Proficient Metabolism of Irinotecan by a Human Intestinal Carboxylesterase

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Abstract

Irinotecan [7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11)] is metabolized by esterases to yield the potent topoisomerase I poison 7-ethyl-10-hydroxy camptothecin. One of the major side effects observed with CPT-11 is gastrointestinal toxicity, and we supposed that this might be due to local activation of CPT-11 within the gut. Carboxylesterase (CE) activity was detected in human gut biopsies, and extracts of these tissues converted CPT-11 to 7-ethyl-10-hydroxy camptothecin in vitro. Expression of a human intestinal CE cDNA in COS-7 cells produced extracts that demonstrated proficient CPT-11 activation and conferred sensitivity of cells to CPT-11. These results suggest that gut toxicity from CPT-11 may be due in part to direct drug conversion by CEs present within the small intestine.

Introduction

CPT-11 is a widely used anticancer agent that has demonstrated remarkable promise in the treatment of solid tumors. It is a prodrug that is activated by esterases to yield SN-38, a topoisomerase I poison (1). CPT-11 has demonstrated good antitumor activity in both preclinical models and patients with refractory disease (2–6) and, as such, has recently been approved for the treatment of colon cancer in adults. The toxicities associated with this agent include a cholinergic syndrome due to direct inhibition of acetylcholinesterase and delayed diarrhea due to intestinal toxicity (7–10). The latter is thought to occur via bacterial-mediated cleavage of SN-38 glucuronide present in the bile to yield SN-38, resulting in local irritation and toxicity to the gut (11).

We have recently demonstrated that mouse small intestine expressed high levels of CEs that could convert CPT-11 to SN-38 (12), and based on these data, we hypothesized that human intestine may also efficiently activate the drug. If so, then activation of CPT-11 in the intestine may result in local toxicity. Hence we assessed the conversion of CPT-11 to SN-38 by sonicates of biopsies of human intestinal mucosa and monitored the ability of a cDNA encoding a hiCE to activate this drug. Our data indicate that high-level expression of esterases occurs in the small intestine and that at least one of these enzymes is proficient at CPT-11 activation.

Materials and Methods

CPT-11. CPT-11 was kindly provided by Dr. J. P. McGovren (Pharmacia Upjohn, Kalamazoo, MI). It was dissolved in methanol and stored at −20°C.

Human Biopsy Material. Biopsies of human intestine and liver were obtained from the Cooperative Human Tissue Network (Birmingham, AL). They were ground under liquid nitrogen, and the resulting powder was sonicated in 50 mM HEPES (pH 7.4) on ice.

CE Assay. CE activity was determined by a spectrophotometric assay using o-NPA as a substrate (13, 14). Briefly, extracts prepared by sonication in 50 mM HEPES (pH 7.4) were incubated in 3 mM o-NPA, and the absorbance at 405 nm was measured at 1-min intervals for 10 min. Protein concentrations in extracts were determined using Bio-Rad protein reagent, with BSA as a standard. CE activity is reported as μmoles of o-NPA converted per minute per milligram of protein.

CPT-11 Conversion Assays. Activation of CPT-11 was determined by incubating samples with either 5 or 25 μM CPT-11 in a total volume of 200 μl of 50 mM HEPES (pH 7.4) at 37°C for up to 20 h. Reactions were terminated by the addition of an equal volume of cold acidified methanol and subsequent centrifugation at 100,000 × g for 30 min; SN-38 in the reaction was quantitated by high-performance liquid chromatography.

Determination of CPT-11 and SN-38. Concentrations of CPT-11 and SN-38 were determined by high-performance liquid chromatography as described previously (15). Detection was achieved using a Jasco FP-920 fluorescence detector; the sensitivity of this system was 20 and 1.5 pg/μl for CPT-11 and SN-38, respectively.

Amplification of hiCE cDNA. The full-length coding sequence of the hiCE (GenBank Accession number Y09916; Ref. 16) was obtained by PCR using oligonucleotide primers that created XhoI restriction sites adjacent to the ATG initiation and TAG termination codons. The cDNA was amplified from human liver cDNA (Clontech, Palo Alto, CA) using Taq polymerase under the following conditions: (a) denaturation at 94°C for 45 s; (b) annealing at 50°C for 1 min; and (c) extension at 72°C for 2 min. After 30 cycles of amplification, products were ligated into pCR-II TOPO and sequenced to verify their identity. One clone containing the bona fide sequence was ligated into pCIneo (pCI-hiCE) for expression in mammalian cells. Plasmids containing hCE1 (pCI-HUMCAR) and rabbit liver CE (pCIRAB) have been described previously (14, 17).

Transfection of COS-7 Cells. COS-7 cells were transfected by electroporation as described previously (14). Extracts were prepared by sonication of cell pellets in minimal volumes of 50 mM HEPES (pH 7.4) on ice 48 h after transfection. Transfection efficiencies of 5–10% were routinely achieved using this method.

Growth Inhibition Assays. Growth inhibition assays were performed with COS-7 cells as described previously (14, 18). Forty-eight h after transfection, 5 × 104 cells were plated into 3.5-cm diameter dishes and allowed to attach overnight. CPT-11 diluted in fresh medium was applied for 2 h, and the cells were allowed to grow for 3 days, equivalent to three cell doublings. Cell number was determined by using a Coulter Multisizer II (Coulter Electronics, Luton, United Kingdom) and growth inhibition curves plotted using Prism software (GraphPad Software, Inc., San Diego, CA). IC50 values were calculated from these curve fits.

Results

Expression of Esterases and Activation of CPT-11 by Human Intestine. To determine the levels of esterase activity in intestinal mucosa biopsies, we monitored the conversion of o-NPA to nitrophe- nol by whole tissue sonicates. Table 1 demonstrates the levels of CE activity present in three small intestine, three colon, and two liver samples. As shown in the table, very high esterase activity was present in liver extracts, whereas lower enzyme levels were detected in the small intestine and colon.
Conversion of CPT-11 to SN-38 by these extracts demonstrated efficient drug activation by the small intestine, but lower yields of SN-38 were observed with colon extracts (Table 1). Interestingly, similar amounts of SN-38 were produced by small intestine and by extracts derived from liver. These data indicate that for the total levels of esterases present in these tissues, the percentage of CPT-11-converting enzymes in the small intestine is greater than that in the liver.

Isolation and Expression of a hiCE. A cDNA encoding a hiCE has recently been isolated (16) and shown to be predominantly expressed in the small intestine. To determine whether this enzyme could activate CPT-11, we isolated the full-length coding sequence by PCR and expressed the protein in COS-7 cells. Table 2 demonstrates the level of CE activity and CPT-11 conversion by sonicates of cells expressing hiCE. As shown in the table, efficient conversion of both o-NPA and CPT-11 was observed. As a comparison, we have included data derived from rabbit and human liver CEs that are efficient and inefficient, respectively, at CPT-11 activation.

No CE activity or CPT-11 conversion was detected in the media of cells transfected with hiCE, indicating that the protein is not secreted from cells (Table 2). This is consistent with the endoplasmic reticulum retention motif (His-Thr-Glu-Leu) present at the COOH terminus of the protein (16).

Expression of hiCE in Mammalian Cells Confers Sensitivity to CPT-11. To determine whether this enzyme could confer sensitivity of cells to CPT-11, we monitored the effect of this drug on the growth of COS-7 cells expressing hiCE. Fig. 1 demonstrates growth inhibition curves for COS-7 cells transiently transfected with either pCI-hiCE or pClneo and treated with CPT-11. The IC_{50} of cells expressing hiCE was approximately 11-fold less than that of cells transfected with the parent plasmid. Because the transient transfections do not yield 100% transfection efficiencies, there is always a population of cells that contribute to the IC_{50} values but do not contain the plasmid of interest. However, it can be seen that even under these transient conditions, a significant reduction in the IC_{50} for CPT-11 was observed for cells expressing hiCE. These data indicate that efficient in vivo activation of CPT-11 by hiCE is sufficient to sensitize cells to this drug.

Sequence Analysis of CEs. We have recently demonstrated that a hCE, hCE1, is very inefficient at CPT-11 activation, despite sharing >80% amino acid homology with a rabbit liver CE that is proficient at drug metabolism (19). Because hCE also converts CPT-11 to SN-38, we aligned the amino acid sequences of the rabbit liver CE, hCE1, and hiCE enzymes. Fig. 2 shows these alignments. Sequence analysis indicates that the rabbit CE demonstrates an 81% identity with hCE1 but only a 47% identity with hiCE. Likewise, hCE1 demonstrates a 49% identity with hiCE.

Although there is clear similarity among the enzymes, particularly the residues adjacent to the active site amino acids Ser-240, Glu-364, and His-478 and a large region from amino acids 122–260, there are significant regions of diversity (Fig. 2). These alignments also indicate that the sequence does not predict the ability of a particular enzyme to activate CPT-11 in that the two enzymes that can metabolize this drug, rabbit CE and hiCE, are the more disparate with respect to amino acid homology.

Discussion

This work reports that high-level activation of CPT-11 occurs in extracts of small intestine and that this is probably due to the expression of the CE hiCE in this tissue. Exogenous expression of this protein in COS-7 cells yielded extracts that were proficient at drug activation, consistent with data observed from the biopsy material. Because the major dose-limiting toxicity of CPT-11 is gastrointestinal damage resulting in diarrhea, our data indicate that local activation of the drug to SN-38 by the gut could contribute to the side effects observed in patients treated with CPT-11.

Previous metabolic and pharmacokinetic studies indicate that SN-38 is conjugated to form SN-38 glucuronide, and this is secreted into the bile (20, 21). Bacterial glucuronidases present within the gut flora can release SN-38 from the conjugate, and it has been proposed that this free drug is responsible for toxicity. Takasuna et al. (11) monitored the conversion of CPT-11 by extracts of the rat digestive tract and demonstrated that the highest conversion was observed in the upper small intestine. They also showed that treatment of these cells...
animals with oral doses of penicillin and streptomycin increased fecal output of CPT-11, confirming the hypothesis that bacterial glucuronidases were involved in the deconjugation of SN-38 glucuronide. Because clinical trials of a combination of CPT-11 and antibiotics are under way, it will be interesting to see to what extent the delayed diarrhea is ameliorated in these patients.

In this report, we now propose an additional mechanism for SN-38 production in the human gut whereby hiCE directly activates CPT-11, leading to local cytotoxicity with subsequent diarrhea. Recently, Slatter et al. (22) have reported that 30% of a dose of CPT-11 has been detected unchanged in human bile after i.v. injection of 125 mg/m² radiolabeled drug. Because the bile duct opens into the proximal duodenum, direct conversion of CPT-11 to SN-38 could occur within the intestine. Hence, both oral and i.v. administration of CPT-11 will result in accumulation of SN-38 within the small intestine by both direct drug activation and bacterial-mediated deconjugation. Because specific esterase inhibitors are not available, it is currently not possible to selectively inhibit hiCE with an aim to reduce gut toxicity.

Recent studies with human small intestine, colon, and liver biopsies have indicated that wide ranges of CPT-11 conversion can be achieved in these tissues (23, 24). Similar levels of drug activation were seen in both liver and colon samples, although there was considerable variability between patients. However, in these studies, CE assays using o-NPA as a substrate were not performed; therefore, the relative amounts and specific activities of both CPT-11-activating and nonspecific enzymes in these tissues are unknown. By identifying a hiCE that can efficiently activate CPT-11, our data provide molecular evidence for drug metabolism and explain the gut toxicity associated with CPT-11.

hiCE cDNA was isolated by Schwer et al. (16) from a small intestine λgt11 library by hybridization screening using a hCE nucleotide sequence. These authors performed Northern analysis of RNA isolated from human tissues and demonstrated significant overexpression of the mRNA in the small intestine. This was further localized to jejenum. Interestingly, they also demonstrated low-level expression of the hiCE mRNA in the liver, raising the possibility that this is the enzyme involved in CPT-11 metabolism in that organ. Recent reports have indicated that another human CE present in the liver, hCE2, may also contribute to CPT-11 activation in vivo (25). Data derived from extracts of small intestine and liver indicate that equally proficient CPT-11 to SN-38 conversion can occur in both tissues (Table 1); however, the overall level of nonspecific CE activity in the former tissue is approximately 10-fold lower. This indicates that the relative proportions of esterases that can activate CPT-11 are 10-fold higher in the small intestine. With the identification of an increasing number of CEs in human tissues that can metabolize CPT-11, the relative involvement of each in drug activation may allow pharmacokinetic guidance of therapeutically relevant doses of SN-38.

The sequence diversity of CEs does not predict the ability of a particular enzyme to metabolize CPT-11. For example, rabbit liver CE and hiCE are both efficient at drug metabolism, yet they share only a 54% similarity and a 47% identity at the amino acid level. In contrast, hCE1 is 86% similar to rabbit CE but metabolizes CPT-11 very inefficiently (19). Preliminary computer modeling studies of the rabbit enzyme and hCE1 indicate that the ability of a CE to activate CPT-11 is dependent on the residues that form the entrance to the active site gorge of these proteins. 4 We are currently purifying these proteins for structural studies to confirm the computer-predicted models.
In conclusion, we have demonstrated efficient activation of CPT-11 by human small intestine and confirmed the ability of hiCE to metabolize this drug when expressed in COS-7 cells. These data provide additional evidence that CPT-11-associated delayed diarrhea may be due to direct activation of this drug by enzymes present within the intestine.

Note Added in Proof
Analysis of the sequences of hiCE and hCE2 (25) shows that the two proteins differ by only 10 amino acids at the N-terminus. The similarity between these enzymes may explain why both proteins metabolize CPT-11 efficiently.

References
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