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Synthesis and Evaluation of Esters and Carbamates to Identify Critical Functional Groups for Esterase-specific Metabolism

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Abstract—In an effort to develop novel prodrugs for viral directed enzyme prodrug therapy (VDEPT) approaches to chemotherapy, eleven esters and carbamates of *o*-nitrophenol, *p*-nitrophenol, and β-naphthol were synthesized and characterized as substrates for rabbit (rCE) and human liver (hCE1) carboxylesterases. All of the esters of *o*-, *p*-nitrophenols, and β-naphthols showed moderate hydrolysis by both rCE and hCE1. Esters of β-naphthols exhibited higher hydrolysis rates compared to esters of *p*-nitrophenols by rCE. Of the carbamates, 4-benzyl-piperazine-1-carboxylic acid 2-nitrophenol showed preferential hydrolysis by rCE compared to hCE1 with a V_{max} of 54.4 μmoles/min/mg, and a K_m value of 1071 μM. Substrate metabolism by a specific CE or inhibition of CEs by each compound depended on several factors, including the types of functional groups and linking moieties.

Introduction

Carboxylesterases (CEs) belong to a group of serine esterases found in virtually all animals and mammalian tissues.[1] Mammalian CEs are usually associated with metabolism of water-soluble drugs and xenobiotics.[2–4] CPT-11, a water-soluble camptothecin analogue used clinically for its anti-tumor activity, is activated by esterases.

CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy camptothecin, irinotecan) is a carbamate prodrug that, when activated, inhibits topoisomerase I (Fig. 1). CPT-11 has a piperidino side chain at the C-10 position that is cleaved by CEs present in human liver and intestine. The cleavage of this side chain by CEs yields SN-38 (7-ethyl-10-hydroxycamptothecin), the active form of this drug.[5] However, CPT-11 is activated very inefficiently by human CEs (hCEs). Depending on the schedule of administration, only 0.1–5% of this prodrug is converted to the active drug SN-38 in vivo,[5] possibly because either human CEs are inefficient at drug catalysis or they are not expressed at high levels in tissues exposed to this prodrug. A rabbit liver CE (rCE) is the most efficient enzyme thus far identified in the activation of CPT-11.[3,6] Human liver microsomal CE 1 (hCE1) is one of the least efficient enzymes in activating this prodrug.[6]

The ability of CE to metabolize CPT-11 depends on the access of the drug to the catalytic site of the enzyme, and this access is restricted by the size of the entrance to gorge containing the active site.[7] The entrance to the gorge containing the active site of rCE is larger than that of hCE1(3.2 and 2.9 Å for rCE and hCE1, respectively), and rCE is 100- to 1000-fold more efficient than hCE1 at converting CPT-11 to SN-38. Therefore it should be possible to synthesize drugs that have access to the catalytic site of rCE, but not hCE1, for use in VDEPT applications.

Viral directed enzyme prodrug therapy (VDEPT) is a type of gene therapy that uses a viral vector to deliver a cDNA encoding an enzyme that converts a nontoxic prodrug to a cytotoxic drug.[8,9] The principal advantage of VDEPT compared to conventional chemotherapy is its potential to be tumor-specific. Our laboratories recently characterized a VDEPT system using adenovirus to deliver the cDNA

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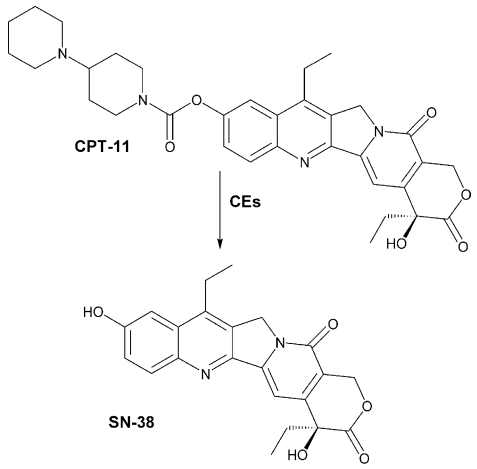


Figure 1. Structure and hydrolysis of CPT-11 by carboxylesterases.

encoding a rCE that efficiently activates CPT-11.[10–13] We now intend to characterize functional groups and linkages specific for activation by rCE, to design additional prodrugs for this VDEPT approach. Our long range goal is to develop tumor-specific combination chemotherapy.

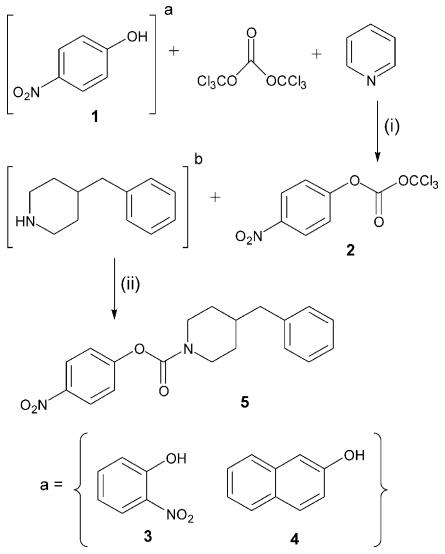
Toward achieving this goal, we determined the specificity of substrates for rCE by using compounds of different chemical classes, and by varying the two main structural features that influence the activity of mammalian CEs: the functional and linking groups of the compounds.

We synthesized a series of esters and carbamates containing *o*- or *p*-nitrophenol or β-naphthol as the parent moiety and tested them as substrates for hCE1 and rCE. The purpose of this study was to determine the feasibility of synthesizing compounds as CE substrates and to identify critical residues that confer preferential activation by specific CEs. This was accomplished by (i) the synthesis of compounds having a variety of secondary amines coupled to different sizes of parent molecules, coupled to side chains of various sizes by carbamate or ester linkages, and (ii) kinetic studies of the synthesized compounds with rCE and hCE1.

Results and Discussion

Synthesis of esters and carbamates of *o*- and *p*- nitrophenol (NP) and β-naphthol (NAP). A series of esters of *o*- and *p*-NP, and β-NAP were prepared in a single step reaction of *o*- or *p*-NP, or β-NAP with benzoyl chloride (or trimethoxy benzoyl chloride) in triethylamine.[14] The synthesis of the carbamate-linked derivatives was accomplished using triphosgene,[15] with subsequent coupling to various amines[16] (Scheme 1). Secondary amines were selected based on a previous study by Tsuji et al. who showed that benzyl piperazine derivatives were preferentially activated by a rat serum CE.[17]

Hydrolysis of esters of *o*- and *p*-nitrophenol (NP) by CEs. One of our specific aims was to identify critical

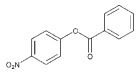
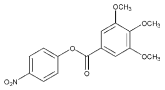
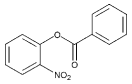
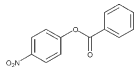
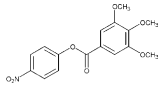
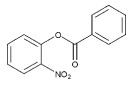


Scheme 1. Synthetic scheme for carbamates. This specific synthesis pictured used *p*-NP and 4-benzyl piperidine as starting materials. All other carbamate-linked derivatives were prepared using this same general scheme. Starting material (a) used included *p*-nitrophenol **1**, *o*-nitrophenol **3**, and β-naphthol **4**. The variety of secondary amines (b) used for coupling included 4-benzyl piperidine, 1-benzyl piperazine, 1-(4-chlorobenzylhydryl) piperazine, and 1-(2-furosyl) piperazine. Reagents: (i) dichloromethane, 1 h, Ar (ii) pyridine, 12 h, Ar.

linking groups that make general CE substrates selective for rCE. The esters of two *p*-, and one *o*-NP (compounds **11**–**13**), and two β-NAP (compounds **14**–**15**) were synthesized and tested for their enzymatic activation by both rCE and hCE1. Table 1 shows the ability of rCE and hCE1 to hydrolyze compounds **11**–**13**. V_{max} values for these compounds ranged from 0.95 to 8.51 μmol/min/mg for hCE1, and from 1.21 to 12.61 μmol/min/mg by rCE (Table 1). All of the NP benzoate (**11**–**13**) were hydrolyzed slightly faster by rCE (1.3, 1.5, and 1.5-fold for **11**, **12**, and **13**, respectively) than by hCE1. Of these esters, *o*-NP benzoate (**13**) had the highest V_{max} values and also showed the greatest difference (~2.7-fold) in K_m (rCE > hCE1) for both enzymes. However, the overall conclusion from these data was that all three ester-linked nitrophenol derivatives were readily activated by both rCE and hCE1, indicating that a single ring structure containing varied ester-linked side chains did not provide selectivity for rCE compared to hCE1. Therefore, we next evaluated two member ring structures, also having ester-linked side chains.

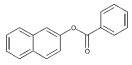
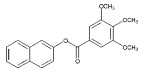
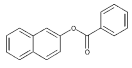
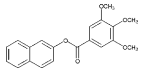
Hydrolysis of esters of β-naphthol by CEs. The benzoic acid naphthalen-2-yl-ester (**14**), showed a slightly greater V_{max} value for rCE compared to hCE1 (2.5-fold), indicating a slightly faster turnover rate by rCE. This is consistent with the k_{cat}/K_m values of 34.1 for rCE and 26 for hCE1, and indicates a better substrate efficiency of about 1.3-fold (Table 2). On the other hand, 3,4,5-trimethoxy-benzoic acid naphthalen-2-yl-ester (**15**), exhibited very similar V_{max} values, and K_m values for both rCE and hCE1, again indicating that, similar to compounds **11**–**13**, an ester linkage did not confer sufficient selectivity for rCE compared to hCE1 to be considered for future prodrug development. Therefore, since the dipiperidino side chain of CPT-11 is

Table 1. Kinetic parameters for the metabolism of three esters of nitrophenol by rCE and hCE1

Enzyme	Compd[a]	Structure	K_m[b] (μM)	V_{max}[c] (μmol/min/mg)	Curve fit (R^2)	k_{cat}/K_m (min^{-1} mM^{-1})
hCE1	**11**		184.5±51.0	0.95±0.09	0.9909	11.1
	12		311.9±238.8	1.71±0.37	0.9652	11.9
	13		98.1±12.9	8.51±0.24	0.9970	187.8
rCE	**11**		431.8±168.0	1.21±0.23	0.9945	9.4
	12		509.9±154.8	2.55±0.41	0.9980	16.7
	13		272.6±22.4	12.61±0.32	0.9994	154.2

[a]Compound synthesized.
[b,c]K_m and V_{max} values were presented as mean±S.E.M. of triplicate experiments. Values were calculated from non-linear regression fit by using GraphPad Prism.

Table 2. Kinetic parameters for the metabolism of two esters of β-naphthol by rCE and hCE1

Enzyme	Compd	Structure	K_m[a] (μM)	V_{max}[b] (μmol/min/mg)	Curve *fit* (R^2)	k_{cat}/K_m (min^{-1} mM^{-1})
hCE1	**14**		1200±485.5	14.44±2.18	0.9232	26.0
	15		918.9±738.7	16.67±7.84	0.9121	39.3
rCE	**14**		3593±3345	36.80±18.52	0.8634	34.1
	15		1180±809	15.46±6.69	0.9980	43.7

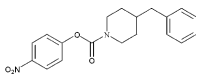
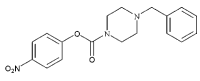
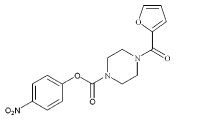
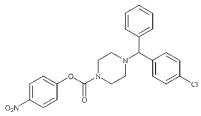
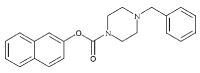
[a,b]K_m, and V_{max} values were presented as mean±S.E.M. of triplicate experiments. Values were calculated from non-linear regression fit by using GraphPad Prism.

linked to the camptothecin moiety by a carbamate linkage, we next evaluted one- and two-ring structures containing carbamate-linked side chains.

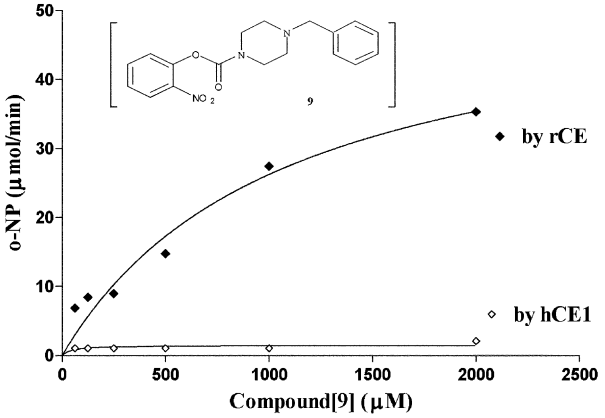
Inhibitory effect of carbamates of *p*-nitrophenol (NP) and β-naphthol (NAP). None of the carbamates of *p*-NP (**5**–**8**) or β-NAP (**10**) were metabolized by either rCE or hCE1, but instead these compounds inhibited the metabolism of the general CE substrate *o*-nitrophenylacetate (NPA) by both enzymes. With the single-ring *p*-NP derivatives, a 10 min pre-incubation of rCE or hCE1 with carbamates of *p*-NP (**5**–**8**) resulted in a concentration-dependent inhibition of esterase activity as measured by metabolism of the general esterase substrate *o*-NPA. Table 3 shows the inhibitory potencies of the carbamates synthesized. The 50% inhibitory concentrations (IC_{50}) of these compounds ranged from 3 nM to 1.35 μM for rCE, and from 21 to 93 nM for hCE1. Similar to results with single ring structures, data with carbamate of β-NAP (**10**) exhibited inhibitory activity for both CEs (Table 3). These data are consistent with published data that carbamates are known inhibitors of CEs.[18,19]

The observation that carbamates inhibited, but were not metabolized by CEs, suggested several possibilities. First, these compounds might have a high affinity for the catalytic site, making them relatively irreversible inhibitors regardless of whether the side chain is cleaved. Second, these compounds may reversibly bind to the active site forming a tetrahedral intermediate[20] However, collapse of this transition state to cleave the phenol could be energetically limiting. Third, the nitro group on *p*-NP may interact with functional groups

Table 3. Inhibitory potencies of carbamates of *p*-nitrophenol and β-naphthol

Compd	Structure	Enzyme	IC_{50}[a] (μM)	Curve fit[b] (R^2)
5		hCE1	0.021	0.9666
		rCE	0.032	0.9989
6		hCE1	0.089	0.9971
		rCE	0.200	0.9947
7		hCE1	0.062	0.8778
		rCE	1.35	0.9985
8		hCE1	0.093	0.9748
		rCE	0.003	0.9967
10		hCE1	316.2	0.9961
		rCE	112.2	0.9999

Control values for *o*-NP produced from 2 mM *o*-NPA were 171.89±5.56, and 46.33±1.67 μmoles/min/mg for rCE and hCE1, respectively.
[a,b]IC_{50} and R^2 values were calculated from non-linear regression fit by using GraphPad Prism.



	K_m[a] (μM)	V_{max}[b] (μmol/min/mg)	K_{cat}/K_m ($min^{-1}mM^{-1}$)
rCE	1071 ± 414.7	54.4 ± 10.4	169.2
hCE1	46.3 ± 55.7	1.5 ± 0.3	68.8

[a,b] K_m and V_{max} values were presented as mean ± SEM of triplicate experiments.

Figure 2. The effect of 4-benzyl-piperazine-1-carboxylic acid 2-nitro-phenyl ester (**9**) hydrolysis by two different CEs (rCE and hCE1).

adjacent to the active site of CEs. This would increase the energy of cleavage for the phenol ring from the parent molecule. Fourth, carbamate derivatives might be metabolized, but the parent compound or the moieties generated by catalysis might bind to sites other than the active site to alter the 3-dimensional structure of the enzyme, thereby inhibiting the access of other substrates to the catalytic gorge. Because CPT-11 contains a carbamate-linked side chain and is metabolized by CEs, we favor the fourth hypothesis. More specifically, as has been proposed for acetylcholinesterases, CEs may have a 'back door' through which cleaved moieties pass and bind transiently.[21] In support of this hypothesis, Bencharit et al.,[21] showed that the structure of rCE is consistent with the presence of a back door for the 4-dipiperidino leaving group after hydrolysis of CPT-11. After CPT-11 binds to the active site, hydrolysis occurs to produce SN-38 by cleaving the 4-dipiperidino moiety; this leaving group is then proposed to pass through the back door to bind adjacent to the Asn residue at position 389 in rCE. Overall, the data indicate that it is essential to optimize both the functional and leaving groups in designing CE prodrugs, to facilitate rapid enzymatic turnover and therefore efficient prodrug activation.

Hydrolysis of 4-benzyl-piperazine-1-carboxylic acid 2-nitro-phenyl ester (9) by CEs. None of the synthesized carbamates of *p*-NP was metabolized by rCE or hCE1. However, the *o*-NP carbamate derivative, 4-benzyl-piperazine-1-carboxylic acid 2-nitro-phenyl carbamate (**9**), was preferentially hydrolyzed by rCE (Fig. 2). The V_{max} value for rCE was 54.4±10.4 μmol/min/mg, ~40-fold higher than that for hCE1 (1.5±0.3 μmol/min/mg). k_{cat}/K_m values were 169.2 and 68.8 $min^{-1}mM^{-1}$ for rCE and hCE1, respectively. Similar to other ester derivatives, a lower K_m value was observed for hCE1 (46.3±55.7 μM) as compared to rCE (1071±414.7 μM) with compound **9**. The data show that 4-benzyl-piperazine-1-carboxylic acid 2-nitro-phenyl ester (**9**) demonstrated preferential hydrolysis by rCE. Of note, the single difference between **6** (which inhibited rCE) and **9** (which was efficiently metabolized by rCE) is that the nitro group in phenol ring was attached at the *para*- versus *ortho*- positions, respectively, suggesting that the minor structural changes greatly influence substrate-enzyme interaction. Collectively, the above results indicate that the parent compounds, linking moieties and leaving groups must be optimized to produce compounds sufficiently selective for a specific enzyme to be useful in VDEPT approaches. Our data also indicate that it is possible to achieve this enzyme selectivity. Specifically, the data suggest that the design of prodrugs for use with rCE should focus on molecules containing a carbamate-linked side chain sufficiently large to inhibit substrate access to human CEs compared to rCE, and that this side chain should be in the ortho position. Further, the data suggest that it may be possible to design selective inhibitors of specific esterases by using carbamate linking moieties.

Conclusion

The lack of tumor-specificity of anti-cancer drugs is a significant drawback in conventional chemotherapy. VDEPT is an approach to render anti-cancer drugs more selective for tumor cells by expressing a prodrug-activating enzyme specifically in tumor cells. Previously, we reported isolation and characterization of a cDNA

encoding a rCE that efficiently converts the prodrug CPT-11 to SN-38.[3] Those studies indicated that rCE/CPT-11 might be useful for VDEPT applications.[6,11,22]

In the current study, we synthesized additional potential substrates for mammalian CEs and investigated their specificity for catalytic hydrolysis by rCE. Since both the functional and linking groups of the substrate structures influence cleavage activity of mammalian CEs, we modified these two features to identify compounds selective for rCE. Our study showed that ester-linked substrates showed little difference in hydrolysis by rCE and hCE1, regardless of substrate size. Further, all carbamates of *p*-NP inhibited both enzymes. However, a carbamate derivative of *o*-NP (**9**) was hydrolyzed preferably by rCE. This information is being used to design prodrugs that, when activated, have mechanisms of cytotoxicity complementary to that of CPT-11, for future VDEPT applications.

Experimental

Synthesis

Materials. All the anhydrous solvents and starting materials were purchased from Aldrich Chemical Company (Wilwaukee, WI). All reagent grade solvents used for chromatography were purchased from Fisher Scientific (Suwanee, GA) and Flash column chromatography silica cartridges (MetaFlash) were obtained from Ansys Technologies Inc. (Lake Forest, CA).

Chemistry general

Triphosgene reaction was performed under a dry argon atmosphere. The reactions were monitored by thin layer chromatography (TLC) on pre-coated Merck 60 F_{254} silica gel plates and visualized using UV light (254 nm). Biotage FLASH 25+ column chromatography system was used to purify mixtures. Melting points were determined on a Thomas Hoover Capillary melting point apparatus (Thomas Scientific, Swedesboro, NJ) and are uncorrected. All ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX-300 (300 and 75 MHz for ^{1}H and ^{13}C NMR, respectively) or Varian INOVA-500 (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) spectrometers. Chemical shifts are reported in ppm (δ) relative to residual solvent peak or internal standard (tetramethylsilane) and coupling constants (*J*) are reported in hertz (Hz). Mass spectra were recorded on a Bruker Esquire LCMS using ESI.

General procedure for carbamates of nitrophenol and naphthol

4-Benzyl-piperidine-1-carboxylic acid 4-nitrophenyl ester (5). To a stirred solution of *p*-nitrophenol (0.5 g, 3.6 mmol), pyridine (1.45 mL, 18 mmol) in dichloromethane (20 mL), triphosgene (1.28 g, 4.3 mmol) was added under argon atmosphere. After 1 h stirring at room temperature, TLC was used to confirm that reactions were complete. 4-benzyl piperidine (1.58 mL, 9 mmol) and pyridine (0.1 mL, 1.25 mmol) were then added to the mixture, and the reaction mixture was stirred for 12 h at room temperature. Dichloromethane was removed in vacuo and the residual reaction mixture was extracted with ethyl acetate (3×30 mL). The organic layer was washed with water (2×30 mL), and dried over anhydrous sodium sulfate. The crude product was purified by column chromatography using petroleum ether/ethyl acetate (8/1) to give **5** as a white solid (0.45 g, 37%). TLC $R_f = 0.6$ (petroleum ether/ethyl acetate, 8/1); mp 92–94 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.3 (q, 2H, $J = 12$, 12 Hz, CH_2CH), 1.78 (d, 2H, $J = 12$ Hz, CH_2CH), 1.8–1.9 (m, 1H, CH$_2$C*H*), 2.6 (d, 2H, $J = 7.5$ Hz, CH_2Ph), 2.83 (t, 1H, $J = 12$ Hz, N-C*H*), 2.96 (t, 1H, $J = 12$ Hz, N-C*H*), 4.25 (br s, 2H, N-CH_2), 7.18 (d, 2H, $J = 7.5$ Hz, Ph-H_2), 7.24–7.35 (m, 3H, Ph-H_3), 7.32 (d, 2H, $J = 9.4$ Hz, ArH_2), 8.25 (d, 2H, $J = 9.4$ Hz, ArH_2); ^{13}C NMR (125 MHz, $CDCl_3$) 39.0, 42.6, 44.6, 122.2, 125.2, 126.2, 128.8, 129.4, 140.0, 144.8, 152.2, 156.8; MS (ESI) *m*/*z* 363.4 $[M + Na]^+$.

The following compounds (**6**–**10**) were prepared in a similar manner as described above for compound **5**.

4-Benzyl piperazine-1-carboxylic acid 4-nitrophenyl ester (6). The reaction of *p*-nitrophenol (0.8 g, 5.8 mmol), pyridine (2.3 mL, 28.5 mmol) in dichloromethane (20 mL), triphosgene (2 g, 6.9 mmol) and 1-benzyl piperazine (2.5 g, 14.4 mmol) gave a crude product. This crude mixture was purified by column chromatography using petroleum ether/ethyl acetate (9/1) to give **6** as a white solid (0.27 g, 14%). TLC $R_f = 0.4$ (petroleum ether/ethyl acetate, 9/1); mp 238–239 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.8 (br s, 2H, N-CH_2), 3.5 (br s, 2H, CH_2Ph), 3.95 (br s, 1H, N-C*H*), 4.18–4.38 (m, 5H, 2× N-CH_2, N-C*H*), 7.3 (d, 2H, $J = 8.3$ Hz, ArH_2), 7.48–7.52 (m, 3H, Ph-H_3), 7.63–7.65 (m, 2H, Ph-H_2), 8.3 (d, 2H, $J = 8.3$ Hz, ArH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 49.87, 59.08, 122.17, 122.37, 124.70, 125.10, 128.57, 128.83, 129.36, 131.28, 144.48, 151.26, 155.59; MS (ESI) *m*/*z* 342.3 $[M + H]^+$.

4-(Furan-2-carbonyl)-piperazine-1-carboxylic acid 4-nitro-phenyl ester (7). The reaction of *p*-nitrophenol (0.8 g, 5.8 mmol), pyridine (2.3 mL, 28.5 mmol) in dichloromethane (20 mL), triphosgene (2 g, 6.9 mmol) and 1-(2-furosyl)-piperazine (2.5 g, 14.4 mmol) gave a crude product. This crude product was recrystalized with methanol to give 7 as off-white solid (0.98 g, 49%). TLC $R_f = 0.25$ (petroleum ether/ethyl acetate, 9/1); mp 157–159 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.7 (d, 4H, $J = 4$ Hz, 2×N-CH$_2$), 3.9 (s, 4H, 2×N-CH$_2$), 6.54 (d, 1H, $J = 3.2$ Hz, furosyl H), 7.12 (d, 1H, $J = 3.2$ Hz, furosyl H), 7.34 (d, 2H, $J = 8.3$ Hz, ArH_2), 7.5 (s, 1H, furosyl H), 8.3 (d, 2H, $J = 8.3$ Hz, ArH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 43.60, 44.17, 46.32, 111.01, 116.71, 116.93, 121.72, 124.54, 143.51, 144.46, 147.04, 151.62, 155.43, 158.64; MS (ESI) *m*/*z* 368.3 $[M + Na]^+$.

4-[(4-Chloro-phenyl)-phenyl-methyl]-piperazine-1-carboxylic acid 4-nitro-phenyl ester (8). The reaction of *p*-nitrophenol (0.35 g, 2.5 mmol), pyridine (0.9 mL, 11.2 mmol) in dichloromethane (20 mL), triphosgene (0.8 g,

2.7 mmol) and 4-(chloro-benzhydryl)-piperazine (1.5 g, 5.2 mmol) gave a crude product. This crude product was purified by column chromatography using petroleum ether/ethyl acetate (9/1) to give **8** as a white solid (0.38 g, 33%). TLC R_f = 0.6 (petroleum ether/ethyl acetate, 9/1); mp 65–66 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.5 (t, 4H, J = 4 Hz, 2×N-CH_2), 3.6 (br s, 2H, N-CH_2), 3.7 (br s, 2H, N-CH_2), 4.3 (s, 1H, CHAr$_2$), 7.25–7.35 (m, 7H, ArH_7), 7.36–7.42 (m, 2H, ArH_2), 7.4 (d, 2H, J = 8.8 Hz, ArH_2), 8.35 (d, 2H, J = 8.8 Hz, ArH_2); ^{13}C NMR (75 MHz, CD_3OD) δ 50.53, 50.72, 74.36, 121.72, 124.07, 125.10, 126.56, 127.06, 127.79, 127.85, 128.60, 131.96, 144.49, 152.00, 155.71; MS (ESI) m/z 474.3 $[M + Na]^+$.

4-Benzyl-piperazine-1-carboxylic acid 2-nitro-phenyl ester (9). The reaction of *o*-nitrophenol (0.8 g, 5.8 mmol), pyridine (2.3 mL, 28.5 mmol) in dichloromethane (20 mL), triphosgene (2 g, 6.9 mmol) and 1-benzyl piperazine (2.5 g, 14.4 mmol) gave a crude product. This crude product was purified by column chromatography using petroleum ether/ethyl acetate (9/1) to give **9** as a white solid (0.29 g, 15%). TLC R_f = 0.3 (petroleum ether/ethyl acetate, 9/1); mp 206–208 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.9 (q, 1H, J = 11.3, 11.3 Hz, N-CH), 3.05 (q, 1H, J = 11.3, 11.3 Hz, N-CH), 3.4 (t, 2H, J = 11.3 Hz, CH_2Ph), 3.9 (t, 1H, J = 12.5 Hz, N-CH), 4.15 (t, 1H, J = 12.5 Hz, N-CH), 4.15–4.4 (m, 4H, 2×N-CH_2), 7.31, 7.54 (dd, 1H, J = 2.2, 8.7 Hz, ArH), 7.43, 7.5 (td, 1H, J = 2.2, 6.5 Hz, ArH), 7.46–7.5 (m, 3H, PhH_3), 7.7, 7.75 (td, 1H, J = 2.2, 6.5 Hz, ArH), 7.69–7.73 (m, 2H, PhH_2), 8.14, 8.18 (dd, 1H, J = 2.2, 8.7 Hz, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 50.46, 60.03, 60.45, 124.31, 124.86, 125.33, 125.50, 126.30, 127.00, 127.26, 128.83, 129.87, 130.83, 134.80, 134.97, 140.77, 143.22, 143.76, 151.45; MS (ESI) m/z 342.3 $[M + H]^+$.

4-Benzyl-piperazine-1-carboxylic acid naphthalene-2-yl ester (10). The reaction of β-naphthol (0.15 g, 1.0 mmol), pyridine (0.5 mL, 6.2 mmol) in dichloromethane (20 mL), triphosgene (0.62 g, 2 mmol) and 1-benzyl piperazine (0.47 g, 2.6 mmol) gave a crude product. This crude mixture was purified by column chromatography using petroleum ether/ethyl acetate (4/1) to give **10** as a white powder (0.065 g, 18%). TLC R_f = 0.25 (petroleum ether/ethyl acetate, 4/1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.5 (t, 4H, J = 4.2 Hz, 2×N-CH_2), 3.6 (s, 2H, CH_2Ph), 3.62 (s, 2H, N-CH_2), 3.72 (s, 2H, N-CH_2), 7.25–7.36 (m, 5H, ArH_5), 7.42–7.52 (m, 2H, ArH_2) 7.58 (d, 1H, J = 2.7 Hz, ArH), 7.78–7.88 (m, 4H, ArH_4); MS (ESI) m/z 347.3 $[M + H]^+$.

General procedure for carboxylic esters of nitrophenol and naphthol

Benzoic acid 4-nitro-phenyl ester (11). Triethylamine (1 mL, 7.2 mmol) was added to a solution of *p*-nitrophenol (1 g, 7.2 mmol) in dichloromethane (30 mL). Benzoyl chloride (3 g, 21.3 mmol) was added slowly to the mixture in an ice bath. The ice bath was then removed, and the reaction mixture was stirred overnight at room temperature. The reaction solvent was then evaporated in vacuo. Excess dichloromethane was added, and the mixture was washed with water (2×30 mL) and dried over sodium sulfate. After the solvent was removed in vacuo, the mixture was subjected to column chromatography with petroleum ether/ethyl acetate (4/1) to give **11** as a white solid (0.3 g, 17%). TLC R_f = 0.8 (petroleum ether/ethyl acetate, 4/1); mp 132–133 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42 (d, 2H, J = 9.1 Hz, PhH_2), 7.55 (t, 2H, J = 7.6 Hz, PhH_2), 7.7 (t, 1H, J = 7.6 Hz, PhH), 8.2 (d, 2H, J = 7.6 Hz, ArH_2), 8.34 (d, 2H, J = 7.5 Hz, ArH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 122.09, 124.72, 128.05, 128.27, 129.79, 133.71, 144.89, 155.23, 163.68; MS (ESI) m/z 266.0 $[M + Na]^+$.

The following compounds (**12**–**15**) were prepared in a similar manner as described above for compopund **11**.

3,4,5-Trimethoxy-benzoic acid 4-nitro-phenyl ester (12). The reaction of *p*-nitrophenol (1 g, 7.2 mmol), triethylamine (1 mL, 7.2 mmol) in dichloromethane (30 mL) and 3,4,5-trimethoxy benzoyl chloride (5 g, 21.6 mmol) gave a crude product. This crude product was purified by column chromatography using petroleum ether/ethyl acetate (9/1) to give **12** as a white solid (0.31 g, 13%). TLC R_f = 0.4 (petroleum ether/ethyl acetate, 9/1); mp 164–165 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.0 (s, 9H, 3×OCH_3), 7.42 (dd, 2H, J = 2.6, 6.6 Hz, ArH_2), 7.46 (s, 2H, ArH_2), 8.36 (dd, 2H, J = 2.6, 6.6 Hz, ArH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ55.85, 60.20, 107.19, 122.51, 122.82, 124.71, 142.77, 144.82, 152.63, 155.34, 163.17; MS (ESI) m/z 356.1 $[M + Na]^+$.

Benzoic acid 2-nitro-phenyl ester (13). The reaction of *o*-nitrophenol (1 g, 7.2 mmol), triethylamine (1 mL, 7.2 mmol) in dichloromethane (30 mL) and benzoyl chloride (3 g, 21.3 mmol) gave a crude product. This crude product was purified by column chromatography using petroleum ether/ethyl acetate (9/1) to give **13** as a off-white solid (0.19 g, 11%). TLC R_f = 0.4 (petroleum ether/ethyl acetate, 4/1); mp 54–56 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44–7.62 (m, 5H, ArH_5), 7.72–7.82 (m, 2H, ArH_2), 8.18–8.32 (m, 2H, ArH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 124.86, 125.29, 126.08, 127.95, 128.17, 128.76, 129.67, 129.99, 133.25, 133.59, 134.08, 143.84, 163.83, 171.26; MS (ESI) m/z 266.0 $[M + Na]^+$.

Benzoic acid naphthalen-2-yl-ester (14). The reaction of β-naphthol (1 g, 6.9 mmol), triethylamine (1 mL, 7.2 mmol) in dichloromethane (30 mL) and benzoyl chloride (3 g, 21.3 mmol) gave a crude product. This crude product was purified by column chromatography using petroleum ether/ethyl acetate (4/1) to give 14 as a white solid (0.8 g, 47%). TLC R_f = 0.6 (petroleum ether/ethyl acetate, 4/1); mp 95–96 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 (dd, 1H, J = 2.3, 8 Hz, ArH), 7.54–7.63 (m, 4H, ArH_4), 7.72 (d, 1H, J = 6.8 Hz, ArH), 7.76 (d, 1H, J = 8 Hz, ArH), 7.9 (d, 1H, J = 6.8 Hz, ArH), 7.94 (d, 1H, J = 6.8 Hz, ArH), 7.98 (d, 1H, J = 8 Hz, ArH), 8.32 (d, 2H, J = 6.8 Hz, ArH_2); ^{13}C NMR (75 MHz, DMSO-d_6) δ 118.62, 121.54, 125.80, 126.64, 127.46, 127.66, 128.90, 129.33, 129.76, 130.27, 131.05, 133.33, 133.98, 134.99, 148.28, 164.74; MS (ESI) m/z 271.1 $[M + Na]^+$.

3,4,5-Trimethoxy-benzoic acid naphthalen-2-yl-ester (15). The reaction of β-naphthol (1 g, 6.9 mmol), triethylamine (1 mL, 7.2 mmol) in dichloromethane (30 mL) and 3,4,5-trimethoxy benzoyl chloride (4.5 g, 19.5 mmol) gave a crude product. This crude product was column chromatographed with petroleum ether/ethyl acetate (9/1) to give **15** as a white solid (1.1 g, 46%). TLC $R_f = 0.4$ (petroleum ether/ethyl acetate, 9/1); mp 114–116 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.0 (s, 9H, 3×OCH_3), 7.38 (dd, 1H, $J = 2.3$, 8.6 Hz, ArH), 7.5–7.58 (m, 4H, ArH_4), 7.7 (d, 1H, $J = 2.3$ Hz, ArH), 7.86 (d, 1H, $J = 8.6$ Hz, ArH), 7.91 (d, 1H, $J = 8.6$ Hz, ArH), 7.94 (d, 1H, $J = 8.6$ Hz, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 55.82, 60.45, 107.03, 118.21, 120.76, 123.91, 125.24, 126.09, 127.14, 127.30, 128.95, 131.02, 133.33, 142.45, 148.15, 152.60, 164.47; MS (ESI) m/z 361.2 $[M + Na]^+$.

Enzymes and assays

Purified carboxylesterases. Pure rCE and hCE1 were prepared from baculovirus-infected cell culture media as described previously.[23]

Hydrolysis of esters of nitrophenol (NP) by CEs. The hydrolysis of esters of NP was determined spectrophotometrically in 96-well plates (Dynex Technologies, Chantilly, VA) as described previously.[24] The incubation mixture contained various concentrations (0.05–2 mM) of substrate and 3 μg/mL of CE in 220 μL of 50 mM HEPES buffer, pH 7.4. The time-dependent liberation of *p*- or *o*-NP was monitored for 0.5 to 10 min by the change in A^{420}. Data were transferred to a computer, and the CE activity was calculated using a Microsoft Excel spreadsheet.

K_m, V_{max}, and k_{cat} values were determined using substrate concentrations of 0.005, 0.05, 0.5, 5, 50, 250, 500, and 1,000 μM. Triplicate experiments were done, and in each experiment all data points were performed in triplicate on the same plate with a matching set of blank CE-free controls and also positive controls (*o*-NPA) with/without CEs. Values were determined from hyperbolic plots of reaction velocities versus substrate concentrations using the Prism software (GraphPad, San Diego, CA).[7] R^2 values were obtained from these curve fits.

Kinetics of metabolism of β-naphthol (NAP) derivatives by rabbit and human CEs. Substrates were dissolved in dimethylsulfoxide (DMSO), and reactions performed in 50 mM HEPES, pH 7.4. The final concentration of DMSO in the reaction never exceeded 1%. Immediately following addition of enzyme to reaction mixtures, hydrolysis rates were quantitated by converting the liberated β-NAP to an azo compound by reaction with Fast Blue RR.[25] Specifically, each well of a 96-well plate contained 150 μL of 50 mM HEPES buffer (pH 7.4), 20 μL CEs (3 μg/mL) or buffer, 100 μL of Fast Blue RR (0.25 mg/mL), and 30 μL of substrate or buffer. Substrate was added last using a multi-channel pipette. Monitoring of the optical density of each well at 470 nm began immediately after Fast blue RR was added. The amount of β-NAP produced was determined from a β-NAP standard curve ($r^2 = 0.975$). Enzyme kinetics were determined using methods modified from those of Huang et al.[26] Initial velocities were recorded as OD/min/mg purified enzyme, and corrected for background (nonenzymatic, time-dependent) hydrolysis. R^2 values were obtained from these curve fits.

Enzymatic inhibition of CEs, by derivatives of *p*-nitrophenol or β-naphthol. Reaction mixtures (300 μL) contained CEs (rCE or hCE1), 2 mM *o*-NPA, and a range (3.1–1000 nM and 78–2500 μM for *p*-NP and β-NAP derivatives, respectively) of concentrations of synthesized compounds in 50 mM HEPES buffer at pH 7.4, 25 °C. Absorbance was monitored spectrophotometrially for 0.5–10 min in 96-well flat bottom clear polystyrene cell culture plates (Corning Costar) at 420 nm using a spectrophotometer (Dynex Technologies, VA), as described above. Enzyme activity was expressed as percent of controls containing no 'inhibitor'. IC_{50} values (inhibition concentration at 50%) were obtained by nonlinear regression fit analysis using GraphPad Prism software. R^2 values were obtained from these curve fits.

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