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HALOKETONE TRANSITION STATE ANALOG INHIBITORS OF CHOLESTEROL ESTERASE

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Summary: The cholesterol esterase-catalyzed hydrolysis of p-nitrophenyl butyrate is reversibly inhibited by four phenyl haloalkyl ketones. Inhibitor potency is greatest for halogenated acetophenones and parallels the extent of hydration of the various ketones in buffered D_2O. These results are consistent with an inhibition mechanism wherein haloketones reversibly form hemiketal adducts at the active site that structurally mimic tetrahedral intermediates of the cholesterol esterase catalytic cycle.

Pancreatic cholesterol esterase (CEase) catalyzes the hydrolysis of ester bonds of emulsified lipids (cholesteryl esters, triacylglycerols and phospholipids) in the alimentary tract [1], and is thought necessary for the absorption of dietary cholesterol through the intestinal mucosa [2,3]. Mechanism-based inhibitors of CEase may prevent absorption of dietary cholesterol into the bloodstream and thereby be of therapeutic value in the treatment of atherosclerosis. With this in mind we have undertaken to characterize the mechanism of CEase catalysis [4] and to use the derived information to design potent mechanism-based inhibitors of the enzyme.

Various investigations suggest that CEase is a serine hydrolase and that CEase catalysis proceeds via an acylenzyme mechanism. These include: a) nucleophilic trapping of acylenzyme intermediates

Abbreviations: CEase, cholesterol esterase; PNPA, p-nitrophenyl acetate; PNPB, p-nitrophenyl butyrate; TX100, triton X-100; DFP, diisopropylfluorophosphate; CDFA, chlorodifluoroacetophenone; TFA, trifluoroacetophenone; PFP, pentafluoropropiophenone; HFB, heptafluorobutyrophenone

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[4,5]; b) inhibition of the enzyme by reagents that modify serine and histidine sidechains [6]; c) active site titration of CEase by DFP [7]; d) inhibition of CEase by carbamates [8] via a carbamyl-enzyme mechanism; e) reversible inhibition of CEase by boronic and borinic acid transition state analogs [9]. Haloalkyl ketones have recently been prepared that are potent inhibitors of the serine hydrolases elastase [10,11], chymotrypsin [11] and acetylcholin-esterase [12-14]. These precedents lead one to speculate that haloketones will be potent inhibitors of CEase. Accordingly, synthesis and evaluation of four phenyl haloalkyl ketone inhibitors of CEase are herein described.

MATERIALS AND METHODS

Materials: Porcine pancreatic CEase (EC 3.1.1.13) was purified to homogeneity by a modification of the procedure of Rudd *et al.* [7], as outlined by Stout *et al.* [4]. Sodium taurocholate, D_2O, PNPA and PNPB were purchased from Sigma Chemical Co. TX100 and anhydrous diethyl ether were obtained from Mallinckrodt and acetophenone, bromobenzene and haloalkyl carboxylic acids were purchased from Aldrich Chemical Co. Buffer components, salts and other solvents were commercially available reagent grade chemicals. Water for buffer preparation was distilled and deionized by passing through a mixed-bed ion exchange column (Barnstead, Sybron Corp.).

Haloketone inhibitors were synthesized by the procedure described by Herkes and Burton [15], which involved reaction of two parts of the Grignard reagent formed from bromobenzene with one part of the requisite haloacid. The haloketones were purified by distillation. Halomethyl ketones were further purified by silica column chromatography, with CH_2Cl_2 as the eluent.

Reaction Kinetics and Data Reduction: Buffers for CEase reactions and reaction conditions are described in Table I. Requisite volumes of stock solutions of PNPA, PNPB and inhibitors in MeCN were injected into reaction buffers via Gilson Pipetmen. CEase was dissolved in 0.1 M sodium phosphate buffer, pH 6.94, that contained 0.1 N NaCl and 1 mM sodium taurocholate. Reactions were initiated by injecting enzyme and monitored by following p-nitrophenoxide formation at 400 nm on Beckman DU40 or Hewlett-Packard 8452 UV-visible spectrophotometers that are interfaced to IBM Personal computers. Reaction temperature was controlled by using VWR-1140 and Brinkman-Lauda RC-3 refrigerated, circulating baths to circulate water through the cell holders of the HP8452 and DU40, respectively.

In the presence of TX100, the timecourses for CEase-catalyzed hydrolysis of PNPB were described by the integrated form of the Michaelis-Menten equation when $[S]_o \geq K_m$ [4]:

$$t = \frac{K_m}{V_{max}} \ln \frac{A_\infty - A_o}{A_\infty - A} + \frac{1}{V_{max}\Delta\varepsilon}(A - A_o) \qquad (1)$$

Table I. Kinetic and Inhibition Constants for CEase Reactions[a]

Compound	K_m, mM	Measured K_i, μM	K_{hyd}[b]	Corrected K_i, μM[c]
PNPA	0.9 ± 0.1 1.4 ± 0.4[d]	-	-	-
PNPB	0.08 ± 0.01 0.14 ± 0.01[d]	-	-	-
CDFA	-	5.1 ± 0.8[e] 17 ± 3[d]	140	0.036
TFA	-	18 ± 2 13 ± 4[d]	52	0.34
PFP	-	90 ± 20	18.6	4.6
HFB	-	230 ± 10	6.1	32

[a] All reactions were done at 25.0 ± 0.1 °C in 1.00 mL of 0.1 M sodium phosphate buffer, pH 7.30, that contained 0.1 N NaCl, 2% MeCN v/v, 1 mM TX100 (unless otherwise noted) and varying concentrations of substrates and inhibitors. Unless otherwise noted, error limits are standard errors generated from the least-squares fits described in MATERIALS AND METHODS.

[b] Equilibrium constant for ketone hydration; estimated uncertainty is ± 5-10%.

[c] The corrected K_i values were calculated by multiplying the measured K_i values by the fraction of ketone in the unhydrated (free) form.

[d] For these determinations reaction media did not contain TX100.

[e] Mean of two determinations; error limit is ± one-half of the range of the two determination.

In this equation A, A_o and A_∞ are absorbances at times t, 0 and ∞, respectively, and Δε is the change in absorptivity constant at 400 nm for the conversion of PNPB to p-nitrophenoxide and butyrate. K_m and V_{max} were calculated by fitting absorbance timecourse data to eq 1 by nonlinear-least squares procedures [16]. The following linear transform of eq 1 was used to determine the form of inhibition of CEase by haloketones:

$$\frac{t_{i+10} - t_i}{A_{i+10} - A_i} = \frac{K_m}{V_{max}} \frac{\ln \frac{A_\infty - A_i}{A_\infty - A_{i+10}}}{A_{i+10} - A_i} + \frac{1}{V_{max}\Delta\varepsilon} \qquad (2)$$

For CEase-catalyzed hydrolysis of PNPA and of PNPB in the absence of TX100, K_m and V_{max} were calculated by fitting initial velocities versus [S] to the differential form of the Michaelis-Menten equation [16]:

$$V_i = \frac{V_{max}[S]}{K_m + [S]} \qquad (3)$$

K_i values were determined by measuring initial velocities at constant [S] and variable [I]; the resultant $\{[I], V_i\}$ pairs were fit to eq 4 [16]:

$$V_i^I = V_i^0 \frac{K_i + \beta[I]}{K_i + [I]} \qquad (4)$$

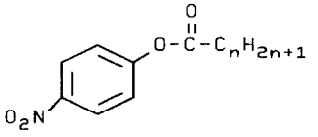
V_i^I is the initial velocity in the presence of inhibitor; K_i, β and V_i^0 are the CEase-inhibitor dissociation constant, the residual fractional activity at saturating [I] and the initial velocity in the absence of inhibitor, respectively, and are the adjustable parameters of the nonlinear-least squares fit [16].

Other Methods: ^{19}F-NMR spectra of haloketones were determined on Bruker WM-360 or Jeol FX90Q spectrometers, with CF_3CO_2H in D_2O as an external reference. Ketones were dissolved in 0.1 M sodium phosphate buffer in D_2O, pD 7.83. The buffer also contained 0.1 N NaCl and 10% v/v DMSO. Hydration equilibrium constants for the haloketones were calculated from the relative intensities of ^{19}F resonances for the unhydrated and hydrated species. Buffer pH values were measured on a Corning model 125 pH meter equipped with a glass combination electrode. For buffers in D_2O, pD values were calculated by adding 0.4 to the pH meter reading [17].

RESULTS AND DISCUSSION

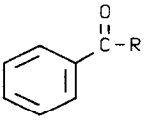
Scheme I shows the structures of substrates and inhibitors used in this study. Initial velocities for CEase-catalyzed hydrolysis of PNPA and PNPB were determined in the presence and absence of TX100. In general, CEase was more stable and reaction kinetics were more reproducible when TX100 was included in the reaction media. K_m values calculated by fitting initial velocities to eq 3 are contained in Table I. In the presence of 1 mM TX100, timecourses for CEase-catalyzed hydrolysis of PNPB were described by the integrated Michaelis-Menten equation (eq 1). K_m values determined by this method agree within experimental error with those determined

SCHEME I



n = 1, PNPA

n = 3, PNPB



R = CF_2Cl, CDFA

R = CF_3, TFA

R = C_2F_5, PFP

R = C_3F_7, HFB

by fitting initial rate data to eq 3. The haloketones of Scheme I are competitive inhibitors of CEase-catalyzed hydrolysis of PNPB. K_i values calculated from fits to eq 4 are contained in Table I. CDFA is the most potent of the inhibitors tested, with a K_i of 5.1 μM. The K_m/K_i ratio for PNPA and CDFA indicates that the haloketone binds 200 to 300-fold more tightly to the enzyme than does the substrate. A similar comparison with PNPB shows that CDFA binds ~20 times more tightly. However, K_m overestimates the affinity of CEase and PNPB in the Michaelis complex by at least an order of magnitude since CEase-catalyzed hydrolysis of PNPB is rate limited by deacylation [4], so that CDFA binds >200-fold more tightly than does PNPB.[1] These comparisons of K_m and K_i suggest that CDFA is a transition state analog inhibitor of CEase [18]. In contrast, acetophenone only inhibits CEase by 10% at 475 μM. Hence, a perhaloalkyl group is an essential feature of inhibitor structure, and is likely involved in electrophilic stabilization of a hemiketal adduct formed with the active site serine.

The data in Table I show a trend toward lower inhibitor potency as the size of the perhaloalkyl function increases. This trend parallels the equilibrium hydration of the haloketones, as determined by ^{19}F-NMR (cf. Table I). It is likely that inhibition occurs via reversible formation of a serine hemiketal at the CEase active site. Formation of this adduct requires the free carbonyl form of the inhibitor, and therefore measured K_i values underestimate the affinity of CEase and haloketones. Corrected K_i values were calculated by dividing measured K_i values by the fraction of inhibitor in the free form and are included in Table I.

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[1] For an acylenzyme mechanism wherein V_{max} is rate limited by deacylation, $K_m = K_s k_3/k_2$, where k_2 and k_3 are rate constants for acylation and deacylation, respectively, and K_s is the Michaelis constant for the ES complex. Since $k_3/k_2 << 1$, $K_s >> K_m$; i.e., K_m overestimates the tightness of CEase-PNPB interaction in ES.

These values demonstrate that aryl haloketones are indeed potent inhibitors of CEase. In addition, there is a linear free energy correlation (plot not shown) between $\ln\{1/K_i\}$ and $\ln\{K_{hyd}\}$:

$$\ln\{1/K_i\} = (2.2\pm0.1)\ln\{K_{hyd}\} + 6.1\pm0.4 \qquad r = 0.998 \qquad (5)$$

Though the data are sparse, the correlation spans a wide range of K_{hyd} and K_i values. The free energy correlation is consistent with an inhibition mechanism wherein nucleophilic addition to the ketone carbonyl is a dominant factor, as expected for formation of a serine hemiketal adduct.

The synthesis and evaluation described in this paper of small molecule inhibitors of CEase represent a logical first step in the design of inhibitors of potential pharmacological usefulness. That aryl haloketones, especially those with smaller haloalkyl functions, are potent CEase inhibitors provides a rationale for the development of haloketone inhibitors that are better biomimics of cholesteryl ester physiological substrates. Accordingly, the synthesis of haloketone analogs of cholesteryl esters is being pursued in our laboratory.

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