Identification of Human Intestinal Carboxylesterase as the Primary Enzyme for Activation of a Doxazolidine Carbamate Prodrug

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Doxazolidine (Doxaz), a formaldehyde−doxorubicin (Dox) conjugate, exhibits markedly increased tumor toxicity with respect to Dox without a concurrent increase in toxicity to cardiomyocytes. Pentyl PABC-Doxaz (PPD) is a Doxaz carbamate prodrug that is hydrolyzed by carboxylesterases. Here, we identify human intestinal carboxylesterase (hiCE) as the agent of activation for PPD. Upon prodrug treatment, cells that express higher levels of hiCE responded with lower IC_{50} values for growth inhibition. Exposing MCF-7 human breast cancer cells, which respond poorly and express little hiCE, to PPD together with hiCE resulted in a dramatic decrease in the IC_{50}, a decrease that was absent when human carboxylesterase 1 was added to prodrug treatment. Finally, U373MG glioblastoma cells overexpressing hiCE displayed ~100-fold reduction in the IC_{50} for PPD compared to cells lacking the carboxylesterase. Overall, our studies indicate that PPD is selectively hydrolyzed to the active metabolite by hiCE.

Introduction

Doxorubicin (Dox[a])is a widely used chemotherapeutic agent that has been successful in the treatment of many different types of tumors. However, Dox-containing treatment regimens are limited by cardiotoxicity and acquired resistance by tumors. Recently, we described a formaldehyde−Dox conjugate, doxazolidine (Doxaz), which exhibits a profound increase in toxicity against tumor cell lines, including those resistant to Dox treatment.[1] Despite the seemingly small change of a single methylene group between the two structures, doxazolidine functions via a distinct mechanism from doxorubicin.[2] Doxorubicin is classified as a topoisomerase II poison, while doxazolidine cross-links DNA and its cytotoxicity is topoisomerase II independent. Importantly, the elevated tumor cell toxicity of doxazolidine was accompanied by no increase in toxicity to rat cardiomyocytes.[1] However, because of both its significant toxicity and its short half-life in aqueous media,[1] development of Doxaz as a therapeutic tool requires the development of a stable, nontoxic Doxaz prodrug that will be enzymatically activated in vivo at the tumor location. Recently, we reported the design, synthesis, and preliminary evaluation of such a prodrug of Doxaz, pentyl PABC-Doxaz (Scheme 1).[3] The design is based on two recent clinical prodrugs, capecitabine and irinotecan (Chart 1), that deliver 5-fluorouracil (5-FU) and 7-ethyl-10-hydroxycamptothecin (**1**, SN-38, Chart 1), respectively, through the actions of the cytoplasmic carboxylesterase (CE) enzymes hCE1 (CES1) and hiCE (CES2), respectively.[4,5]

hCE1 and hiCE are promiscuous serine hydrolase enzymes implicated in detoxification of various xenobiotics, such as cocaine and heroin, as well as in cholesterol transesterification.[6–8] Activation of capecitabine and irinotecan by hCE1 and hiCE, respectively, occurs by hydrolysis of the carbamate moiety, a site recognized by the enzymes as a substrate but an uncommon motif in the human body. In spite of significant structural differences between capecitabine and irinotecan, including a reversal of the orientation of the carbamate, both prodrugs can be activated by both enzymes.[9,10] Generally, however, hCE1 is viewed as the primary agent for activation of capecitabine, while irinotecan hydrolysis is primarily mediated by hiCE.[10,11]

Structurally more similar to capecitabine than irinotecan, our prodrug is a membrane permeable, CE-activated prodrug of Doxaz (pentyl PABC-Doxaz, Chart 1). It utilizes two carbamate groups: one to stabilize the oxazolidine nitrogen, the primary cause of the aqueous instability of Doxaz, and a second to function as a CE hydrolysis site. This latter carbamate is divorced from the bulky anthracycline ring system by a self-eliminating Katzenellenbogen spacer.[12] Upon enzymatic cleavage at the distal carbamate, liberation of Doxaz proceeds by three spontaneous steps as illustrated in Scheme 1. In our previous characterization, neither hCE1 nor hiCE was conclusively identified as the primary enzymatic activator.[3] Further experimentation, presented here, allows the identification of hiCE, rather than hCE1, as the primary enzymatic effector of pentyl PABC-Doxaz hydrolysis and release of doxazolidine.

Results and Discussion

Nonhuman Mammalian Carboxylesterase Activity. Pentyl PABC-Doxaz has been demonstrated to be quite stable under a wide range of conditions, and the activation of pentyl PABC-Doxaz proceeds through release of Doxaz, which quickly degrades in aqueous environments to Dox (Scheme 1). Thus, activation of pentyl PABC-Doxaz can be assayed by monitoring the production of Dox. Initially, in an effort to identify enzymes that cleaved pentyl PABC-Doxaz efficiently, we screened three mammalian liver CEs: horse, pig, and rabbit. Rabbit liver CE is widely recognized as a highly effective activator of irinotecan[13] and was expected to demonstrate significant activity against pentyl PABC-Doxaz. Surprisingly, however, reaction

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[a]Abbreviations: CE, carboxylesterase; Dox, doxorubicin; Doxaz, doxazolidine; FBS, fetal bovine serum; hCE1, human carboxylesterase 1; hiCE, human intestinal carboxylesterase; PABC, *p*-aminobenzyl carbamate; PLE, pig liver esterase; PPD or pentyl PABC-Doxaz, pentyl carbamate of *p*-aminobenzyl carbamate of doxazolidine.

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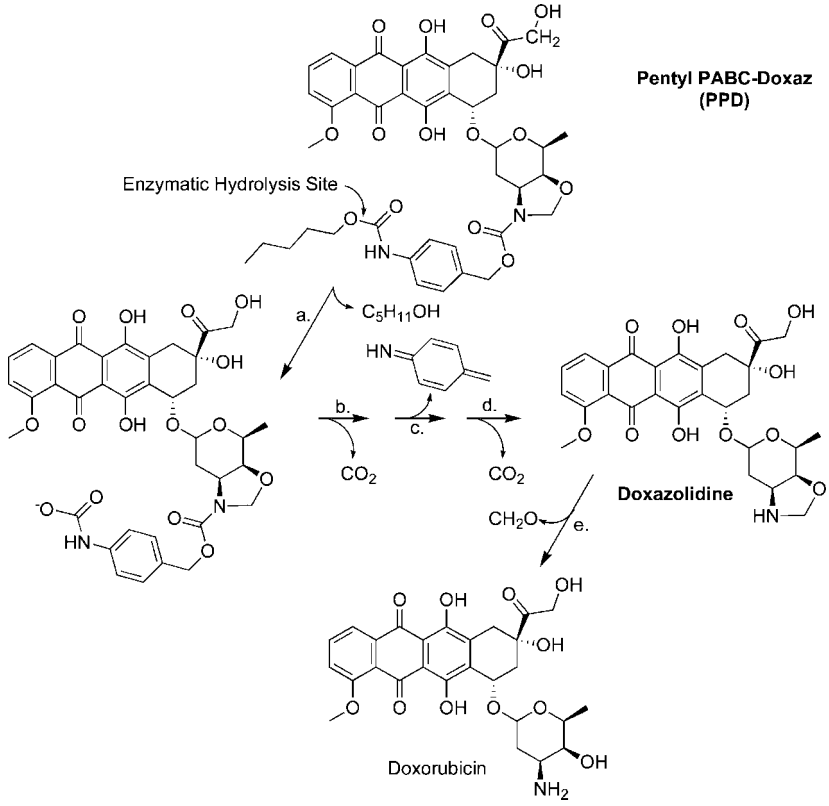
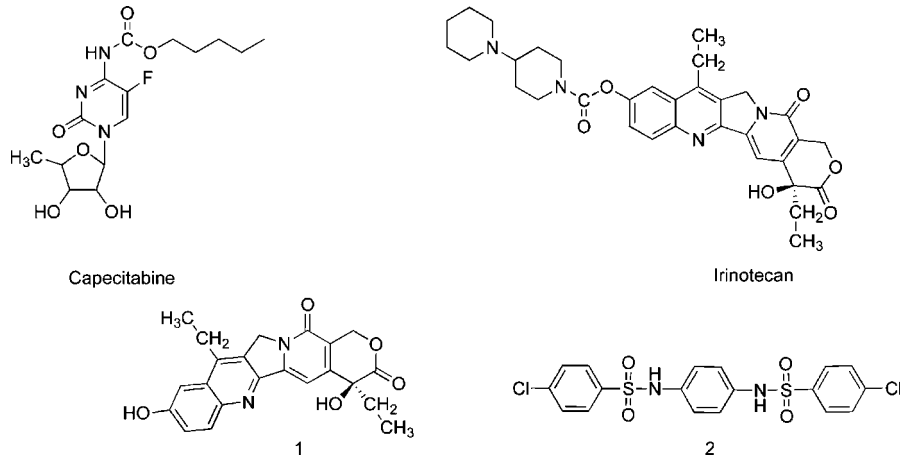
Scheme 1[a]



[a] Enzymatic cleavage of pentyl PABC-Doxaz (PPD) likely occurs at the distal carbamate via a serine hydrolase mechanism, releasing pentanol and the PABC-Doxaz carbamic acid (a). Three spontaneous steps follow to produce free, toxic doxazolidine: (b) decarboxylation, (c) 1, 6-elimination of the Katzenellenbogen spacer, and (d) a second decarboxylation. Once produced in aqueous conditions, doxazolidine decomposes to doxorubicin and formaldehyde (e) with a half-life of approximate 3 min.[27] Although the molecule contains two carbamate moieties, inclusion of the Katzenellenbogen spacer improved response of cells to the prodrug,[3] suggesting that cleavage preferentially occurs at the carbamate further removed from the bulky, hydrophobic anthracycline ring system.

Chart 1. Structures for the CE-Activated Clinical Prodrugs Capecitabine and Irinotecan, the Product of Activation of Irinotecan, 7-Ethyl-10-hydroxycamptothecin (**1**), as Well as the hiCE-Specific Inhibitor 4-Chloro-*N*-(4-(((4-chlorophenyl)sulfonyl)amino)phenyl)-benzenesulfonamide[21] (**2**)



of 50 μM pentyl PABC-Doxaz with either rabbit or horse CE resulted in no detectable Dox. Pig liver esterase, however, produced significant levels of Dox after 5–10 min. Additionally, the production of Dox by pig liver esterase proceeded approximately 3-fold more quickly when FBS was present in the reaction (Figure 1A) and the maximum specific activity was calculated to be 24.4 $\pm$ 1.0 units/mg of total protein, where 1 unit is defined as the amount of enzyme that produces 1 μM Dox per hour (Table 1).

In addition to observing activation of pentyl PABC-Doxaz and the production of Dox by HPLC analysis, cell death due to the presence of Doxaz upon enzymatic cleavage of the prodrug was also used as an indicator of enzyme activity. Shown in Figure 1B, treatment of MCF-7 cells with pentyl PABC-Doxaz alone resulted in little or no cytotoxicity as measured by cell proliferation. However, MCF-7 cells treated with pig liver esterase and pentyl PABC-Doxaz depressed the IC_{50} to 4 nM. Taken together, these results indicate that pentyl PABC-Doxaz

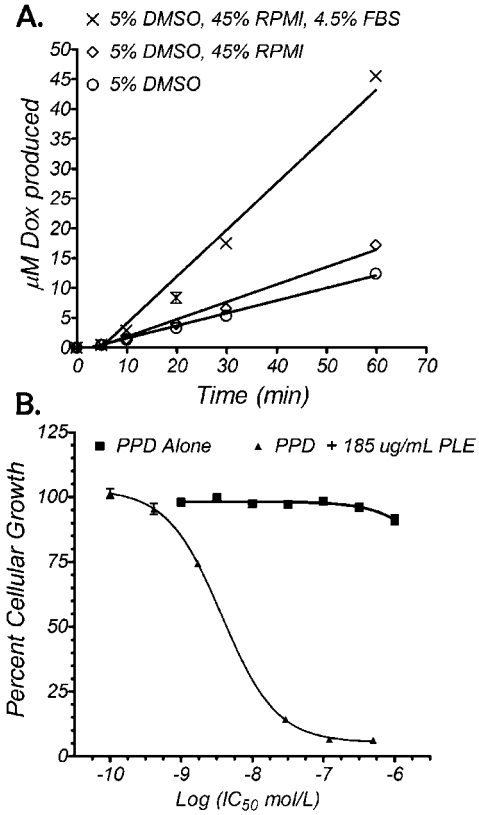


Figure 1. (A) HPLC results for the formation of Dox in the presence of pig liver esterase under different reaction conditions. Pentyl PABC-Doxaz (50 μM) was reacted in either 4.5% FBS/45% RPMI/45.5% PBS (crosses), 45% RPMI/50% PBS (open diamonds), or 95% PBS (open circles). All reactions contained 5% DMSO and 37 μg/mL pig liver esterase. (B). Dose–response curves for MCF-7 cells treated with pentyl PABC-Doxaz (PPD, black squares) or pentyl PABC-Doxaz plus 30 μg/mL of pig liver esterase (black triangles). Data points and error bars are the mean ± standard deviation of three repetitions for part A and six measurements for part B.

Table 1. Cellular Response to Pentyl PABC-Doxaz Treatment in Serum-Free Media[a]

cell line	pentyl PABC-Doxaz log(IC_{50}, mol/L)
MCF-7	-5.6 ± 0.3[b]
MCF-7/Adr	-6.5 ± 0.06[b]
SK-HEP-1	-6.0 ± 0.06[b]
Hep G2	-7.3 ± 0.2[b]
N-Hep G2	-7.7 ± 0.08
H9c2(2-1)	-6.2 ± 0.2[b]

[a] IC_{50} values are expressed as the log of the concentration in mol/L that inhibits half the growth. [b] Reference 3.

is capable of being efficiently hydrolyzed to Doxaz, yielding significant growth inhibition of MCF-7 cells. Additionally, the presence of FBS increased the rate of the enzymatic hydrolysis reaction. The reason for this is not completely clear but may involve the well-known ability of serum albumin to aid in the solubilization and transport of drug agents, including Dox.[14–20] Because of the physiological relevance of serum and albumin in these reactions, further growth inhibition experiments and enzymatic assays were all done in the presence of FBS, despite the prior observation that serum inhibits uptake of pentyl PABC-Doxaz.[3]

Western Blot Analysis and IC_{50} Correlation. Previous results suggested that the observed IC_{50} values for cells treated with pentyl PABC-Doxaz correlated better with cellular hCE1 mRNA expression, as opposed to hiCE.[3] Therefore, we determined whether a similar correlation existed for hCE1 or hiCE protein levels. Using Western analysis, we evaluated the expression of these proteins in a panel of cell lines (MCF-7, MCF-7/Adr, SK-HEP-1, Hep G2, N-Hep G2, and H9c2(2–1); Figure 2) and compared it to the IC_{50} values for these cell lines (Table 1). As can be seen, high hCE1 expression was found in Hep G2 and SK-HEP-1, with much lower levels being observed in MCF-7/Adr and N-Hep G2 cells, while strong expression of hiCE was observed in N-Hep G2 cells, with lower levels in Hep G2 and SK-HEP-1 (Figure 2A). When protein expression is compared to drug response (Figure 2B), increasing levels of hiCE tend to correlate with increased drug response and decreased IC_{50}. This correlation is absent when hCE1 expression is compared to drug response. These data demonstrate that increased expression of hiCE is a determinant for increased drug response, suggesting that hiCE is the primary enzymatic activator of pentyl PABC-Doxaz.

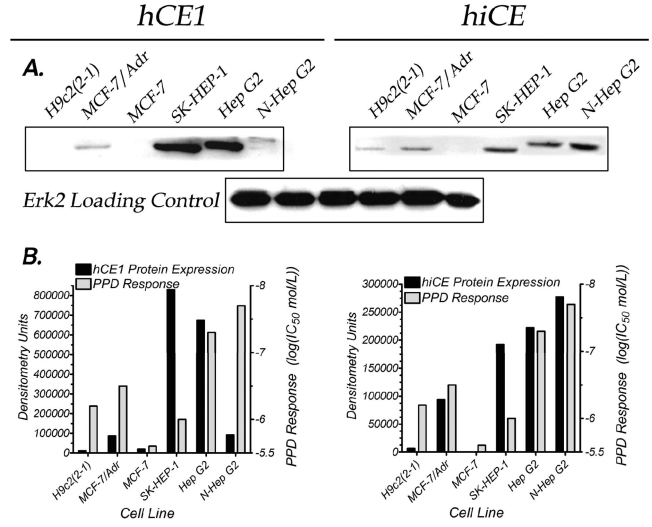


Figure 2. Western blot of SDS−PAGE on 15 μg of total protein from whole cell lysates from rat cardiomyocytes (H9c2(2-1)) or the cancer cell lines MCF-7/Adr, MCF-7, SK-HEP-1, Hep G2, and N-Hep G2. Cell lysates were probed for hCE1 or hiCE, and Erk2 is included to control for loading (A). The density of the carboxylesterase band was then compared to drug response, as measured by IC_{50} values from Table 1 (B).

While the above conclusion holds true for the majority of the cell lines examined, deviation from the trend can be observed in the cell lines SK-HEP-1 and H9c2(2-1). SK-HEP-1 cells exhibit poor response to pentyl PABC-Doxaz in spite of relatively robust hiCE expression. This is likely due to an inherent property of SK-HEP-1 cells that decreases their sensitivity to treatment, either by limiting enzymatic access to the prodrug or via reduced response to the final active product. Conversely, the heightened response from H9c2(2-1) cells relative to the rat homologue of hiCE could be indicative that the rat version of hiCE is significantly more effective at hydrolyzing pentyl PABC-Doxaz than the human form. The mobility difference in the hiCE band among the cell lines is probably due to differences in post-translational modifications; recombinant hiCE runs identically to the higher mobility band (data not shown). Since the two cell lines that express lower-mobility hiCE are related (B. Knowles, unpublished data), it is likely that a difference in post-translational processing was carried over from Hep G2 into the N-Hep G2 cell line.

Activity of Recombinant hCE1 and hiCE against Pentyl PABC-Doxaz. As demonstrated above, the correlation between the levels of CE expression and the IC_{50} values for

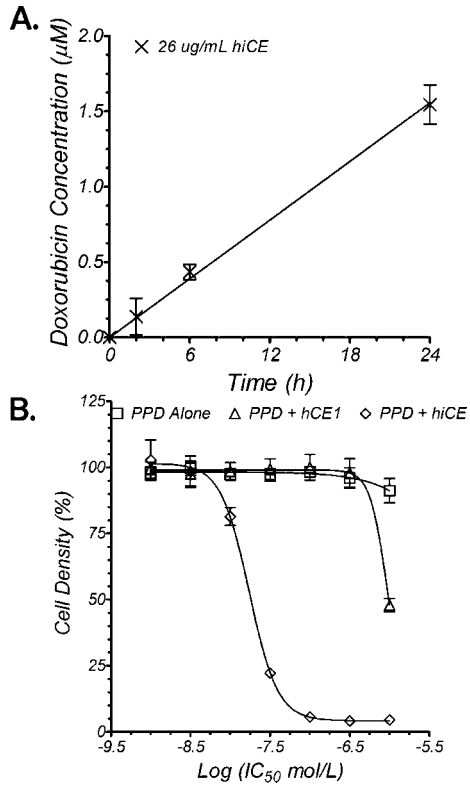


Figure 3. HPLC analysis of enzymatic hydrolysis reactions and comparison of cell growth inhibition for pentyl PABC-Doxaz in the presence of hCE1 or hiCE. (A) 26 μg/mL recombinant hCE1 or hiCE was reacted with a 50 μM solution of pentyl PABC-Doxaz (PPD). No production of Dox was observed in the presence of hCE1. (B) Dose–response curves for MCF-7 cells treated with pentyl PABC-Doxaz alone (open squares), with 2500 ng/mL hCE1 (open triangles), or with 250 ng/mL hiCE (open diamonds). Data points and error bars represent the mean ± standard deviation of three measurements for part A and six measurements for part B.

Table 2. Enzyme Activity against Pentyl PABC-Doxaz

enzyme	units (μM Dox produced/h)	specific activity (U/mg protein)[a]
pig liver esterase	47.04 ± 1.92	24.4 ± 1.0
hCE1	no reaction observed	
hiCE	0.065 ± 0.002	20.8 ± 0.6

[a] 1 unit (U) produces 1 μM Dox per hour.

pentyl PABC-Doxaz appeared better for hiCE than for hCE1. To directly test the ability of each enzyme to hydrolyze pentyl PABC-Doxaz, the drug was incubated with recombinant purified CEs. As shown in Figure 3A, Dox was observed following incubation of 50 μM pentyl PABC-Doxaz with hiCE (26 μg/mL) but not with a similar concentration of hCE1. Similar to pig liver esterase, hydrolysis was more efficient in the presence of FBS (data not shown) and the maximal specific activity was determined to be 20.8 ± 0.6 units/mg protein (Table 2). For comparison, the specific activity when using irinotecan as a substrate was measured to be 11 100 ± 400 units/mg protein in serum-containing media.

Exogenous addition of 250 ng/mL hiCE to the culture medium resulted in a significant decrease of more than 63-fold in the IC_{50} for MCF-7 cells with pentyl PABC-Doxaz (Figure 3B). Surprisingly, addition of 2500 ng/mL hCE1 to the treatment media also produced an increase in prodrug toxicity, but the change in the IC_{50} value was ~2 logs less than that observed with hiCE, suggesting that hCE1 can poorly activate pentyl PABC-Doxaz. Dox-sensitive MCF-7 and Dox-resistant MCF-7/Adr cells showed a similar response profile, exhibiting large reductions in the IC_{50} values upon exogenous addition of hiCE but a markedly reduced effect in the presence of hCE1 (Table 3). Therefore, we conclude that pentyl PABC-Doxaz is preferentially activated by hiCE. Further, despite the lack of any observable Dox product from the HPLC analysis of the reaction between the prodrug and hCE1, the enzyme is able to induce an elevation in prodrug toxicity. This discrepancy between HPLC and tissue culture experiments is likely due to the exquisite toxicity of Doxaz; cells exhibit dramatic responses to Doxaz at concentrations below 5 nM.[1] Such concentrations are far below the threshold of detection of both the HPLC diode array detector and the fluorescence detector (approximately 100 nM; data not shown). Thus, activation of pentyl PABC-Doxaz by hCE1 likely occurs in our HPLC activity assays, but detection of that activation requires a much more sensitive readout, such as the observed toxicity in the cellular assays described here.

Activity of N-Hep G2 Lysate against Pentyl PABC-Doxaz. N-Hep G2 cells exhibited the lowest IC_{50} value of any cell line tested and expressed high levels of hiCE but little hCE1. Thus, N-Hep G2 lysate would be predicted to demonstrate activity against pentyl PABC-Doxaz in both biochemical and tissue culture assays. Therefore, we produced lysates from N-Hep G2 cells and incubated them with 50 μM pentyl PABC-Doxaz. Although no HPLC-detectable Dox was observed, these samples decreased IC_{50} values approximately 4- to 6-fold compared to treatment with prodrug alone when applied to MCF-7 or MCF-7/Adr cells (Table 3). To demonstrate that this increase in toxicity was due to hiCE, the specific inhibitor 4-chloro-*N*-(4-(((4-chlorophenyl)sulfonyl)amino)phenyl)benzenesulfonamide (**2**, Chart 1)[21] was added. After addition of the hiCE specific inhibitor, the IC_{50} for pentyl PABC-Doxaz was elevated from 150 to 200 nM to approximately 1 μM.

Cellular Overexpression of Carboxylesterases and Prodrug Response. We have presented data indicating that pentyl PABC-Doxaz is preferentially cleaved by hiCE, These CEs, however, are cytoplasmic enzymes, and their activity may be affected by other components of their cellular environment. To determine whether this was the case, we performed growth inhibition assays in cell lines engineered to express the human CEs, U373MG glioblastoma cells expressing basal levels (U373IRES), high levels of hCE1 (UhCE1), or high levels of hiCE (UhiCE).[22] As shown in Figure 4A and Table 4, U373IRES cells exhibit little to no sensitivity to pentyl PABC-Doxaz. While both UhCE1 (Figure 4B) and UhiCE (Figure 4C) display decreased IC_{50} values relative to U373IRES, the IC_{50} for pentyl PABC-Doxaz in UhiCE cells is more than 1 log lower than that for UhCE1, despite displaying reduced total CE activity relative to UhCE1 cells. As predicted from previous work,[22] addition of the membrane permeable CE inhibitor benzil diminished the activity of pentyl PABC-Doxaz toward UhiCE by approximately 16-fold (Figure 5). This suggests that the hydrolysis of pentyl PABC-Doxaz by hiCE in a cellular cytoplasmic environment is effectively unchanged relative to enzymes present in tissue culture media and agrees with the previously presented Western analyses. We conclude that elevated cellular hiCE is a strong determinant for a significant response to treatment with pentyl PABC-Doxaz. These data also agree with the above conclusion that extremely high levels of hCE1 are able to activate the prodrug. This activation, however,

Table 3. Growth Inhibition of MCF-7 and MCF-7/Adr Cells in Complete Media[a]

treatment	log(IC_{50} mol/L)		fold change in IC_{50} value	
	MCF-7	MCF-7/Adr	MCF-7	MCF-7/Adr
PPD	>-6	>-6	1	1
PPD + PLE	-8.4 ± 0.2		>100	
PPD + hiCE	-7.8 ± 0.1	-7.9 ± 0.2	>63	>79
PPD + N-Hep G2 lysate	-6.6 ± 0.2	-6.8 ± 0.2	>4	>6
2	>-6	>-6	ND[b]	ND[b]
PPD + N-Hep G2 lysate + **2**	-6.1 ± 0.1	-6.1 ± 0.1	>1	>1
PPD + hCE1	-6.0 ± 0.1	-6.1 ± 0.2	>1	>1

[a] The log of the IC_{50} growth values in mol/L for the breast carcinoma cell lines MCF-7 (Dox-sensitive) and MCF-7/Adr (Dox-resistant) in response to 24 h of treatment with various combinations of pentyl PABC-Doxaz (PPD), 30 μg/mL pig liver esterase (PLE), 0.250 μg/mL of recombinant hiCE, 83.5 μg of total protein/mL of N-Hep G2 lysate, 10 μg/mL hiCE-specific inhibitor **2** (Chart 1), and 2.5 μg/mL recombinant hCE1. Cell survival was measured by staining with crystal violet. Fold changes in IC_{50} values are relative to prodrug treatment alone for each cell line. [b] Not determined.

is quite inefficient, because the IC_{50} in UhCE1 cells is nearly 10-fold higher than that in UhiCE cells.

Conclusions

Pentyl PABC-Doxaz, a CE-activated prodrug of doxazolidine, is hydrolyzed primarily by hiCE compared to hCE1. Cell response, as exhibited by growth arrest in response to treatment with pentyl PABC-Doxaz, correlates with hiCE levels as determined by Western analyses, and biochemical assays demonstrate higher rates of formation of Dox upon reaction of the prodrug with hiCE, compared to hCE1. Additionally, exogenous addition of hiCE to culture medium reduces the IC_{50} by greater than 100-fold, and co-treatment of cells with N-Hep G2 lysate and pentyl PABC-Doxaz results in a reduction in the IC_{50} which can be ameliorated by addition of the hiCE specific inhibitor **2**. Finally, U373MG cells that have been transfected with hiCE yield a lower IC_{50} for the drug than either wild-type cells or those transfected with hCE1.

We note that the HPLC detection system was insufficient to detect the small amount of prodrug activation that would lead to a toxic level of Doxaz. As evidenced by N-Hep G2 cell lysates, significant toxicity can be observed in the highly sensitive cell growth assay while HPLC analysis would predict no activity. Further, treatment with prodrug alone in serum-free media results in lower IC_{50} values than treatment in complete media, while the presence of FBS increases the rate of enzyme activity. This confirms that, as previously reported,[3] FBS inhibits cellular uptake of the prodrug. Also, since this trend is seen in cells that express little or no hiCE, it is likely that while hiCE is the preferred activator of pentyl PABC-Doxaz, other enzymes, such as hCE1, can still hydrolyze the prodrug and release Doxaz. While cellular activation of pentyl PABC-Doxaz by hCE1 was demonstrated in the overexpression system described above, activation by enzymes other than hiCE is not observed in the HPLC assays because of low activity and the high detection threshold. Therefore, alternative pathways for activation of the prodrug may play a minor role in activation of the prodrug in cells and, ultimately, in animal models.

Doxorubicin treatment, which has enjoyed a long and successful tenure as a mainstream chemotherapeutic regimen, is not without its drawbacks. Doxazolidine, despite differing minimally in structure from Dox, introduces a distinct cytotoxic mechanism but also introduces problems of stability. Pentyl PABC-Doxaz offers a stable system that is highly effective at remaining invisible to cells in which hiCE is absent but delivers its highly toxic product to cells high in hiCE. Tumors that match this profile, such as human hepatocellular carcinoma (HCC) or renal cancer, are therefore likely candidates for success under pentyl PABC-Doxaz therapy. Incidences of such tumors are on the rise both locally and globally, and this increase is predicted to accelerate in coming years.[23–25]

Experimental Methods

1. General Remarks. Pentyl PABC-Doxaz (PPD, pentyl carbamate of *p*-aminobenzyl carbamate of doxazolidine; complete chemical name, pentyl 4-(*N*-doxazolidinylcarbonyloxymethyl)-phenyl carbamate) was synthesized as previously described.[3] Recombinant enzymes hCE1 and hiCE were generated using a baculoviral expression system as previously reported.[26] The synthesis and characterization of the hiCE-specific inhibitor 4-chloro-*N*-(4-(((4-chlorophenyl)sulfonyl)amino)phenyl)benzenesulfonamide (**2**, Chart 1) has been previously discussed.[21] All tissue culture media and additives were purchased from Gibco Life Techologies (Grand Island, NY), and tissue culture plates were from Sarstedt, Inc. (Newton, NC). All cells, except as noted below, were obtained from the American Type Culture Collection (Rockville, MD). N-Hep G2 cells were received as a gift from Barbara Knowles (Jackson Laboratory, Barr Harbor, ME), and MCF-7/Adr cells were received from William Wells (Michigan State University, East Lansing, MI). Chemicals, unless otherwise noted, were purchased from Sigma-Aldrich (Milwaukee, WI). Antibodies used for Western blot analysis were obtained from Philip Potter (St. Jude Children's Research Hospital, Memphis, TN).

2. General Tissue Culture. The human carcinoma cell lines SK-HEP-1 (liver), MCF-7, and MCF-7/Adr (breast) were cultured in RPMI 1640 media, supplemented with 10% v/v fetal calf serum and 1% v/v penicillin−streptomycin. The liver lines Hep G2 and N-Hep G2 and the rat cardiomyocyte H9c2(2-1) were cultured in Dulbecco's modified Eagle media (DMEM) with 10% v/v fetal calf serum and 1% v/v penicillin−streptomycin. U373MG (glioblastoma) derived cell lines were cultured in the same media with the exception of the antibiotics. All cells were grown at 37 °C in a humidified incubator under an atmosphere of 5% CO_2, 95% air.

3. HPLC Enzyme Activity Assays. Concentrations of stock solutions of drug in DMSO were established by measuring the optical density at 480 nm of dilutions in 75% DMSO, 25% water, assuming a molar extinction coefficient for the Dox chromophore of 11 500 M^{-1} cm^{-1}. To assay a range of mammalian esterase enzymes for activity against pentyl PABC-Doxaz, 50 μg/mL horse liver esterase, 5 μg/mL pig liver esterase (both from the Esterase Basic Kit, Fluka Biochemika, Buchs, Switzerland), and 425 μg/mL rabbit liver esterase (Sigma-Aldrich, Milwaukee, WI) were incubated with 50 μM pentyl PABC-Doxaz in 5% DMSO, 95% PBS at 37 °C. Aliquots as a function of time were taken, quenched in an equal volume of 100% ethanol, and incubated at −20 °C overnight, then centrifuged for 5 min at 10000*g* to precipitate protein. The supernatant was then subjected to HPLC analysis to test for the presence of prodrug and/or Dox. Analytical HPLC was performed with an Agilent 1050/1100 hybrid instrument equipped with a 1050 autoinjector and 1100 UV–vis diode array absorbance and a 1046A fluorescence detector. Chromatography was performed on an Agilent Zorbax, 4.6 mm i.d. × 150 mm, 5 μm reverse-phase octadecylsilyl (ODS) column, eluting at 1.0 mL/min with a gradient of room temperature acetonitrile/20 mM triethylammonium acetate

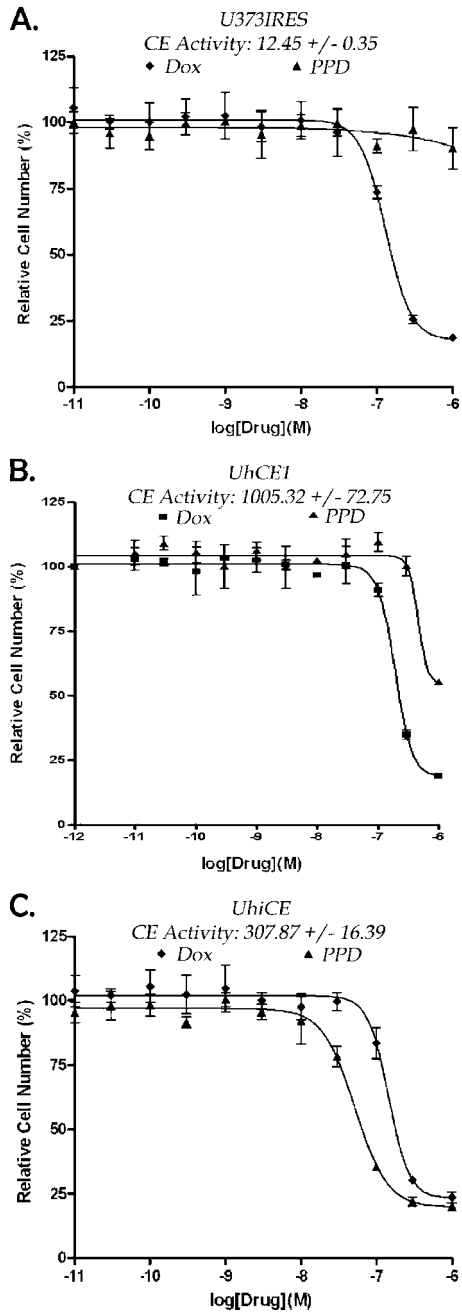


Figure 4. Growth inhibition analyses of U373MG glioblastoma cells expressing human CEs. Cell lines contained the parental plasmid (U373IRES, A), hCE1 (UhCE1, B), or hiCE (UhiCE, C) with Dox (closed diamonds or squares) or pentyl PABC-Doxaz (PPD, closed triangles). CE activity is the activity of the cell lysate using *o*-nitrophenyl acetate as a substrate in units of nmol of *o*-nitrophenol produced per minute per milligram of protein. Growth curves for both Dox and pentyl PABC-Doxaz (PPD) are shown, with data points representing the mean ± standard deviation for six measurements.

buffer, pH 6.0. Buffer was ramped down from 80% to 20% over 10 min, held isocratic until 14.5 min, and then returned to 80% by 16 min. The presence of Dox-chromophore-containing compounds in the eluent was monitored by absorbance at 480 nm and fluorescence at 550 nm (excitation at 480 nm). Production of Dox was observed only upon addition of pig liver esterase, so more enzyme was purchased from Aldrich and used for further experimentation. All data presented here are from this second purchase, which was of approximately 30-fold lower specific activity. Lysates of N-Hep G2 cells were made by probe sonication of 35 million cells in 50 mM HEPES, pH 7.4 buffer. Activity assays were routinely performed using 50 μM drug and 5% DMSO, varying

Table 4. Growth Inhibition in U373MG Glioblastoma Cells Expressing Empty Plasmid (U373IRES) or Plasmid Containing hCE1 (UhCE1) or hiCE (UhiCE) upon 1 h of Treatment with Dox or Pentyl PABC-Doxaz (PPD)[a]

cell line	log(IC_{50}, mol/L) Dox	log(IC_{50}, mol/L) PPD	fold change in PPD IC_{50}
U373IRES	-6.8 ± 0.03	>-6	1
UhCE1	-6.8 ± 0.03	>-6	ND[b]
UhiCE	-6.7 ± 0.02	-7.3 ± 0.04	>20
UhiCE + benzil		-6.4 ± 0.1	>2.5

[a] The fold change in PPD IC_{50} values is relative to U373IRES cells. [b] Not determined.

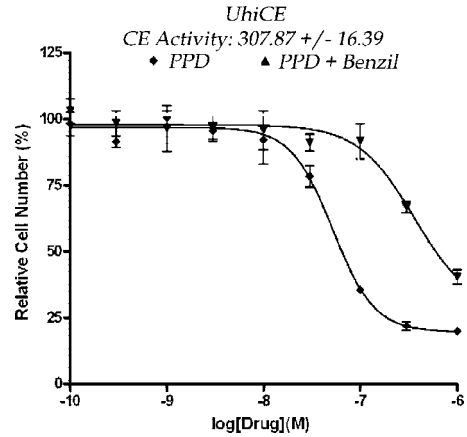


Figure 5. Growth inhibition analysis of UhiCE cells treated with pentyl PABC-Doxaz alone (black diamonds) or in the presence of 10 μM benzil (black triangles). CE activity is the activity of the cell lysate using *o*-nitrophenyl acetate as a substrate (nmoles of *o*-nitrophenol produced per minute per milligram of protein). Data points represent the mean ± standard deviation of six measurements.

the concentrations of FBS/RPMI, N-Hep G2 lysate/enzyme (hCE1 or hiCE), and PBS, pH 7.4. Aliquots at various time points were quenched, the protein was precipitated, and the supernatant was analyzed as indicated above. Following identification of hiCE as the major enzyme of activation, its activity against irinotecan was determined for comparison. A sample of 50 μM irinotecan in DMSO was incubated with 26 μg/mL hiCE in 10% FBS, 45% RPMI-1640, 45% PBS and reacted for various amounts of time. Aliquots were quenched as before and analyzed by HPLC, measuring optical density at 384 nm to observe elution of irinotecan and its hydrolysis product **1**.

4. IC_{50} Measurements. Growth inhibition of U373MG glioblastoma cells was performed as described previously;[22] cells were treated with various concentrations of pentyl PABC-Doxaz for 1 h. For the benzil treatments, the cells were incubated with 10 μM benzil for 2 h prior to drug introduction. All other IC_{50} experiments were performed as described below. Cells were seeded in complete media into 96-well plates and allowed to adhere overnight. The medium was then replaced with media containing various combinations of (1) no addition, (2) 250 ng/mL recombinant hiCE, (3) 83.5 μg of total protein/mL of N-Hep G2 lysate in 50 mM HEPES, pH 7.4, or (4) 10 μg/mL of specific inhibitor **2**. Various concentrations of drug dissolved in DMSO were then added to the cells such that the final DMSO concentration was 1%. The cells were incubated for 24 h, after which the treatment medium was replaced with fresh, complete medium. The cells were then grown in the incubator until the cell confluency in the no-drug control lanes reached 80% (3–5 days). The cellular densities were then quantified by crystal violet staining, solublization in 70% ethanol, and measurement of the optical density at 588 nm. Percent optical density data, relative to the no-drug control lanes, were fit to sigmoidal dose–response curves by nonlinear regression analysis using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

Initially, all growth inhibition experiments were performed in serum-free media because of the reduction in cellular uptake of pentyl PABC-Doxaz when cells were tested in the presence of serum versus serum-free conditions.[3] However, following the observation that both pig liver esterase and hiCE reacted much more quickly in complete media, all subsequent assays were performed in complete media.

5. Western Blot Analyses. Total lysates from MCF-7, MCF-7/Adr, SK-HEP-1, Hep G2, N-Hep G2, and H9c2(2-1) cells were made by scraping the cells into a denaturing lysis buffer (100 mM Tris, pH 7.5, 4% SDS) and protein concentrations assayed by BCA (Pierce, Inc., Rockford, IL). Total protein (15 μg) was subjected to SDS−PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, Inc., Piscataway, NJ). The membrane was blocked with 5% w/v powdered milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Antibodies for hCE1 and hiCE were used at 1:2000 and 1:5000 dilutions in TBS-T, respectively, and incubated for 2 h at room temperature. HRP-conjugated goat-α-rabbit-IgG was used as a secondary antibody, diluted 1:5000 in TBS-T, and incubated for 2 h at room temperature. The secondary antibody was visualized using chemiluminescence with Durawest (Pierce, Inc., Rockford, IL) and developed using BioMax Light film (Eastman Kodak Company, Rochester, NY). Equal loading of lanes was demonstrated by probing with α-Erk2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:1000).

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