Comparison of Activation of CPT-11 by Rabbit and Human Carboxylesterases for Use in Enzyme/Prodrug Therapy[1]

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ABSTRACT

Several recent studies have examined the possibility of producing tumor-specific cytotoxicity with various enzyme/prodrug combinations. The enzymes are targeted to tumor cells either with antibodies (ADEPT, antibody directed enzyme prodrug therapy) or with viruses (VDEPT). The goal of the present study was to identify an appropriate enzyme for use in activating the prodrug 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11). In this study, we compared the efficiency of CPT-11 metabolism by rabbit and human carboxylesterases in *in vitro* and *in situ* assays. Although the rabbit and human enzymes are very similar (81% identical; 86% homologous) and the active site amino acids are 100% identical, the rabbit enzyme was 100-1000-fold more efficient at converting CPT-11 to SN-38 *in vitro* and was 12–55-fold more efficient in sensitizing transfected cells to CPT-11. *In vivo*, Rh30 rhabdomyosarcoma cells expressing the rabbit carboxylesterase and grown as xenografts in immune-deprived mice were also more sensitive to CPT-11 than were control xenografts or xenografts expressing the human enzyme. Each of the three types of xenografts regressed when the mice were treated with CPT-11 given i.v. at 2.5 mg of CPT-11/kg/daily for 5 days/week for 2 weeks [(dx5)2] (one cycle of therapy), repeated every 21 days for a total of three cycles. However, following cessation of treatment, recurrent tumors were detected in seven of seven mice bearing control Rh30 xenografts and in two of seven mice bearing Rh30 xenografts that expressed the human enzyme. No tumors recurred in mice bearing xenografts that expressed the rabbit carboxylesterase. We conclude that rabbit carboxylesterase/CPT-11 may be a useful enzyme/prodrug combination.

INTRODUCTION

Several approaches to achieve tumor cell-specific activation of prodrugs have been reported recently. One approach is to target enzymes that activate chemotherapeutic agents by coupling the enzymes to antibodies that recognize epitopes expressed on the surface of tumors (ADEPT;[3] Refs. 1 and 2). A second approach is to transduce the cDNA for an activating enzyme using retroviral vectors (VDEPT; Refs. 3 and 4). A modification of this approach is to use adenoviral vectors to transduce the cDNA encoding a drug-activating enzyme, the expression of which is under the control of a transcription factor overexpressed by a particular tumor (5–8).

The first consideration in designing potential treatment schema based on the above approaches is the choice of an appropriate enzyme. With each enzyme/prodrug combination, an enzyme efficient in converting the prodrug to the active compound *in situ* must be identified. Enzymes most commonly used in previous studies have been *Escherichia coli* cytosine deaminase in combination with 5-fluorocytosine and HSVtk to activate ganciclovir (9–12). We have recently isolated and sequenced a cDNA encoding a rabbit liver carboxylesterase that converts the topoisomerase I inhibitor CPT-11 to its active metabolite SN-38 (13, 14).

The therapeutic advantage to overexpressing cytosine deaminase or thymidine kinase derives from unique characteristics of mammalian cells or enzymes compared with the bacterial or viral enzymes. Mammalian cells do not express cytosine deaminase (12, 15); the overexpression of the *E. coli* enzyme is thought, therefore, to confer selective cytotoxicity of 5-fluorocytosine by converting it to 5-fluorouridine in tumor cells induced to express the exogenous deaminase (16). In contrast, mammalian cells do express thymidine kinase. However, the affinity of the human enzyme for ganciclovir is 4 to 15 times lower than that of the HSVtk (17). Ganciclovir is thereby preferentially phosphorylated by HSVtk, inhibiting DNA synthesis and producing selective toxicity in cells that overexpress the viral enzyme (18, 19). Similarly, both human and rabbit

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[3] The abbreviations used are: ADEPT, antibody directed enzyme prodrug therapy; VDEPT, virus directed enzyme prodrug therapy; HSVtk, *Herpes simplex* virus thymidine kinase; APC, 7-ethyl-[4-*N*-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin; CPT-11, irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; HPLC, high performance liquid chromatography; HA, hemagglutinin; IC_{50}, concentration of drug that inhibits the growth of cells in tissue culture by 50%; o-NPA, *ortho*-nitrophenyl acetate; SN-38, 7-ethyl-10-hydroxycamptothecin.

Table 1 Comparison of deduced amino acid residues of human and rabbit carboxylesterases at the four positions for which the reported sequences of the human enzymes differ

Tissue of origin	Aligned amino acid residues[a]			
	#18	#57	#363	#538
Human (99% overall identity)				
Macrophage (I57004)[b]		Alanine	Glutamine	Alanine
Liver (1) (A48809)[b]	Alanine	Glycine	Glutamine	Glycine
Liver (2) (I61085)[b]	Glycine	Glycine		Glycine
Rabbit (81% identical to human)				
Liver		Alanine		Alanine
Human (enzyme sequence used in this study)				
Liver		Alanine		Alanine

[a] Amino acid sequences of all five carboxylesterases were aligned and numbered using the reported sequence for liver (1) carboxylesterase.
[b] PIR accession number.

carboxylesterases are known to convert CPT-11 to SN-38 (13, 14, 20, 21), but unlike the thymidine kinase enzymes, the relative efficiency of catalysis by each enzyme is not known.

In patients, SN-38 is detectable in the plasma within minutes after CPT-11 is administered (22). The amount of CPT-11 that is converted to SN-38 is <5%, as determined by concentrations of each compound in the plasma (22). *In vitro*, overexpression of a human liver enzyme sensitized the A549 human lung tumor cell line to CPT-11 17-fold (23), and overexpression of the rabbit liver enzyme sensitized human rhabdomyosarcoma and glioma cell lines to CPT-11 by 9- and 56-fold, respectively (13, 14). A side-by-side comparison of the two enzymes to demonstrate the relative efficiencies of each enzyme in activating CPT-11 has not been reported. To facilitate the choice of an enzyme for use in ADEPT and VDEPT, we compared the amino acid sequences of rabbit and human carboxylesterases, the activation of CPT-11 by each enzyme *in vitro* and *in situ*, the relative ability of each enzyme to sensitize Rh30 rhabdomyosarcoma cells and U-373 MG glioma cells to CPT-11, and the ability of each enzyme to sensitize human tumor xenografts to CPT-11 in a preclinical *in vivo* model.

MATERIALS AND METHODS

Chemicals. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. CPT-11, APC, and SN-38 were generous gifts from Dr. J. P. McGovren (Pharmacia Upjohn Co., Kalamazoo, MI). Each drug was dissolved in methanol or DMSO and stored at −20°C at a concentration of 10 mM. Immediately prior to use, dilutions were made with water (CPT-11 and APC) or DMSO (SN-38).

Cell Lines. U-373 MG glioblastoma cells were purchased from American Type Culture Collection. U-373 MG cells and Rh30 rhabdomyosarcoma cells were grown in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 2 mM L-glutamine and kept at 37°C in a humidified atmosphere of 5% CO_2, as reported previously (13, 14). Each of the above cell lines was transfected with the mammalian expression vector pIRESneo (Rh30pIRES and U373pIRES; Clontech, Palo Alto, CA) or pIRESneo containing the cDNA encoding a rabbit liver carboxylesterase (U373pIRES$_{rabbit}$ and Rh30pIRES$_{rabbit}$) as reported previously (14). Briefly, 10^7 cells were electroporated with 20 μg of plasmid DNA in a volume of 200 μl of 0.9% NaCl using a Bio-Rad (Hercules, CA) electroporator and a capacitance extender. U-373 MG and Rh30 cells were also transfected with pIRESneo containing the coding sequence for a human carboxylesterase (U373pIRES$_{human}$ and Rh30pIRES$_{human}$) by the same method as above, also as reported previously (14).

Isolation and Characterization of the Human Carboxylesterase cDNA. This cDNA was obtained by designing PCR primers based on the published sequence for human alveolar macrophage carboxylesterase (GenBank Accession No. M73499) and performing PCR reactions with cDNA prepared from human liver poly(A)$^+$ RNA mRNA (Clontech) using random primers and a cDNA cycle kit (Invitrogen, Carlsbad, CA). Pfu polymerase (Stratagene, La Jolla, CA) was used to minimize PCR-induced mutations. Preparation of plasmid DNA, ligations, and transformations have all been described previously in detail (14). cDNA sequences of selected clones were characterized by automated sequencing in the St. Jude Center for Biotechnology.

HPLC Analysis for CPT-11 and SN-38. This procedure has been reported previously (22). Briefly, a Nova-Pak C_{18} column was equilibrated with 75 mM ammonium acetate, 25% acetonitrile, pH 4.0, and acidified methanolic extracts of reaction mixtures or cell pellets were eluted at a flow rate of 1 ml/min. Under these conditions, CPT-11 and SN-38 eluted at 5.2 and 7.5 min, respectively. Each compound was detected with a Jasco 821-FP fluorescence detector at an excitation wavelength of 375 nm and an emission wavelength of 550 nm. Products were detected with a Jasco 821-FP fluorescence detector. Data were analyzed using System Gold software. The limit of detection for CPT-11 and SN-38 was 20 and 2 pg/μl, respectively (24). Quantitations of CPT-11 and SN-38 produced by this method are reported as total number of fluorescence units/peak based on HPLC internal standards or as pg or molar concentrations based on standard curves of fluorescence peak areas generated with solutions of known concentrations of each drug. The variability of the assay between duplicate samples and among replicate experiments was <1%.

Carboxylesterase Activity. Carboxylesterase activity was quantitated as described previously using o-NPA as a substrate (14, 25). The reaction monitored was the conversion of o-NPA to nitrophenol. Production of nitrophenol was detected spectrophotometrically at 420 nm. Activity is expressed as μmol of nitrophenol produced/mg protein/min. One unit of enzyme

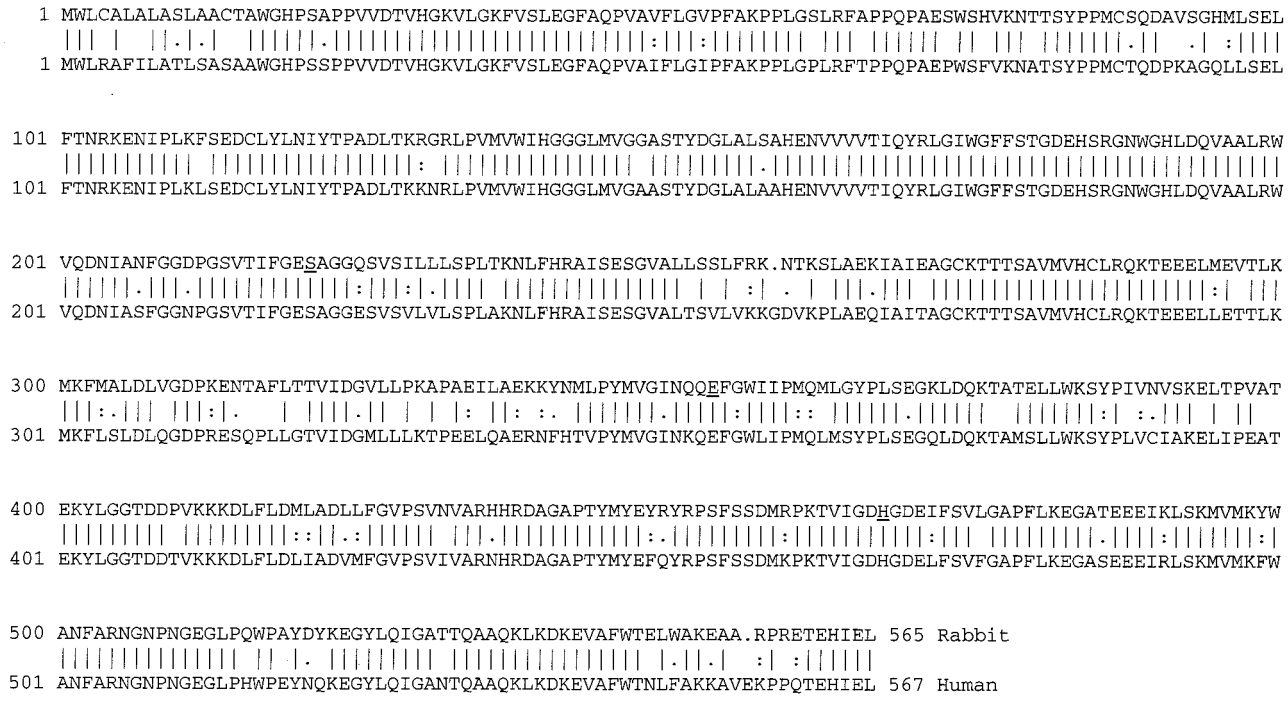

Fig. 1 Comparison of the amino acid sequences of the human and rabbit carboxylesterase proteins used in this study. The three reported active site residues (serine 221, glutamic acid 353, and histidine 467) are *underlined*. The cDNA encoding each enzyme was isolated from liver poly(A)$^+$ RNA as detailed in "Materials and Methods" and sequenced by automated methods.

activity is defined as mg of protein required to produce 1 μmol of nitrophenol/min.

Enzymatic Conversion of CPT-11 to SN-38. These analyses were carried out as described previously (24). In brief, cell sonicates were incubated with 25 μM CPT-11 in a final volume of 200 μl of 50 mM HEPES (pH 7.4) at 37°C for 22 h. The reaction was terminated by the addition of one volume of acidified methanol, vortexed, and centrifuged at 14,000 × *g* for 2 min. SN-38 in the supernatant was quantitated by HPLC (22).

Growth Inhibition Assays. These assays were performed as reported previously (13, 14). Cells were plated at a density of 2×10^4 cells/ml, 2 ml/dish. The cells were allowed to attach overnight, and drug was added in a volume of 20 μl/2-ml tissue culture medium. After control cultures had doubled two to three times, the number of cells remaining in each dish was determined using a Coulter Counter. Data were analyzed using the program for a sigmoidal dose response with a variable or fixed slope of GraphPad Prism software. The concentration of CPT-11 required to inhibit growth of cells by 50% compared with vehicle-treated controls is defined as the IC_{50}.

SDS-PAGE and Immunoblot Analyses. SDS-PAGE and immunoblot analyses of whole-cell sonciates were performed as described previously (26–28). Gels were either stained with Coomassie Blue R-250, or proteins were transferred by electroblotting to Immobilon-P (Millipore, Bedford, MA) for Western analysis. Filters were incubated with primary antibody, and specific immunoreactivity was detected by chemiluminescence (ECL; Amersham, Arlington Heights, IL).

Xenografts Studies. Methods for immune deprivation of CBA/CaJ mice, establishment of human tumor cell lines as xenografts, administration of CPT-11, and statistical analysis of xenograft studies were performed as published previously (29). Parental Rh30 cells or Rh30 cells transfected with the pIRES plasmid encoding either the rabbit or the human carboxylesterase were established *in vitro* and subsequently as xenografts by injection 10^7 cells s.c. in the mice. Drug treatment was begun when the tumors had reached an average volume of ~0.5–1.0 cm^3. Any tumor <0.2 cm^3 in size at the time drug treatment was initiated was excluded from the analysis. Tumor size at the beginning of the experiment was equal among all six control and drug-treated groups shown in Fig. 5; tumor volumes of each group are specified in the legend to this figure.

Mice bearing advanced s.c. tumors each received CPT-11 2.5 mg/kg/day for three treatment cycles as detailed in "Results." The third treatment cycle ended at week 8, and mice were monitored for an additional 6 weeks to assess tumor recurrence. Tumors were measured by obtaining two perpendicular diameters at 7-day intervals using digital Vernier calipers interfaced with a computer. Tumor volumes were calculated assuming the tumors to be spherical, using the formula $[(\pi/6) \times d^3]$, where d is the mean diameter.

RESULTS

Amino Acid Sequences of the Human and Rabbit Carboxylesterases. A review of the literature showed that three sequences have been reported for carboxylesterases of human origin: one isolated from alveolar macrophages, and two from liver microsomes. These three sequences are remarkably similar, differing in only 4 of 569 amino acids as summarized in

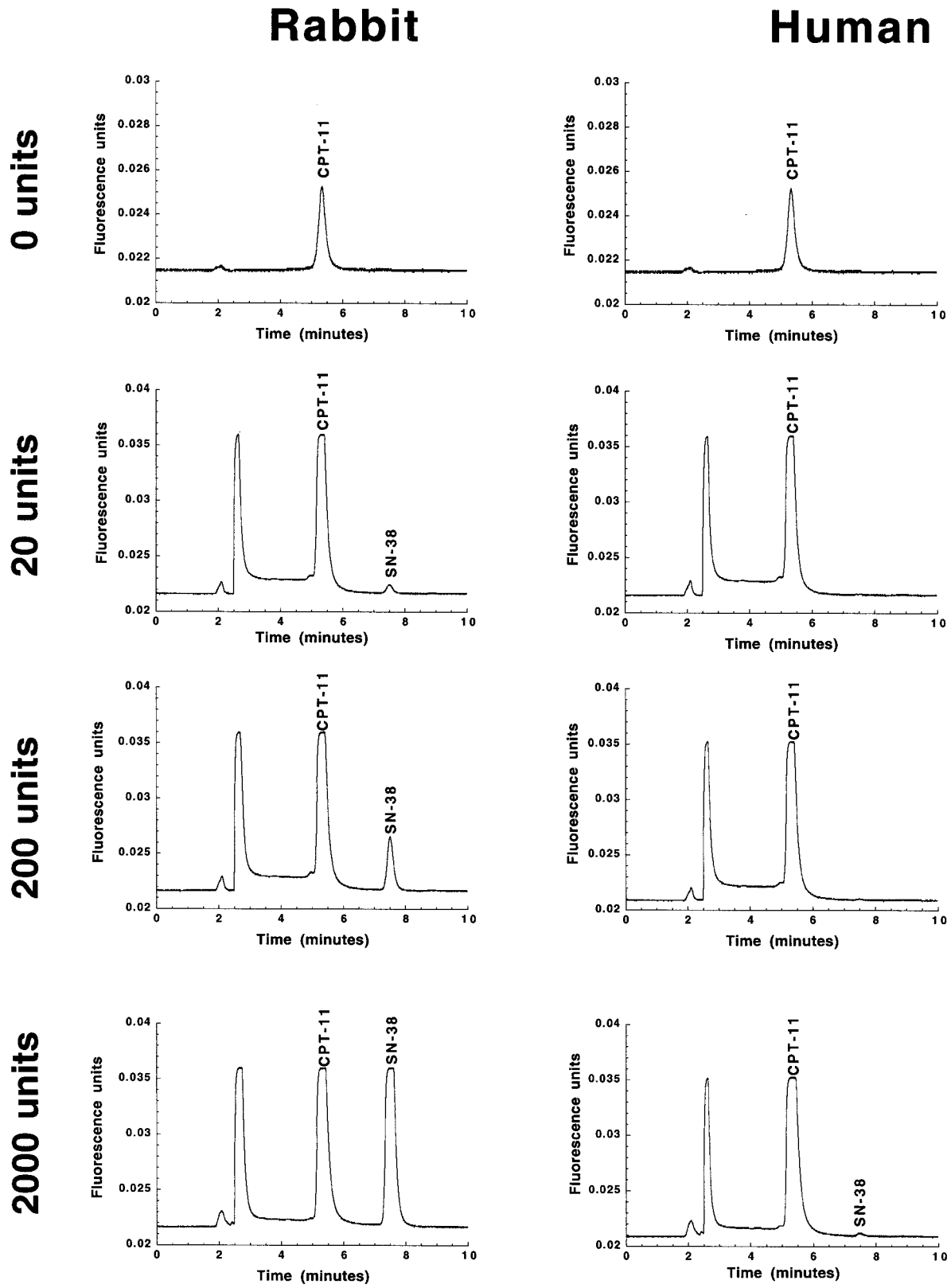

Fig. 2 HPLC profiles of CPT-11 and SN-38 produced by incubating cell lysates of Rh30pIRES$_{human}$ and Rh30pIRES$_{rabbit}$ cells with 25 μM CPT-11 at 37°C for 22 h. The amount of cell lysate used contained 20, 200, or 2000 units of carboxylesterase activity, as determined by incubation of each lysate with the classic carboxylesterase substrate o-NPA. The method is detailed in "Materials and Methods."

Table 1. These four amino acids occur in areas of the protein that are not in the active site of the enzyme. The cDNA sequences of the three human enzymes are >99% identical (data not shown). The sequence of the rabbit liver carboxylesterase, which has been published recently (14), is 86% homologous to each of the three human enzymes (see below).

The cDNAs of both rabbit and human origin that were used in this study were derived from liver RNA of each species. The high degree of homology among carboxylesterases as well as the method and primers used to isolate the cDNA (14) made it necessary to sequence each complete cDNA to determine precisely which isozyme, if indeed several exist, had been isolated. The sequence of the human carboxylesterase cDNA isolated from human liver RNA as described above and used in this study corresponded exactly to that reported for human alveolar macrophage carboxylesterase with the exception of a glutamine residue deleted at position 363 (Table 1; Ref. 30). This human carboxylesterase is 81% identical and 86% similar to the rabbit enzyme at the amino acid level. The regions of greatest disparity between the rabbit and human enzymes occur between amino acids 250 and 272 and 303 and 344 (Fig. 1). The three reported active site residues (serine 221, glutamic acid 353, and histidine 467) are identical in all previously reported carboxylesterases, as well as in the carboxylesterases used in this study.

Catalysis of o-NPA and CPT-11 by Human and Rabbit Carboxylesterases. Rh30 rhabdomyosarcoma cells expressing human or rabbit carboxylesterases were harvested, resuspended in 50 mM HEPES buffer, and sonicated to prepare lysates. Each lysate was then incubated with 3 mM o-NPA, and the conversion of o-NPA to nitrophenol was monitored spectrophotometrically. Equal amounts of enzymatic activity (20, 200, or 2000 units) from sonicates of $Rh30pIRES_{human}$ or $Rh30pIRES_{rabbit}$ cells were then incubated with 25 μM CPT-11 for 22 h. Methanolic extracts of each reaction mixture were analyzed by HPLC (Fig. 2) to quantitate the enzymatic conversion of CPT-11 to SN-38. Quantitation of data in HPLC profiles (Fig. 3) shows a concentration-dependent increase from 70 nM to 17 μM SN-38 in reaction mixtures containing CPT-11 and increasing amounts of rabbit carboxylesterase activity. In each case, the concentration of CPT-11 decreased commensurately with an increase in SN-38 production. In contrast, with sonicates from cells expressing the human carboxylesterase, only ~15 nM SN-38 was detected in reaction mixtures containing 200 or 2000 units of carboxylesterase activity. Similar results were obtained with enzymes expressed in baculovirus and with cell extracts from other carboxylesterase transfected cell lines and xenografts (data not shown). The kinetics of conversion of CPT-11 to SN-38 by the rabbit enzyme were as reported previously by Guichard *et al.* (24). It was not possible to calculate kinetic parameters for activation of CPT-11 by the human enzyme due to the very low level of SN-38 produced.

The above comparison was normalized to an equal amount of enzymatic activity based on catalysis of the classic carboxylesterase substrate o-NPA by each enzyme. To verify that such a comparison was valid, we compared the efficiency of the enzymes to metabolize o-NPA based on amount of carboxylesterase, as quantitated from immunoblots. Because of the high degree of homology among carboxylesterases, it was necessary to tag each enzyme with an 11-amino acid HA sequence that is

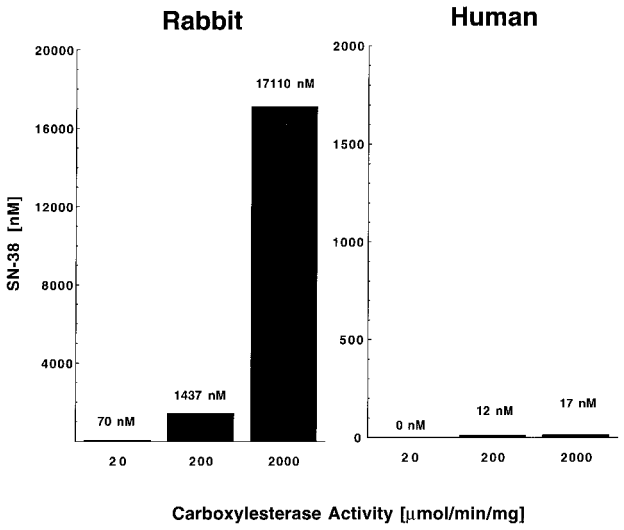

Fig. 3 Quantitation of the amount of SN-38 produced in reactions of cell lysates of $Rh30pIRES_{human}$ or $Rh30pIRES_{rabbit}$ cells with 25 μM CPT-11, as shown in Fig. 2.

detectable with a specific, high affinity antibody (27). Proteins of whole-cell sonicates of Rh30, $Rh30pIRES_{rabbitHA}$, and $Rh30pIRES_{humanHA}$ cells were separated by SDS-PAGE and stained with Coomassie Blue to show approximate equal protein loading (Fig. 4, *PAGE*), and immunoblots with anti-HA antibody (Fig. 4, *HA*) and anti-tubulin (data not shown) were done. Specific HA-reactive and TUB-reactive bands were quantitated, and the amount of immunodetectable tagged carboxylesterase was normalized to amount of tubulin. The levels of tagged carboxylesterase expressed by $Rh30pIRES_{rabbitHA}$ and $Rh30pIRES_{human\ HA}$ cells (bands indicated by *arrow*) differed by <2-fold. One nonspecific immunoreactive band was also detected in sonicates from all three cell lines. Because it is already known that $Rh30pIRES_{rabbitHA}$ and $Rh30pIRES_{human\ HA}$ cells have equal levels of enzymatic activity (Ref. 27 and Fig. 4 legend), the results indicate that the ability of the two enzymes to metabolize o-NPA was very similar. Taken together, the above results show that the rabbit carboxylesterase activates CPT-11 more efficiently than the human carboxylesterase when compared either on the basis of units of enzymatic activity using o-NPA as a substrate or on the basis of amount carboxylesterase protein.

***In Situ* Conversion of CPT-11 to SN-38 by Human and Rabbit Carboxylesterases.** $U373pIRES_{human}$ and $U373pIRES_{rabbit}$ cells were grown as monolayers, harvested by trypsinization, and resuspended in medium without serum at a concentration of 2×10^5 cells/ml. An aliquot of each cell suspension was sonicated and incubated with o-NPA as described above to determine specific activity of carboxylesterase, expressed as μmol of nitrophenol produced/min/number of cells. A second aliquot of cells was incubated for 2 h with 0, 2.5, 25, or 250 μM CPT-11. This aliquot was then centrifuged, and methanolic extracts were made of the cell pellets. These extracts were analyzed by HPLC to quantitate the amount of SN-38 produced intracellularly by the overexpressed human or rabbit

Table 2 Amount of SN-38 produced *in situ* in U373pIRES$_{rabbit}$ and U373pIRES$_{human}$ cells incubated with 250 μM CPT-11

Results of three individual experiments are shown.

pg of SN-38/350 units of CE activity		Relative efficiency
U373pIRES$_{rabbit}$	U373pIRES$_{human}$	rabbit/human
489	72	6.8
134	16	8.6
239	35	6.6
		7.4 + 1.1[a]

[a] Mean + SD of the relative efficiency of the rabbit compared with the human enzyme.

carboxylesterase. Results were normalized to levels of carboxylesterase activity and are expressed as pg of SN-38 produced per 350 units of carboxylesterase activity. When 2.5 or 25 μM CPT-11 was used as the substrate, SN-38 was detected only with cells expressing the rabbit enzyme (data not shown). Data in Table 2 compare the amount of SN-38 produced by U373pIRES$_{rabbit}$ and U373pIRES$_{human}$ cells incubated with 250 μM CPT-11. The data indicate that in intact cells, the rabbit carboxylesterase is ~7-fold more efficient than the human carboxylesterase in metabolizing CPT-11.

Relative Increase in Sensitivity to CPT-11 in Rh30 and U-373 Cells Expressing Rabbit or Human Carboxylesterase, as Assessed by Growth Inhibition Experiments. Levels of expression of carboxylesterase *per se* were not assessed by Western analysis because the high degree of homology among members of the carboxylesterase family of proteins prevents the detection of specific carboxylesterases. However, data published previously (13, 14) and presented above indicated that the level of human carboxylesterase activity expressed in Rh30pIRES$_{human}$ cells or in U373pIRES$_{human}$ cells, as quantitated by metabolism of o-NPA, was similar to or slightly greater than the level of the rabbit carboxylesterase activity in Rh30pIRES$_{rabbit}$ and U373pIRES$_{rabbit}$ cells. U373pIRES$_{rabbit}$ and U373pIRES$_{human}$ cells expressed activity levels of 807 and 970 μmol/min/mg protein, respectively (14). Rh30pIRES$_{human}$ cells expressed 3–4-fold higher levels of carboxylesterase activity than Rh30pIRES$_{rabbit}$ cells (1100 and 330 μmol/min/mg protein, respectively). Having determined that levels of carboxylesterase activities in each pair of cell lines were approximately comparable, we assessed the sensitivity of the transfected cell lines to a 2-h exposure to CPT-11 by growth inhibition assays and report the data as the IC$_{50}$ of each cell line expressing the human enzyme divided by the IC$_{50}$ of the cell line expressing the rabbit enzyme. This ratio reflects the relative efficiency of the two enzymes to metabolize CPT-11 *in situ*. The IC$_{50}$s of the U373pIRES$_{rabbit}$ cells and Rh30pIRES$_{rabbit}$ cell lines were 55- and 12-fold lower, respectively, than the those for the U373pIRES$_{human}$ and Rh30pIRES$_{human}$ cell lines. Expression of the human carboxylesterase sensitized neither Rh30 cells nor U-373 MG cells to CPT-11, because the IC$_{50}$s of Rh30pIRES$_{human}$ and U373pIRES$_{human}$ were not significantly different from vector controls (13, 14). We conclude that expression of equivalent or slightly lower levels of rabbit carboxylesterase activity sensitized human tumor cells to CPT-11 more efficiently than did the human enzyme.

Sensitization of Rh30 Xenografts to CPT-11 by Rabbit or Human Carboxylesterase in a Preclinical Mouse Model. Because the long-range goal of these experiments is the application of an appropriate enzyme for use in ADEPT or VDEPT, we next determined whether expression of carboxylesterases could sensitize Rh30 rhabdomyosarcoma human tumor cells grown as xenografts in immune-deprived mice to CPT-11. In this preclinical model, expression of the transfected cDNAs produced carboxylesterase activities of 212 μmol/min/mg for the human enzyme and 132 μmol/min/mg protein for the rabbit enzyme when xenografts were assessed at weeks 7–8, indicating that roughly equivalent amounts of both enzyme activities were present throughout the treatment cycle. Carboxylesterase activity is also readily detectable in xenografts that have been propagated s.c. for as long as a year, with the level of enzyme activity in xenografts expressing the human carboxylesterase cDNA being consistently slightly higher (2–3-fold) than the xenografts expressing the rabbit cDNA (data not shown). Importantly, tumors were advanced (an average of 0.5–1.0 cm^3 in volume) before CPT-11 treatment was begun. Mice bearing xenografts (seven animals per group; two tumors/mouse) were treated with 2.5 mg of CPT-11/kg/day for 5 days each week for 2 weeks (one cycle of therapy), repeated every 21 days for a total of three cycles (over 8 weeks). Week 8 was the final week of treatment. Mice were observed for an additional 6 weeks after cessation of treatment to assess tumor recurrence. Using this regimen, all tumors regressed. However, xenografts not expressing either the rabbit or the human carboxylesterase (Rh30) recurred in seven of seven mice immediately after cessation of CPT-11 administration (Fig. 5, *arrow*). Xenografts expressing the human carboxylesterase regressed, but tumors recurred in two of seven mice. In contrast, xenografts expressing the rabbit carboxylesterase regressed completely and did not regrow during the 14 weeks of the study. We conclude that although the human carboxylesterase does activate CPT-11, the rabbit carboxylesterase is more efficient *in vitro*, *in situ*, and *in vivo*.

DISCUSSION

The success of therapy based on enzyme activation of prodrugs will require identification of enzymes that confer a therapeutic advantage based either on relative level of expression or efficiency of substrate conversion, compared with endogenous enzymes. HSVtk and *E. coli* cytosine deaminase are candidate enzymes for this approach (5–11). We report here the novel finding that a carboxylesterase isolated from rabbit liver may also be useful for therapeutic application to activate the prodrug CPT-11, based on its greater efficiency of conversion of CPT-11 to SN-38 relative to the human enzyme. In *in vitro* and *in situ* assays, the rabbit enzyme was consistently more efficient than a human homologue, although the primary amino acid sequences of the two carboxylesterases were remarkably similar (81% identity). Consistent with these data, comparison of the two CEs in a preclinical mouse xenograft model showed that the rabbit liver carboxylesterase more efficiently sensitized Rh30 human rhabdomyosarcoma cells to CPT-11, as reflected by more rapid tumor regression as well as, importantly, the prevention of tumor recurrence.

In addition to the enzymes used in this study, several other

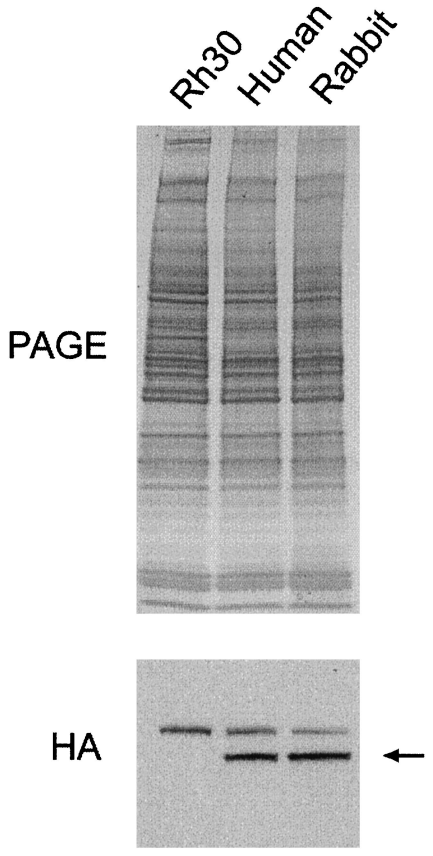

Fig. 4 SDS-PAGE and immunoblot analysis of HA-tagged rabbit and human carboxylesterases in whole-cell sonicates of Rh30pIRES$_{rabbitHA}$ and Rh30pIRES$_{humanHA}$ cells. Proteins were separated by SDS-PAGE (*PAGE*). Filters were probed with an anti-HA antibody (*HA*; *arrow*). As reported previously (26), the Rh30pIRES$_{rabbitHA}$ cells and the Rh30pIRES$_{humanHA}$ cells had 221 and 202 units of carboxylesterase activity (μmol of nitrophenol produced/min/mg protein), respectively.

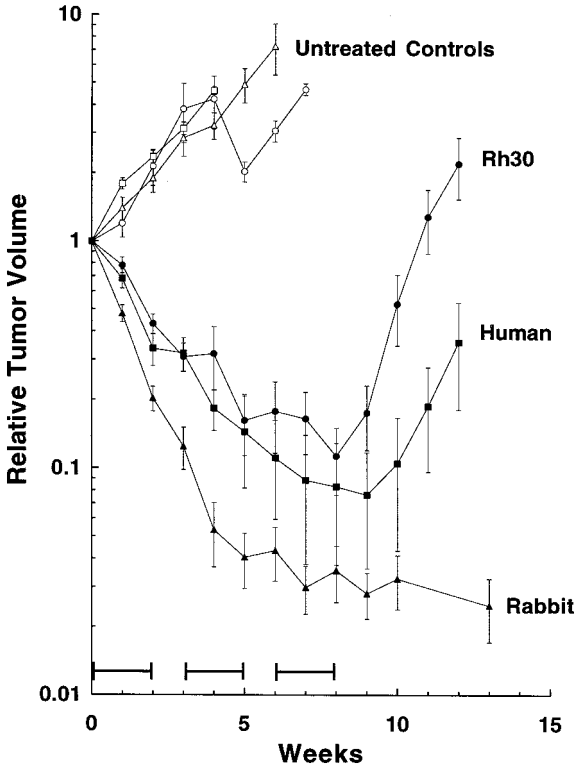

Fig. 5 Responses of Rh30 and Rh30pIRES$_{rabbit}$, and Rh30pIRES$_{human}$ xenografts, to CPT-11. CPT-11 was administered to mice bearing advanced xenografts. Drug (2.5 mg/kg/day) was administered i.v. daily × 5, each week for 2 consecutive weeks. This cycle was repeated every 21 days for a total of three cycles. By this treatment regimen, mice received drug during weeks 1–2, 4–5, and 7–8 (as indicated by the *horizontal bars* along the *x-axis*). Control animals received injections of vehicle only on the same schedule. Each *line* represents the average size of 14 tumors in seven mice (2 tumors/mouse). Drug-treated xenografts: ■, Rh30; ●, Rh30pIRES$_{rabbit}$; ▲, Rh30pIRES$_{human}$. Untreated xenografts: □, Rh30; ○, Rh30pIRES$_{rabbit}$; △, Rh30pIRES$_{human}$. The mean volume in cm^3 of the tumors at the beginning of treatment (time 0) in the control groups were: Rh30, 0.85 ± 0.23; Rh30pIRES$_{rabbit}$, 0.59 ± 0.41; Rh30pIRES$_{human}$, 0.57 ± 0.23; *bars*, SD. The volumes of tumors at time 0 in the drug-treated groups were: Rh30, 0.67 ± 0.31; Rh30pIRES$_{rabbit}$, 0.90 ± 0.42; Rh30pIRES$_{human}$, 0.54 ± 0.23; *bars*, SD.

carboxylesterases have been partially purified or their cDNAs isolated. The purified or partially purified enzymes that catalyze CPT-11 include those from human liver microsomes (21) and rat serum (20, 31); but the specific enzyme(s) or isozyme(s) that activate CPT-11 *in vivo* has not been determined. Neither have cDNAs encoding these proteins been isolated. The coding sequences for 13 carboxylesterases are, however, available in the GenBank database. As part of our long-range goal to identify enzymes useful in ADEPT or VDEPT, we also examined the expression in human tumor cells of the cDNAs encoding a rat serum carboxylesterase and a mouse carboxylesterase. The methods used were identical to those detailed in this report, and the sequences of those carboxylesterases were, as with the sequences above, remarkably homologous to the sequences shown in Fig. 1. However, for reasons unknown, repeated efforts to establish cells lines that stably or transiently expressed either the rat or the mouse protein in human tumor cells lines were unsuccessful. Because it was not possible to express these proteins, we could not assess the ability of either the mouse or the rat protein to metabolize CPT-11.

Additionally, a human liver carboxylesterase has been reported recently to sensitize A549, H157, and SK-MES1 cells ~6–17-fold to CPT-11 (23) but to have a minimal effect in eight other human tumor cell lines (ΔIC_{50} ~1.2–2.5). It is not known whether the cDNA used in that study corresponded to the liver (1) or liver (2) enzyme as designated in Table 1. It remains to be determined, because of the minimal differences among the sequences reported for the three human enzymes, whether these different sequences represent products of three separate genes, polymorphisms, or mutations or were introduced by *in vitro* manipulations. The >99% identity among these proteins indicates that the possibility that they represent products of unique genes is somewhat unlikely.

Present studies focus on delivery of the rabbit liver carboxylesterase cDNA with adenoviral vectors and induction of

expression of this enzyme by tumor-specific transcription factors to achieve tumor-specific toxicity.

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