Characterization of inhibitors of specific carboxylesterases: Development of carboxylesterase inhibitors for translational application

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Abstract

Carboxylesterases, expressed at high levels in human liver and intestine, are thought to detoxify xenobiotics. The anticancer prodrug 7-ethyl-10-[4-1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) is also metabolized by carboxylesterases to produce the active drug 7-ethyl-10-hydroxycamptothecin. Activation of CPT-11 by human intestinal carboxylesterase (hiCE) in the human intestine may contribute to delayed onset diarrhea, a dose-limiting side effect of this drug. The goal of this study was to develop small molecule inhibitors selective for hiCE to circumvent or treat the toxic side effects of CPT-11. A secondary goal was to develop molecules that specifically inhibit activation of CPT-11 by a rabbit liver carboxylesterase (rCE). rCE is the most efficient CPT-11–activating enzyme thus far identified, and this enzyme is being developed for viral-directed enzyme prodrug therapy applications. Based on *in vitro* assays with partially purified hiCE and rCE proteins and on growth inhibition assays using U373MG human glioma cells transfected to express hiCE or rCE (U373pIREShiCE or U373pIRESrCE), we identified specific inhibitors of each enzyme. Lead compounds are derivatives of nitrophenol having 4-(furan-2-carbonyl)-piperazine-1-carboxylic acid or 4-[(4-chlorophenyl)-phenyl-methyl]-piperazine-1-carboxylic acid substitutions in the *p* position. Kinetic analysis of each compound for hiCE compared with rCE showed that the K_i values of the most selective of these inhibitors differed by 6- to 10-fold. In growth inhibition assays, nontoxic, low micromolar concentrations of these inhibitors increased the EC_{50} of CPT-11 for U373pIREShiCE or U373pIRESrCE cells by 13- to >1,500-fold. The four compounds characterized in this study will serve as lead compounds for a series of inhibitors to be constructed using a combinatorial approach. [Mol Cancer Ther 2004;3(8):903–9]

Introduction

Carboxylesterases comprise a family of enzymes ubiquitously expressed in animals and mammalian tissues (1). These enzymes are associated with metabolism of xenobiotics such as pesticides, nerve gasses, heroin, and many drugs including some prodrugs used in chemotherapy (1, 2). Esterases are usually classified according to the substrates they metabolize, but there is significant overlap in substrate and inhibitor ''specificity'' among esterase families. In cancer patients treated with 7-ethyl-10-[4-1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11), carboxylesterases convert CPT-11 to its active form 7-ethyl-10-hydroxycamptothecin (SN-38); SN-38 is a potent inhibitor of topoisomerase I (3). The purpose of this study was to develop small molecule inhibitors of specific carboxylesterases that activate and therefore mediate the antitumor efficacy and toxicity of the antitumor agent CPT-11.

Three carboxylesterases have been identified that activate CPT-11 relatively efficiently (4–7). This study focuses on the two carboxylesterases that are most likely to impact on the clinical use of CPT-11. The first of these is human intestinal carboxylesterase (hiCE), which is expressed at high levels in the small intestine and at ~10-fold lower levels in the liver (also called hCE2; refs. 5, 7). Because of the high level of expression of hiCE in the intestine, we postulate that this enzyme is likely to be at least partly responsible for producing high local levels of SN-38 and to contribute to the gastrointestinal toxicity seen in patients treated with CPT-11. The second enzyme known to activate CPT-11 is a rabbit liver carboxylesterase (rCE) that is being developed for viral-directed enzyme prodrug therapy approaches to chemotherapy (8–10). rCE activates CPT-11 most efficiently of all carboxylesterases thus far characterized. Because of the inevitable variability in endogenous (hiCE) or exogenous (viral-directed enzyme prodrug therapy with rCE) levels of enzymes, small molecules that specifically inhibit the activity of the enzyme(s) responsible for CPT-11 activation might be used to ameliorate or circumvent toxic effects of CPT-11 therapy. Therefore, our long-term goal is to develop small molecule inhibitors of hiCE or rCE. The study described in this article confirms that it is possible to develop small molecules with such specificity and identifies lead compounds that selectively inhibit hiCE or rCE.

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Materials and Methods

CPT-11

CPT-11 was kindly provided by Dr. J.P. McGovren (Pharmacia, Peapack, NJ).

Cell Lines

U373MG glioblastoma cells were obtained from the American Type Culture Collection (Rockville, MD). Construction of plasmids and the methods used to establish U373MG transfectants that stably expressed hiCE or rCE have been published, as have conditions under which these transfectants and pIRESneo-transfected vector controls were maintained in culture (4, 5).

Partially Purified Carboxylesterases

rCE and hiCE were prepared from baculovirus culture medium as reported previously (11). The rationale for focusing on rCE and hiCE (hCE2) for this study is explained above.

Synthesis of Inhibitors

The synthesis of the four inhibitors was accomplished using triphosgene, with subsequent coupling to various amines, and has been reported in a separate article (12).

Carboxylesterase Assays

General carboxylesterase activity was determined spectrophotometrically using the carboxylesterase substrate *o*-nitrophenyl acetate (*o*-NPA) as described previously (13). Briefly, cell sonicates were incubated with 3 mmol/L substrate in 50 mmol/L HEPES (pH 7.4), and generation of *o*-nitrophenol (*o*-NP) was monitored at 420 nm and quantitated from its extinction coefficient (13.4×10^3 mol/L^{-1} cm^{-1}). Results are expressed as nanomoles of *o*-NP produced per minute per milligram of protein. The reproducibility of this assay is $\pm$2%.

Conversion of CPT-11 to SN-38

Also as published previously (14), cell sonicates or recombinant proteins were incubated with 5 μmol/L CPT-11 at 37°C for 1 hour. Reactions were terminated with an equal volume of ice-cold acidified methanol. Reaction mixtures were centrifuged at 12,000 $\times$ *g* for 15 minutes, and SN-38 concentrations in the supernatants were determined by high-performance liquid chromatography. Ability of the synthesized compounds to inhibit carboxylesterase activity in intact cells was assessed by preincubating U373pIRESneo, U373pIREShiCE, or U373pIRESrCE cells with 5 μmol/L ''inhibitor.'' To quantitate inhibition of carboxylesterase activity *in situ*, each cell pellet was harvested and sonicated, and 85 μL of each cell sonicate were assayed immediately using *o*-NPA as a substrate. As controls, an equal volume of sonicates from cells that had not been incubated with inhibitors was assayed immediately in the presence and absence of inhibitors. Under these conditions, none of the compounds, when added directly to sonicates of U373 sublines, inhibited carboxylesterase activity within the 30 seconds to 2 minutes required to perform the assay.

Determination of K_i Values

K_i values for the inhibitors were determined with 50 μmol/L CPT-11 as the substrate. At least eight inhibitor concentrations were used for each analysis, and data were fitted to a one-site competition curve using the GraphPad Prism software. Routinely, r^2 values for these curve fits exceeded 0.99. K_i values were calculated from the curves using the method described by Cheng and Prusoff (15). This model assumes competitive inhibition (16), and calculations are based on the following equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{Km}}$$

Growth Inhibition Assays

Cells in log-phase growth were plated in six-well plates (Costar, Corning, NY) and allowed to adhere overnight. Plates were exposed to 5 μmol/L inhibitor for 1 hour and/or a range of concentrations of CPT-11 for an additional hour. Cell monolayers were cultured in drug-free medium for five cell doubling times, and the number of cells was quantitated with a Coulter counter. Results were expressed as the percentage of surviving cells compared with untreated controls and the concentration of CPT-11 required to inhibit cell growth by 50% (EC_{50}) using GraphPad Prism software as published previously (13).

Results

Schematic Figure Showing Carboxylesterase-Catalyzed Metabolism of *o*-NPA and CPT-11

o-NPA is a general esterase substrate (Fig. 1) and provides a means to quickly and easily quantitate esterase activity by quantitating cleavage of the ester-linked side chain as measured spectrophotometrically by the production of *o*-NP. In contrast, CPT-11 is metabolized efficiently only by hiCE, rCE, and esterase activities in mouse and rat sera that have not yet been well characterized (4–7).

Of the library of compounds synthesized, the four compounds shown in Fig. 2 were found to inhibit rCE and hiCE activity at nanomolar or low micromolar concentrations. These compounds were characterized more fully for their enzyme specificity.

Inhibition of Recombinant hiCE and rCE *In vitro*

Three hundred to 400 units of recombinant hiCE or rCE activity, defined as the amount of enzyme that catalyzed 300 to 400 nanomoles of *o*-NPA per milligram of protein per minute, were used in each reaction mixture. Enzymes either were preincubated with inhibitor for 1 hour and CPT-11 was added for an additional hour (Fig. 3A) or were added to reaction mixtures containing both inhibitor and CPT-11 (Fig. 3B). SN-38 production was quantitated following the hour of coincubation of CPT-11 and inhibitor. Inhibitors were used at concentrations of 0.5, 5, and 50 μmol/L with 5 μmol/L CPT-11 in the reaction mixtures in these *in vitro* screening assays to identify compounds that might be worth further evaluation in intact cells. The reproducibility of assays shown in Figs. 3 and 4 was $\pm$2% for all enzyme preparations.

Results in Fig. 3A show that preincubation of 4-benzylpiperidine-1-carboxylic acid 4-nitrophenyl ester **1** with rCE and hiCE inhibited catalysis of CPT-11 by >98% at

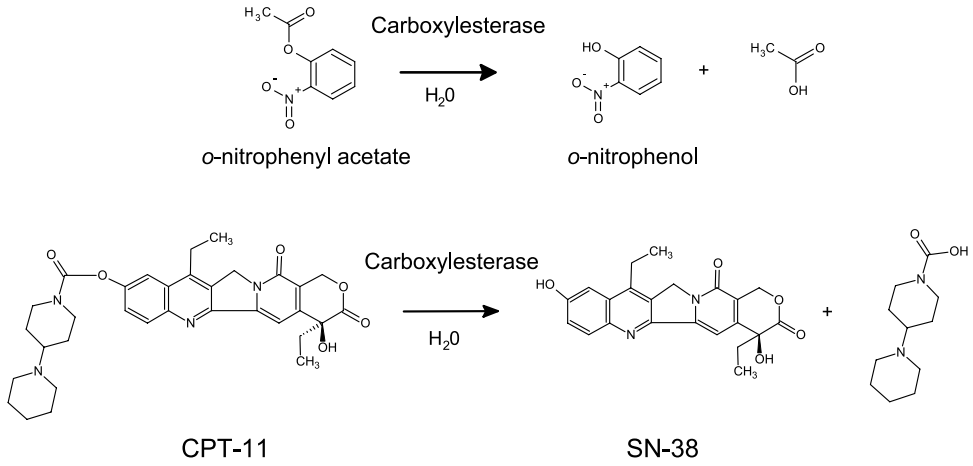

Figure 1. Schematic representation of reactions catalyzed by carboxylesterases, with the general carboxylesterase substrate *o*-NPA or the anticancer prodrug CPT-11. SN-38 is the active form of CPT-11.

0.5 μmol/L but showed no specificity for either enzyme. Preincubation of 4-benzyl-piperazine-1-carboxylic acid 4-nitrophenyl ester **2** showed similar results, with 97% inhibition of rCE and 92% inhibition of hiCE at 0.5 μmol/L. The third compound, 4-(furan-2-carbonyl)-piperazine-1-carboxylic acid 4-nitrophenyl ester **3**, was a more potent inhibitor of hiCE than rCE, with 97% inhibition of hiCE compared with 67% inhibition of rCE following preincubation with 50 μmol/L of this inhibitor. Conversely, the fourth compound, 4-[(4-chlorophenyl)-phenylmethyl]-piperazine-1-carboxylic acid 4-nitrophenyl ester **4**, was a more potent inhibitor of rCE than hiCE, with 13% of hiCE activity remaining under conditions (5 μmol/L inhibitor) that completely inhibited rCE activity. A dose response was evident with each compound.

In translational applications, it would be essential that compounds inhibit CPT-11–activating enzymes after CPT-11 had already been administered. Therefore, we repeated the above inhibition assays but with CPT-11 and inhibitor added simultaneously. With all four compounds, the specificities of the inhibitors were identical to those seen in Fig. 3A: compound **3** selectively inhibited hiCE, and compound **4** preferentially inhibited rCE (Fig. 3B).

Compounds **1** and **2** were evaluated at lower concentrations, because the lowest concentration in the screening assay (Fig. 3) almost completely inhibited both hiCE and rCE activity. Three additional concentrations, 0.01, 0.05, and 0.1 μmol/L, were used to evaluate compounds **1** and **2** with simultaneous addition of inhibitor and CPT-11 *in vitro*. At these lower concentrations, compounds **1** and **2** inhibited carboxylesterase activity from 25% to 96%, but hiCE and rCE were inhibited equally by all three concentrations of both compounds. Because all four compounds inhibited the metabolism of CPT-11, we next determined K_i values for each inhibitor with either hiCE or rCE to determine whether the K_i might reflect enzyme specificity or the lack thereof.

K_i Values of Each Inhibitor for rCE and hiCE

K_i values were determined using the method described by Cheng and Prusoff (15), because the K_m values for

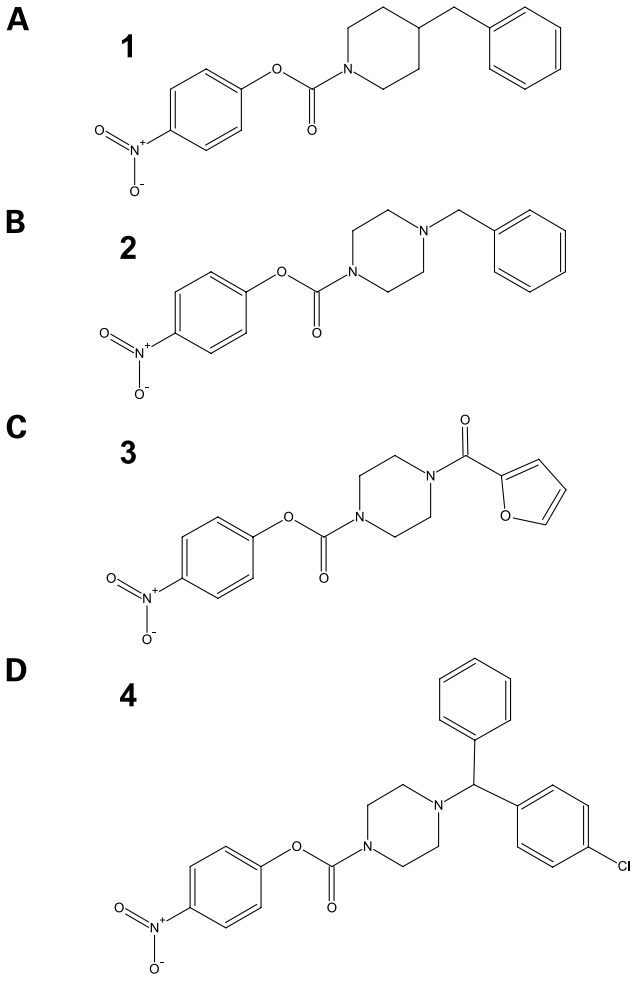

Figure 2. Structure of the four nitrophenyl derivatives characterized in this study for their potency and selectivity as inhibitors of hiCE and rCE. **A,** 4-Benzyl-piperidine-1-carboxylic acid 4-nitrophenyl ester; **B,** 4-benzyl-piperazine-1-carboxylic acid 4-nitrophenyl ester; **C,** 4-(furan-2-carbonyl)-piperazine-1-carboxylic acid 4-nitrophenyl ester; and **D,** 4-[(4-chlorophenyl)-phenylmethyl]-piperazine-1-carboxylic acid 4-nitrophenyl ester.

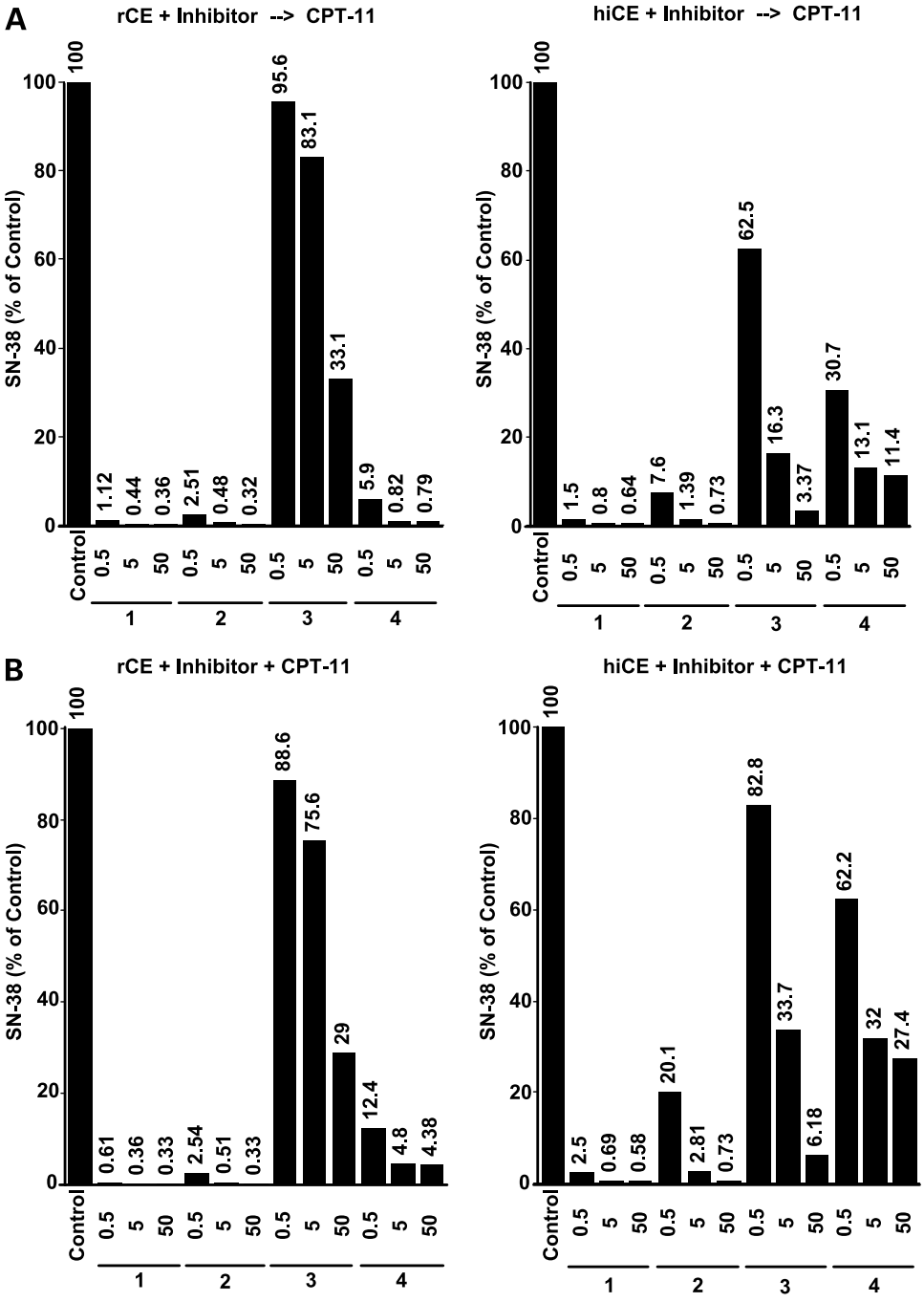

Figure 3. Inhibition of carboxylesterase-mediated catalysis of CPT-11. **A,** enzymes were incubated with 5 μmol/L CPT-11 with 0.5, 5.0, or 50 μmol/L inhibitor for 1 hour and SN-38 levels were determined. **B,** enzymes were preincubated with 0.5, 5.0, or 50 μmol/L inhibitor for 1 hour prior to addition of CPT-11. *Columns,* percentage SN-38 produced for each enzyme in the absence of inhibitor.

rCE and hiCE with CPT-11 have been determined previously. Using 5 μmol/L CPT-11 as a substrate and a range of inhibitor concentrations from 1 nmol/L to 100 μmol/L, the K_i values were calculated from inhibition curves generated by GraphPad Prism software. As shown in Table 1, K_i values for compounds **1**, **2**, and **4** were ~2-, 3-, and 6-fold lower for rCE than for hiCE, respectively. Conversely, compound **3** had a K_i ~10-fold lower for hiCE than for rCE. These results suggested that a lower K_i value did reflect enzyme specificity and that it is possible to develop inhibitors of specific carboxylesterases.

Inhibition of hiCE or rCE in Stably Transfected U373MG Glioma Cells

To determine whether compounds **1** to **4** crossed the cell membrane and inhibited intracellular carboxylesterase

activity, we preincubated intact cells that express rCE or hiCE with 5 μmol/L inhibitor for 1 hour, harvested and sonicated the cells, and immediately quantitated the *o*-NPA metabolizing activity remaining in cell sonicates. Cell lines used for these assays included U373MG glioma cells that had been transfected with a control pIRES plasmid (U373pIRESneo; Clontech, Palo Alto, CA) or this plasmid encoding hiCE (U373pIREShiCE) or rCE (U373pIRESrCE). U373pIRESneo, U373pIREShiCE, and U373pIRESrCE cells express ~10, 200, and 1,000 units, respectively, of carboxylesterase activity per milligram of protein per minute. Data from the cell lines transfected to express hiCE or rCE show that compounds **1** to **4** inhibited carboxylesterase activity in sonicates (Fig. 4), supporting the *in vitro* data in Fig. 3 and confirming that these inhibitors cross the cell membrane. It was also apparent that compound **3** preferentially inhibited hiCE (14% carboxylesterase activity remaining) compared with rCE (30% carboxylesterase activity remaining) and compound **4** selectively inhibited rCE (2% carboxylesterase activity remaining) compared with hiCE (35% carboxylesterase activity remaining). The endogenous carboxylesterase activity in the vector-transfected U373pIRESneo cell line is minimal; therefore, the effect of inhibitors could not be reliably quantitated in this cell line.

Inhibition of hiCE or rCE in Intact Glioma Cells Assessed by Increases in EC_{50}s of CPT-11 by Compounds 1 to 4

In the second series of experiments to determine the effect of inhibitors on carboxylesterase activity *in situ*, cells were preincubated with one of the four inhibitors (5 μmol/L) for 1 hour, and a range of concentrations of CPT-11 was added to the culture medium for an additional hour (Table 2). The EC_{50} values for CPT-11 were determined in growth inhibition assays. Results in Figs. 3 and 4 would predict that preincubation of all four compounds with cell lines expressing carboxylesterases that activate CPT-11 would increase the EC_{50} of this drug. As expected, all compounds made the U373pIREShiCE and U373pIRESrCE cell lines less sensitive to CPT-11 and induced little or no change in the EC_{50} of CPT-11 for the U373pIRESneo cells. Data in Fig. 3 and Table 1 would also predict that compound **3** would preferentially increase the EC_{50} of CPT-11 for U373pIREShiCE cells and compound **4** would selectively increase the EC_{50} for U373pIRESrCE cells. Data in Table 2 support these predictions.

In the absence of inhibitor, the EC_{50} values for CPT-11 were 10, 0.75, and 0.06 μmol/L for U373pIRESneo, U373pIREShiCE, and U373pIRESrCE cells, respectively. Preincubation with compounds **1** to **4** increased the EC_{50} values for CPT-11 in U373pIREShiCE and U373pIRESrCE from 13- to >1,500-fold. These results show that *in situ* all four compounds inhibited conversion of CPT-11 to SN-38

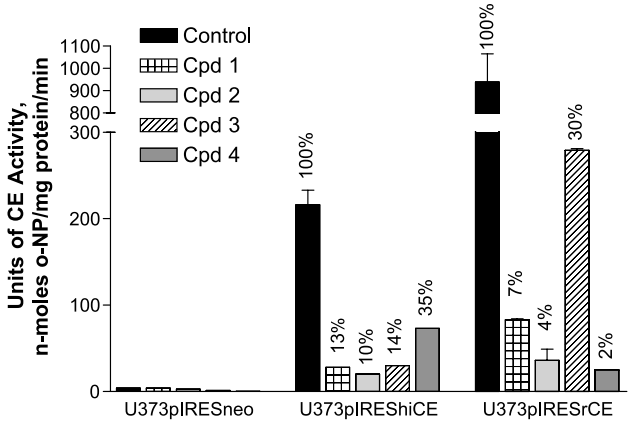

Figure 4. Inhibition of carboxylesterase activity in intact cells as assessed by quantitation of enzyme activity using the general carboxylesterase substrate *o*-NPA. Intact U373pIRESneo, U373pIREShiCE, or U373pIRESrCE cells were incubated with 5 μmol/L of the indicated inhibitor for 1 hour. Cell pellets were harvested and sonicated, and carboxylesterase activity in the sonicates was quantitated within 30 seconds of sonication. *Columns,* nmol *o*-NP produced/mg protein/min. The percentage notations reflect the percentage of enzyme activity compared with ''no inhibitor'' controls for each cell line (enzyme). For the U373pIREShiCE cells, 100% activity was 211 ± 17 units; for the U373pIRESrCE cells, 100% activity was 1,144 ± 289 units. U373pIRESneo cells express 4 units of carboxylesterase activity.

Table 1. K_i values of inhibitors

Enzyme	Inhibitor	K_i (nmol/L)	r^2
hiCE	**1**	71	0.999
	2	160	0.999
	3	2,000	0.999
	4	430	0.994
rCE	**1**	35	0.997
	2	49	0.994
	3	21,400	0.985
	4	74	0.995

NOTE: Values were obtained from sigmoidal curve fits of data following incubation with the different inhibitors. K_i values were calculated from these curves using GraphPad Prism software. Refer to Materials and Methods for details of the method.

Table 2. Inhibition of hiCE or rCE in intact glioma cells

U373 Cell Line	No Inhibitor	+**1**	+**2**	+**3**	+**4**
		EC_{50} CPT-11 (μmol/L)			
pIRESneo	10	70	49	33	8.1
pIREShiCE	0.75	>100	>100	>100	9.8
pIRESrCE	0.06	>100	83	29	5.9
		Fold increase in EC_{50}			
pIRESneo	1	7×	5×	3×	0.8×
pIREShiCE	1	>130×	>130×	>130×	13×
pIRESrCE	1	>1,660×	1,380×	480×	98×

NOTE: U373MG cells were transfected with a control plasmid (U373pIRESneo) or pIRES encoding hiCE or rCE (U373pIREShiCE or U373pIRESrCE, respectively). Growth inhibition experiments were done by preincubating each cell line with 5 μmol/L of compounds **1**–**4** for 1 hour and a range of concentrations of CPT-11 for an additional hour. Results are expressed as the concentration of CPT-11 required to inhibit the growth of each cell line by 50% (EC_{50}).

by both hiCE and rCE as measured by an increase in the EC_{50} of cell lines transfected to express carboxylesterases that activate CPT-11. Data in Table 2 also show that compound **4** is a more potent inhibitor of rCE than hiCE and that this preferential inhibition is particularly notable because U373pIRESrCE cells express ~5-fold more carboxylesterase activity than the U373pIREShiCE cells. These data corroborate data in Figs. 3 and 4. Additionally, we noted that, whereas compound **4** was more potent than compound **3** in *in vitro* assays (Fig. 3), compound **4** increased the CPT-11 EC_{50} in intact cells by the smallest increment compared with the other three inhibitors (Table 2). This observation suggests that intracellular levels or distribution of compound **4** may limit its ability to inhibit carboxylesterases in intact cells.

Discussion

This study partially characterizes four nitrophenol derivatives predicted to inhibit carboxylesterases. These compounds were the most potent carboxylesterase inhibitors of a series of compounds with increasingly large carbamate-linked side chains attached in the *p* position to a nitrophenyl ring. The four compounds on which this study focused were relatively lipophilic, with ClogP values ranging from 1.7 to 5.7, and were all expected to diffuse passively across cell membranes. The data document the novel finding that selective inhibition of CPT-11 metabolism by two specific carboxylesterases, hiCE and rCE, can be achieved. These two enzymes activate CPT-11 more efficiently than any other known enzymes and are being characterized extensively as part of a long-range goal to develop small molecule inhibitors that might be used to treat toxic side effects of CPT-11 by inhibiting its conversion to SN-38. Compounds **3** and **4** are considered to be lead compounds in the development of specific inhibitors for hiCE and rCE and are the first reported inhibitors having this selectivity.

Whereas many inhibitors of acetylcholinesterase have been developed (17–22) and some of these are used clinically, the single class of inhibitors reported to be selective for a specific carboxylesterase is the benzodioxaphosphorines, bomin-1, bomin-2, and bomin-3 (Patent USSR 06.22.1985, No. 1187444). These three compounds were found to inhibit rat liver carboxylesterase at ~10-fold lower concentrations than human erythrocyte acetylcholinesterase and horse serum butyrylcholinesterase. The benzodioxaphosphorines did not inhibit recombinant rCE at concentrations up to 100 μmol/L.[3] We have previously characterized compounds **1** to **4** with respect to their ability to inhibit general carboxylesterase activity (12).

Based on published data (11, 12, 16, 23), we proposed that nitrophenol derivatives would have access to the catalytic gorge and affinity for the essential amino acids (Ser, Glu, and His; amino acids 221, 353, and 467 for rCE and amino acids 240, 364, and 478 for hiCE) of carboxylesterases (11). We also postulated that access of inhibitors to the catalytic gorge of specific carboxylesterases could be modulated by the overall size of the inhibitor (23), the linkage by which moieties are attached to nitrophenol (12), and the position at which side chains are attached (12). Because a series of *p*-NPs was found to have higher affinities for rCE than similar *o*-NPs, we focused on a series of potential inhibitors composed of side chains linked to NP in the *p* position attached via a carbamate group. We anticipated that compounds **1** and **2** would be less selective for specific carboxylesterases than compounds **3** and **4**, as their relatively small size would not restrict their entry into the catalytic pocket of carboxylesterases (23). It was unknown whether the compounds would be inhibitors or substrates for carboxylesterases. None of the four compounds was metabolized by hiCE or rCE (data not shown). As mentioned above, previously published data indicate that overall size and the linkage and the position of side chains determine at least in part the interaction of small molecules with carboxylesterases. According to these known criteria, we hypothesized that compounds **1** and **2** would efficiently inhibit both enzymes, and this prediction is supported by data in Table 1. Further, we also proposed that, based on overall size and bulkiness, compound **4** would inhibit both rCE and hiCE. However, the data show that compound **4** inhibited rCE 6-fold more effectively than hiCE. This specificity is likely due to the differences in dimensions of the entrance to the gorge containing the catalytic amino acids (23). However, unexpectedly, compound **3** showed a K_i value 10-fold lower for hiCE compared with rCE.

Because the linkage and position of the side chains in compounds **3** and **4** are identical, these two criteria cannot explain the selectivity of compound **3** for hiCE. Further, the size of the furan-2-carbonyl moiety in compound **3** would not preclude entry of this molecule into the rCE active site. The data suggest that a previously unrecognized molecular characteristic likely influences interaction of these inhibitors with rCE and hiCE. In this regard, we note that compounds **3** and **4** have ClogP values of 1.7 and 5.7, respectively, indicating that the hydrophobicity of these two compounds differs by ~4 logs. Therefore, as has been reported for carboxylesterase substrates (23), we postulate that hydrophobicity is also a determinant of enzyme specificity for carboxylesterase inhibitors.

For these types of inhibitors to be of eventual use clinically, it will be essential that they be absorbed by intestinal epithelial cells but poorly absorbed systemically. This strategy has the potential to modulate SN-38 production specifically in the gastrointestinal tract. Such an approach would be predicted to improve the therapeutic index of CPT-11 for the treatment of solid tumors, with the exception of the rare tumors of the small intestine.

In summary, the long-range goal of this work is to develop inhibitors that can be used to reverse toxic side effects following administration of the prodrug CPT-11 to

[3] P.M. Potter, data not shown.

cancer patients. This is the first report of selective inhibitors of hiCE and rCE, the most efficient enzymes known to activate CPT-11. These inhibitors are nitrophenyl derivatives that have carbamate-linked side chains of increasing size and are considered lead compounds for development of this class of compounds for translational use.

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