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ORIGINAL ARTICLE

An improved human carboxylesterase for enzyme/prodrug therapy with CPT-11

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CPT-11 is a potent antitumor agent that is activated by carboxylesterases (CE) and intracellular expression of CEs that can activate the drug results in increased cytotoxicity to the drug. As activation of CPT-11 (irinotecan-7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) by human CEs is relatively inefficient, we have developed enzyme/prodrug therapy approaches based on the CE/CPT-11 combination using a rabbit liver CE (rCE). However, the *in vivo* application of this technology may be hampered by the development of an immune response to rCE. Therefore, we have developed a mutant human CE (hCE1m6), based on the human liver CE hCE1, that can activate CPT-11 approximately 70-fold more efficiently than the wild-type protein and can be expressed at high levels in mammalian cells. Indeed, adenoviral-mediated delivery of hCE1m6 with human tumor cells resulted in up to a 670-fold reduction in the IC_{50} value for CPT-11, as compared to cells transduced with vector control virus. Furthermore, xenograft studies with human tumors expressing hCE1m6 confirm the ability of this enzyme to activate CPT-11 *in vivo* and induce antitumor activity. We propose that this enzyme should likely be less immunogenic than rCE and would be suitable for the *in vivo* application of CE/CPT-11 enzyme/prodrug therapy.
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Introduction

Carboxylesterases (CE) are ubiquitous enzymes responsible for the detoxification of xenobiotics.[1] They cleave carboxylesters (RCOOR′) into the corresponding alcohol (R′OH) and the carboxylic acids (RCOOH). Since numerous clinically used agents contain the ester group, many drugs are metabolized by CEs. This includes the anticancer agents, capecitabine and CPT-11 (irinotecan-7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin). CPT-11 is a carbamate that is hydrolyzed by CEs to yield SN-38 (7-ethyl-10-hydroxycamptothecin), a potent topoisomerase I poison.[2] The latter compound is highly toxic and results in cell death at low nanomole concentrations. As CPT-11 has demonstrated remarkable antitumor activity in both preclinical models and in Phase I/II clinical trials,[3–5] this drug is being tested against a variety of human malignancies. At present, CPT-11 is approved for use against colon cancer.

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When CPT-11 is administered into humans, typically less than 5% of the drug is converted into SN-38. This is in contrast to mice where more than 50% of the CPT-11 is hydrolyzed to SN-38 within the first hour after dosing.[6,7] This may be either due to the different levels of CEs expressed in these species or due to the proficiency of drug hydrolysis of the different CEs. Since the activation of CPT-11 by humans is relatively inefficient, we have proposed and developed an enzyme/prodrug therapy approach, using a rabbit liver CE (rCE), that can proficiently activate the drug.[8–14] Consequently, expression of rCE in human tumor cells, grown either in culture or as xenografts in immune-deprived mice, results in increased sensitivity to CPT-11.[8,12]

However, the application of rCE to an enzyme/prodrug therapy approach in humans may be limited due to the potential immunogenicity of the lagomorph protein. Although the activation of CPT-11 in humans appears to be catalyzed by the human intestinal CE (hiCE),[15,16] *in vitro* studies indicate that this enzyme is not as efficient at drug activation as rCE. Also, sensitization of cells to CPT-11 expressing hiCE although has been achieved,[16–20] studies in our laboratory indicate that the levels and duration of hiCE expressed are considerably lower than that can be achieved with rCE.

Therefore, we have developed a human CE that can activate CPT-11 as efficiently as rCE and can be expressed at high levels in mammalian cells. This was based on the structural similarity observed between rCE and a human liver CE, hCE1. The latter enzyme is very inefficient at CPT-11 activation (100- to 1000-fold lower than rCE), but demonstrates 81% amino-acid identity[8] and only an approximately 1.0 Å RMSD over 455 residues of the α-carbon trace for rCE.[8,21,22] By comparison of the rCE and hCE1 crystal structures, we identified two loops at the entrance to the active site of rCE, which we predicted to be flexible. Our studies reveal that by substituting amino acids in the hCE1 loops with the corresponding residues from rCE, resulted in an hCE1 variant that can efficiently activate CPT-11. We believe that this protein would be the preferred CE for use in enzyme/prodrug therapy approaches with CPT-11 in clinical studies.

Materials and methods

Cell lines, plasmids and adenoviral vectors

Cell lines were grown in 10% fetal bovine serum and 2 mM glutamine in an atmosphere of 10% CO_2 at 37 °C.

Plasmids containing the cDNAs encoding hCE1, hiCE and rCE have been described previously.[8,10,16] The Genbank accession numbers for these sequences are M73499,[23] Y09616[24] and AF036930,[10] respectively. All of the plasmids, cell lines and adenoviral vectors used in these studies are described in Table 1.

Analysis of carboxylesterase crystal structures

The X-ray crystal structures of rCE (PDB 1K4Y[21]) and hCE1 (PDB 1MX5)[22,26] were overlaid and examined using ICM Pro software (Molsoft, San Diego, CA). All structural figures presented in this manuscript were drawn using this program.

Site-directed mutagenesis

Site-directed mutagenesis was achieved using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with customized primers designed to produce the desired mutations. All mutants were subjected to DNA sequencing to confirm the identity of the clones.

Carboxylesterase assays

Carboxylesterase activity was determined using a spectrophotometric assay with 3 mM *o*-nitrophenyl acetate as a substrate.[10,12,27] Data were expressed as nanomoles *O*-nitrophenol produced per minute per milligram of protein. To correct for differences in CE expression within transfected cells, the enzyme activity values were corrected for the level of immuno reactive CE protein as determined from western blot analyses (see below).

Table 1 Description of the plasmids, cell lines and adenoviral vectors used in this study

Name	*Plasmid (P) cell line (C) adenovirus (Ad)*	*Description*	*Details*
pCIneo	P	Mammalian expression vector	Obtained from Promega
pCIhCE1	P	pCIneo containing wild-type hCE1 cDNA	From Danks *et al*.[8]
pCIrCE	P	pCIneo containing wild-type rCE cDNA	From Potter *et al*.[10]
pCIhCE1m2	P	pCIneo containing mutant hCE1 cDNA	M363L, L364M
pCIhCE1m3	P	pCIneo containing mutant hCE1 cDNA	M363L, L364M, K459R
pCIhCE1m4	P	pCIneo containing mutant hCE1 cDNA	M363L, L364M, K459R, F448Y, Q449R
pCIhCE1m5	P	pCIneo containing mutant hCE1 cDNA	M363L, L364M, K459R, F448Y, Q449R, L357I, S365G
pCIhCE1m6	P	pCIneo containing mutant hCE1 cDNA	M363L, L364M, K459R, F448Y, Q449R, L357I, S365G, Q361 insertion
pIRESneo	P	Mammalian expression vector	Contains G418 resistance gene coupled to an IRES sequence
pIRESrCE	P	pIRESneo containing rCE cDNA	From Potter *et al*.[10]
pIREShCE1	P	pIRESneo containing hCE1 cDNA	From Danks *et al*.[8]
pIREShiCE	P	pIRESneo containing hiCE cDNA	Expresses hiCE following transfection and selection with G418
pIREShCE1m6	P	pIRESneo containing hCE1m6 cDNA	Contains mutations as listed above for pCIhCE1m6
COS-7	C	African green monkey kidney cell line	Obtained from the American Type Culture Collection
U373MG	C	Human astrocytoma cell line	Obtained from the American Type Culture Collection
U373IRES	C	U373MG transfected with pIRESneo	G418 resistant but lacking exogenous CE expression
U373hCE1	C	U373MG transfected with pIREShCE1	U373MG expressing hCE1
U373rCE	C	U373MG transfected with pIRESrCE	U373MG expressing rCE
U373hiCE	C	U373MG transfected with pIREShiCE	U373MG expressing hiCE
U373hCE1m6	C	U373MG transfected with pIREShCE1m6	U373MG expressing hCE1m6
293	C	Human embryo kidney cell line	Obtained from the American Type Culture Collection
Rh30	C	Rhabdomyosarcoma cell line	From Douglass *et al*.[25]
SK-N-AS	C	Neuroblastoma cell line	Obtained from the American Type Culture Collection
AdVC	Ad	Adenovirus vector based upon Ad5	E1, E3-deleted Ad vector
AdCMVrCE	Ad	Adenovirus containing rCE cDNA	Expresses high levels of rCE under control of CMV promoter
AdCMVhCE1m6	Ad	Adenovirus containing hCE1m6 cDNA	Expresses high levels of hCE1m6 under control of CMV promoter

Transfection of cell lines

Transient transfection of COS-7 cells was achieved by electroporation.[10] For the generation of stable cell lines, cDNAs were ligated into pIRESneo and U373MG cells were electroporated under similar conditions. Transfectants were selected in a media containing 400 μg ml^{-1} of G418. Since the CE cDNA is linked via an internal ribosome entry sequence to the neomycin gene, selection of individual G418-resistant clones was not necessary. Routinely, whole-cell sonicates obtained from these pooled populations of cells contained 200–500 nmoles min^{-1} mg^{-1} of CE activity.

CPT-11 conversion assays

Conversion of CPT-11 into SN-38 was monitored by incubating cell extracts with 5 μM CPT-11 for 1 h in 50 mM Hepes (pH 7.4) at 37 °C. An equal volume of acidified methanol was added to terminate the reactions and particulate matter was removed by centrifugation at 100 000 *g* for 5 min at 4 °C. Concentrations of both drugs in the supernatant were then determined by high-performance liquid chromatography.[28,29]

Protein purification

Secreted forms of hCE1, rCE and hCE1m6 were expressed in *Spodoptera frugiperda* Sf9 cells and purified from serum-free culture media as described previously.[30] For hiCE, an alternative purification procedure was developed using DEAE chromatography and elution with a pH/salt gradient (MJ Hatfield and PM Potter, manuscript in preparation).

Determination of kinetic parameters for substrate hydrolysis

K_m, V_{max} and k_{cat} values for the hydrolysis of CPT-11 by the recombinant purified proteins were determined as described previously.[31]

Western analysis

Cell extracts were separated in 4–20% pre-cast SDS-PAGE gels (Invitrogen, Carlsbad, CA), and following transfer to Immobilon-P membranes by electroblotting,[32] western blot analysis was performed as described previously.[33] CEs were identified using an anti-peptide antibody raised against the C-terminal amino acids CEKPPQTEHIEL of hCE1, and ECL detection (Amersham Life Sciences, Arlington Heights, IL). In all experiments, membranes were reprobed with an anti-TFIID antibody to confirm equal loading and to correct for any differences in total protein. The molecular weight of immunoreactive bands was determined using pre-stained molecular weight protein markers (Pierce, Rockford, IL). Densitometric quantitation of CE expression was performed using One-Dscan gel analysis software (Scanalytics Inc, Fairfax, VA).

Construction of adenovirus

Replication-deficient adenovirus expressing hCE1m6 or rCE were constructed as described previously.[12] Multiplicity of infection was defined as the number of plaques produced in 1×10^6 293 cells in a total volume of 1 ml of media after incubation with virus for 1 h. Typically, to determine the concentration of drug required to inhibit cell growth by 50% (IC_{50} values), a multiplicity of infection of 5 was used; however, using these conditions in U373MG cells resulted in very high levels of transgene expression, leading to toxicity. Therefore a multiplicity of infection of 1 was used for this cell line.

Growth inhibition assays

Growth inhibition assays using CPT-11 were performed in triplicate in six-wellplates as described previously.[34] IC_{50} values were calculated using Prism software (GraphPad Software, San Diego, CA).

Human tumor xenograft studies

To generate xenografts from the U373MG cell lines, 1×10^7 cells in 100 μl of phosphate-buffered saline were injected into the flanks of severe combined immunodeficiency (SCID) mice. When tumors reached approximately 1 cm,[3] they were excised and transplanted into esterase-deficient SCID mice[6,29] using the previously described methods.[5] Animals were then treated with CPT-11 given i.v., daily five times repeated for 2 weeks in a 3-week cycle at dosages ranging from 2.5 to 10 mg kg^{-1} day^{-1}. Routinely, five animals per group were used and growth of tumors was measured by monitoring volumes at weekly intervals using digital vernier calipers. Statistical analysis of xenograft data were then performed as described previously.[5] All animal studies were performed in accordance with the St Jude Children's Research Hospital Animal Care and Use Committee.

Results

Selection of amino-acid residues for mutagenesis

To identify regions within hCE1 that might enhance the hydrolysis of CPT-11, we overlaid the α-carbon traces of rCE and hCE1.[8,21,22] This identified two loops in rCE (amino acids 356–371 and 450–465) that were apparently missing from the crystal structure (Figures 1a and b). These loops formed the entrance to the active site of the protein, and we presumed that the structures of these domains could not be determined due to enhanced flexibility and thermal motion. Since these domains would likely influence the substrate specificity of the CEs, we developed a series of hCE1 mutants that contained multiple amino-acid substitutions in these loop regions, such that the sequence was identical to rCE (Figure 1c). In all, five-mutant hCE1 cDNAs were constructed (hCE1m2–hCE1m6; see Table 1 for details on the mutations).

Carboxylesterase and CPT-11-converting activity of hCE1 mutants

To assess CPT-11 activation by the hCE1 mutant proteins, we ligated the cDNAs into the mammalian expression vector pCIneo and transiently produced the proteins in COS-7 cells. As indicated in Table 2, following

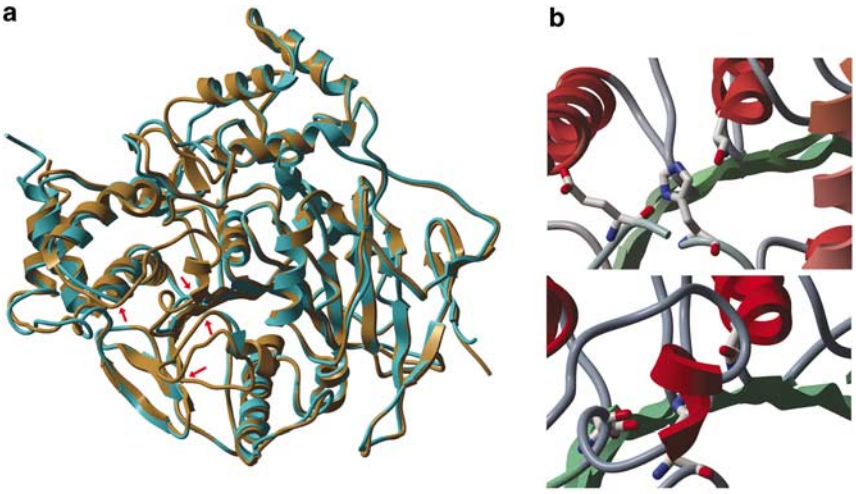

Figure 1 (**a**) Overlay of the rCE (cyan) and the hCE1 (gold) crystal structures (PDB codes 1K4Y and 1MX5, respectively). Red arrows indicate the termini of the loops that were missing in the rCE structure. (**b**) Close-up view of the entrance to the active site gorge. The upper diagram represents rCE and the lower hCE1. In both cases, the catalytic amino acids (Ser, Glu and His) are displayed in stick format. (**c**) Alignment of the rCE and hCE1 amino-acid sequences, that form the missing loops from the former enzyme, and a list of the hCE1 mutants that were constructed. Bold residues indicate amino acids that were substituted by mutagenesis. CE, carboxyesterase; hCE1, human carboxyesterase; rCE1, rabbit liver carboxyesterase.

Table 2 Carboxylesterase and CPT-11-converting activities of CE containing plasmids following transfection into COS-7 cells

Plasmid	*CE activity (nmol min^{-1} mg^{-1} ± s.d.)*	*CPT-11-converting activity (pmol h^{-1} mg^{-1} CE)*
pCIneo	5.5 ± 0.1	ND
hCE1	119.0 ± 5.0	ND
hCE1m2	267.2 ± 65.2	ND
hCE1m3	158.4 ± 20.2	ND
hCE1m4	450.8 ± 45.7	ND
hCE1m5	369.5 ± 26.8	ND
rCE (wt)	332.7 ± 17.2	41
hCE1m6	202.8 ± 10.3	40.5

Abbreviations: CE, carboxyesterase; CPT-11, irinotecan-7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; hCE1, human carboxyesterase; rCE1, rabbit liver carboxyesterase; ND, not detected.

transfection with hCE1-containing plasmids, all cell extracts demonstrated CE activity. In addition, all had similar levels of CE protein as determined by western analysis using an anti-hCE1 antibody (data not shown). However, only hCE1m6 could convert CPT-11 into SN-38.

Comparison of the CPT-11-converting activity of hCE1m6 and rCE

To directly compare the ability of hCE1m6 and rCE to activate CPT-11, we expressed both proteins in COS-7 cells and assessed the ability of extracts to hydrolyze the drug. In these studies, the levels of CPT-11 activation were corrected for the amounts of CE protein in the cell extracts by western analysis (Figure 2a). This was necessary since it was unclear whether the mutations would influence the ability of the CEs to metabolize *o*-nitrophenyl acetate, which is used as a measure of CE enzyme activity. As indicated in Table 2, hCE1m6 and rCE were essentially equally efficient at CPT-11 hydrolysis.

Kinetic parameters of hCE1m6

To directly compare the abilities of the mammalian CEs to hydrolyze CPT-11, we determined the K_m, V_{max}, k_{cat} and k_{cat}/K_m values for the purified enzymes. As indicated in Table 3, hCE1m6 was approximately70-fold more efficient at CPT-11 hydrolysis than hCE1. In addition, hCE1m6 was almost as effective at CPT-11 hydrolysis as hiCE. It should be noted that both hiCE and hCE1m6 were less efficient at drug activation than Rce; however,

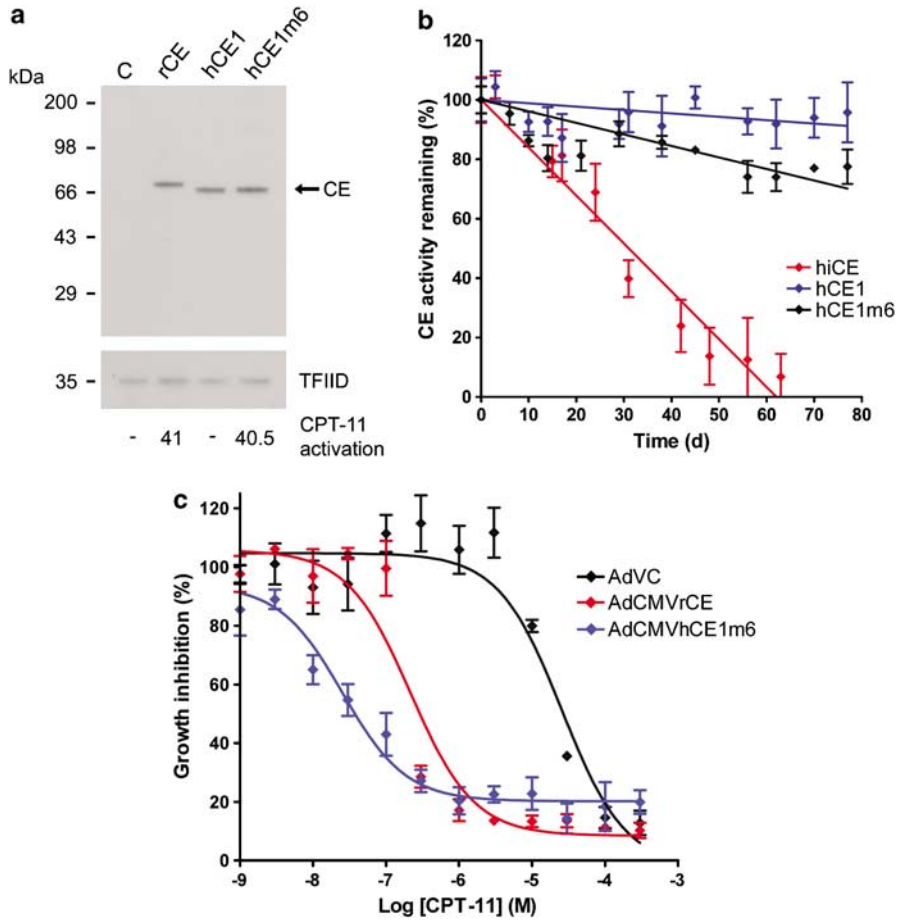

Figure 2 (**a**) COS-7 cells were transfected with pCIneo (C), pCIrCE (rCE), pCIhCE1 (hCE1) or pCIhCE1m6 (hCE1m6) and the expression was evaluated using an anti-CE antibody. TFIID was used as a loading control. The ability of the extracts to hydrolyze CPT-11 is indicated below each lane, where the data are expressed as pmol SN-38 produced per hour per milligram of CE. (**b**) *In vitro* stability of hCE1 (blue line), hCE1m6 (black line) and hiCE (red line). Enzymes were aliquoted in 50 mM Hepes, stored at 20 °C and CE activities were determined at various time intervals, up to 11 weeks. Data are expressed as the amount of active CE remaining as compared to day 0. (**c**) Growth inhibition curve for U373MG cells transduced with AdVC (black line), AdCMVrCE (red line) or AdCMVhCE1m6 (blue line), following treatment with CPT-11. The IC_{50} values for these cells with CPT-11 are 26.8, 0.3 and 0.04 μM, respectively. CE, carboxyesterase; CPT-11, irinotecan-7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; hCE1, human carboxyesterase; hiCE, human intestinal carboxylesterase; IC_{50}, the concentration of drug that produced a 50% inhibition of cell growth; rCE1, rabbit liver carboxyesterase; SN-38, 7-ethyl-10-hydroxycamptothecin.

Table 3 Kinetic parameters for the conversion of CPT-11 to SN-38 by hCE1, hCE1m6, rCE and hiCE

Enzyme	K*m* (μM)	V*max* (nmol min^{-1} mg^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	*Ratio* k_{cat}/K_m *as compared to hCE1*
hCE1[a]	82.8 ± 9.6	0.36 ± 0.017	0.28	1
hCE1m6	6.25 ± 0.59	2.11 ± 0.06	19.8	71
rCE[a]	6.20 ± 0.63	18 ± 0.9	180.0	650
hiCE[b]	3.35 ± 0.34	1.49 ± 0.04	25.2	91

Abbreviations: CE, carboxyesterase; CPT-11, irinotecan-7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; hCE1, human carboxyesterase; hCE1m6, hCE1 containing 8 site specific mutations; hiCE, human intestinal carboxylesterase; rCE1, rabbit liver carboxyesterase; SN-38, 7-ethyl-10-hydroxycamptothecin.
[a]Data are taken from Wadkins *et al.*[31]
[b]Data are taken Hatfield and Potter (manuscript in preparation).

since hiCE has effectively been used in prior enzyme/prodrug therapy approaches,[17–20] we presumed that hCE1m6 would be efficacious in CPT-11 activation *in vivo*.

In vitro *and* in vivo *stability of hCE1, hiCE and hCE1m6*

In previous biochemical experiments, we noted that *in vitro* purified hiCE was much less stable than hCE1. Therefore, we evaluated the loss of CE activity in

preparations of these proteins, as well as hCE1m6, which had been stored at room temperature. In these studies, purified protein was aliquoted in 50 mM Hepes (pH7.4), and enzyme activity was determined over a period of 12 weeks. As indicated in Figure 2b, hiCE was the least stable protein, losing 50% of its activity by approximately 30 days. In contrast, both hCE1m6 and hCE1 were relatively stable under these conditions with predicted half-lives of 129 and 440 days, respectively.

In *in vivo* studies, we observed that expression of hiCE following plasmid-mediated transfection was frequently lower than that seen with rCE or hCE1. Therefore, we developed U373MG cells expressing hiCE, hCE1 or hCE1m6 using the plasmid pIRESneo, and monitored CE expression in the derived lines over an extended period of time. The average levels of CE activity in U373hiCE, U373hCE1 and U373hCE1m6 cells were 287.6 ± 76.6, 1077.1 ± 77.5 and 466.5 ± 52.6 nmoles min^{-1} mg^{-1}, respectively. Since high-level expression of the prodrug-activating protein would be necessary for effective application of enzyme/prodrug therapy, we envisage that hiCE would not be the best CE for activating CPT-11.

Growth inhibition curves for human tumor cells expressing hCE1m6

To determine whether the expression of hCE1m6 in mammalian cells sensitized them to CPT-11, we generated a panel of transfected U373MG cell lines expressing the appropriate enzyme and evaluated their dose–response to the drug. As indicated in Table 4, cells expressing hCE1m6, hiCE or rCE were sensitized to CPT-11 due to intracellular conversion of the drug to SN-38. Cells expressing hCE1 were not sensitized, consistent with the lack of CPT-11 hydrolysis observed in the biochemical studies. Furthermore, U373MG cells expressing hCE1m6 were equally as sensitive to CPT-11 as cells expressing rCE, with IC_{50} values ranging from 0.18 to 0.40 μM. These values were approximately 18- to 86-fold less than that of cells expressing wild-type hCE1 (IC_{50} value = 15.5 μM). Since all the cell lines used in these studies expressed approximately similar amounts of CE, these results indicate that hCE1m6 can efficiently convert CPT-11 to SN-38 intracellularly and sensitize cells into the drug.

Studies with adenovirus expressing hCE1m6

For the success of any enzyme/prodrug therapy approach, high levels of tumor-specific expression of the prodrug-activating enzyme will be required. Since plasmid transfection is unlikely to be a viable option for this approach, we generated E1A, the E3-deleted replication-deficient adenovirus expressing hCE1m6, and compared the ability of this vector to sensitize cells to CPT-11. As shown in Table 4, in all human tumor cell lines, expression of hCE1m6 significantly decreased the CPT-11 IC_{50} values as compared to vector-transduced cells. In SK-N-AS and Rh30 cell lines, the CPT-11 sensitivity was comparable to cells transduced with AdCMVrCE (adenovirus containing the rabbit liver CE cDNA) with IC_{50} values of 2 and 0.6 nM, respectively. However, the human astrocytoma cell line U373MG was 7.5-fold more sensitive to CPT-11 after AdCMVhCE1m6 transduction than after exposure to AdCMVrCE. This resulted in an overall reduction in IC_{50} for CPT-11 of approximately 670-fold in U373MG cells (Figure 2c), the greatest sensitization we have ever observed using this enzyme/prodrug approach. Overall, these results indicate that AdCMVhCE1m6 can sensitize cells to CPT-11 as effectively as AdCMVrCE and that the former vector should be suitable for enzyme/prodrug therapy with this drug.

Table 4 Growth inhibition studies of U373MG cells expressing different CEs to CPT-11

Cell line	*Adenovirus*	*Enzyme expressed*	*CE activity (nmol min^{-1} mg^{-1} ± s.d.)*	*CPT-11 IC_{50} (μM)*[a]	*Fold decrease in IC_{50} value as compared to U373hCE1 or cell line+AdVC*
U373IRES	—	None	10.0 ± 0.3	24.0	—
U373hCE1	—	hCE1	1016.4 ± 45.5	15.5	—
U373hiCE	—	hiCE	408.2 ± 5.6	0.84	18
U373rCE	—	rCE	601.0 ± 20.5	0.40	39
U373hCE1m6	—	hCE1m6	437.3 ± 37.5	0.18	86
U373MG	AdVC	None	10.0 ± 0.5	26.8	—
	AdCMVrCE	rCE	1076.8 ± 67.3	0.30	89
	AdCMVhCE1m6	hCE1m6	5999.8 ± 162.5	0.04	670
Rh30	AdVC	None	4.6 ± 0.3	64.3	—
	AdCMVrCE	rCE	665.6 ± 52.0	3.40	29
	AdCMVhCE1m6	hCE1m6	2757.6 ± 87.5	2.00	32
SK-N-AS	AdVC	None	6.9 ± 0.3	31.7	—
	AdCMVrCE	rCE	2150.3 ± 105.9	0.60	53
	AdCMVhCE1m6	hCE1m6	6225.0 ± 113.4	0.50	63

Abbreviations: Ad, Adenovirus; CE, carboxyesterase; CPT-11, irinotecan-7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; hCE1, human carboxyesterase; hCE1m6, hCE1 containing 8 site specific mutations; hiCE, human intestinal carboxylesterase; IC_{50}, the concentration of drug that produced a 50% inhibition of cell growth; rCE1, rabbit liver carboxyesterase.
[a]Results are a representative data set from three individual experiments.

Sensitization of human tumor xenografts expressing hCE1m6 to CPT-11

Having determined that cells expressing hCE1m6 were sensitized *in vitro* to CPT-11, we wished to evaluate whether similar results could be obtained *in vivo*. Therefore, we established xenografts from the transfected U373MG cell line (U373MGhCE1m6) by injection into the flanks of SCID mice. Following transplantation into esterase-deficient SCID mice[6,29] tumors obtained from these animals as well as those generated from U373MG cells, were treated with CPT-11 at dosages ranging from 0 to 10 mg kg^{-1} and growth was monitored weekly for up to 12 weeks. As indicated in Figures 3 a and b, all untreated tumors grew very rapidly and animals were euthanized within 2 weeks. CPT-11-treated animals demonstrated increasing delays in tumor growth as the drug dose increased (Figures 3c, e and g). However, xenografts expressing hCE1m6 (Figures 3d, f and h) were all considerably more sensitive to the drug to the extent that mice treated with either 5 or 10 mg kg^{-1} CPT-11 demonstrated complete elimination of the tumors. This was not observed in mice containing parental U373MG tumors.

Discussion

Enzyme/prodrug therapy approaches have previously involved enzymes derived from viruses (for example, Herpes simplex virus thymidine kinase) or bacteria (for example, *Escherichia coli* cytosine deaminase). However, it is likely that these enzymes would be very immunogenic when expressed in humans. Originally, we had developed an enzyme-prodrug system using CE/CPT-11 with rCE as the activating enzyme. Although rCE is likely to be less immunogenic than the bacterial or viral proteins, potentially, this CE could induce unwanted immune responses that may hinder the application of this approach. Therefore, we have developed a human CE that is proficient at CPT-11 activation, and can be

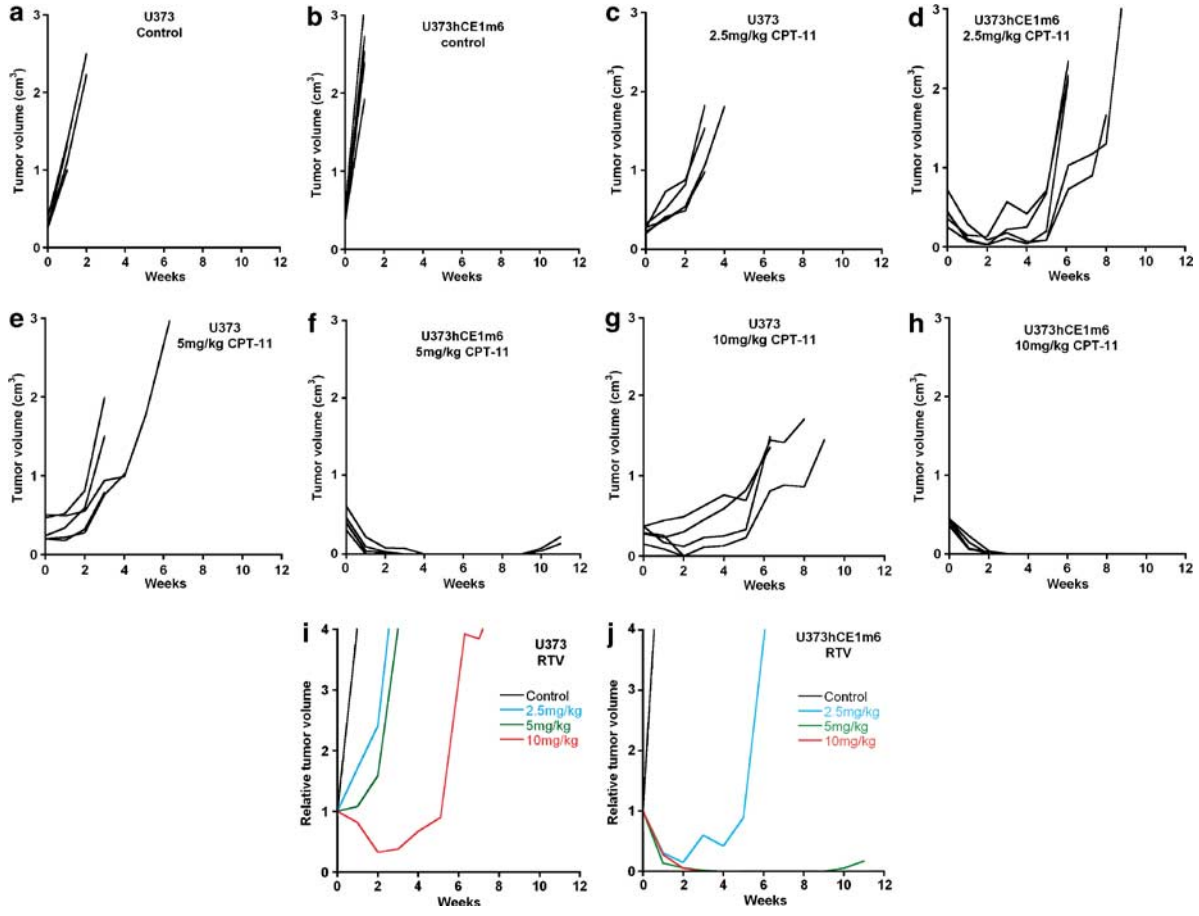

Figure 3 Xenograft studies with U373MG tumors either lacking (U373; left panels) or expressing (U373hCE1m6; right panels) hCE1m6, treated with CPT-11. Control tumors (**a** and **b**) were not treated with drug. The doses of drug used were 2.5, 5 and 10 mg kg^{-1} for panels (**c** and **d**), (**e** and **f**) and (**g** and **h**), respectively. Drug was given daily five times repeated for 2 weeks in a 3-week cycle (((d × 5)2)3) and each graph represents the growth of five individual tumors in five different animals. The bottom panel in each column (**i** and **j**) provides a composite of all of the data in the preceding graphs, expressed as relative tumor volumes. CPT-11, irinotecan-7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; hCE1, human carboxyesterase; hCE1m6, hCE1 containing 8 site specific mutations.

expressed at high levels in human tumor cells. This was achieved by examining the crystal structures of rCE and hCE1 and identifying domains that may influence CPT-11 hydrolysis.

In initial studies, we subjected a region of the protein thought to be involved in the 'side door' of the CEs to site-directed mutagenesis. This domain is adjacent to the active site and thought to act as an exit pore for the products of enzymatic hydrolysis.[22,26] However, a series of hCE1 mutants containing changes in residues that form the side door did not alter CPT-11 activation (data not shown). Since we have previously reported that the entrance to the active site significantly influences substrate hydrolysis,[31] we examined these domains in the crystal structures of rCE and hCE1. In rCE, the loops that formed the active site entrance were missing, suggesting considerable flexibility of these regions of the protein. This suggests that motion within these loops may be necessary to accommodate large substrates within the active site gorge. Since these loops were visible in the hCE1 crystal structure, we hypothesized that this lack of movement, and presumably flexibility within this domain, may impede the access of bulky substrates, such as CPT-11, to the catalytic residues that are buried at the bottom of the gorge.

Based on this hypothesis, we developed a series of hCE1 mutants containing multiple mutations that would make the amino-acid sequence of these loops identical to those seen in rCE. The final clone hCE1m6, that contained a total of eight mutations, metabolized CPT-11 as efficiently as hiCE. In addition, expression of this protein in COS-7 cells resulted in sensitivity to CPT-11, yielding IC_{50} values similar to that observed with rCE. Interestingly, all eight mutations were required to produce a protein that could hydrolyze CPT-11, with no change in SN-38 production being observed in mutants, hCE1m2–hCE1m5 (Table 2). This indicates that it is the combination of amino-acid substitutions that results in altered substrate specificity for hCE1. Whether these residues originally evolved for a specific function of hCE1 *in vivo* is unclear, especially since it is currently unknown if there are endogenous substrates for this enzyme. However, it is apparent that mutagenesis to alter substrate specificity for CEs can be achieved.

While site-specific mutagenesis can influence the ability of CEs to hydrolyze different substrates, selection of the amino acids necessary to alter such specificity could not be achieved by the analysis of the primary sequence of the proteins. This is exemplified by the fact that in the homology alignments of the amino acids in hCE1 and rCE, there are 104 differences between the two enzymes.[8] Hence, randomly or selectively, choosing eight amino acids from these alignments to change, in an attempt to alter substrate specificity, would be impossible. Indeed, we were only able to perform these mutagenesis studies by guidance from the X-ray crystal structures of the proteins. Our results suggest that to alter the substrate specificity of CEs, either a detailed knowledge of the structure of the enzyme is necessary or a selection system for evolution of a desired catalytic activity is required.[35,36]

As mentioned above, the hCE1m6 protein that contains eight amino-acid substitutions can metabolize CPT-11 with approximately the same efficiency as hiCE and about 9-fold less than rCE (based on the k_{cat}/K_m values). However, the converse eight mutations generated in the rCE protein did not completely abolish the CPT-11-converting ability of this CE. These observations suggest that although the loop structures that form the entrance of the active site gorge are important, they are not the sole determinants of the ability of the enzyme to metabolize CPT-11. Alternative factors that are likely involved in substrate specificity include the dimensions of the active site gorge, the dynamics and flexibility of the protein as a whole, and the hydrophobicity of the catalytic domain. We have not examined these parameters in detail, but preliminary studies using the *Bacillus subtilis* pnbA protein (a homolog of both hCE1 and rCE[34]) indicate that the motion of one domain of the enzyme significantly impacts substrate hydrolysis.[37] We are currently extending these studies to determine the contribution of these factors toward CE substrate selectivity.

Another human CE, hiCE, has been demonstrated to be proficient at CPT-11 hydrolysis and hence this would seem to be a more likely choice for any *in vivo* enzyme/prodrug therapy approach. However, in preliminary studies, we have observed that the expression of hiCE in cells is variable and lower than that observed for hCE1 or rCE. In addition, hCE1 and hCE1m6 are exceptionally stable (indefinitely at $-80\,^{\circ}C$ or freeze dried, and for up to several years at $4\,^{\circ}C$ in solution), whereas hiCE loses activity (Figure 2b). However, perhaps the most compelling reason that hCE1m6 might be more suitable for enzyme/prodrug therapy approaches with CPT-11 is that in *in vivo* studies using both plasmid transfection and adenoviral transduction (Table 4), the degree of sensitization to the drug was greater as compared to cells expressing hiCE or rCE. Indeed, using adenovirus expressing hCE1m6, we observed the largest enhancement in CPT-11 sensitivity, greater than that seen in the previous studies with rCE. The exact reasons why hCE1m6 is better than rCE in *in situ* CPT-11 activation are unclear, but may result from a combination of factors including enzyme stability and/or protein folding. Furthermore, xenograft studies using CPT-11-resistant human tumors indicate that expression of hCE1m6 can result in significant growth delays and complete regression of xenografts with higher drug doses (Figure 3). Overall, our results suggest that hCE1m6 would be the preferred choice for enzyme/prodrug therapy with CPT-11.

In the studies presented here, we used adenovirus as a delivery vector to ensure high-level expression of hCE1m6 in mammalian cells. Clearly, this approach would not be used clinically since any reduction in immunogenicity achieved using hCE1m6 (versus rCE) would be negated by the highly immunogenic adenoviral capsid proteins. Rather, we used this viral vector to demonstrate 'proof of principle' for enzyme/prodrug therapy with CPT-11, and to allow direct comparison of the results obtained using hCE1m6 with previously constructed virus expressing

rCE.[12] Future studies will assess whether the modifications we have generated in hCE1m6 result in reduced immunogenicity in both *in vitro* (for example, lymphocyte activation) and *in vivo* assays. We anticipate that these experiments will determine whether the modified hCE1 protein is indeed less immunogenic than rCE and suitable for clinical application of enzyme/prodrug therapy in combination with CPT-11.

Recently we have developed a novel approach to treat metastatic cancer using neural progenitor cells.[13,14] These studies demonstrated that 90% of mice bearing multiple neuroblastoma metastases were disease-free, following treatment with neural progenitor cells expressing rCE and CPT-11 administration. In addition, no increase in toxicity was observed in these animals and doses of CPT-11 were used that are tolerable in cancer patients. Hence, this approach that we have termed NDEPT (neural progenitor cell-directed enzyme prodrug therapy) may have utility in the treatment of metastatic lesions in patients. However, one of the drawbacks of this approach is that rCE was used as the drug-activating enzyme. Since this is likely to be immunogenic in humans, we believe that hCE1m6 will be a more suitable candidate CE for CPT-11 activation in *in vivo* applications. We are currently assessing the applicability of these techniques.

Abbreviations

CE, carboxylesterase; CPT-11, irinotecan,7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; hCE1, human carboxylesterase 1; hCE1m6, hCE1 containing 8 site specific mutations; hiCE, human intestinal carboxylesterase; IC_{50}, the concentration of drug that produced a 50% inhibition of cell growth; rCE, rabbit liver carboxylesterase; SN-38, 7-ethyl-10-hydroxycamptothecin.

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