, Gatley (23) indicated that both (S)-(−)-cocaethylene and (R)-(−)-cocaine had similar affinities for the serum cholinesterase, but the rate of hydrolysis of the benzoyl ester of (+)-cocaine was several orders of magnitude faster compared with (−)-cocaine, the natural isomer. This observation agrees with a study comparing the cytotoxic potential of (R)-(−)-cocaethylene to (S)-(−)-cocaine and (S)-(−)-pseudococaine in cultured rat hepatocytes, which indicates that the stereoselective differences in the rates of hydrolysis primarily account for the cytotoxic potency of cocaine (24).

The kinetic data define, in part, structure-binding relationships between the cocaine-like molecules and hCE-1. There appears to be a preference for the methyl ester group at the R_3 position versus ethyl ester or propyl ester groups, as indicated by the ~10 fold greater K_i for cocaethylene and benzoylcegonine propyl ester versus cocaine. Removing the ester at this position to form tropacocaine results in a 240-fold greater inhibition constant. K_i values similar to tropacocaine were obtained with the antimuscarinic agent atropine (25). Ecgonine methyl ester, the de-esterified derivative with a hydroxyl group at R_3, has virtually no affinity for hCE-1 with a K_i >10 mM. Hence, the ester linkage at R_3 is very important for binding to hCE-1. Bulky groups at the C3 position display intermediate binding affinities (0.01–2.2 mM) as shown by the WIN and RTI compounds (table 1). Compounds #1 and #2 (table 1) that contain an ester-like phosphorothionate linkage show remarkably high affinity with K_i values of 0.01 mM and 0.03 mM, respectively. The phosphorothionate inhibitor (#2) might be useful as a ligand to purify hCE-1 by affinity chromatography. Removal of the N-methyl group decreases affinity of cocaine, benzoylecgonine, and cocaethylene to the carboxylesterase since the K_i values were substantially higher for the demethylated derivatives norcocaine, benzylnorecgonine, and norcocaethylene (fig. 1).

Many of the cocaine isomers, metabolites, and structurally related compounds included in this study were previously used to investigate binding to the cocaine receptor. A study by Carroll et al. (26) demonstrated the cocaine binding site at the dopamine transporter is stereoselective; the potency of natural (R)-(−)-cocaine was 60–670 fold greater than that of its isomers. The K_i values reported for the transporter with cocaine, cocaethylene, and benzoylecgonine propyl ester differed by less than 2-fold while a nearly 2000-fold difference in values was obtained between cocaine and benzoylecgonine (27). The two RTI compounds listed in table 1 displayed enhanced pharmacological activity relative to cocaine and were approximately 80-fold more potent in binding to the receptor protein than cocaine (28). Hence, both the receptor and hCE-1 exhibit similar stereoselectivity for cocaine isomers but differing specificities for other analogs.

The K_i values for heroin and 6-acetylmorphine were determined because hCE-1 is one of three enzymes that catalyzes the decactylation of heroin to form 6-acetylmorphine (7). A K_i of 0.3 mM was determined for both heroin and 6-acetylmorphine (table 2) suggesting the ester group at the 3 position contributes very little to enzyme binding. By contrast, the ester group at the 6 position was crucial to binding capability, since the K_i value for morphine was >10 mM (table 2).

The concurrent use of alcohol and cocaine is a common practice among drug abusers. In the presence of ethanol, hCE-1 catalyzes the ethyl transesterification of cocaine to form the active metabolite cocaethylene. The K_M for cocaine was 116 ± 17 μM (2). The K_i for cocaethylene was 10-fold higher than the value obtained for cocaine. The lower affinity of cocaethylene for hCE-1 is consistent with the longer elimination half-life reported for cocaethylene, which is 24% longer than that of cocaine in humans (29). Since cocaethylene is more potent than cocaine in mediating lethality in mice (30, 31), the combined use of cocaine and ethanol may present a greater health risk than cocaine alone.

Carboxylesterases play an important role in cocaine elimination since benzoylecgonine and ecgonine methyl ester (fig. 1) are the major metabolites that eventually appear in urine (5). Studies indicate that a minor pathway involving cytochrome P450 is largely responsible for cocaine-induced hepatotoxicity (32). This route involves the N-demethylation of cocaine to norcocaine (fig. 1), which then undergoes subsequent oxidation reactions in the endoplasmic reticulum. The presence of ethanol or other drugs that disrupt the hCE-1-mediated hydrolysis of cocaine may alter the formation of the cytotoxic metabolites of cocaine. The identification of combinations of drugs that can alter cocaine metabolism is of considerable interest.
The competitive, spectrophotometric assay used in this study represents a fast and effective method for evaluating the binding affinities of a series of compounds to the enzyme.

References