Structure-Activity Relationships for Substrates and Inhibitors of Mammalian Liver Microsomal Carboxylesterases

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Purpose. Carboxylesterases are important in the detoxification of drugs, pesticides and other xenobiotics. This study was to evaluate a series of substrates and inhibitors for characterizing these enzymes.

Methods. A series of novel aliphatic esters and thioureas were used in spectral assays to monitor human, murine and porcine esterases. A series of transition state mimics were evaluated as selective esterase inhibitors.

Results. Several α-alkyl thioacetatoximes were found to be ~2 to 11-fold superior to commonly used substrates for monitoring carboxylesterase activity. Insertion of a heteroatom in the acid portion of these esters in the β or γ position relative to the carbonyl had a dramatic effect on enzyme activity with S or O substituents often improving the Kcat/Km ratio of the substrate and N decreasing it. Several α,α'-bis(2-oxo-3,3,3-trifluoropropyl)alkanes proved to be potent selective transition state mimics of the esterase activity with IC50's from 10^-7 to 10^-4 M.

Conclusions. This library of substrates and inhibitors is useful for research tools for characterizing the numerous isozymes of carboxylesterases present in mammalian tissues.

KEY WORDS: carboxylesterases; mammalian liver; thioester substrates; trifluoromethylketone inhibitors; structure-activity relationships.

INTRODUCTION

Mammalian liver microsomal carboxylesterases (EC 3.1.1.1) are a group of isozymes located mainly in the endoplasmic reticulum. These enzymes are involved in the metabolism of a wide variety of xenobiotics containing ester, thioester and amide functional groups (1-5).

In the present study, a series of methyl and phenyl α-alkyl thioacetatoximes and related compounds were developed as selective, spectrophotometric substrates of mammalian liver microsomal carboxylesterases. In order to study substrate selectivity adequately, it is important to characterize the substrate selectivity of carboxylesterase in the crude as well as the purified state. The substrates reported in this study are potentially useful diagnostic tools for monitoring and distinguishing the different isozymes of carboxylesterase in mammalian tissues. One of these substrates, methyl β-(1-pentylthio)propionoate (MBPTP, 15), is a potentially useful diagnostic substrate for mammalian serum carboxylesterase (7), and has been used to monitor carboxylesterase activity in the affinity purification of mammalian liver carboxylesterases (8).

In a previous study (9), we reported the inhibition of mammalian liver carboxylesterases by a series of potent selective substituted trifluoromethylketone (TFKs) as “transition state” mimics. These compounds have proven very useful in studying the catalytic mechanism and biological roles of a variety of esterases (9-12). To further characterize the important hydrolytic carboxylesterase enzymes, we investigated the inhibitory effects of a series of α,α'-bis(2-oxo-3,3,3-trifluoropropylthio)alkanes (bis-TFKs) due to their potency as transition state inhibitors and suspected higher water solubility than the monosubstituted TFKs as discussed previously (9).

MATERIALS AND METHODS

Reagents

The synthesis and characterization of the substrates reported in this study are described in McCutchen et al. (6). Technical grade (95%) malathion, obtained from American Cyanamid Corp. (Princeton, NJ) was purified by thin layer chromatography. p-Nitrophenol, p-nitrophenyl acetate (p-NpAc) were purchased from Aldrich Chemical Co. (Milwaukee, WI). α-Napthol, α-napthyl acetate (α-NA), p-iodonitrophenyltetrazolium violet (INT), alcohol dehydrogenase, NAD and NAD diaphorase, bovine serum albumin (fraction V), n-octyl-β-D-glucopyranoside (octyl glucoside), and porcine liver carboxylesterase (EC 3.1.1.1) with 200 units/mg protein were purchased from Sigma Chemical Co. (St. Louis, MO). BCA reagent was purchased from Pierce Chemical Co. (Rockford, IL). 3-Octylthio-1,1,1-trifluoro-propanone (OTFP) and α,α'-bis(2-oxo-3,3,3-trifluoropropylthio)alkanes were available or prepared according to previously reported methods (9,11).

Preparation of Liver Microsomes

Liver microsomal fractions from human and mouse were prepared as described previously (2,7). Microsomes were solubilized in 0.1 M Tris-HCl buffer, pH 7.5 containing 1% octyl glucoside, at a final protein concentration of 4.0 mg/mL. The mixtures were gently shaken for 1 hr on a rotating wheel at approx. 50 rpm and afterwards centrifuged at 100,000 g for 1 hr. The supernatant fractions were collected and dialyzed against the same buffer to obtain the solubilized microsomes, which was used for enzyme assays.

Esterase Assays with Spectrophotometric Substrates

The rates of hydrolysis of the thioesters, 1-21, by the various mammalian liver carboxylesterases were measured in a continuous assay using 96-well microtiter plates (Dynatech Laboratories, Inc., Virginia, VA) with a Vmax plate reader (Molecular Devices, Palo Alto, CA).

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Unless otherwise specified, the buffer used in the assay of porcine liver carboxylesterase was 0.1 M Tris-HCl, pH 7.5 containing 50 μg/ml BSA and for the assay of human and mouse liver carboxylesterases, the buffer used was 0.1 M Tris-HCl, pH 7.5. The esterase assays with the thioesters are based on a modification of the Ellman method (13) by colorimetric determination at 405 nm of the free chromophoric product (5-thiolate-2-nitro benzoic acid) formed after reacting with 0.015% 5,5'-dithiobis-(2-nitro benzoic acid) (DTNB). In a typical assay, 278 μl of 0.015% DTNB in buffer and 20 μl of the enzyme solution were added to individual wells. In the nonenzymatic reaction, 20 μl buffer instead of the enzyme solution was added. The reaction was started by the injection of 2 μl of the substrates (in ethanol) to give a final concentration of 2 × 10^{-4} M. The hydrolytic rates were monitored at 405 nm for 2 mins at 23°C and corrected for nonenzymatic rates.

Previous reported assay procedures were followed for the assays of substrates 22–28 (12), p-NpAc and α-NA (2,7), and malathion (2,7). The enzyme assays were carried out under conditions where the initial hydrolytic rates were linear with time for the protein and substrate concentrations, used. The assay was carried out in quadruplicate. The measured rates were corrected for the spontaneous hydrolysis of the substrate where this was significant. Specific activities were determined using a substrate concentration which was >10 times the K_m for the compounds. Protein concentrations were determined using the standard protocol version of the Pierce BCA assay modified for use in a 96-well plate reader. BSA was used as a protein standard.

**Determination of Kinetic Constants**

The apparent K_m and V_max were calculated as described previously (2). Eleven substrate concentrations (5 × 10^{-6} – 2 × 10^{-4} M) were used with the partially purified porcine liver carboxylesterase. The maximal velocities (V_max) are expressed in micromoles of substrate hydrolyzed per mg protein in 1 min under the conditions described.

**Optimum pH of Porcine Liver Esterase**

Buffers (286 μl at pH 6.0–10.0 in 0.2 or 0.5 pH unit increments) were added to 10 μl of porcine esterase and incubated at room temperature for 10 min in individual wells. In control samples, the final pH of the reaction mixture was verified using a pH meter. DTNB (2 μl solution of a 2.25% in acetone, w/v) was injected to give a final concentration of 0.015%. The esterase activity was measured after injecting 2 μl of the substrates (in ethanol) to give a final concentration of 2 × 10^{-4} M. Nonenzymatic rates for each substrate were subtracted from the reported enzymatic rates. The ionic strength of all buffers was 0.1 M. A phosphate buffer was used for pH 6.0–8.0, Tris-HCl for pH 8.0–9.0, and glycine-NaOH for 9.5–10.0.

**Inhibition Studies**

The inhibition of carboxylesterase activity by the TFKs was determined according to previously described procedures (9). For measuring the inhibition of malathion carboxylesterase, the inhibitors (in acetone) were added in 1.0 μl to wells containing 100 μl enzyme solutions. After preincubation of the enzyme with the inhibitor for 10 min at 23°C, the reagent mixture (200 μl) was added followed by injection of 1.0 μl of an acetone solution of malathion to give a final concentration of 3 × 10^{-4} M. For measuring the inhibition of carboxylesterase acting on p-NpAc and 15, the inhibitors (in acetone or ethanol) were added in 1.0 μl to wells containing 300 μl enzyme solutions diluted in 0.1 M Tris-HCl buffer pH 7.5, or the same buffer containing 0.015% DTNB reagent, respectively. After preincubation of the enzyme with the inhibitor for 10 min at 23°C, the substrate p-NpAc or 15 in 2.0 μl was added and the hydrolytic rates were monitored for 2 min at 405 nm. Separate experiments indicated that the solvents used (<1% of acetone or ethanol) had little effect on enzyme activity.

The inhibitors were screened at concentrations ranging from 1 × 10^{-5} to 1 × 10^{-10} M. For determination of the concentration required for 50% inhibition of the enzyme, triplicate incubations at four or five different concentrations of the inhibitor were used. The IC_{50} values were calculated from semi-log plots using the linear portion of the curve by least-squares regression analysis.

**RESULTS AND DISCUSSION**

**Substrate Selectivity of Mammalian Liver Microsomal Carboxylesterases**

Significant differences in the substrate selectivity of liver microsomal carboxylesterases of human, porcine and mouse were observed (Table I). This may be due to species differences and/or unequal quantities of specific isozymes involved in the hydrolysis of these substrates. The higher specific activity exhibited by the porcine liver carboxylesterase acting on these substrates probably indicate the greater purity of this enzyme preparation.

Among a homologous series of unbranched aliphatic thioesters (1–12) (Table I), the mammalian liver carboxylesterases showed a preference for substrates with shorter acyl chains 1 or 2 (i.e. C_5 or C_7). Substitution of a S or O in the β position of the acyl group (9–12), did not significantly alter the hydrolytic rates compared to the corresponding carbon analogues, although the ether analog (14), was about 2.6-fold better than its carbon analog (3) when tested with the human enzyme. However, in terms of kinetic characteristics, the heteroatom (S or O) in the β position of the acyl group of 10 and 14, lowered the K_{cat} of the substrates by about 5-fold when compared to substrate 2 (Table II). An increased binding to the enzyme resulted in decreased turnover rates (lower V_{max}/K_{cat}) for 10 and 14. Unlike the methyl thioesters, the insertion of a S or O in the β position of the acyl moiety in the phenyl thio-and carbonyl esters significantly enhanced the hydrolytic rates catalyzed by the mammalian liver carboxylesterases e.g. 18 and 26 (Table I). However, 18 and 26, have different binding affinities and turnover rates (Table II). Although 26 has a very high specificity (V_{max}/K_{cat}) ratio, its usefulness as a substrate may be limited due to its high liability under basic conditions (Table III). Compound 26 is the most labile to base hydrolysis among the substrates tested.

The presence of a S in the γ position of the acyl moiety in the methyl (15) and phenyl γ-thioethers (21 and 28) significantly enhanced the substrate specificity profile of these compounds. Compound 15 was approximately 2.3 to 2.6-fold more active