What can protective drug metabolism proteins teach us about the treatment of human disease? That sometimes even a promiscuous protein has to get specific

Keynote review:

Mammalian carboxylesterases: from drug targets to protein therapeutics

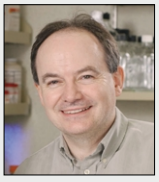
Matthew R. Redinbo and Philip M. Potter

Our understanding of the detailed recognition and processing of clinically useful therapeutic agents has grown rapidly in recent years, and we are now able to begin to apply this knowledge to the rational treatment of disease. Mammalian carboxylesterases (CEs) are enzymes with broad substrate specificities that have key roles in the metabolism of a wide variety of clinical drugs, illicit narcotics and chemical nerve agents. Here, the functions, mechanism of action and structures of human CEs are reviewed, with the goal of understanding how these proteins are able to act in such a non-specific fashion, yet catalyze a remarkably specific chemical reaction. Current approaches to harness these enzymes as protein-based therapeutics for drug and chemical toxin clearance are described, as well as their uses for targeted chemotherapeutic prodrug activation. Also included is an outline of how selective CE inhibitors could be used as co-drugs to improve the efficacy of clinically approved agents.

MATTHEW R. REDINBO

Matthew R. Redinbo is Associate Professor of Chemistry, Biochemistry and Biophysics at the University of North Carolina, Chapel Hill (USA), where he also serves as a member of the Lineberger Comprehensive Cancer Institute. Redinbo researches the action of human therapeutics, from receptors and enzymes to complex DNA manipulation systems. He was trained at the University of California, Los Angeles (USA), and the University of Washington (USA), and his research is funded by the National Institutes of Health (NIH) and the Burroughs Wellcome Fund.

PHILIP M. POTTER



Philip M. Potter is an Associate Member in the Department of Molecular Pharmacology at St Jude Children's Research Hospital. Potter is a leader in the field of experimental therapeutics and pharmacology, having expertise in subjects such as DNA repair, ribozymes, adenoviral-mediated gene delivery and the design and synthesis of small molecules for improving therapy. He was trained at the Paterson Institute for Cancer Research in Manchester (UK) and at St Jude Children's Research Hospital, Memphis (TN, USA). His research is funded by the NIH and the American Lebanese Syrian Associated Charities.

Matthew R. Redinbo*
Department of Chemistry,
Department of Biochemistry and Biophysics,
University of North Carolina,
Chapel Hill,
NC 27599-3290, USA
Philip M. Potter
Department of Molecular Pharmacology,
St Jude Children's Research Hospital,
Memphis,
TN 38105-2794, USA
*e-mail: redinbo@unc.edu

Carboxylesterases (CEs) are ubiquitous proteins identified in species ranging from bacteria to man that, as their name implies, are members of the esterase class of proteins (EC 3.1.1.1 for CEs; [1,2]). CEs cleave carboxylic esters into the corresponding alcohol and carboxylic acid. Currently, the Esther database (http://bioweb.ensam.inra.fr/ESTHER/general?what=index) lists 5,237 nucleotide sequences for genes encoding esterases, of which 318 are CEs. However, because many esterases demonstrate catalytic activity towards a variety of substrates, [e.g. acetyl- and butyryl-cholinesterase can hydrolyze the simple esterified substrate *o*-nitrophenyl acetate (NPA)], the naming and categorization of these proteins has been relatively confusing. Typically, substrate specificity has been the primary means of naming these enzymes,

1359-6446/04/$ – see front matter PII: S1359-6446(04)03383-0

however, location within particular organs, and/or physical parameters (e.g. molecular weight and isoelectric point) have also been used. This has resulted in numerous names for the same CE depending on the investigator who isolated the protein [2,3]. Moreover, the recent genomics and proteomics revolution, which has led to the identification of the mRNAs that encode many of these enzymes, has exacerbated this situation. However, because definitive assignment of proteins purified from tissues of different species to the corresponding mRNA has yet to be accomplished, the literature is replete with examples of multiple names for the same enzyme.

Here, the types, catalytic function, structures and biological roles of CEs are examined, with a focus on human CEs, although in many cases the wealth of data collected on rodent enzymes is relied on. Also included are outlines of current proposals for the use of these non-specific enzymes for the treatment of a variety of specific human diseases, from cancer to narcotic drug overdose. Understanding of the mechanism of action of this class of enzymes, and their role in drug metabolism, is rapidly expanding, such that changes in drug design and improvements in the application of these agents will be achieved.

Molecular characteristics of human carboxylesterases

There are two key human CEs, human liver CE (hCE1), which is primarily produced in the liver but can also be isolated in macrophages and human lung epithelia [4], and human intestinal CE (hiCE), which is expressed more widely with mRNA detected in the small intestine, liver, kidney, heart and skeletal muscle [5]; hiCE shares 99% sequence identity to a liver isoform – hCE2 [5]. A third, brain-specific CE was isolated in 1999 and termed isoform 3 (hCE3, hBr3; [6]); however, relatively little characterization of this CE has been presented.

Tissue distribution of human carboxylesterases

Numerous CEs have been named after the tissues from which they were originally isolated. Hence, the literature contains numerous reports of purified liver, serum, macrophage and alveolar CEs [2,3,4]. In addition, because many colorimetric esterase substrates exist, histopathologists have used these reagents to examine expression of esterases in many different organs. However, in these instances, it is not possible to distinguish accurately between CEs and esterases. Therefore, in recent years, molecular biology methods have been used to attribute gene expression to specific tissues. It was the application of these techniques that resulted in the identification of the two major human CEs – hCE1 (GenBank accession number for gene encoding hCE1 is NM_001266) and hiCE (GenBank accession number for gene encoding hiCE is Y09616). hCE1 and hiCE share 49% amino acid sequence identity and are predicted to be glycoproteins of ~62 kDa. hCE3 (GenBank accession number for gene encoding hCE3 is AB025028) has been reported to be expressed exclusively in the brain, although little is known about its pattern of tissue distribution and/or its substrate specificity [6]. hCE3 shares 77% and 49.5% sequence identity with hCE1 and hiCE, respectively.

At present, although the definitive homologues of these proteins in lower mammals are unclear, it is probable that, as with other molecular protection systems, there are enigmatic but distinct differences in the substrate specificities of such proteins from different organisms (e.g. as seen with the nuclear pregnane X receptor, a xenobiotic receptor [7]): this is presumably a consequence of the variant evolutionary pressures placed on each species by different chemical, and perhaps biological, exposures.

Typically, expression is maximal in the epithelia of most organs, suggesting that these proteins could play a protective role against xenobiotics [4,8]. In addition, although high levels of CE activity can be detected in the blood of the majority of mammals, no such activity is detected in the blood of humans [9,10], which suggests evolutionary pressure either for small mammals to maintain this CE or for humans to dispense with this enzyme. However, the pattern of expression of CEs is consistent with a protective function.

Carboxylesterase reactions

Ester hydrolysis

The catalysis of the cleavage of esters by CEs is base-mediated, requiring water as a co-reactant. This reaction is achieved via a triad of catalytic amino acids (Ser, His and Glu) that are all essential for enzymatic activity. In the two-stage reaction, a ping-pong mechanism occurs that consists of two identical steps. At neutral pH, the active site glutamic acid exists as the charged form, which facilitates removal of a proton from histidine. This loss subsequently results in transfer of a proton from the adjacent serine to the opposing nitrogen of histidine, generating an oxygen nucleophile that can attack the carbonyl carbon of the substrate (Figure 1). After formation of the tetrahedral intermediate, the reverse reaction occurs to release the alcohol product and reform the carbonyl group, which is then attacked in an identical fashion with water acting as the nucleophile, leading to release of the carboxylic acid and return of the catalytic amino acids to their original state. This mechanism has been conserved in virtually all esterases and has been modified by proteases, which use aspartic acid in place of glutamic acid.

Substrate transesterification

CEs can also perform transesterification reactions, and perhaps the most well-researched example of this is the formation of cocaethylene in individuals abusing both cocaine and alcohol [11–13]. Under these conditions, the ethyl group from ethanol replaces the methyl group of cocaine to produce cocaethylene (Figure 1). Because cocaethylene is more potent than cocaine, and has an

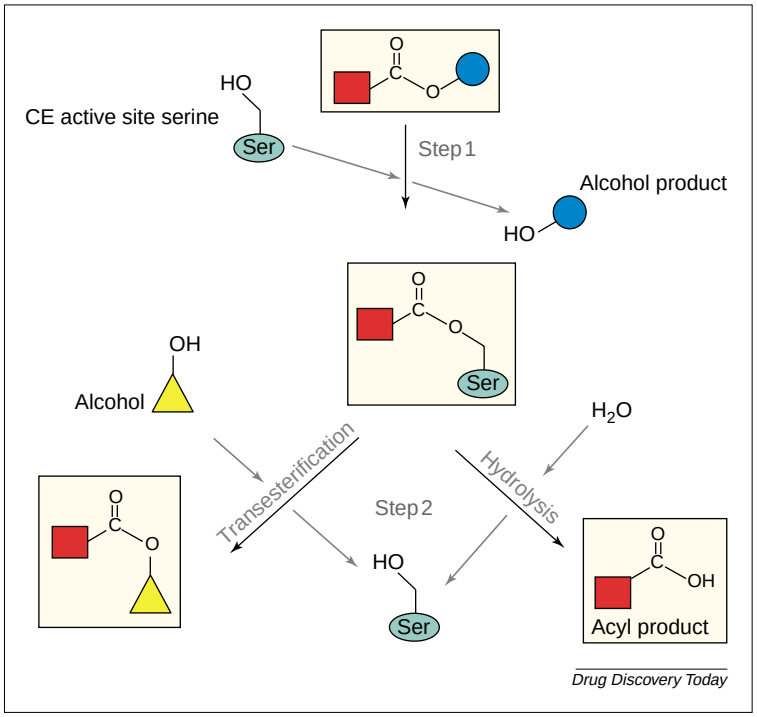


FIGURE 1

The two-step catalytic mechanism of mammalian carboxylesterases. Because the enzymes considered here are relatively non-specific, the substituent groups on the substrate are indicated as a nondescript square (red) and circle (blue). In the first catalytic step, the active site serine amino acid side chain (green) attacks the carboxylester linkage in the substrate to generate the alcohol product and the covalent acyl–enzyme intermediate. The second step typically involves hydrolysis to yield the carboxylic acid-containing acyl product. However, when an alcohol (yellow) is present in abundance, the enzyme can facilitate a transesterification reaction to generate a new ester product.

increased plasma half-life compared with the parent drug, even low efficiency transesterification can result in accumulation of toxic levels of the metabolite. Similar transesterification reactions appear to have a role in the ability of CEs to generate transesterification products from endogenous compounds. For example, hCE1 generates fatty acid ethyl esters (FAEEs) from fatty acyl-Coenzyme A (CoA) and ethanol [14–16]. FAEEs are toxic to numerous tissues and are thought to be associated with the necrotic decay of the liver and other tissues related to chronic alcohol abuse [17,18]. Because several CEs have fatty acyl CoA hydrolysis activity, in the presence of ethanol, the transesterification to FAEEs appears to replace the typical hydrolysis reaction.

Substrate specificity

The catalysis of substrates by CEs is dependent on several factors, including the size of the substrate, the accessibility of the ester group, the logP of the compound and interaction of the molecule with residues within the active site [2,19]. Because the catalytic amino acids of hCE1 and hiCE are buried at the bottom of a long deep gorge, the molecule must travel a considerable distance down this cleft [2,19] for substrate hydrolysis to occur. To aid this movement, an electrostatic gradient is created that effectively 'sucks' the substrate towards the catalytic amino acids. Additionally, the active site is lined with aromatic residues, and thus hydrophobic molecules will preferentially localize within this domain. Hence, in a series of nitrophenyl and naphthyl esters, lower K_m values are observed as the logP of the molecule increases [2,19]. However, turnover (V_{max}) of the substrate also decreases, presumably because the hydrophobic moiety does not exit the active site as rapidly. Finally, using this same series of substrates, size constraints enforced by the domains at the entrance to the active site limited the access of the compounds to the catalytic residues. Computer modeling of hCE1, hiCE and a rabbit liver CE (rCE; GenBank accession number of the gene encoding rCE is AF036930) validated these results, indicating the role of these regions in substrate specificity [2,19]. In general, hCE1 removes smaller adducts (e.g. methyl and ethyl groups), whereas hiCE prefers larger moieties (e.g. benzyl). This is exemplified in the metabolism of cocaine, in which the methyl group is removed by hCE1 to yield benzyl ecgonine, and the benzyl group is cleaved by hiCE to produce methyl ecgonine.

A comparison of various substrates of CEs highlights the promiscuous nature of these enzymes (Figure 2). CEs act on small compounds, such as NPA, moderately sized compounds, for example, naphthyl esters and cocaine, and large molecules like the anticancer drug CPT-11. Furthermore, although hCE1 and hiCE exhibit preferences with respect to the size of the substituent group on one side of the ester linkage, each of these enzymes can act on carboxyl, thio and amide ester linkages. For example, hCE1 hydrolyzes simple ester substrates such as NPA, the insecticide permethrin (M. Ross and P. Potter, unpublished), as well as fatty acyl CoA compounds with thioesters. In addition, transesterifications by CEs are not limited to those reactions involving small alcohols such as ethanol. For example, hCE1 has been suggested to have acyl-CoA:acyl transferase (ACAT) activity, in which a fatty acid from a fatty acyl CoA substrate is linked to cholesterol to create a cholesterol ester [20]. Thus, CEs appear to be able to perform a variety of protective functions via catalytic hydrolysis, and might serve as crucial 'back-up' systems during particularly stressful situations.

Biological roles of carboxylesterases

Drug and xenobiotic processing

Drug metabolism

Many clinically useful drugs contain ester moieties and, hence, are subject to catalysis by CEs. Such compounds include the anticancer drugs CPT-11 and capecitabine, the narcotics heroin and cocaine and a variety of other agents, for example, flumazenil, procaine, oxybutynin and delapril. In the majority of cases, esterase-mediated hydrolysis results in inactivation of the drug. However, with CPT-11 and capecitabine, the ester group was

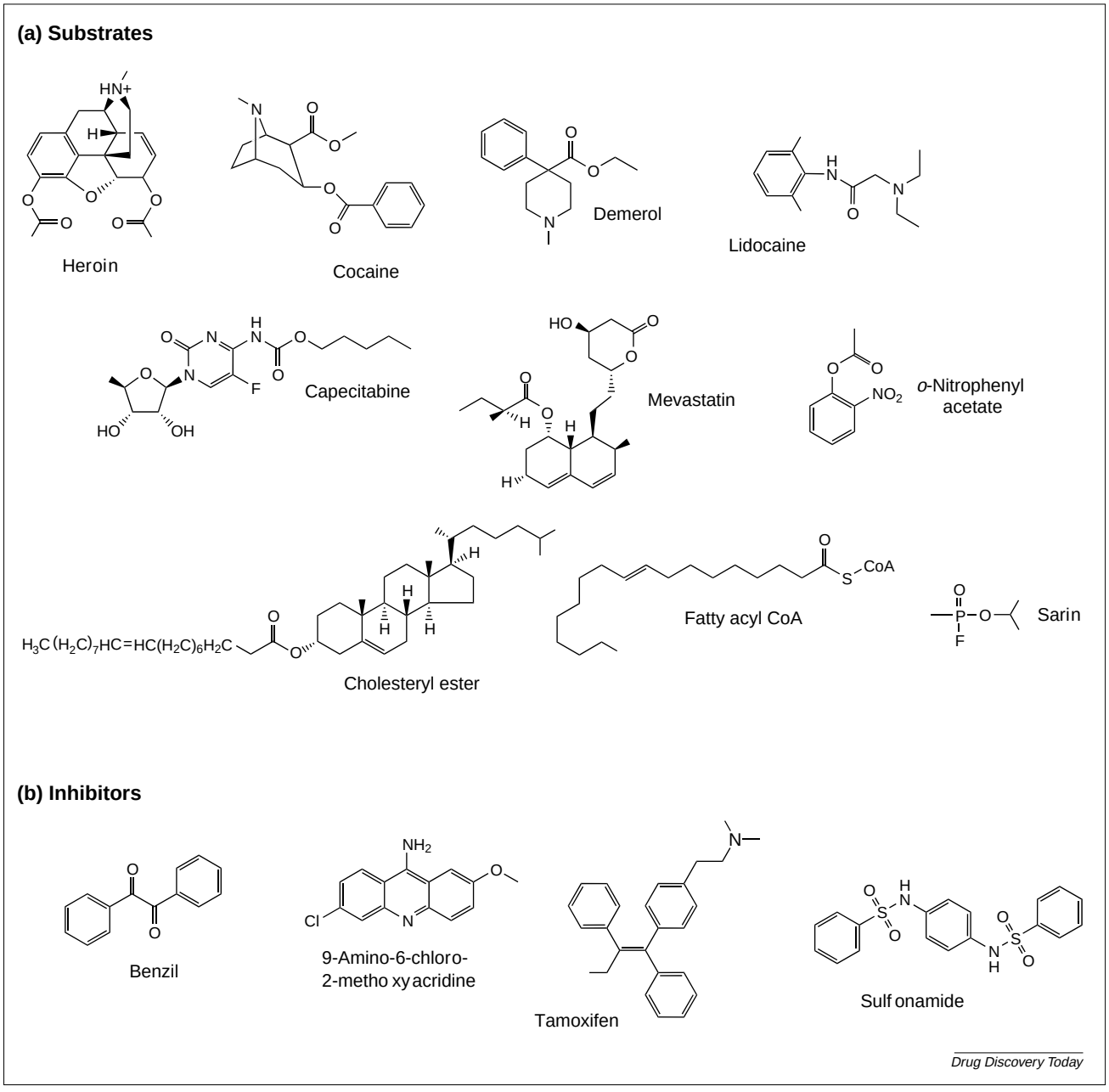


FIGURE 2

Carboxylesterase substrates and inhibitors vary widely in size and chemical composition.

deliberately engineered into the molecule to improve their water solubility [21–23]. Therefore, hydrolysis is essential for these drugs to be effective antitumor agents [24,25]. CPT-11 is a camptothecin-derived prodrug, the hydrolysis of which yields SN38, a potent topoisomerase I inhibitor [26]. This prodrug has demonstrated remarkable antitumor activity in animal models and has been approved for the treatment of colon cancer in adults [27–35]. CPT-11 is primarily activated by hiCE [36]. However, the dose-limiting toxicity of the drug is delayed diarrhea [28,37,38]. This can be attributed to the catalytic activity of hiCE expressed within the small intestine – hiCE converts the drug to SN38, which produces direct intestinal damage, and hence diarrhea [5,39–41]. Therefore, in this situation, although hiCE is required for conversion of the drug to the active metabolite, it also contributes to the toxicity of the agent. By understanding the pattern of expression and substrate specificity of CEs that are involved in drug metabolism, it is possible to predict, and potentially improve, the efficacy of therapy. The advent and development of proteomics and pharmacogenomics will greatly increase the knowledge required to administer and manage effectively the toxicities associated with old and new drugs.

Prodrug activation

The prodrugs CPT-11 and capecitabine require enzymatic hydrolysis to produce the active metabolites. With CPT-11,

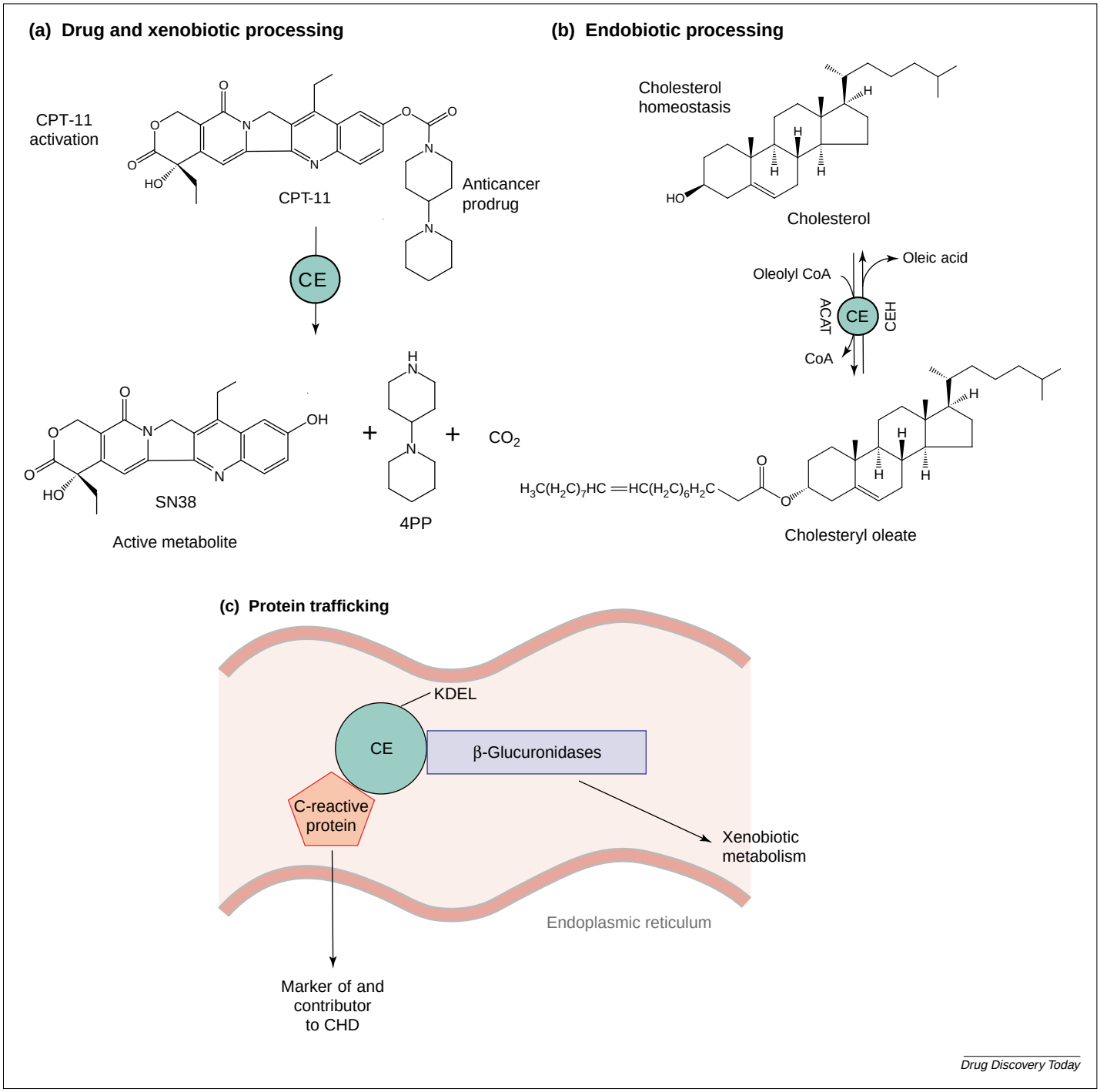


FIGURE 3

The biological functions of mammalian carboxylesterases. (a) CEs catalytically process a variety of drugs and xenobiotics to either active or inactive metabolites. (b) CEs are involved in processing endogenous compounds, having, for example, ACAT and CEH activities. (c) CEs bind to proteins within the ER and appear to regulate protein trafficking, including the release of the CRP, which is a sensitive marker for the development of CHD. Abbreviations: CHD, coronary heart disease; 4pp, 4-piperidino-piperidine

the dipiperidino moiety present at the 10-position of the molecule is removed to yield SN38 (Figure 3a). SN38 effects the formation of a covalent DNA–protein complex that ultimately leads to cell death, presumably by inhibition of DNA transcription [26]. When CPT-11 is administered to cancer patients as a single bolus, typically less than 5% of the drug is converted to SN38 [42,43], which suggests that the level and/or efficiency of the esterases that activate the drug are low. Biochemical analysis of the activation of this drug by purified human CEs indicates that hiCE is the most probable candidate responsible for CPT-11 hydrolysis *in vivo* [36]: hiCE is at least 60-fold more efficient than hCE1 at catalyzing the conversion of CPT-11 to SN38.

In clinical trials, CPT-11 prolongs the survival of patients with drug-resistant and naïve colon tumors [44–46]. As a

consequence, this agent is now used for front-line therapy of this disease. Indeed, CPT-11 is considered one of the most effective new anticancer drugs identified in the past 30 years, and it is likely to be approved for the treatment of a variety of solid tumors in coming years.

Endobiotic processing

In addition to the well-established roles of CEs in drug and xenobiotic metabolism, these enzymes have recently been recognized for their involvement in processing endobiotic compounds, for example, with roles in cholesterol homeostasis and fatty acid metabolism.

Cholesterol homeostasis

Cholesterol, which has an essential role in the structural integrity of plasma membranes, is potentially toxic if present in excess. Thus, systemic trafficking of cholesterol is crucial to the delivery and clearance of this compound. Fatty acids are added to the 3-position of cholesterol to create cholesteryl esters, and these product compounds are then packaged with high-, intermediate- and low-density lipoproteins for systemic delivery and clearance. Cholesteryl esters are formed in macrophages and monocytes after the phagocytic destruction of damaged endothelial cells. Macrophages and monocytes use an ACAT activity to remove fatty acids from fatty acyl CoA molecules and subsequently attach them to free cholesterol. Two isoforms of ACAT enzymes have been cloned and shown to act not just in macrophages and monocytes, but also in liver, intestine and other tissues. Although ACATs are distinct in sequence from CEs, in 1994, hCE1 was shown to have ACAT activity (Figure 3b; [20]). This observation suggests that, at particular stages, macrophages and monocytes could rely on enzymes other than the two ACAT isoforms to handle the load of phagocytosed cholesterol and convert it to cholesteryl esters. However, the validity of assigning an ACAT role to hCE1 has been challenged by more recent studies [47]. The participation of hCE1 in the ACAT-type conversion of cholesterol into cholesteryl esters remains uncertain.

The mechanism of ACAT activity of a CE, such as hCE1, would involve an example of a transesterification reaction. The fatty acid moiety from the fatty acyl CoA substrate would have to remain covalently attached to the catalytic serine residue and then cholesterol would attack, thus acting as the alcohol group.

In cells where cholesteryl esters are abundant, a catalytic function that is the opposite to that of an ACAT hydrolyzes cholesteryl esters to cholesterol and free fatty acids. Such a cholesteryl ester hydrolase (CEH) activity is particularly important in the liver, which receives the majority of the cholesteryl ester-laden lipoprotein particles that arise from endogenous and exogenous sources of cholesterol. hCE1 has also been proposed to contain an efficient CEH activity in macrophages, monocytes and hepatocytes (Figure 3b; [48–50]). A structural basis for this action has been proposed involving a secondary channel in the enzyme. Taken together with the potential ACAT activity assigned to these enzymes, CEs could potentially have a role as a back-up system to reduce cellular levels of cholesteryl esters or free cholesterol. For example, in macrophages and monocytes, as cholesterol from a phagocytosed cell is processed, CE-mediated metabolism of cholesterol to cholesteryl esters could occur. Conversely, CEs might also eliminate excess cholesteryl esters formed during the packaging of lipid droplets and high-density lipoproteins. Overly taxed macrophages and monocytes transform into foam cells, the first step towards atherosclerosis. Thus, understanding the detailed roles of CEs in cholesterol processing in these cells could open novel avenues for the detection and treatment of this disease.

Generation of fatty acid ethyl esters

The build-up of FAEEs in a variety of tissues, particularly liver, is a hallmark of chronic alcohol abuse [17,18]. These compounds are toxic and cause necrotic tissue decay, presumably by uncoupling oxidative phosphorylation in the inner membrane of mitochondria [17,18]. There are four enzymes that are known to act as FAEE synthases, and hCE1 was identified as one of the four and named FAEE synthase IV [14–16]. The mechanism of FAEE generation by hCE1 involves the transesterification of a fatty acid by ethanol. A fatty acid from either a fatty acyl CoA or cholesteryl ester covalently binds to the serine and after the first catalytic step would be attacked by ethanol in the subsequent step. Hence, hCE1 transesterifies an endogenous compound (a fatty acid) with an exogenous one (ethanol). Such a reaction highlights the promiscuous action of these non-specific enzymes in substrate use and in biological role.

Protein trafficking and retention in the endoplasmic reticulum

CEs contain an N-terminal hydrophobic signal peptide that marks them for trafficking through the endoplasmic reticulum (ER). In addition, a Lys-Asp-Glu-Leu (KDEL) sequence present at the C-terminus of the protein ensures retention within the cell [51]. Typically, CEs are glycosylated in the ER with *N*-linked carbohydrates that appear to be necessary for catalytic activity [52]. The KDEL sequence can be cleaved off in the ER after protein processing, which facilitates secretion of the enzyme [51,53]. The specific deletion of these residues and overexpression of the truncated protein in cells has enabled the facile purification of these enzymes [53].

It has recently been determined that while in the ER, hCE1 and hiCE form what appear to be crucial interactions with other proteins that have an impact on the trafficking and release of these proteins from the cell (Figure 3c). For example, the C-reactive protein (CRP), which is the most sensitive serum marker for coronary heart disease, is released from the liver in response to tissue damage within the body [54]. However, while in the hepatic ER, CRP forms direct

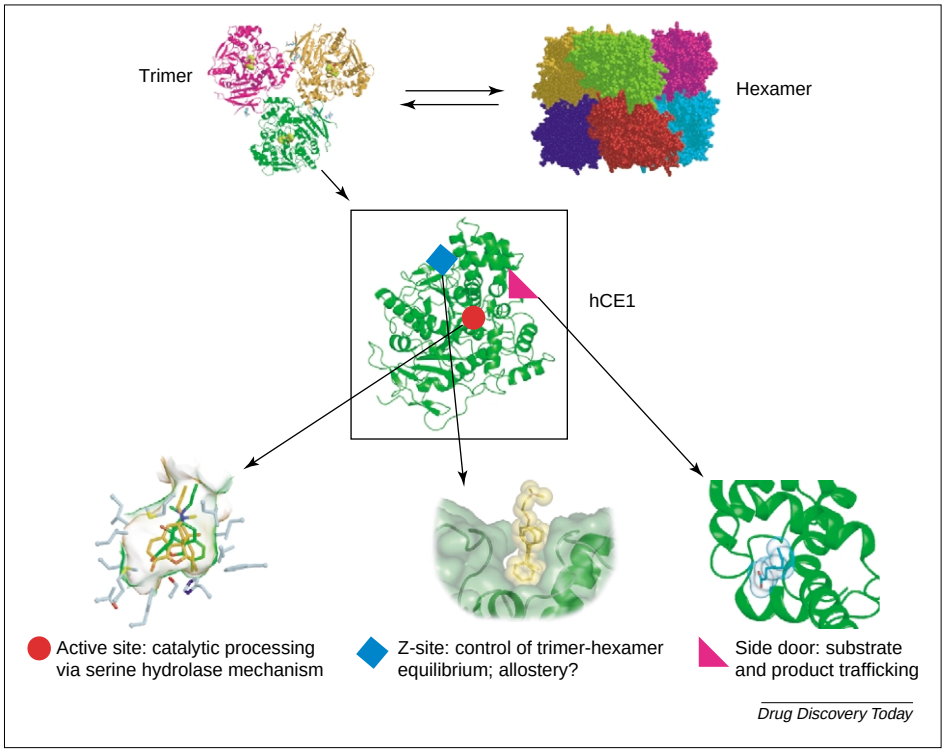


FIGURE 4

Structural features of human carboxylesterase 1. The enzyme exists in a trimer–hexamer equilibrium, and each protein monomer contains three ligand binding sites. The AS (red) incorporates the catalytic triad of the enzyme and facilitates the docking of structurally distinct substrates; in some cases, substrates dock in more than one orientation simultaneously, as illustrated for the heroin analogue naloxone (green and gold). The ZS (blue) is a surface ligand binding groove that might be allosteric and controls the trimer–hexamer equilibrium of the enzyme. It is relatively non-specific, and is shown here with the anticancer drug tamoxifen (yellow) bound. The SD (magenta) is a secondary pore to the active site that could facilitate the release of product [as indicated here for a fatty acid (cyan)] or the entrance of substrate. Abbreviations: AS, active site; SD, side door; ZS, Z-site.

contacts with two CEs – hiCE and, to a lesser extent, hCE1 [55]. Both CEs use a region of amino acid sequence adjacent to the 'side door', which is comprised of the loop between α15 and β18, to contact CRP. These CEs could hold a small reservoir of CRP within the ER, and then release it on the detection of tissue injury. It is possible that these are the first CRP molecules sent forth in response to tissue damage present in the body.

It has also been shown that CEs directly interact with β-glucuronidase enzyme in the ER [56]. β-Glucuronidases remove glucuronic acid moieties that are typically conjugated to drugs and endobiotics by the Phase II drug-metabolizing UDP-glucuronosyltransferase (UGT) enzymes. The contact between CEs and β-glucuronidases appears to involve the active site region of the CEs and a serine protease inhibitor domain on the β-glucuronidase [57–59]. The interaction of β-glucuronidases with CEs indicates that compounds that bind to one enzyme could serve as more effective substrates of the other enzyme. Such a situation could occur with the anticancer drug CPT-11, which is activated by a CE but inactivated by glucuronidation [60]. The interaction of CPT-11 with a CE–β-glucuronidase complex could provide an efficient 'one-stop' activation of numerous forms of CPT-11.

Structural analyses of carboxylesterases

The first crystal structure of a mammalian CE, that of rCE, was reported in 2002 [61] and revealed that CEs share the serine hydrolase fold observed in other esterases. The structure of rCE was soon followed by that of hCE1 in complexes with several human drugs [62,63]. rCE and hCE1 share 81% sequence identity and a 0.68 Å root-mean-square deviation (rmsd) over equivalent Cα positions, but exhibit somewhat distinct substrate preferences. For example, rCE efficiently activates the anticancer drug CPT-11, whereas hCE1 does not [64]. hCE1 shares a 1.2 Å rmsd, but moderate sequence identity (38%), with human acetylcholinesterase (hAcChE).

Overall structure

The serine hydrolase fold exhibited by the CEs and esterases includes a central 15-stranded β-sheet surrounded by numerous α-helices and β-strands (Figure 4). The active site cavity is ~15 Å deep and is located at the interface of several protein domains. Although the overall folds of the serine hydrolase enzymes from humans are similar to those of CEs, the details of their active site architecture are distinct between the specific hAcChE and human butyrylcholinesterase (hBuChE) enzymes and the non-specific CEs. rCE and hCE1 are glycoproteins that contain at least one *N*-linked glycosylation site and biochemical studies have shown that the carbohydrate modifications are required for enzyme activity [52]. rCE and hCE1 also form trimers in their respective crystal structures; in addition, hCE1 is capable of forming a hexamer consisting of two stacked trimers. A surface ligand binding site in hCE1, termed the Z-site, was identified, and it has been proposed that this domain can regulate the oligomeric state of the protein and could act as an allosteric site.

Carboxylesterase–drug complex structures

hCE1 has been crystallized with numerous different endobiotic and xenobiotic compounds bound within the catalytic gorge of the protein [61–63] (Figure 4). Two regions, 88–103 (α1) and 353–366 (α10′), cover the entrance to the active site in hCE1. In the crystal structure of rCE, which contained no ligand bound at the active site, the same loops were disordered, suggesting that they might remain unstructured until binding of the substrate. The active site cavity of hCE1 is large (~1,300 Å^3 in volume) and is lined predominantly by hydrophobic amino acids, with the exception of residues in the catalytic triad. Examination of the structures of hCE1 in complex with several chemically distinct ligands suggests that there is a specific region

responsible for the binding of small linkages (e.g. methyl and ethyl groups) and a domain that can accommodate much larger functional groups. For example, the structure of hCE1 in complex with the cocaine analogue homatropine revealed that the specific region of the catalytic pocket aligns the methyl ester linkage in a position suitable for hydrolysis [63]. The structure also revealed that cocaethylene, the toxic metabolite of cocaine, would not align as productively for catalysis, helping to explain the extended serum half-life of this compound.

A structural comparison of the active site of hCE1 with that of hAcChE bound to the Alzheimer's drug tacrine established that hAcChE contains a much smaller and more highly selective active site cavity that is designed to bind to acetylcholine [62]. By contrast, hCE1 has a considerably larger and more featureless pocket, suitable for non-specific substrate binding. Indeed, it appears from the structures of hCE1 that if a substrate has a small ester-type linkage and can fit into the catalytic site, it will probably be cleaved by the protein. Efforts are underway to introduce targeted mutations in the active site of hCE1 to convert it to a more selective and catalytically efficient enzyme for use in a variety of clinical applications.

Functional domains

Although the Z-site identified on hCE1 is located ~15 Å away from the active site, only the α10′ helix that helps to close over the entrance to the catalytic gorge of the enzyme (Figure 4) separates the two features. When ligands bind to the Z-site, hCE1 is a trimer, when ligands are unable to bind, the enzyme can form a hexamer [63]: these observations have been confirmed using crystallographic studies and atomic force microscopy [62]. Indeed, the Z-site name arose from the shape of the interdigitated protein monomers at this position, which resembled the letter Z (Figure 4). Binding at the Z-site appears to be non-specific, and only the size of the ligand seems to have a key role. Studies to determine whether the Z-site functions in an allosteric fashion are currently ongoing. An interesting preliminary observation is that tamoxifen, which is a weak inhibitor of hCE1 (K_i of 15.2 μM) and was found to bind at the active site and Z-site of hCE1, acts in a 'mixed inhibitor' fashion, suggesting that there is more than one binding site available for this compound (C. Fleming, P. Potter and M. Redinbo, unpublished).

The side door region of the CEs was first identified in the structure of rCE, which contained a product of CPT-11 metabolism bound at this surface position [61]. Recently, it has been observed that larger compounds, including fatty acids, also bind at this site in hCE1 (Figure 4) (S. Bencharit, P. Potter and M. Redinbo, unpublished). The side door is reminiscent of the 'back door' proposed for the hAcChE, although the side door is in a distinct position relative to the proposed back door. The side door in the CEs is separated from the active site cavity by a thin wall composed of four amino acids [61]. It has been proposed that the side door allows products or substrates to enter the active site, gated by one or more of these small residues. There are several attractive features of this hypothesis. For example, the ability of hCE1 to act as a CEH would require that the fatty acyl region of a cholesteryl ester extrude from the catalytic gorge of the enzyme to align the labile ester linkage for hydrolysis by the enzyme. In addition, this pore might allow the access of small substrates, such as ethanol, to the covalent acyl–enzyme intermediate to facilitate the transesterification reaction.

Overall, the numerous crystal structures of CEs in complex with a variety of small molecules have provided detailed insights into the mechanism and function of these proteins in many different biological processes. It is probable that future studies to validate the functions of the domains identified will provide additional information in clarifying the roles of CE in endobiotic and xenobiotic metabolism.

Inhibition of carboxylesterases

Because CEs are responsible for the metabolism of a whole host of different therapeutically useful drugs, the use of selective inhibitors of these proteins might be valuable in modulating the efficacy of such agents. For example, for an esterified drug that loses activity after CE-mediated hydrolysis, co-administration of a specific CE inhibitor might delay detoxification of the drug and thereby increase its half-life. Conversely, for an agent that is selectively activated by CEs, such as CPT-11, CE inhibitors might be valuable in ameliorating the toxicity associated with the drug. Finally, the use of specific inhibitors of these proteins might prove invaluable as research tools to facilitate understanding of the mechanism of drug catalysis and the role of discrete domains within the protein (e.g. the side door) for substrate hydrolysis.

Identification of selective carboxylesterase inhibitors

Wadkins and colleagues [41] embarked on the isolation and characterization of specific CE inhibitors that might have applications as research reagents and as tools for modulating drug bioavailability. Using the Target-Related Affinity Profiling (TRAP™) approach developed by Telik, a series of compounds were screened against three mammalian CEs – rCE, hCE1 and hiCE [41]. Typically, 70 compounds were screened against all three proteins using *o*-NPA as a substrate and inhibition was detected using a spectrophotometric assay [41]. After four rounds of screening, several chemical classes of compounds were identified that had different inhibitory properties.

Because the ultimate goal of this research was to identify hiCE-specific inhibitors for the amelioration of CPT-11-induced toxicity, a series of benzene sulfonamides (Figure 2) that were potent inhibitors of this protein was initially developed [41]. These compounds act as partially competitive, reversible inhibitors of hiCE, and K_i values as low as 50 nM have been obtained using *o*-NPA as a substrate.

In addition, these inhibitors prevent the cleavage of CPT-11 by hiCE to afford SN38, suggesting that they might have some therapeutic utility. More importantly, these compounds were entirely selective for hiCE, demonstrating no inhibition of hAcChe, hBuChE or hCE1 at concentrations of 100 μM [41]. This is the first description of selective CE inhibitors, suggesting that, although these proteins are highly related at the amino acid and structural level, compounds that demonstrate selectivity for different enzymes can be identified.

Whereas the sulfonamide chemotype demonstrates similarity to the ester function (Figure 2), it is unlikely that the serine nucleophile can attack this group in a manner analogous to the carbonyl carbon. A more probable explanation is that the exposed oxygen atoms on the inhibitor form hydrogen bonds with the hydrophobic residues present within the active site gorge of the protein. Such a mechanism has been observed for the inhibition of thrombin by sulfonamide-based inhibitors of this protein [65,66].

General inhibitors of carboxylesterases

During the TRAP-screening procedure, compounds that could inhibit all the CEs assayed were also identified. Again, these novel inhibitors did not inhibit hAcChe or hBuChE and had K_i values in the low nM range. Analysis of the structures of these compounds indicated that they belonged to the aromatic dione family, with benzil being the prototypical inhibitor (Figure 2). As with the sulfonamides, these compounds acted as partially competitive reversible inhibitors. Inhibitory activity was shown to be dependent on the dione structure, and potency towards different CEs was related to the substituents appended to the benzene rings. For example, 4,4′-dibromobenzil was considerably more potent at inhibiting the CEs compared with benzil. Because the active sites of these proteins are highly hydrophobic, this increase in activity is presumably a consequence, in part, of the increase in the hydrophobicity of the inhibitor, which is afforded by the bromine atoms. Over 30 benzil analogues have been examined and a wide variability in the ability of these compounds to inhibit different CEs has been observed. However, it is clear that this represents a novel class of highly potent enzyme inhibitors that will have applications as research reagents, and could potentially prove useful in modulating CE activity *in vivo*. Interestingly, benzil has been used for many years in the polymer industry (with little associated toxicity) and animal studies indicate that this compound is non-toxic to small mammals. Hence, the development of clinically useful inhibitors based on this structure seems possible.

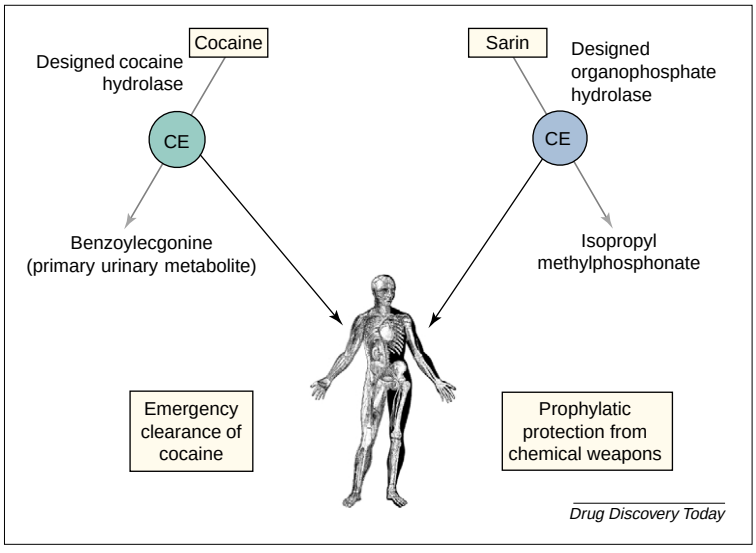


FIGURE 5

Protein-based therapeutics involving designed, highly efficient forms of carboxylesterases targeted to cocaine or chemical weapons could provide novel and safe methods for the treatment of drug overdose or nerve agent attack.

Clinical applications of carboxylesterases

Protein-based therapeutics

Treatments for drug overdose or addiction

Because CEs are responsible for the metabolism of numerous esterified compounds, including agents such as heroin and cocaine [11,67–69], strategies to modulate the levels of these drugs in patients who have overdosed on these substances can be proposed. For example, hCE1 converts cocaine into benzoylecgonine, a non-toxic derivative that is the primary urinary metabolite of the drug [63,68,69]. The administration of hCE1 to individuals containing high levels of cocaine in their bloodstream would rapidly reduce the levels of the active compound in the body (Figure 5). Such an approach might be useful in cases where drug levels are extremely high and would result in significant clinical problems that cannot be resolved by standard emergency hospital care. In addition, using the existing structural data showing cocaine bound to hCE1, the enzyme could be engineered to be an even more efficient cocaine hydrolase for such clinical applications.

Alternatively, considering that heroin is metabolized to morphine by CEs [63,68,69], the use of selective CE inhibitors might prove useful in delaying the production of the active metabolite, thereby extending the window available for physicians to manage the symptoms of overdose that are associated with this drug. Such approaches would typically be used in emergency situations and might not be suitable for repeated applications, particularly with the administration of hCE1 or hiCE protein, because of potential antibody responses that might be acquired. However, in life-and-death situations, it is not unreasonable to assume that these therapeutic modalities might be viable alternatives for treatment.

Chemical weapons

The detoxification of numerous organophosphate and carbamate analogues is achieved by CEs [70]. Hence, agents such as the pesticides, malathion and Dursban® (Dow Agrosciences), as well as the chemical weapons sarin, tabun

and soman, are all detoxified by these enzymes [70]. Although these proteins act as stoichiometric scavengers of the poisons that result in inactivation of the enzyme, it has been proposed that hydrolytic variants (i.e. proteins that can catalytically detoxify the agents) can be developed. However, until recently, such approaches have been limited, in part, as a result of the lack of high-resolution crystal structures of CEs in complex with these compounds.

Recently, the crystal structure of hCE1 bound to a non-toxic organophosphate analogue was determined (C. Fleming and M. Redinbo, unpublished). These structures clearly demonstrate that a covalent adduct is formed after the first step of hydrolytic cleavage of this particular compound. A crucial hurdle in engineering an efficient organophosphate hydrolase is preventing the 'aging' reaction at the hydrolase active site, in which the covalent acyl enzyme intermediate loses an alcohol group to produce a permanent covalent adduct and inactive enzyme. The crystal structure of the hCE1–organophosphate complex provides a suitable scaffold for the assessment of the effect of specific amino acid mutations, with the ultimate goal of developing an efficient organophosphate hydrolase. Now, it is possible to develop site-specific mutants derived from these structures that could enable the generation of proteins that have catalytic activity towards these poisons (Figure 5). Ultimately, we envisage the construction and high-level expression of hCE1 mutants that are highly effective in hydrolyzing substrates such as sarin and tabun. These proteins could be used for protection of personnel who could potentially be exposed to such agents, and for the surface decontamination of humans and sensitive equipment.

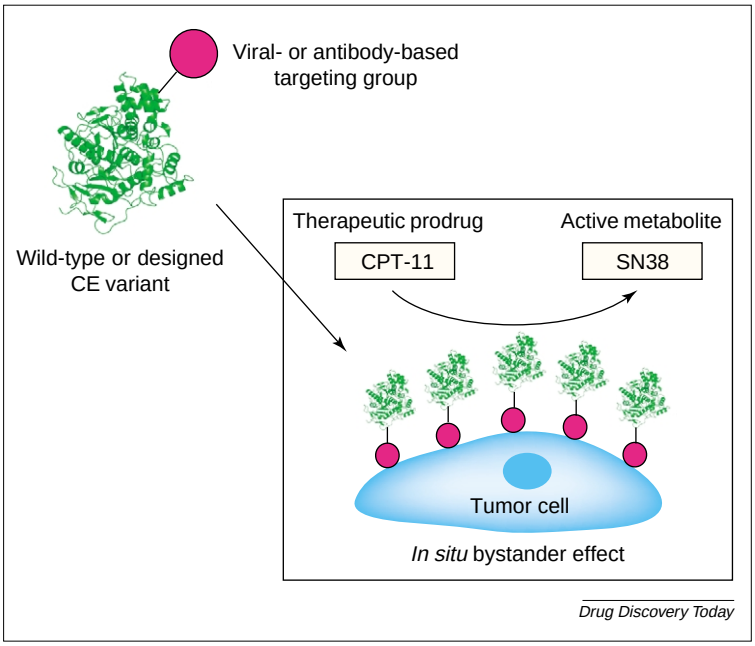


FIGURE 6

Carboxylesterases that activate prodrugs could be used in VDEPT or ADEPT approaches to treat cancer or other diseases via selective targeting or gene expression.

Enzyme prodrug therapy

CPT-11 is poorly activated in humans, thus the potential exists for the development of an enzyme prodrug therapy approach for this drug [71]. Such a strategy exploits the ability of enzymes to selectively activate inactive prodrugs to produce specific toxicity in desired cell types. For CPT-11, an rCE was identified that is ~5-fold and ~1000-fold more efficient than hiCE and hCE1, respectively, at drug activation [25,51,64,71]. Expression of rCE in mammalian cells results in sensitivity to CPT-11 and complete regression of human tumor xenografts grown in immune-deprived mice [64]. Two different approaches can be applied using the enzyme prodrug therapy methodology (Figure 6).

Viral-directed enzyme prodrug therapy (VDEPT) uses a viral vector to achieve delivery of the cDNA encoding the rCE to tumor cells. The resulting expression of the protein in these cells leads to cytotoxicity on addition of CPT-11. To demonstrate the effectiveness of the approach, a method was developed to purge human neuroblastoma cells from the bone marrow of cancer patients [72,73]. Replication-deficient adenovirus-expressing rCE is used to transduce bone marrow aspirates at a multiplicity of infection of 50 and after 24 h CPT-11 is added. After 48 h, the cells are harvested and assessed for tumor cell contamination by RT–PCR. This procedure is effective because bone marrow stem cells are not transduced by adenovirus (whereas tumor cells are), and hence high-level rCE expression only occurs in the neuroblastoma cells. Using this approach, tumor cells have been successfully purged from at least 12 patient samples with no loss of viability of the bone marrow stem cells [72,73]. Viral vectors are currently in development that should be suitable for use against solid tumors *in vivo*. In addition, efforts to develop hiCE as the activating enzyme are being pursued.

An alternate method for enzyme prodrug therapy involves targeting of an antibody–enzyme fusion protein to tumor cells. In this antibody-directed enzyme prodrug therapy (ADEPT) approach, an antigen that is specifically expressed on tumor cells is used to selectively deliver an enzyme that can activate a prodrug [74–79]. After administration of a prodrug, antitumor activity should occur because high levels of active metabolite will be produced within the milieu of the tumor. This methodology has been applied to activate CPT-11 using hiCE and rCE against a melanoma tumor model [77]. However, little success has been achieved with this strategy, which could be a consequence of several factors, including the loss of enzyme activity after coupling to the antibody, the affinity of the antibody for the antigen and the relatively inefficient activation of the drug. However, it is possible that with the development of reagents with improved specificity and/or enzyme activity, this approach could yield novel approaches to cancer therapy.

Use of carboxylesterase inhibitors

CE inhibitors could be used to modulate the distribution

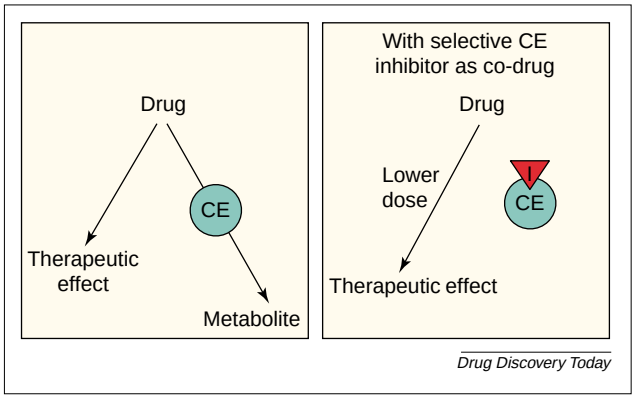


FIGURE 7

Selective carboxylesterase inhibitors might be effective co-drugs that could reduce drug doses, side effects and drug–drug interactions.

of drugs that are metabolized by these enzymes. A general CE inhibitor, such as benzil, might prove effective in preventing the formation of morphine following heroin overdose. In addition, many drugs are detoxified by CEs, thus the co-administration of drug plus inhibitor might improve the bioavailability of the drug. For example, tamoxifen is known to bind to and act as a weak inhibitor of hCE1, thus the use of a potent hCE1 inhibitor might improve the trafficking of tamoxifen through the liver, perhaps even at a lower clinical dose (Figure 7).

Conclusions

Protection of tissues from xenobiotics that vary widely in their chemical structure requires either a non-specific or promiscuous enzyme. Promiscuity is the exception to the classic rule in biology – typically, highly specific molecular interactions are involved in metabolism, signaling and homeostasis. Until relatively recently, there was no detailed understanding of how proteins with wide substrate specificities performed their tasks without sacrificing enzyme activity. It has become apparent that promiscuous receptors and enzymes use large binding cavities that are structurally flexible to enable the protein to conform around chemically distinct compounds. In some cases, these proteins also enable the docking of ligands in multiple orientations simultaneously, which provides direct evidence of binding promiscuity.

With the CEs as a model, the use of these non-specific enzymes to treat specific human diseases can now be assessed. The enzymes can be converted to efficient and specific hydrolases by targeted site-directed mutagenesis using crystallographic data as a guide. For example, mammalian CEs designed to hydrolyze narcotics, chemical weapons or chemotherapeutic compounds could be applied to a wide range of situations. In addition, selective CE inhibitors could be used as co-drugs to impact the bioavailability of primary therapeutics, and perhaps limit side effects. However, it is ironic that the clinical applications envisioned for these non-specific CEs all involve one overriding theme – specificity. The design of enzyme variants that are specific for a particular compound, or of inhibitors that bind exclusively to a target enzyme, reveals that the reality of life at the molecular level can rarely be escaped. To get a particular job done, even a promiscuous protein must get specific.

Acknowledgements

We wish to thank Chris Fleming, Sompop Bencharit and Denise Teotico for assistance with the Figures, and all laboratory members, past and present, who contributed ideas and data to this review. This work was supported by NIH grants CA98468 (M.R.R.), CA76202, CA79763 and P30 CA21765 (P.M.P.), by a Burroughs Wellcome Fund Career Award in the Biomedical Sciences (M.R.R.) and by the American Lebanese Syrian Associated Charities (P.M.P.).

References

1 Cashman, J. *et al.* (1996) Pharmacokinetics and molecular detoxification. *Environ. Health Perspect.* 104, 23–40
2 Satoh, T. and Hosokawa, M. (1998) The mammalian carboxylesterases: from molecules to function. *Annu. Rev. Pharmacol. Toxicol.* 38, 257–288
3 Satoh, T. and Hosokawa, M. (1995) Molecular aspects of carboxylesterase isoforms in comparison with other esterases. *Toxicol. Lett.* 82–83, 439–445
4 Munger, J.S. *et al.* (1991) A serine esterase released by human alveolar macrophages is closely related to liver microsomal carboxylesterases. *J. Biol. Chem.* 266, 18832–18838
5 Schwer, H. *et al.* (1997) Molecular cloning and characterization of a novel putative carboxylesterase, present in human intestine and liver. *Biochem. Biophys. Res. Commun.* 233, 117–120
6 Mori, M. *et al.* (1999) cDNA cloning, characterization and stable expression of novel human brain carboxylesterase. *FEBS Lett.* 458, 17–22
7 Goodwin, B. *et al.* (2002) Regulation of CYP3A gene transcription by the pregnane X receptor. *Annu. Rev. Pharmacol. Toxicol.* 42, 1–23
8 Stadnyk, A.W. *et al.* (1990) Characterization of nonspecific esterase activity in macrophages and intestinal epithelium of the rat. *J. Histochem. Cytochem.* 38, 1–6
9 Guemei, A.A. *et al.* (2001) Human plasma carboxylesterase and butyrylcholinesterase enzyme activity: correlations with SN-38 pharmacokinetics during a prolonged infusion of irinotecan. *Cancer Chemother. Pharmacol.* 47, 283–290
10 Morton, C.L. *et al.* (2000) Activation of CPT-11 in mice: identification and analysis of a highly effective plasma esterase. *Cancer Res.* 60, 4206–4210
11 Brzezinski, M.R. *et al.* (1994) Purification and characterization of a human liver cocaine carboxylesterase that catalyzes the production of benzoylecgonine and the formation of cocaethylene from alcohol and cocaine. *Biochem. Pharmacol.* 48, 1747–1755
12 Dean, R.A. *et al.* (1991) Human liver cocaine esterases: ethanol-mediated formation of ethylcocaine. *FASEB J.* 5, 2735–2739
13 Redinbo, M.R. *et al.* (2003) Human carboxylesterase 1: from drug metabolism to drug discovery. *Biochem. Soc. Trans.* 31, 620–624
14 Beckemeier, M.E. and Bora, P.S. (1998) Fatty acid ethyl esters: potentially toxic products of myocardial ethanol metabolism. *J. Mol. Cell. Cardiol.* 30, 2487–2494
15 Bora, P.S. *et al.* (1996) Purification and characterization of human heart fatty acid ethyl ester synthase/carboxylesterase. *J. Mol. Cell. Cardiol.* 28, 2027–2032
16 Diczfalusy, M.A. *et al.* (2001) Characterization of enzymes involved in formation of ethyl esters of long-chain fatty acids in humans. *J. Lipid Res.* 42, 1025–1032
17 Bora, P.S. *et al.* (1996) Myocardial cell damage by fatty acid ethyl esters. *J. Cardiovasc. Pharmacol.* 27, 1–6

18 Lange, L.G. and Sobel, B.E. (1983) Mitochondrial dysfunction induced by fatty acid ethyl esters, myocardial metabolites of ethanol. *J. Clin. Invest.* 72, 724–731

19 Wadkins, R.M. *et al.* (2001) Structural constraints affect the metabolism of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) by carboxylesterases. *Mol. Pharmacol.* 60, 355–362

20 Becker, A. *et al.* (1994) Purification, cloning, and expression of a human enzyme with acyl coenzyme A: cholesterol acyltransferase activity, which is identical to liver carboxylesterase. *Arterioscler. Thromb.* 14, 1346–1355

21 Bajetta, E. *et al.* (1996) A pilot safety study of capecitabine, a new oral fluoropyrimidine, in patients with advanced neoplastic disease. *Tumori* 82, 450–452

22 Matsuzaki, T. *et al.* (1988) Inhibition of spontaneous and experimental metastasis by a new derivative of camptothecin, CPT-11, in mice. *Cancer Chemother. Pharmacol.* 21, 308–312

23 Kunimoto, T. *et al.* (1987) Antitumor activity of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin, a novel water-soluble derivative of camptothecin, against murine tumors. *Cancer Res.* 47, 5944–5947

24 Danks, M.K. *et al.* (1998) Overexpression of a rabbit liver carboxylesterase sensitizes human tumor cells to CPT-11. *Cancer Res.* 58, 20–22

25 Potter, P.M. *et al.* (1998) Isolation and partial characterization of a cDNA encoding a rabbit liver carboxylesterase that activates the prodrug Irinotecan (CPT-11). *Cancer Res.* 58, 2646–2651

26 Tanizawa, A. *et al.* (1994) Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. *J. Natl. Cancer Inst.* 86, 836–842

27 Abad-Zapatero, C. *et al.* (1987) Refined crystal structure of dogfish M4 apo-lactate dehydrogenase. *J. Mol. Biol.* 198, 445–467

28 Abigerges, D. *et al.* (1994) Irinotecan (CPT-11) high-dose escalation using intensive high-dose loperamide to control diarrhea. *J. Natl. Cancer Inst.* 86, 446–449

29 Armand, J.P. *et al.* (1995) CPT-11 (irinotecan) in the treatment of colorectal cancer. *Eur. J. Cancer* 31A, 1283–1287

30 Furman, W.L. *et al.* (1999) Direct translation of a protracted irinotecan schedule from a xenograft model to a Phase I trial in children. *J. Clin. Oncol.* 17, 1815–1824

31 Houghton, J.A. *et al.* (1996) Evaluation of irinotecan in combination with 5-fluorouracil or etoposide in xenograft models of colon adenocarcinoma and rhabdomyosarcoma. *Clin. Cancer Res.* 2, 107–118

32 Houghton, P.J. *et al.* (1993) Therapeutic efficacy of the topoisomerase I inhibitor 7-ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyloxy-camptothecin against human tumor xenografts: lack of cross-resistance *in vivo* in tumors with acquired resistance to the topoisomerase I inhibitor 9-dimethylaminomethyl-10-hydroxycamptothecin. *Cancer Res.* 53, 2823–2839

33 Houghton, P.J. *et al.* (1995) Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. *Cancer Chemother. Pharmacol.* 36, 393–403

34 Thompson, J. *et al.* (1997) Efficacy of systemic administration of irinotecan against neuroblastoma xenografts. *Clin. Cancer Res.* 3, 423–431

35 Thompson, J. *et al.* (1997) Efficacy of oral irinotecan against neuroblastoma xenografts. *Anticancer Drugs* 8, 313–322

36 Humerickhouse, R. *et al.* (2000) Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. *Cancer Res.* 60, 1189–1192

37 de Forni, M. *et al.* (1994) Phase I and pharmacokinetic study of the camptothecin derivative irinotecan, administered on a weekly schedule in cancer patients. *Cancer Res.* 54, 4347–4354

38 Masuda, N. *et al.* (1993) Phase I and pharmacologic study of irinotecan in combination with cisplatin for advanced lung cancer. *Br. J. Cancer* 68, 777–782

39 Wu, M.H. *et al.* (2003) Characterization of multiple promoters in the human carboxylesterase 2 gene. *Pharmacogenetics* 13, 425–435

40 Khanna, R. *et al.* (2000) Proficient metabolism of CPT-11 by a human intestinal carboxylesterase. *Cancer Res.* 60, 4725–4728

41 Wadkins, R.M. *et al.* (2004) Identification of novel selective human intestinal carboxylesterase inhibitors for the amelioration of irinotecan-induced diarrhea: synthesis, quantitative structure–activity relationship analysis, and biological activity. *Mol. Pharmacol.* 65, 1336–1343

42 Rivory, L.P. *et al.* (1997) Pharmacokinetic interrelationships of irinotecan (CPT-11) and its three major plasma metabolites in patients enrolled in PhaseI/II trials. *Clin. Cancer Res.* 3, 1261–1266

43 Slatter, J.G. *et al.* (2000) Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following i.v. infusion of [14C]CPT-11 in cancer patients. *Drug Metab. Dispos.* 28, 423–433

44 Van Cutsem, E. *et al.* (1997) Clinical benefit of irinotecan (CPT-11) in metastatic colorectal cancer (CRC) resistant to 5-FU. *Annual Meeting of the American Society of Clinical Oncology* 17–20 May 1997, Denver, CO, USA 16, A950

45 Rougier, P. *et al.* (1997) Phase II study of irinotecan in the treatment of advanced colorectal cancer in chemotherapy-naive patients and patients pretreated with fluorouracil-based chemotherapy. *J. Clin. Oncol.* 15, 251–260

46 Rothenberg, M.L. *et al.* (1997) A Phase II multicenter trial of alternating cycles of irinotecan (CPT-11) and 5-FU/LV in patients with previously untreated metastatic colorectal cancer (CRC). *Annual Meeting of the American Society of Clinical Oncology* 17–20 May 1997, Denver, CO, USA 16, A944

47 Diczfalusy, M.A. *et al.* (1996) Acyl-coenzyme A: cholesterol O-acyltransferase is not identical to liver microsomal carboxylesterase. *Arterioscler. Thromb. Vasc. Biol.* 16, 606–610

48 Ghosh, S. (2000) Cholesteryl ester hydrolase in human monocyte/macrophage: cloning, sequencing, and expression of full-length cDNA. *Physiol. Genomics* 2, 1–8

49 Ghosh, S. and Natarajan, R. (2001) Cloning of the human cholesteryl ester hydrolase promoter: identification of functional peroxisomal proliferator-activated receptor responsive elements. *Biochem. Biophys. Res. Commun.* 284, 1065–1070

50 Ghosh, S. *et al.* (2003) Mobilization of cytoplasmic CE droplets by overexpression of human macrophage cholesteryl ester hydrolase. *J. Lipid Res.* 44, 1833–1840

51 Potter, P.M. *et al.* (1998) Cellular localization domains of a rabbit and a human carboxylesterase: influence on irinotecan (CPT-11) metabolism by the rabbit enzyme. *Cancer Res.* 58, 3627–3632

52 Kroetz, D.L. *et al.* (1993) Glycosylation-dependent activity of baculovirus-expressed human liver carboxylesterases: cDNA cloning and characterization of two highly similar enzyme forms. *Biochemistry* 32, 11606–11617

53 Morton, C.L. and Potter, P.M. (2000) Comparison of *Escherichia coli, Saccharomyces cerevisiae, Pichia pastoris, Spodoptera frugiperda* and COS7 cells for recombinant gene expression: application to a rabbit liver carboxylesterase. *Mol. Biotechnol.* 16, 193–202

54 Kushner, I. (1982) The phenomenon of the acute phase response. *Ann. N. Y. Acad. Sci.* 389, 39–48

55 Yue, C.C. *et al.* (1996) Identification of a C-reactive protein binding site in two hepatic carboxylesterases capable of retaining C-reactive protein within the endoplasmic reticulum. *J. Biol. Chem.* 271, 22245–22250

56 Zhen, L. *et al.* (1993) The signal for retention of the egasyn-glucuronidase complex within the endoplasmic reticulum. *Arch. Biochem. Biophys.* 304, 402–414

57 Li, H. *et al.* (1990) The propeptide of beta-glucuronidase. Further evidence of its involvement in compartmentalization of beta-glucuronidase and sequence similarity with portions of the reactive site region of the serpin superfamily. *J. Biol. Chem.* 265, 14732–14735

58 Medda, S. *et al.* (1987) Involvement of the esterase active site of egasyn in compartmentalization of beta-glucuronidase within the endoplasmic reticulum. *Cell* 50, 301–310

59 Novak, E.K. *et al.* (1991) Expression of egasyn-esterase in mammalian cells. Sequestration in the endoplasmic reticulum and complexation with beta-glucuronidase. *J. Biol. Chem.* 266, 6377–6380

60 Gupta, E. *et al.* (1994) Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. *Cancer Res.* 54, 3723–3725

61 Bencharit, S. *et al.* (2002) Structural insights into CPT-11 activation by mammalian carboxylesterases. *Nat. Struct. Biol.* 9, 337–342

62 Bencharit, S. *et al.* (2003) Crystal structure of human carboxylesterase 1 complexed with the Alzheimer's drug tacrine. From binding promiscuity to selective inhibition. *Chem. Biol.* 10, 341–349

63 Bencharit, S. *et al.* (2003) Structural basis of heroin and cocaine metabolism by a promiscuous human drug-processing enzyme. *Nat. Struct. Biol.* 10, 349–356

64 Danks, M.K. *et al.* (1999) Comparison of activation of CPT-11 by rabbit and human carboxylesterases for use in enzyme/prodrug therapy. *Clin. Cancer Res.* 5, 917–924

65 Brandstetter, H. *et al.* (1992) Refined 2.3 Å X-ray crystal structure of bovine thrombin complexes formed with the benzamidine and arginine-based thrombin inhibitors NAPAP, 4-TAPAP and MQPA. A starting point for improving

antithrombotics. *J. Mol. Biol.* 226, 1085–1099
66 Sturzebecher, J. *et al.* (2002) Thrombin. In *Proteinase and Peptidase Inhibition: Recent Potential Targets for Drug Development* (Smith, H.J. and Simmons, C., eds), pp. 185–201, Taylor & Francis
67 Dean, R.A. *et al.* (1995) Tissue distribution of cocaine methyl esterase and ethyl transferase activities: correlation with carboxylesterase protein. *J. Pharmacol. Exp. Ther.* 275, 965–971
68 Kamendulis, L.M. *et al.* (1996) Metabolism of cocaine and heroin is catalyzed by the same human liver carboxylesterases. *J. Pharmacol. Exp. Ther.* 279, 713–717
69 Pindel, E.V. *et al.* (1997) Purification and cloning of a broad substrate specificity human liver carboxylesterase that catalyzes the hydrolysis of cocaine and heroin. *J. Biol. Chem.* 272, 14769–14775
70 Maxwell, D.M. and Brecht, K.M. (2001) Carboxylesterase: specificity and spontaneous reactivation of an endogenous scavenger for organophosphorus compounds. *J. Appl. Toxicol.* 21, S103–S107
71 Danks, M.K. and Potter, P.M. (2004) Enzyme-prodrug systems: carboxylesterase/CPT-11. *Methods Mol. Med.* 90, 247–262
72 Meck, M. *et al.* (2001) A VDEPT approach to purging neuroblastoma cells from hematopoeitic cells using adenovirus encoding rabbit carboxylesterase and CPT-11. *Cancer Res.* 61, 5083–5089
73 Wagner, L.M. *et al.* (2002) Efficacy and toxicity of a virus-directed enzyme prodrug therapy purging method: preclinical assessment and application to bone marrow samples from neuroblastoma patients. *Cancer Res.* 62, 5001–5007
74 Hellstrom, K.E. and Senter, P.D. (1991) Activation of prodrugs by targeted enzymes. *Eur. J. Cancer* 27, 1342–1343
75 Senter, P.D. (1990) Activation of prodrugs by antibody–enzyme conjugates: a new approach to cancer therapy. *FASEB J.* 4, 188–193
76 Senter, P.D. (1990) Antitumor effects of antibody enzyme conjugates in combination with prodrugs. *Front. Radiat. Ther. Oncol.* 24, 132–141
77 Senter, P.D. *et al.* (2001) Identification and activities of human carboxylesterases for the activation of CPT-11, a clinically approved anticancer drug. *Bioconjug. Chem.* 12, 1074–1080
78 Senter, P.D. and Springer, C.J. (2001) Selective activation of anticancer prodrugs by monoclonal antibody–enzyme conjugates. *Adv. Drug Deliv. Rev.* 53, 247–264
79 Senter, P.D. *et al.* (1991) Activation of prodrugs by antibody–enzyme conjugates. *Adv. Exp. Med. Biol.* 303, 97–105