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HYDROLYSIS OF IRINOTECAN AND ITS OXIDATIVE METABOLITES, 7-ETHYL-10-[4-*N*-(5-AMINOPENTANOIC ACID)-1-PIPERIDINO] CARBONYLOXYCAMPTOTHECIN AND 7-ETHYL-10-[4-(1-PIPERIDINO)-1-AMINO]-CARBONYLOXYCAMPTOTHECIN, BY HUMAN CARBOXYLESTERASES CES1A1, CES2, AND A NEWLY EXPRESSED CARBOXYLESTERASE ISOENZYME, CES3

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ABSTRACT:

Carboxylesterases metabolize ester, thioester, carbamate, and amide compounds to more soluble acid, alcohol, and amine products. They belong to a multigene family with about 50% sequence identity between classes. CES1A1 and CES2 are the most studied human isoenzymes from class 1 and 2, respectively. In this study, we report the cloning and expression of a new human isoenzyme, CES3, that belongs to class 3. The purified recombinant CES3 protein has carboxylesterase activity. Carboxylesterases metabolize the carbamate prodrug 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11; irinotecan) to its active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38), a potent topoisomerase I inhibitor. CYP3A4 oxidizes CPT-11 to two major oxidative metabolites, 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin (APC) and 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin (NPC). In this study, we investigate whether these oxidative metabolites, NPC and APC, can be metabolized to SN-38 by purified human carboxylesterases, CES1A1, CES2, and CES3. We find that CPT-11, APC, and NPC can all be metabolized by carboxylesterases to SN-38. CES2 has the highest catalytic activity of 0.012 min^{-1} μM^{-1} among the three carboxylesterases studied for hydrolysis of CPT-11. NPC was an equally good substrate of CES2 in comparison to CPT-11, with a catalytic efficiency of 0.005 min^{-1} μM^{-1}. APC was a very poor substrate for all three isoenzymes, exhibiting a catalytic activity of 0.015 × 10^{-3} min^{-1} μM^{-1} for CES2. Catalytic efficiency of CES3 for CPT-11 hydrolysis was 20- to 2000-fold less than that of CES1A1 and CES2. The relative activity of the three isoenzymes was CES2 > CES1A1 ≫ CES3, for all three substrates.

CPT-11[1] is a water-soluble carbamate prodrug of camptothecin and is activated in vivo to SN-38, a potent topoisomerase I inhibitor (Kunimoto et al., 1987). CPT-11 either alone or in combination with other chemotherapeutic agents has shown promising clinical activity against several solid tumors (Rothenberg, 2001). Currently, CPT-11 in combination with 5-fluorouracil and leucovorin is approved by the U.S. Food and Drug Administration as first-line therapy in the treatment of metastatic carcinoma of colon or rectum [Camptosar (irinotecan hydrochloride) package insert, Pharmacia & Upjohn Co., 2002 May].

CPT-11 undergoes extensive hepatic metabolism as shown in Fig. 1. Two major human liver carboxylesterases (E.C.3.1.1.1), CES1A1 and CES2,[2] can hydrolyze CPT-11 to generate the active form of the drug, SN-38. CES2 is 64 times more efficient in metabolizing CPT-11 than CES1A1 (Humerickhouse et al., 2000). Human plasma esterases can hydrolyze CPT-11 in vitro (Kehrer et al., 2000; Morton et al., 2000). However, the peak plasma CPT-11 concentration is about 50 times greater than that of SN-38 after intravenous administration of drug (Slatter et al., 2000); thus, hydrolysis in plasma seems ineffi-

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[1] Abbreviations used are: CPT-11, irinotecan or 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin; CES, carboxylesterase; SN-38, 7-ethyl-10-hydroxycamptothecin; APC, 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin; NPC, 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin; SN-38G (10-*O*-glucoronyl-SN-38; UGT, uridine diphosphate glucuronosyltransferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; kb, kilobase(s); nt, nucleotide(s); α-NA, α-naphthyl acetate.

[2] Carboxylesterase Nomenclature: Human carboxylesterases are named according to the phylogenetic alignment of genes from several species into four major classes (Satoh and Hosokawa, 1998). CES1A1 (also called hCE-1, GI: 16905523) belongs to class 1, CES2 belongs to class 2 (also called hCE-2, GI: 37622884), and CES3 belongs to class 3 (GI: 7019977).

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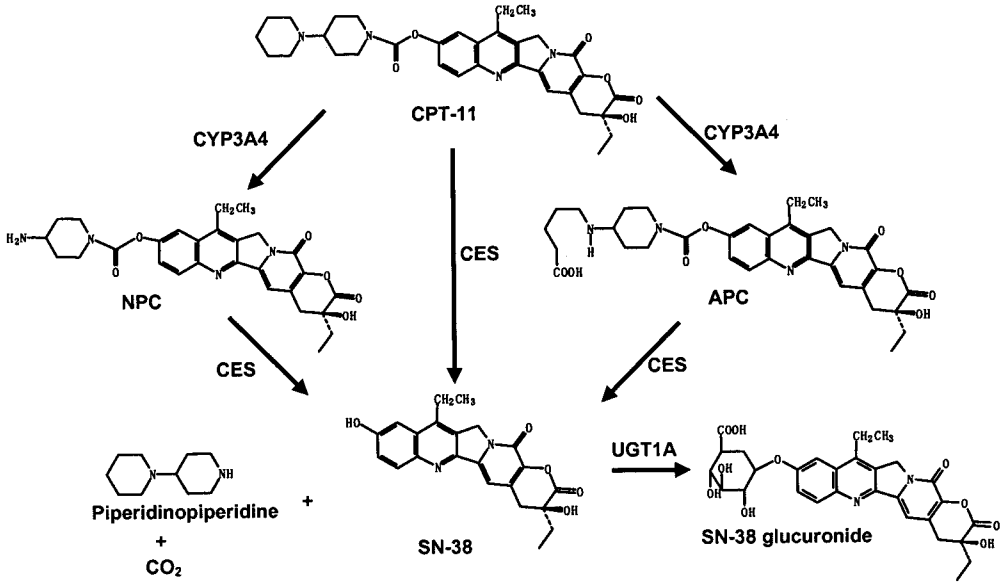

FIG. 1. *CPT-11 metabolism.*

CPT-11 is oxidized by cytochrome P450 3A4 (CYP3A4) isoenzyme to produce 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin (APC) and 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin (NPC). NPC, APC, and CPT-11 are metabolized by carboxylesterases (CES) to produce active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38). SN-38 is inactivated by UDP-glucuronosyltransferase isoform 1A1 to SN-38 glucuronide.

cient. Oxidative metabolism of CPT-11 by cytochrome P450 isoenzymes results in formation of two major metabolites, APC and NPC (Dodds et al., 1998; Haaz et al., 1998). In vitro studies demonstrate that among the cytochrome P450 isoenzymes, only CYP3A4 can oxidize CPT-11 to APC and NPC (Santos et al., 2000). SN-38 is inactivated by glucuronidation to form SN-38 glucuronide (SN-38G). Several uridine diphosphate glucuronosyltransferase (UGT) isoforms were studied, and UGT1A1 was found to be at least 10 times more active than other isoforms (Hanioka et al., 2001). In vitro studies suggested that NPC, but not APC, is metabolized by liver microsomes and/or CES1A1 to produce SN-38 (Rivory et al., 1996; Dodds et al., 1998).

Upon intravenous administration of [^{14}C]CPT-11, 30% of the dose was recovered in the urine and 62% of the dose was excreted through feces (Slatter et al., 2000). The major excretion product was unchanged CPT-11, accounting for 55% of the administered dose, followed by APC (10.5%), SN-38 (8.7%), SN-38G (3.3%), and NPC (1.5%) (Slatter et al., 2000). Interindividual variation in CPT-11 pharmacokinetics is reported in several studies (Sparreboom et al., 1998; Kehrer et al., 2000; Slatter et al., 2000). Analysis of 24 human primary and metastatic colon tumor samples indicated a 56-fold variation in CPT-11 hydrolase activity (Sanghani et al., 2003). This variation in activity correlated significantly with CES2 but not CES1A1 gene expression (Sanghani et al., 2003). The major dose-limiting toxicities of CPT-11 therapy are diarrhea and leukopenia. Mick et al. (1996) showed that the predicted biliary index of SN-38 correlated with the intestinal toxicity associated with CPT-11 therapy. The biliary index is the product of total CPT-11 area under the curve and the relative area ratio of SN-38 to SN-38G. The expression of CPT-11 carboxylesterases (Ahmed et al., 1999) and UGT1A1 (Iyer et al., 2001) in gastrointestinal tissue will determine the local conversion of CPT-11 to SN-38 and SN-38 glucuronide. Therefore, the intestinal levels of CPT-11 carboxylesterases and UGT1A1 may be determinants of the diarrhea side effect of CPT-11 therapy. Significant response has been observed in patients with low plasma SN-38 concentrations, and therefore, it has been suggested that tumor CPT-11 hydrolase activity may be important for efficacy of CPT-11 (Ratain, 2000). Hence, the expression of CPT-11 carboxylesterases may be a determinant of both the therapeutic efficacy and toxicity of CPT-11 therapy for colorectal cancer.

Carboxylesterases belong to a multigene family. These isoenzymes are responsible for metabolism of a variety of ester, carbamate, thioester, and amide drug compounds (Satoh and Hosokawa, 1998). Although substrate specificity of these isoenzymes is overlapping, they do show substrate preference (Bosron and Hurley, 2002). Human CES1A1 prefers substrates with a smaller alcohol moiety and larger acyl substituent such as meperidine or methylphenidate, whereas CES2 prefers a large alcohol and small acyl moieties such as CPT-11 or heroin (Satoh et al., 2002). Multi-tissue Northern blot analysis in human (Satoh et al., 2002) and rat (Sanghani et al., 2002) shows that carboxylesterase isoenzymes are most abundantly expressed in the liver, but some isoenzymes are expressed in a tissue-specific manner.

The overall goal of this study was to determine the relative contribution of individual carboxylesterases in SN-38 formation. We are reporting the cloning and expression of a new human carboxylesterase isoenzyme, CES3. Purified CES3 has carboxylesterase activity and is expressed in human liver and colon tissues by Northern blot analysis. The role of human liver carboxylesterases CES1A1, CES2, and CES3 in hydrolysis of CPT-11, APC, and NPC was investigated by steady-state kinetics.

Materials and Methods

Cloning of CES3. The CES3 gene (GI: 7019977) was cloned from cDNA generated by reverse transcription of human liver RNA (OriGene Technologies, Inc., Rockville, MD) using oligo-dT primer. A 50-μl reverse transcription reaction containing 2.5 μg of total human liver RNA was performed using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). The reaction contained 2.5 μM oligo-dT primer, 5 mM magnesium, 1 mM concentration of each deoxynucleotide triphosphate, 50 U of RNase inhibitor, and 2.5 U/μl final concentration of murine leukemia virus reverse transcriptase. The reverse transcription conditions were 10 min at room temperature, 60 min at 42°C, 10 min at 68°C, and 5 min at 95°C. The cDNA was purified with a QIAquick PCR purification kit (QIAGEN, Valencia, CA). The CES3 gene was amplified from human liver cDNA using the following forward 5′-GAACCAGTTGTAAGGAGAATGGAGA-3′ and reverse 5′-CTGGGACTCTGCCACCACTT-

GAA-3′ primers. The 1.8-kb PCR product was gel purified with a QIAquick gel extraction kit (QIAGEN) and cloned into pCR-Blunt TOPO II vector (Invitrogen, Carlsbad, CA). Clones 11, 13, and 18 were sequenced in both directions.

Expression and Purification of CES3. The full-length 1.8-kb CES3 gene was obtained by complete digestion of clone 18 with BamHI and partial digestion with PstI, and was inserted into the pAcMP2 transfer vector (BD Biosciences PharMingen, San Diego, CA). The pAcMP2-CES3 transfer vector, 2.5 μg, was cotransfected with 0.5 μg of linearized Baculogold DNA into 2.5×10^6 Sf9 cells using the Baculogold kit (BD Biosciences PharMingen). The recombinant viruses were collected on day 5 and purified by plaque assay. One virus plaque was amplified, titered by plaque assay, and used to infect log phase Sf9 cells in serum-free medium (Cambrex Corp., East Rutherford, NJ) at a multiplicity of infection of 1.

CES3 was purified from Sf9 cell extracts by a two-step purification protocol involving concanavalin A affinity chromatography followed by preparative nondenaturing gel electrophoresis. Briefly, Sf9 cells were harvested at 65 to 69 h postinfection, and immediately frozen in liquid nitrogen and stored at −70°C until further use. The frozen cell pellet from a 1-liter culture was resuspended in 40 ml of 20 mM Tris buffer, pH 7.4, containing 0.1% Triton X-100, 1 mM benzamidine, and 1 μM leupeptin. The cells were lysed by sonication and the lysate was centrifuged at 40,000*g* for 45 min. One millimolar concentration each of Ca^{2+}, Mg^{2+}, and Mn^{2+} ions was added to the clear supernatant and loaded onto a 20-ml concanavalin A (Sigma-Aldrich, St. Louis, MO) column equilibrated in 20 mM Tris buffer, pH 7.4, with 0.2 M NaCl (buffer A). The affinity resin was washed with buffer A and the enzyme was eluted with a linear gradient of 150 ml of buffer A and 150 ml of buffer A containing 0.1 M methyl-α-D-mannopyranoside. The activity was monitored by enzyme assay using α-naphthyl acetate substrate, the active fractions were pooled and concentrated, and buffer was exchanged into 20 mM Tris buffer, pH 7.4. Concentrated protein from concanavalin A was separated by preparative nondenaturing gel electrophoresis on an 8-cm column of 6% polyacrylamide running gel and 1 cm of 4% polyacrylamide stacking gel prepared in a 28-mm-diameter tube for Bio-Rad Prep Cell model 491 (Bio-Rad, Hercules, CA) (Sanghani et al., 2002). Proteins were eluted in 20 mM Tris buffer, pH 7.4 containing 10% ethylene glycol. Active fractions were pooled, concentrated, and stored at 4°C.

Purified CES3 was analyzed by SDS-PAGE with Coomassie Blue protein staining. The 60-kDa protein bands were cut out of the gel and subjected to in-gel digest with either endoproteinase Glu-C or trypsin. Peptides in the digested gel bands were analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (INGEN Proteomics Core Facility, Indiana University School of Medicine).

CES1A1 and CES2 Purification. CES1A1 and CES2 were purified by a modification of the method of Humerickhouse et al. (2000). Briefly, 85 g of human liver, obtained with institutional review board approval at autopsy, was homogenized in 200 ml of 50 mM Hepes buffer, pH 6.8, containing 1 mM benzamidine and dithiothreitol (buffer B). The extract was centrifuged at 35,000*g* for 40 min. The supernatant was added to 170 g of DE52 resin equilibrated with buffer B and the ion-exchange resin was washed with approximately 1 liter of buffer B until the filtrate was colorless. The carboxylesterases were eluted from DE52 resin with 20 mM Tris, pH 7.4, containing 0.2 M NaCl and a 1 mM concentration each of dithiothreitol and benzamidine (buffer C). The eluant was loaded onto a 50-ml concanavalin A column equilibrated in buffer C, washed with 500 ml of buffer C, and glycoproteins were eluted with buffer C containing 0.5 M methyl-α-D-mannopyranoside. Fractions with carboxylesterase activity were pooled, concentrated, and buffer exchanged to 20 mM Tris, pH 7.4, containing 10% ethylene glycol. CES1A1 and CES2 were separated on preparative nondenaturing gel electrophoresis in the Bio-Rad Prep Cell as described above.

Northern Blot analysis. A human multi-tissue blot (Origene Technologies, Inc.) was probed with a CES3 specific probe generated by PCR from 919 to 1234 nt of the CES3 gene (GI: 7019977). The probe was radiolabeled with ^{32}P-dCTP using the random primed labeling kit (Roche Diagnostics, Indianapolis, IN). The blot was prehybridized for 30 min in Quikhyb solution (Stratagene, La Jolla, CA) at 65°C. The denatured probe and 100 μl of sonicated salmon sperm DNA (Stratagene) were added to the prehybridized blot. After 2 h of hybridization at 65°C, the blot was washed twice with 2× standard saline citrate containing 0.1% SDS for 15 min at room temperature, followed by one 30-min wash at 58°C with 0.1× standard saline citrate containing 0.1% SDS. The autoradiograph was generated by exposing the X-OMAT AR film (Kodak, Rochester, NY) to the radioactive blot for 8 days at −70°C.

4-Methylumbelliferyl Acetate Hydrolase Assay. Total carboxylesterase activity was monitored by a spectrophotometric method described by Brzezinski et al. (1997). Briefly, the enzyme was incubated at 37°C with 0.5 mM 4-methylumbelliferyl acetate in 90 mM KH_2PO_4, 40 mM KCl, pH 7.4. The formation of product, 4-methylumbelliferone, was monitored spectrophotometrically at 350 nm, $\epsilon_{350} = 12.2$ mM $^{-1}$ cm^{-1}. Protein was quantitated by the Bradford dye-binding method with bovine serum albumin as standard (Bio-Rad). The specific activity is expressed as μmol min^{-1} mg^{-1}. CES1A1 purified from human liver exhibited a specific activity of about 7 U/mg (Brzezinski et al., 1994) and CES2 exhibited a specific activity of about 140 U/mg (Humerickhouse et al., 2000) with 4-methylumbelliferyl acetate as substrate.

***α*-Naphthyl Acetate Hydrolase Assay.** A coupled, simultaneous assay using a diazonium dye was employed (Johnston and Ashford, 1980). Briefly, an assay cocktail was made with 20 ml of 100 mM phosphate buffer, pH 6.5, 20 mg of bovine serum albumin, 4 mg of Fast Violet Blue, and the total volume was adjusted to 27 ml with water. For each 1-ml assay, 960 μl of cocktail was mixed with 10 μl of enzyme and 30 μl of 50 mM α-naphthyl acetate solution in acetone. Formation of the azo dye was monitored at 490 nm, $\epsilon_{490} = 18.33$ mM^{-1} cm^{-1}.

Steady-State Kinetics with CPT-11, NPC, and APC. The ability of carboxylesterases to hydrolyze CPT-11 and its metabolites was investigated by detecting the product, SN-38, using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA). Purified carboxylesterases were incubated with CPT-11 (0–200 μM), NPC (0–200 μM), or APC (0–1000 μM) in 20 mM Hepes buffer, pH 7.0, with 10% ethylene glycol at 37°C from 2 to 24 h in 250 μl of reaction volume. The reaction was stopped by addition of 250 μl of acetonitrile. Ten microliters of 0.14 mM camptothecin (internal standard) was added, and the mixture was centrifuged at 1400*g* for 8 min. We found that the APC stock was contaminated with camptothecin; therefore, a control with no enzyme was studied for each concentration of APC analyzed. For CPT-11 and NPC, 100 μl of 2.5% perchloric acid was added to 400 μl of supernatant to convert the drugs to the lactone form and extracted with 2×5 ml of $CHCl_3$. The $CHCl_3$ phase from each extraction was pooled and dried under nitrogen. For APC, a solid phase extraction method was used after quenching the reaction with 670 μl of 0.1 N HCl. The mixture was loaded on an Oasis HLB column (Waters, Milford, MA) that had been prepared by washing twice with methanol and equilibrated in water. The columns were washed twice with water and 40% methanol before eluting SN-38 with 1.6 ml of methanol. The samples from all the assays were dried under nitrogen and reconstituted in 100 μl of 0.005% perchloric acid containing 23% acetonitrile. SN-38 standards were prepared using the same procedure as described for the samples, and 45 μl was injected onto a 5-μm C18, 150 × 4.6 mm Luna column (Phenomenex, Torrance, CA). The mobile phase was 28.5% acetonitrile in 0.1 M KH_2PO_4, pH 4.0, with 3 mM heptane sulfonic acid. The compounds were eluted at a flow rate of 1 ml/min and monitored by fluorescence (excitation = 375 nm, emission = 560 nm). The data were fit to the Michaelis-Menten equation (GraFit 4.0; Erithacus Software Ltd., Surrey, UK), and the K_M and k_{cat} values for the three isoenzymes with the three substrates were determined.

Results

Cloning, Expression, and Purification of CES3. The CES3 gene was recently identified (GI: 7019977) during the New Energy and Industrial Technology Development Organization human cDNA sequencing project. We amplified the CES3 gene from human liver cDNA to generate a 1.8-kb PCR product including the start and stop codons. Sequencing in both directions confirmed that it was identical to the reported sequence (GI: 7019977). The *Baculovirus* expression system was used to overexpress the CES3 protein in Sf9 insect cells. CES3 was purified to homogeneity by a two-step procedure involving concanavalin A affinity chromatography and preparative nondenaturing PAGE (Table 1). Most of the activity in the cell lysate was from

TABLE 1

Purification of recombinant CES3

	Volume	α-NA Activity[a]	Total Activity	Protein	Total Protein	Specific Activity	Fold Purification
	ml	*U/ml*	*U*	*mg/ml*	*mg*	*U/mg*	
Cell Lysate 100	38	1.26	48	9.4	356	0.13	1
Concanavalin A concentrate	0.7	5.6	3.9	4.0	2.8	1.4	10
Prep Cell concentrate	0.4	9.3	3.9	1.6	0.67	5.8	44

[a] The activity was monitored by α-napthylacetate (α-NA) hydrolase with Fast Violet Blue. One unit is defined as 1 μmol of product formed per minute.

insect cell esterases. Concanavalin A column selectively binds the CES3 protein, and the insect cell esterases were separated in the flow-through. The specific activity of purified CES3 was 5.8 U/mg with α-naphthyl acetate as substrate. We used α-naphthyl acetate as substrate because 4-methylumbelliferyl acetate was found to be a very poor substrate for CES3. The protein yield from 1 liter of insect cell culture was ~0.7 mg (Table 1). The carboxylesterase was purified 44-fold from the transfected Sf9 cell extract, and it exhibited a major band (>90% purity) of 60 kDa on SDS-PAGE (Fig. 2). Endoproteinase Glu-C or trypsin cleavage of this 60-kDa protein after SDS-PAGE (Sanghani et al., 2002) and MALDI-TOF mass spectroscopic analysis of peptides (data not shown) positively confirmed that the purified enzyme was CES3. The peptides identified by MALDI-TOF are underlined in Fig. 3.

Purification of CES1A1 and CES2. As shown in Table 2, CES1A1 and CES2 were purified from human liver by a modification of the method of Humerickhouse et al. (2000). The substitution of preparative nondenaturing PAGE for hydroxyapatite ion-exchange chromatography resulted in enzyme with similar specific activity (Table 2) but required less time and produced greater yield.

Tissue-Specific Expression of CES3. Human multi-tissue Northern blot studies investigating CES3 gene expression showed two major bands at 2 and 4 kb. Multiple bands were seen on Northern blot for CES2 gene (Sanghani et al., 2003), and they arise from multiple transcriptional start sites (Wu et al., 2003). The reason for multiple bands for the CES3 gene is presently unknown. The CES3 gene was largely expressed in three tissues, and its relative abundance was liver > colon > small intestine (Fig. 4).

Steady-State Kinetic Studies for Hydrolysis of CPT-11, NPC, and APC by Carboxylesterases. Steady-state kinetic analysis for the hydrolysis of CPT-11, NPC, and APC was performed with purified carboxylesterase isoenzymes CES1A1, CES2, and CES3. As shown in Table 3, CPT-11 was most efficiently hydrolyzed by CES2, with the lowest K_M (~1 μM) and highest k_{cat} (0.013 min^{-1}). It was 100 times more efficient than CES1A1 and 2000 times more efficient than CES3. The k_{cat} of CES2 for CPT-11 is less than that reported by Humerickhouse et al. (2000) (0.16 min^{-1}) and may represent protein heterogeneity that affects CPT-11 kinetics. A similar trend in catalytic efficiency was seen in the case of NPC hydrolysis, where CES2 was about 200 times more efficient than CES1A1 and about 9000 times more efficient than CES3. The k_{cat}/K_M values for NPC were consistently lower than that for CPT-11, for each isoenzyme. The steady-state kinetic study of CES2 with APC indicated a K_M of 270 μM and a catalytic efficiency of 0.015×10^{-3} min^{-1} μM^{-1}, which is more than 2 orders of magnitude less than NPC and CPT-11 with CES2. The estimated k_{cat} of CES1A1 and CES3 with APC was between 10^{-5} and 10^{-6} min^{-1}; therefore, kinetic analysis was not feasible.

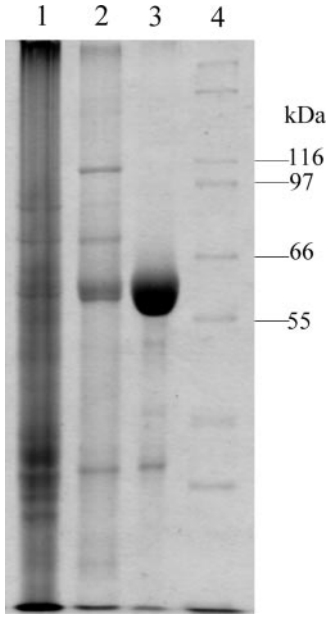

FIG. 2. *SDS-PAGE of samples at various stages of purification of the recombinant human CES3 isoenzyme expressed in Sf9 insect cells.*

Lane 1, 40 μg of cell lysate; lane 2, 5 μg of protein after concanavalin A chromatography; lane 3, 5 μg of protein after preparative nondenaturing gel; lane 4, molecular weight markers.

Discussion

Carboxylesterases belong to a multigene family (Satoh and Hosokawa, 1998), and the two main human carboxylesterase isoenzymes, CES1A1 (GI: 16905523) and CES2 (GI: 37622884), have been well characterized in human liver (Brzezinski et al., 1994; Pindel et al., 1997; Satoh et al., 2002). The CES3 gene was identified in colon tissue during the New Energy and Industrial Technology Development Organization human cDNA sequencing project (GI: 7019977). However, the expression of CES3 has not been reported. Hence, this is the first report of the cloning of the entire coding region of CES3 gene from human liver cDNA, expression of the CES3 carboxylesterase in insect cells, and characterization of CES3 catalytic activity with CPT-11, NPC, and APC as substrates.

CES1A1 and CES2 are mapped to human chromosome 16, q13–q22.1 and q21, respectively. The National Center for Biotechnology Information's Spidey mRNA-to-genomic alignment program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html) was used to identify the location of CES3 gene (GI: 7019977). The mRNA mapped to the *Homo sapiens* chromosome 16 (GI: NT_010478) locus with no mismatches in the coding region. Thirteen exons were identified spanning about 12 kb of genomic sequence near the CES2 gene. The exon size and location of cDNA sequences are shown in Fig. 5. Sequence identity analysis of carboxylesterase isoenzymes showed that CES3 has about 40% identity with both CES1A1 and CES2 isoenzymes. Phylogenetic analysis of CES3 indicates that it belongs to a new class of human carboxylesterase isoenzyme, and it has 63% identity with mouse esterase 31 (GI: 38511890), which was classified as Class 3 by Satoh and Hosokawa (1995). Hence, we call this isoenzyme CES3.

*MERAVRVESGVLVGVVCLLLACPAT*ATGPEVAQPEVDTTLGRVRGRQVGVKGTDRLVNVF 60
LGIPFAQPPLGPDRFSAPHPAQPWEGVRDASTAPPMCLQDVESMNSSRFVLNGKQQIFSV 120
SEDCLVLNVYSPAEVPAGSGRPVMVWVHGGALITGAATSYDGSALAAYGDVVVVTVQYRL 180
GVLGFFSTGDEHAPGNQGFLDVVAALRWVQENIAPFGGDLNCVTVFGGSAGGSIISGLVL 240
SPVAAGLFHRAITQSGVITTPGIIDSHPWPLAQKIANTLACSSSSPAEMVQCLQQKEGEE 300
LVLSKKLKNTIYPLTVDGTVFPKSPKELLKEKPFHSVPFLMGVNNHEFSWLIPRGWGLLD 360
TMEQMSREDMLAISTPVLTSLDVPPEMMPTVIDEYLGSNSDAQAKCQAFQEFMGDVFINV 420
PTVSFSRYLRDSGSPVFFYEFQHRPSSFAKIKPAWVKADHGAEGAFVFGGPFLMDESSRL 480
AFPEATEEEKQLSLTMMAQWTHFARTGDPNSKALPPWPQFNQAEQYLEINPVPRAGQKFR 540
EAWMQFWSETLPSKIQQWHQKQKNRKA*QEDL* 571

FIG. 3. *Predicted protein sequence for CES3 gene (GI: 7019977).*

This protein has several characteristics of the carboxylesterase family; the N-terminal signal peptide and the potential C-terminal endoplasmic reticulum retention signal both are shown in italics. The active-site residues Ser229, Glu347, and His460 predicted from sequence alignment with other isoenzymes are shown in bold face, and the positions of the four conserved cysteine residues are marked by ∗. One potential glycosylation site, Asn105, is shown in bold and underlined. The peptides that were identified by MALDI-TOF mass spectrometry of Glu-C and tryptic peptides of the recombinant protein are underlined.

TABLE 2

Purification of CES1A1 and CES2 from human liver

	Volume	4-MUA[a] Activity	Total Activity	Protein	Total Protein	Specific Activity
	ml	*U/ml*	*U*	*mg/ml*	*mg*	*U/mg*
Liver homogenate	220	5.29	1164	18.8	4136	0.28
Concentrate after DE52	123	8.6	1057	16.7	2054	0.51
Concanavalin A concentrate	4	186	742	8.8	35	21.2
Prep Cell concentrate CES1A1	2	77	154	11.6	23.2	6.6
Prep Cell concentrate CES2	0.5	814	407	6.8	3.4	120

[a] The 4-methylumbelliferyl acetate (MUA) activity was measured at 37°C in 90 mM KH_2PO_4, 40 mM KCl, pH 7.4 buffer with 0.5 mM 4-methylumbelliferyl acetate. The formation of product, 4-methylumbelliferone, was monitored spectrophotometrically at 350 nm.

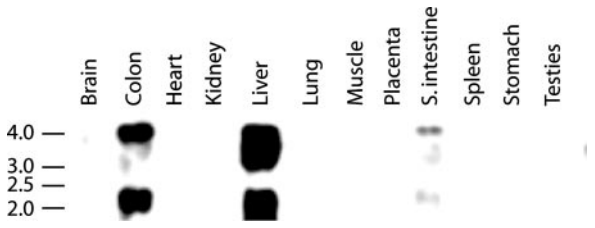

FIG. 4. *Human multi-tissue Northern blot probed for CES3.*

A CES3-specific probe was generated by PCR across 919-1234 nt of CES3 gene (GI: 7019977). ^{32}P-dCTP was used for random primed labeling of the probe, and the blot was hybridized overnight. The autoradiograph was developed after 8 days of exposure.

The predicted amino acid sequence of the CES3 gene is shown in Fig. 3. The active site residues Ser229, Glu347, and His460 and the four conserved cysteine residues were identified by alignment with rat and human carboxylesterase isoenzymes (Omiga 1.1; Accelrys, San Diego, CA). Analysis of the CES3 protein with ScanProsite (http://us.expasy.org/tools/scanprosite/) identified a conserved carboxylesterase motif, PS00122 (amino acids 216–231), and one potential glycosylation site at Asn105, shown in bold and underlined in Fig. 3. During purification, recombinant CES3 protein binds to concanavalin A, verifying that it is glycosylated. Consistent with other carboxylesterases, a 26-amino acid N-terminal signal peptide was identified by Signal P 1.1 software (http://www.cbs.dtu.dk/services/SignalP/). In support of this, the most N-terminal peptide was identified during MALDI-TOF mass spectrometry of the tryptic peptides but not the leader sequence (Fig. 3). Mammalian carboxylesterases have a C-terminal microsomal retention sequence that is usually a variation of "HXEL" (Robbi and Beaufay, 1991). CES3 is the first carboxylesterase isoenzyme with a "QEDL" C-terminal retention signal. This C-terminal tetra peptide QEDL does, however, function as an endoplasmic reticulum retention signal in the case of the microsomal protein ERp (Mazzarella et al., 1994). It is not clear whether QDEL functions more commonly as a microsomal retention signal. Northern-blot analysis (Fig. 4) showed that the tissue distribution of CES3 is very similar to that of CES2 (Satoh et al., 2002), with liver > colon > small intestine. Based on the exposure time and determination of relative expression in colon tissue (Sanghani et al., 2003), we conclude that the abundance of CES3 is much less than that of CES2 in colon. In the same study, we did not find any correlation between CPT-11 hydrolase activity and CES3 message in colon tumor samples.

In vitro cytotoxicity studies show that the IC_{50} for CPT-11 is about 10^3 higher (less efficient) than for the active form of the drug, SN-38 (Sparreboom et al., 1998). The oxidative metabolism of CPT-11 by the action of CYP3A4 (Fig. 1) generates two major metabolites, APC and NPC (Santos et al., 2000). The IC_{50} values of NPC and APC are also about 10^3 higher than SN-38 (Rivory et al., 1996; Dodds et al., 1998). Enzymes involved in the glucuronidation of SN-38 (Fig. 1) have been extensively studied with respect to inactivation of SN-38 (Hanioka et al., 2001; Tukey et al., 2003). Hence, overall SN-38 concentration is precisely determined by a balance between generation of SN-38 from CPT-11, APC, and NPC and inactivation by glucuronidation.

The identity and roles of specific carboxylesterases in the in vivo activation of NPC or APC to form SN-38 have not been established. We find that CPT-11, NPC, and APC are converted to SN-38 by all three human liver carboxylesterases but with different catalytic efficiencies, CES2 > CES1A1 > CES3 (Table 3). In vitro studies demonstrated that CPT-11 is metabolized by carboxylesterases and that CES2 is 64 times more efficient than CES1A1 (Humerickhouse et al., 2000). In this study, CPT-11 is similarly found to be hydrolyzed about 100 times more efficiently by CES2 than by CES1A1 (Table 3). However, the k_{cat} values of CES1A1 and CES2 with CPT-11 are less than that reported previously (Humerickhouse et al., 2000). CES3 can hydrolyze CPT-11, but the relative rate is much lower than those of CES2 and CES1A1 (Table 3). The relative abundance of carboxyles-

TABLE 3

Steady state kinetic studies for carboxylesterase isoenzymes with CPT-11, NPC, and APC as substrates

The substrates, CPT-11 (0–200 μM), NPC (0–200 μM), or APC (0–1000 μM) were incubated in 20 mM Hepes buffer, pH 7.0, with 10% ethylene glycol at 37°C for 2–24 h in 250 μl of reaction volume. The product, SN-38, was extracted with 2 × 5 ml $CHCl_3$ or with Oasis HLB columns and quantitated on an Agilent 1100 HPLC equipped with a fluorescence detector, with excitation set at 375 nm and emission monitored at 560 nm. The concentration of SN-38 was determined from the standard curve generated under identical conditions with each experiment. The substrate consumption was always less than 6%.

Isoenzyme	Substrate	K_M[a]	k_{cat}	Catalytic Efficiency	Normalization of Catalytic Efficiency to CES2 for Each Substrate
		μM	$10^{-3}\ min^{-1}$	$min^{-1}\ mM^{-1}$	
CES1A1	CPT-11	39 ± 3	4.9 ± 0.1	0.13	1
CES2	**CPT-11**	**1.1 ± 0.1**	**13 ± 0.2**	**12**	**100**
CES3	CPT-11	137 ± 11	0.9 ± 0.04	0.0063	0.05
CES1A1	NPC	80 ± 6	1.8 ± 0.06	0.023	0.5
CES2	**NPC**	**3.2 ± 0.2**	**16 ± 0.2**	**5**	**100**
CES3	NPC	460 ± 50	0.26 ± 0.01	0.00056	0.01
CES2	**APC**	**270 ± 50**	**2.45**	**0.015**	

[a] Independent estimations of K_M and k_{cat} values were made for each enzyme and substrate. The values for one such experiment with standard error for the fit are reported. The estimations were made by nonlinear regression analysis of the data to the Michaelis-Menten equation (GraFit 4.0; Erithacus Software Ltd.).

FIG. 5. *The exon-intron map of CES3 gene on human chromosome 16 (Contig; GI: NT_010478) as determined by Spidey software.*

One hundred percent of the cDNA sequence (GI: 7019977) matched with the genomic sequence. The cDNA was distributed over 13 exons (shown as boxes) with 100% identity and with no gaps and no mismatches. The mRNA coordinates based on GI: 7019977 and genomic coordinates based on Contig; GI: NT_010478 for each exon are as follows: exon 1, 1–100, 632,917–633,016; exon 2, 101–305, 634,806–635,010; exon 3, 306–444, 635,118–635,256; exon 4, 445–578, 635,429–635,562; exon 5, 579–732, 635,984–636,137; exon 6, 733–837, 636,250–636,354; exon 7, 838–939, 637,871–637,972; exon 8, 940-1080, 638,352–638,492; exon 9, 1081–1161, 641,306–641,386; exon 10, 1162–1309, 642,799–642,946; exon 11, 1310–1459, 643,983–644,132; exon 12, 1460–1538, 644,295–644,373; exon 13, 1539–2093, 644,481–645,035. Blasting the human genome with the GI: 7019977 also identified this gene on human chromosome 16 and adjacent to CES2 gene.

terase genes was determined by Northern blot analysis of normal colon tissue and was found to be CES2 > CES1A1 ≥ CES3 (Sanghani et al., 2003). Hence, CES3 will not play a significant role in metabolism of CPT-11 and its metabolites in colon tissue. Future studies are needed to uncover the importance of this isoenzyme in metabolism of other drug compounds. Incubation of NPC with liver microsomes results in SN-38 production that is linear with time (Dodds et al., 1998; Haaz et al., 1998). Formation of SN-38 upon incubation with purified CES1A1 has been reported as 4-fold lower with NPC than with CPT-11 (Dodds et al., 1998). In agreement with this, we find that the k_{cat}/K_M of NPC with CES1A1 is about 6-fold less than that with CPT-11 (Table 3). Comparing the catalytic activities of carboxylesterases for hydrolysis of NPC, we find that CES2 is about 200 times more active than CES1A1. We find that APC is hydrolyzed slowly by all three purified carboxylesterases. The catalytic efficiency of CES2 for APC is 800-fold lower than that for CPT-11 and about 300-fold lower than for NPC. Efficient metabolism of NPC by carboxylesterases may account for lower serum levels of NPC (1.5%) in comparison with APC (11%) upon intravenous administration of CPT-11, as reported by Slatter et al. (2000). We expect that human microsomes should slowly hydrolyze APC. However, Rivory et al. (1996) reported that APC was not metabolized by human liver microsomal carboxylesterase. We could not obtain kinetic constants of APC with CES1A1 and CES3 because of low activity.

Large interindividual variation in SN-38 area under the curve (about 70%) has been reported among cancer patients treated with CPT-11 (Canal et al., 1996; Slatter et al., 2000). Part of this variability could be due to the differences in the expression and activity of CPT-11-metabolizing enzymes, especially CES2, CYP3A4, and UGT1A1. In the case of CES2, multiple transcripts are detected in a tissue-dependent fashion (Satoh et al., 2002) that arise from three different transcription start sites (Wu et al., 2003). The two longer transcripts have an in-frame upstream ATG, resulting in CES2 protein with 64 additional N-terminal amino acids. However the importance and characteristics of this longer protein are currently unclear (Wu et al., 2003). The CES2 transcript levels in colon tumor samples and their ability to hydrolyze CPT-11 were significantly correlated (Sanghani et al., 2003). Hence, studying the heterogeneity, specificity, and distribution of the CPT-11-metabolizing enzymes is undoubtedly important to understanding its overall metabolism.

This study conclusively shows that CPT-11 and its metabolites, APC and NPC, are metabolized by carboxylesterases to SN-38. We also find that the new isoenzyme, CES3, is expressed in liver and colon tissue but has very poor activity for CPT-11, APC, and NPC and therefore will not play a significant role in CPT-11 metabolism. Based on the steady-state kinetics results and the tissue distribution and abundance of carboxylesterases determined by Northern analysis (Sanghani et al., 2003), we predict that CES2 isoenzyme is the most important isoenzyme in CPT-11 metabolism.

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