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Hydrolysis of Capecitabine to 5′-Deoxy-5-fluorocytidine by Human Carboxylesterases and Inhibition by Loperamide

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ABSTRACT

Capecitabine is an oral prodrug of 5-fluorouracil that is indicated for the treatment of breast and colorectal cancers. A three-step in vivo-targeted activation process requiring carboxylesterases, cytidine deaminase, and thymidine phosphorylase converts capecitabine to 5-fluorouracil. Carboxylesterases hydrolyze capecitabine's carbamate side chain to form 5′-deoxy-5-fluorocytidine (5′-DFCR). This study examines the steady-state kinetics of recombinant human carboxylesterase isozymes carboxylesterase (CES) 1A1, CES2, and CES3 for hydrolysis of capecitabine with a liquid chromatography/mass spectroscopy assay. Additionally, a spectrophotometric screening assay was utilized to identify drugs that may inhibit carboxylesterase activation of capecitabine. CES1A1 and CES2 hydrolyze capecitabine to a similar extent, with catalytic efficiencies of 14.7 and 12.9 min^{-1} mM^{-1}, respectively. Little catalytic activity is detected for CES3 with capecitabine. Northern blot analysis indicates that relative expression in intestinal tissue is CES2 > CES1A1 > CES3. Hence, intestinal activation of capecitabine may contribute to its efficacy in colon cancer and toxic diarrhea associated with the agent. Loperamide is a strong inhibitor of CES2, with a K_i of 1.5 μM, but it only weakly inhibits CES1A1 (IC_{50} = 0.44 mM). Inhibition of CES2 in the gastrointestinal tract by loperamide may reduce local formation of 5′-DFCR. Both CES1A1 and CES2 are responsible for the activation of capecitabine, whereas CES3 plays little role in 5′-DFCR formation.

Capecitabine is a recently developed oral prodrug of 5-fluorouracil that has shown anticancer activity in a variety of solid tumor types and is approved in the United States for the treatment of metastatic breast and colorectal cancers. Capecitabine is as effective in treating colorectal cancer as 5-fluorouracil/leucovorin (Mayo regimen) (Scheithauer et al., 2003). Patients taking capecitabine experienced a remarkably lower incidence of diarrhea, stomatitis, nausea/vomiting, alopecia, and neutropenia than those receiving 5-fluorouracil/leucovorin (Scheithauer et al., 2003). However, patients receiving capecitabine had a greater frequency of hand-foot syndrome (Scheithauer et al., 2003). This toxicity is not life-threatening and can be managed by dosage adjustment. Capecitabine has the additional benefit of being orally administered, which reduces hospitalization time and cost of treatment compared with 5-fluorouracil/leucovorin (Jansman et al., 2004).

Capecitabine undergoes a three-step activation process in vivo that preferentially targets formation of 5-fluorouracil to the tumor (Miwa et al., 1998). The first step of this process, hydrolysis of the carbamate side chain of capecitabine to produce 5′-deoxy-5-fluorocytidine (5′-DFCR), occurs primarily in the liver by carboxylesterases (Miwa et al., 1998; Shimma et al., 2000; Tabata et al., 2004a,b). Subsequently, 5′-deoxy-5-fluorouridine (5′-DFUR) is formed by cytidine deaminase in the liver and tumor tissue. 5-Fluorouracil is ultimately formed from 5′-DFUR by thymidine phosphorylase, which is highly expressed in tumor tissue (Miwa et al., 1998). This three-step targeted prodrug approach was developed to increase the bioavailability of 5-fluorouracil by reducing the catabolism of 5-fluorouracil in the liver by dihydropyrimidine dehydrogenases and targeting 5-fluorouracil formation to the tumor. Pharmacokinetic analyses indicate high interpatient variability in the exposure to capecitabine and its metabolites in plasma (Reigner et al., 2003).

A 60-kDa carboxylesterase is reportedly responsible for the

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ABBREVIATIONS: 5′-DFCR, 5′-deoxy-5-fluorocytidine; 5′-DFUR, 5′-deoxy-5-fluorouridine; CES, carboxylesterase; MS, mass spectroscopy; LC, liquid chromatography; 4-MUA, 4-methylumbelliferylacetate.

formation of 5′-DFCR (Miwa et al., 1998). Three 60-kDa carboxylesterase isozymes have been identified in humans, CES1A1 (gi: 179927), CES2 (gi: 21536284), and CES3 (gi: 7019977). These enzymes are responsible for the metabolism of ester-, amide-, and thioester-containing compounds to their free acid forms (Satoh and Hosokawa, 1998). Although they exhibit overlapping substrate specificity, selectivity is observed for therapeutic drugs like irinotecan (Sanghani et al., 2004) and methylphenidate (Sun et al., 2004).

In cancer treatment, multiple agents are often combined to obtain the greatest therapeutic effect. For example, capecitabine has been used in combination with irinotecan, vincristine, and oxaliplatin. Irinotecan is activated by CES2 (Humerickhouse et al., 2000; Sanghani et al., 2004). Other anticancer agents that contain ester moieties may also be substrates for the enzymes responsible for capecitabine activation. Also, drugs used to prevent or treat toxicities, such as atropine, corticosteroids, and serotonin antagonists, may affect the activity of carboxylesterases. Drug-drug interactions often influence the efficacy of agents metabolized by the cytochrome P450 enzyme system. However, little information regarding inhibition of carboxylesterase enzymes is available. Drugs administered concomitantly with capecitabine may reduce its effectiveness by inhibiting the carboxylesterase enzymes.

The purpose of this study is to examine the steady-state kinetics of the human carboxylesterase isozymes for capecitabine hydrolysis and to determine the carboxylesterases responsible for hydrolysis of capecitabine to 5′-DFCR. Using a spectrophotometric assay, we investigated whether drugs commonly coadministered with capecitabine can bind to the carboxylesterase isozymes, potentially altering the bioavailability of 5′-DFCR.

Materials and Methods

Materials. Capecitabine (Xeloda; Hoffman-LaRoche, Inc., Nutley, NJ) tablets were provided by Dr. David Potter (Indiana University, Indianapolis, IN). Pure 5′-DFCR standard was kindly provided by Roche Molecular Systems Inc. All other chemicals and reagents were of the purest form available and obtained from Fisher Scientific Co. (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Carboxylesterases. Recombinant CES1A1, CES2, and CES3 were expressed in Sf9 cells and purified as previously described (Sanghani et al., 2004; Sun et al., 2004). Briefly, CES1A1, CES2, and CES3 cDNAs were cloned into baculovirus expression vectors, and recombinant proteins were expressed in Sf9 insect cells. Expressed proteins were purified by concanavalin A affinity chromatography followed by preparative nondenaturing polyacrylamide gel electrophoresis. For the inhibition assays, native CES1A1 and CES2 were purified from human liver as previously described (Sanghani et al., 2004). The properties and enzyme kinetics of native and recombinant enzymes are very similar (Sun et al., 2004).

Capecitabine Purification. Capecitabine was purified from Xeloda tablets. Tablets were crushed and dissolved in water. The mixture was centrifuged to remove nonsoluble excipients and the supernatant extracted through a 20-ml Bond Elut Mega BE-C18 column (Varian, Inc., Palo Alto, CA) that had been pre-equilibrated with methanol and water. The columns were washed with 1-column volume of water, and purified capecitabine was eluted with methanol. The methanol was evaporated, and the drug was resuspended in water and lyophilized to dryness. The ultraviolet absorption spectrum of capecitabine purified by this method was identical to that of capecitabine purchased from Sequoia Research Products Ltd. (Oxford, UK) (ϵ_{304} = 8.5 mM^{-1}). Moreover, the mass spectroscopy (MS) spectrum of purified capecitabine exhibited the expected peak at *m/z* of 359.95 with no contaminating peaks (data not shown).

Steady-State Kinetics of Capecitabine Hydrolysis. Capecitabine was dissolved in water and diluted to 0.25 to 10 mM final concentration in 125 μl of 20 mM HEPES, pH 7.4, containing 10% ethylene glycol to stabilize the enzyme. Purified recombinant CES1A1 (56 μg/ml), CES2 (30 μg/ml), or CES3 (30 μg/ml) enzyme was added. After a 5- (CES1A1) or 30- (CES2 and CES3) min incubation at 37°C, the reaction was quenched with 125 μl of acetonitrile and placed on ice. The mixture was centrifuged for 1 min at 16,400*g*. Ten microliters of the supernatant were diluted in 490 μl of liquid chromatography (LC)/MS mobile phase (described below). A standard curve was constructed by diluting 5′-DFCR standard in 20 mM HEPES, pH 7.4, containing 10% ethylene glycol/acetonitrile (50:50). Standards were further diluted 1:50 in LC/MS mobile phase.

LC/MS Detection of Capecitabine and 5′-DFCR. Capecitabine and 5′-DFCR concentrations were determined using LC/MS. Analysis was conducted using a Shimadzu LC-MS 2010 equipped with an atmospheric pressure chemical ionization probe operated in positive ion mode. The column was a Luna Synergi Polar-RP (4 μm, 250- × 2-mm inner diameter column; Phenomenex, Torrance, CA). Mobile phase consisted of 55% aqueous and 45% organic at a flow rate of 0.2 ml/min. The aqueous phase was nanopure water containing 0.1% trifluoroacetic acid. The organic phase was a 50:50 mixture of acetonitrile/methanol. The atmospheric pressure chemical ionization probe was operated at 450°C, the curved desolvation line temperature was 250°C, and the heat block was set at 200°C. Nitrogen was used as the nebulizer gas with a constant flow rate of 2.5 l/min. Compounds were detected using selected ion monitoring in positive mode with *m/z* of 245.9 for 5′-DFCR and 359.95 for capecitabine. The amount of 5′-DFCR formed was determined by comparing peak areas for 5′-DFCR with peak area ratios for standards. The standard curve was linear from 0.02 to 4.0 μM of 5′-DFCR. The rate of 5′-DFCR produced was plotted versus capecitabine concentration and fitted to a Michaelis-Menten equation to obtain estimates of K_m and V_{max} for each enzyme using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA).

Screening Assay for Drug Interactions. Drugs commonly administered with capecitabine and irinotecan, e.g., other antineoplastic agents and drugs that alleviate side effects, were evaluated for their ability to inhibit CES1A1 or CES2 activity. CES1A1 or CES2 purified from human liver was incubated with 0.5 or 0.2 mM 4-methylumbelliferyl acetate, respectively, and varying concentrations of inhibitors in buffer consisting of 90 mM KH_2PO_4 and 40 mM KCl, pH 7.4. Formation of 4-methylumbilliferone from 4-methylumbelliferyl acetate was monitored spectrophotometrically at 350 nm using a Cary 50 UV-Vis Spectrophotometer (Varian, Inc.). Activity of CES1A1 or CES2 in the absence of inhibitor was adjusted to ~3.6 units/ml. The data were fit to an inhibition model, percent inhibition = $1 + 10^{(\log[I]\ -\ \log\ IC50)-1}$, where [I] is the concentration of inhibitor, to estimate the inhibitor concentration at which the carboxylesterase activity is reduced by 50% (IC_{50}) using GraphPad Prism 4.0.

The mechanism of inhibition for loperamide was determined by incubating varying concentrations of 4-methylumbelliferyl acetate (4-MUA) (0.10, 0.15, 0.20, and 0.50 mM) with varying concentrations of the inhibitor agent, loperamide (0–8 μM), in the presence of purified CES1A1 or CES2. The data were fit to models for competitive, noncompetitive, or uncompetitive inhibition (Segel, 1975) using GraphPad Prism 4.0. The preferred model was selected based on statistical analysis and comparison of the Akaike Information Criterion value for each curve fit.

Gastrointestinal Expression of CES1A1, CES2, and CES3. A human digestive system 12-lane MTN blot (BD Biosciences Clontech, Palo Alto, CA) was probed with specific cDNA probes for CES1A1, CES2, and CES3. The CES1A1 and CES2 genes had been cloned into pCR-topo blunt and pVL1392 vectors, respectively. For CES1A1, the vector was digested with SapI in NEB4 buffer at 37°C for 4 h. The digested fragments were electrophoresed onto an agarose gel, and the band at 0.425 kbp was isolated and purified using a QIAquick gel extraction kit (QIAGEN, Valencia, CA). This band corresponds to nucleotides 1060 to 1485 of the CES1A1 gene. For CES2, the sequence between nucleotide 1111 and 1451 was isolated in a similar method by digesting the CES2 containing vector with NcoI and EcoRI and extracting a 341-kbp band from the gel. The CES3 specific probe was generated by PCR from 919 to 1234 nucleotides of the CES3 gene (gi: 7019977). Approximately 27 ng of each DNA probe was labeled with α^{32}P-dCTP using the High Prime labeling kit (Roche Diagnostics, Indianapolis, IN). The denatured probe and 100 μl of sonicated salmon sperm DNA (Stratagene, La Jolla, CA) were added to the blot, which had been prehybridized at 65°C for 30 min in Quikhyb solution (Stratagene). 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 a 30-min wash with 0.1× standard saline citrate containing 0.1% SDS at 58°C. The blot was exposed to Biomax MS film (Eastman Kodak, Rochester, NY) overnight at −70°C, and an autoradiograph was developed.

Results

Capecitabine Hydrolysis. Steady-state kinetics analysis for capecitabine hydrolysis by purified human carboxylesterase isozymes was performed using recombinant CES1A1, CES2, and CES3. Capecitabine underwent slow nonenzymatic hydrolysis in the assay buffer at a rate of 0.050 ± 0.008 μM/min. CES1A1 and CES2 were both capable of hydrolyzing capecitabine (Fig. 1; Table 1). The K_m values (1.3 and 1.0 mM for CES1A1 and CES2, respectively) were not significantly different. CES1A1 exhibited a significantly higher K_{cat} (18.8 min^{-1}) than CES2 (13.5 min^{-1}). Hence, the catalytic efficiency, K_{cat}/K_m, of CES1A1 for capecitabine, 14.7 min^{-1} mM^{-1}, was slightly greater than that of CES2, 12.9 min^{-1} mM^{-1}. The rate of 5′-DFCR formation by CES3 was approximately twice that of nonenzymatic hydrolysis. The activity of CES3 was 0.34 nmol/min/mg at a capecitabine concentration of 6 mM. This is approximately 1000-fold lower than the V_{max} of CES1A1 and CES2 (Table 1). Due to low activity, catalytic efficiency of CES3 for capecitabine could not be determined.

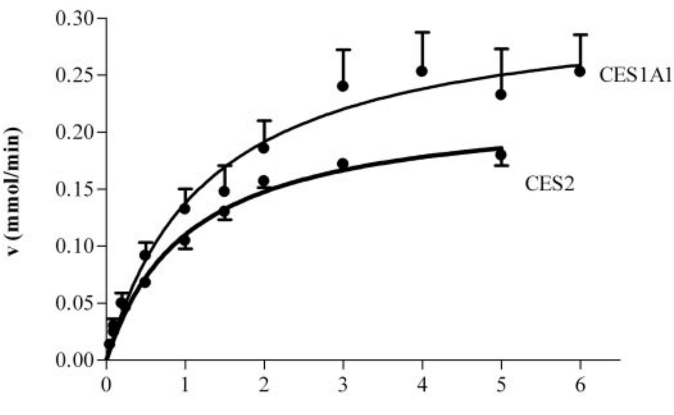

Fig. 1. Michaelis-Menten plot of capecitabine hydrolysis by CES1A1 and CES2. Capecitabine (0.25–6 mM) was incubated in the presence of purified CES1A1 or CES2 enzymes for 5 or 30 min, respectively. Formation of 5′-DFCR was detected using LC/MS and quantified by standard curve analysis.

TABLE 1

Steady-state kinetic constants for formation of 5′-DFCR from capecitabine

Capecitabine (0–10 mM) was incubated with purified recombinant carboxylesterases in 20 mM HEPES buffer, pH 7.4, containing 10% ethylene glycol at 37°C for 5 to 30 min in 125 μl of reaction volume. The product, 5′-DFCR, was quantitated using a Shimadzu LC-MS 2010. The concentration of 5′-DFCR was determined from a standard curve generated under identical conditions with each experiment. Values are presented as mean ± S.E. from at least three independent experiments.

	K_m	V_{max}	K_{cat}	Catalytic Efficiency
	mM	*μmol/min/mg*	*min^{-1}*	*min^{-1} mM^{-1}*
CES1A1	1.3 ± 0.38	0.31 ± 0.03	18.8	14.7
CES2	1.0 ± 0.11	0.22 ± 0.01	13.5	12.9

Inhibition Assays. Inhibition of CES1A1 and CES2 activity by drugs commonly coadministered with irinotecan was screened using 4-MUA as substrate (Brzezinski et al., 1997). Results are presented in Table 2. Loperamide exhibited the greatest inhibition of CES2, with an IC_{50} of 0.38 μM. The CES1A1 inhibition constant was approximately 1000-fold less (IC_{50} = 0.44 mM) than CES2. The other agents examined inhibited CES1A1 and CES2 to similar extents. For both enzymes, the IC_{50} was greater than 0.3 mM for dolasetron mesylate, followed by docetaxel and capecitabine (IC_{50}, 0.7–5 mM). Atropine and dexamethasone were very poor inhibitors (IC_{50} > 7 mM) of both CES1A1 and CES2.

The mechanism of loperamide inhibition of CES2 was further characterized by varying the concentration of both the substrate (4-MUA) and inhibitor (loperamide). Data were fit to equations for competitive, noncompetitive, and uncompetitive inhibition (Segel, 1975). The data best fit the competitive inhibition equation, with an r^2 of 0.96, producing a K_i of 1.5 μM (Fig. 2).

Gastrointestinal Expression of Carboxylesterase Isozymes. Expression of carboxylesterases within the gastrointestinal tract was examined by probing a multitissue Northern blot with specific probes for CES1A1, CES2, and CES3. All three mRNAs are observed in most gastrointestinal tissues with multiple forms of CES2 and CES3 present in liver and gastrointestinal tissues. All three isozymes were expressed to the greatest extent in liver (Fig. 3). All three CES mRNAs were expressed in the jejunum, ileum, ileocecum, and duodenum, as well as in the ascending, descending, and transverse segments of the colon with CES2 > CES1A1 > CES3. Expression of CES1A1 and CES2 shows the following pattern of expression: small intestine, colon > rectum, esophagus > cecum, and stomach.

TABLE 2

Drug inhibition of 4-MUA hydrolysis by CES1A1 and CES2

4-MUA was incubated in the presence of varying inhibitors in 90 mM KH_2PO_4 and 40 mM KCl, pH 7.4. Formation of the product, 4-methylumbelliferone, was monitored spectrophotometrically at 350 nm. Data were fit to an inhibition model to estimate the IC_{50}.

Inhibitor	CES1A1 IC_{50} (95% CI)	CES2 IC_{50} (95% CI)
		mM
Loperamide	0.44 (0.36–0.53)	3.8×10^{-4} (2.7×10^{-4}–5.4×10^{-4})
Dolasetron mesylate	0.33 (0.26–0.42)	0.50 (0.36–0.70)
Docetaxel	0.68 (0.59–0.77)	0.79 (0.62–1.0)
Capecitabine	4.9 (4.2–5.5)	4.8 (4.1–5.5)
Atropine	7.0 (6.7–7.2)	9.0 (8.5–9.5)
Dexamethasone	N.D.	22 (17–29)

N.D., not determined; 95% CI, 95% confidence interval.

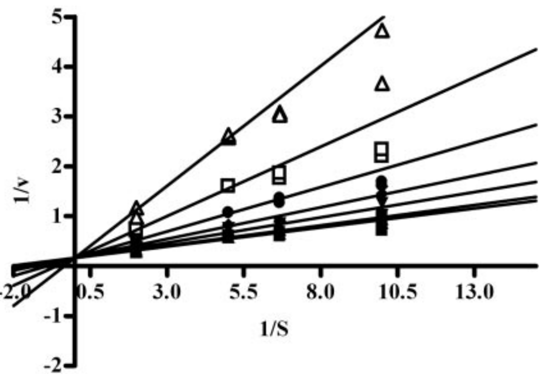

Fig. 2. Competitive inhibition of CES2 by loperamide. Formation of 4-methylumbelliferone was monitored at 350 nm in the presence of varying substrate and inhibitor concentrations. Symbols correspond to 1/observed rates, and lines correspond to 1/predicted rates derived from nonlinear regression fit of the data to the equation for competitive inhibition. The K_i, calculated from the fit of data to an equation for competitive inhibition, for loperamide was 1.5 ± 0.14 μM. The concentrations of loperamide were 0 (■), 0.1 (▲), 0.5 (▼), 1 (◆), 2 (●), 4 (□), and 8 (△) μM.

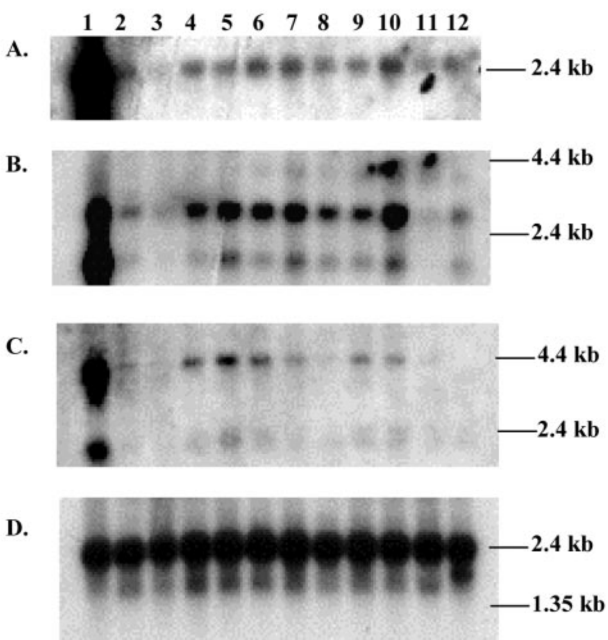

Fig. 3. Human multitissue Northern blot. Specific cDNA probes for CES1A1 (A), CES2 (B), CES3 (C), and β-actin (D) were labeled with α^{32}P-dCTP, a human digestive system 12-lane MTN blot from BD Biosciences Clontech was hybridized for 2 h, and autoradiographs were developed after 20 to 24 h of exposure to film. Lanes are: 1, liver; 2, rectum; 3, cecum; 4, transverse colon; 5, descending colon; 6, ascending colon; 7, jejunum; 8, ileum; 9, ileocecum; 10, duodenum; 11, stomach; and 12, esophagus.

Discussion

Capecitabine undergoes a three-step targeted activation process in vivo to deliver high concentrations of 5-fluorouracil to tumor tissue and to reduce toxicity to normal tissue. The first step of capecitabine's activation process is reportedly catalyzed by a 60-kDa liver carboxylesterase (Miwa et al., 1998). Steady-state kinetic analysis of purified recombinant human carboxylesterase isozymes indicates that CES1A1 and CES2 have similar catalytic efficiencies for capecitabine (Table 1). Hence, both isozymes are equally able to form 5′-DFCR. The activity of CES3 with 6 mM capecitabine is approximately 1000-fold lower than the saturating maximal velocity of CES1A1 and CES2 (Table 1). Hence, it is unlikely that CES3 plays a role in the activation of capecitabine in vivo.

Tabata et al. (2004a) reported the specific activities of CES1A1 (HU1) to be 275 pmol/min/mg and CES2 (HU3) to be 9.4 pmol/min/mg at 100 μM capecitabine. This concentration, although of physiological significance, is 10-fold lower than the K_m values for CES1A1 and CES2 in Table 1. We calculate that CES1A1 and CES2 would have specific activities of 23 and 20 nmol/min/mg, respectively, at 100 μM capecitabine from our kinetic constants. These values do not agree with the specific activities reported by Tabata et al. (2004a). A cytosolic enzyme capable of hydrolyzing capecitabine was recently isolated by Tabata et al. (2004b). However, the catalytic efficiency of this enzyme was more than 50-fold lower than that found for CES1A1 or CES2 in Table 1. Based on partial protein sequence analysis, Tabata et al. (2004b) determined that this enzyme was similar in sequence to CES1A1. It is unclear how this CES1A1-like enzyme appears in the cytosol because CES1A1 has microsomal leader and retention sequences.

The X-ray structure of CES1A1 has been reported (Bencharit et al., 2003). Capecitabine was manually docked into the CES1A1 structure using the observed position of homotropine as a guide (Fig. 4). Capecitabine fits well into the large site and can be modeled such that the interactions with surrounding amino acid side chains are constructive in nature and consistent with the known volume characteristics of the distinct alcohol and acyl group binding sites in CES1A1 (Bosron and Hurley, 2002; Bencharit et al., 2003). The CES2 and CES3 structures are not available for similar docking studies.

The tissue expression pattern of carboxylesterase isozymes will impact the site of activation of prodrugs such as capecitabine. All three isozymes are expressed in the liver, with CES1A1 expression being greater than that of CES2 and CES3 (Satoh et al., 2002). Human multitissue Northern blots have previously shown that CES2 is expressed in gastrointestinal tissue (Satoh et al., 2002; Wu et al., 2003). However, these reports do not describe localization of carboxylesterase isozymes along various sections of the gastrointestinal tract. Therefore, we examined the expression of the three CES mRNAs among various gastrointestinal tissues by Northern blot analysis (Fig. 3). The pattern of expression was somewhat similar for all three isozyme mRNAs with expression in different regions of the colon and small intestine. Expression was small intestine > rectum, esophagus > cecum, and stomach for CES1A1 and CES2. In these gastrointestinal tissues, CES2 expression was greater than CES1A1, which was substantially greater than CES3. The expression of CES2 generally agreed with the immunoblot analysis performed by Zhang et al. (2002).

Multiple mRNA bands were identified for CES2 and CES3 (Satoh et al., 2002; Sanghani et al., 2004). CES2 is known to have multiple transcription start sites (Wu et al., 2003) and to undergo alternative splicing, which may lead to multiple-sized mRNA and different protein products. The molecular basis for multiplicity of CES3 mRNAs is not known. It is not known whether the active protein products of these splice variants are expressed in liver or intestine. The estimation of expression of CES1A1 and CES2 in liver and small intestinal from Northern blot (Satoh et al., 2002) indicates that the

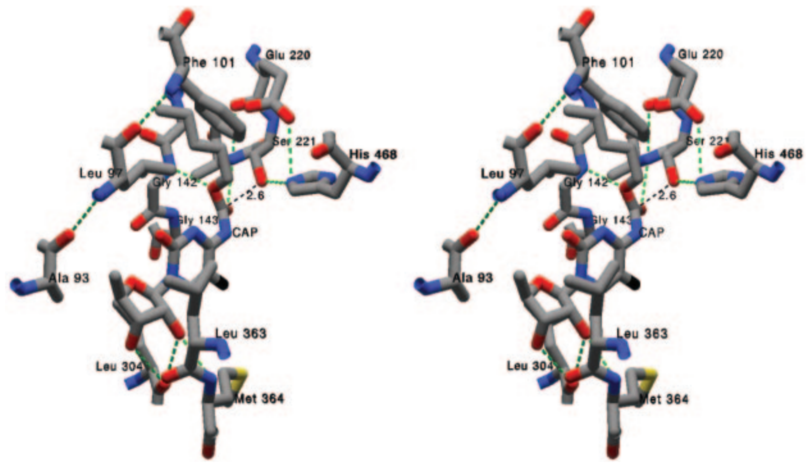

Fig. 4. Capecitabine bound to CES1A1. Capecitabine (CAP) was modeled into the CES1A1 active site using the crystal structure of CES1A1 complexed with homatropine as a guide (Bencharit et al., 2003), and the stereodiagram is presented. The structure for capecitabine was obtained from the 3DPSD database (http://www.ps.toyaku.ac.jp/dobashi) and converted to a PDB-formatted file. The program O (Jones et al., 1991) was used to dock the compound into the active site of CES1A1. CES1A1 was treated as a rigid molecule for the docking. Manual adjustment of dihedral rotations with the capecitabine molecule was used to optimize the fit to CES1A1 and complies with standard dihedral rotation values. The distance between the hydroxyl group of Ser 221 and the carbonyl carbon of capecitabine is 2.6 Å and is consistent in both distance and orientation for nucleophilic attack.

proteins are expressed at higher level in liver than intestine by approximately 100- and 10-fold, respectively. Additionally, capecitabine has been shown to be hydrolyzed about 19 times more efficiently by crude carboxylesterase preparations from human liver than from human intestine (Shimma et al., 2000). We believe that liver is the major site for activation of capecitabine. It is possible that orally administered capecitabine could be activated within the gastrointestinal tract. However, the catalytic activity of the CES2 protein for capecitabine in intestinal mucosa needs to be verified.

Pharmacokinetic analyses demonstrate that plasma exposure to capecitabine and its metabolites, especially 5′-DFCR, are highly variable. Following doses of 1250 to 1255 mg/m^2, the mean area under the curve of 5′-DFCR ranged from 6.51 to 14.1 mg/h/l with a coefficient of variation up to 77% (Reigner et al., 2003). According to our data, 5′-DFCR is equally well formed by CES1A1 and CES2. Both CES1A1 and CES2 are highly expressed in the liver and to a lesser extent in intestinal tissue (Fig. 3). Thus, first pass activation of capecitabine undoubtedly occurs in the liver and may occur in gastrointestinal tissue as well. We have previously reported that the expression of CES2 in colon tissue exhibits high interpatient variability (Sanghani et al., 2003). Variability in CES2 expression in liver and gastrointestinal tissues may influence the variability of capecitabine and 5′-DFCR plasma concentrations. These variations in carboxylesterase activity may be due to genetic, splice variant, or environmental influences. Several polymorphisms have been reported for CES1A1 and CES2 (Marsh et al., 2004; Wu et al., 2004). Environmental factors are also important for the in vivo activity of many drug metabolizing enzymes. For instance, concomitant use of certain foods, drugs, or herbal preparations may inhibit or induce expression of the cytochrome P450 isozymes. It has been reported that dexamethasone is an inducer of CES1A1 and CES2 (Zhu et al., 2000).

We employed a screening assay utilizing 4-methylumbelliferyl acetate to identify agents that are commonly coadministered with irinotecan or capecitabine that may inhibit drug activation by carboxylesterases. Hydrolysis of 4-MUA to 4-methylumbelliferone is easily monitored and serves as a marker for carboxylesterase activation. Inhibition of 4-MUA activity may indicate inhibition of capecitabine activity. Inhibition of prodrug activation may lead to reduced patient response. Several agents that contain ester or amide substituents were evaluated. Docetaxel was chosen as an ester-containing chemotherapeutic agent that may be administered with capecitabine or irinotecan. Dexamethasone and dolasetron mesylate are administered to prevent chemotherapy-induced nausea and vomiting. Loperamide and atropine are often required to treat chemotherapy-associated diarrhea. Of the drugs screened, the greatest degree of CES2 inhibition was observed with loperamide (IC_{50} = 0.38 μM). The remaining drugs had IC_{50} values that were at least 1000-fold higher, ranging from 0.4 to 22 mM (Table 2). Loperamide's inhibition of CES2 was further characterized by monitoring the rate of 4-MUA hydrolysis at varying concentrations of substrate and inhibitor. The data were fit to models for competitive, noncompetitive, and uncompetitive inhibition. Based on statistical analysis, loperamide was found to be a competitive inhibitor of CES2, with a K_i of 1.5 ± 0.14 μM (Fig. 2). Rivory et al. (1996) reported that a purified human carboxylesterase (probably CES1A1) was significantly inhibited by loperamide at concentrations from 50 to 200 μM.

In the clinical setting, loperamide is administered at high doses, up to 2 mg every 2 h, to treat chemotherapy-associated diarrhea. The C_{max} of loperamide in healthy volunteers following a single 16-mg dose has been reported as 7.8 to 8.6 nM (Doser et al., 1995; Tayrouz et al., 2001). However, loperamide is also a potent P-glycoprotein substrate (Wandel et al., 2002), and studies in laboratory animals have shown that it accumulates in the small intestine (Wuster and Herz, 1978; Lavrijsen et al., 1995). Therefore, CES2 in the intestinal epithelium may be exposed to a higher and potentially inhibitory concentration of loperamide. Activation of capecitabine within gastrointestinal tissue may be partially responsible for diarrhea associated with the drug. Both cytidine deaminase and thymidine phosphorylases activities are observed in colorectal tissues (Miwa et al., 1998), allowing for localized formation of 5-fluorouracil. Blockade of CES2 in the gastrointestinal tract by concomitant administration of loperamide may prevent the first step of local capecitabine activation. This may decrease the extent of 5-fluorouracil formation in intestinal tissue. Thus, loperamide may not only reduce diarrhea through its anticholinergic properties but also by inhibiting CES2 hydrolysis of capecitabine. Since CES2 is the major carboxylesterase isozyme expressed in the gastrointestinal tract, its inhibition would effectively reduce the local concentration of 5′-DFCR. However, CES1A1 activity in the liver would be unaffected by loperamide. Thus, systemic 5′-DFCR concentrations would likely be unchanged. In patients being treated for breast cancer or other tumors outside of the gastrointestinal tract, inhibition of CES2 by loperamide would be of therapeutic benefit. However, for patients with colorectal carcinomas, local inhibition of CES2 may also reduce the concentration of 5-fluorouracil in the tumor. Appropriate use of capecitabine and similar prodrugs requires a thorough understanding of the activating enzymes. Toxicity and response to capecitabine may be affected by the variability in the expression and activity of CES1A1 and CES2 in gastrointestinal and liver tissue.

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