The Anticancer Prodrug CPT-11 Is a Potent Inhibitor of Acetylcholinesterase but Is Rapidly Catalyzed to SN-38 by Butyrylcholinesterase

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ABSTRACT

Patients treated with high doses of CPT-11 rapidly develop a cholinergic syndrome that can be alleviated by atropine. Although CPT-11 was not a substrate for acetylcholinesterase (AcChE), in vitro assays confirmed that CPT-11 inhibited both human and electric eel AcChE with apparent \( K_m \)s of 415 and 194 nM, respectively. In contrast, human or equine butyrylcholinesterase (BuChE) converted CPT-11 to SN-38 with \( K_m \)s of 42.4 and 44.2 \( \mu \)M for the human and horse BuChE, respectively. Modeling of CPT-11 within the predicted active site of AcChE and BuChE corroborated experimental results indicating that, although the drug was oriented correctly for activation, the constraints dictated by the active site gorge were such that CPT-11 would be unlikely to be activated by AcChE.

INTRODUCTION

CPT-11 [Irinotecan; 7-ethyl-10-\( \beta \)-(1-piperidino)-1-piperidino]carbonyloxy camptothecin] is a prodrug that is activated by esterases to generate SN-38 (7-ethyl-10-hydroxycamptothecin), a potent topoisomerase I poison (1). CPT-11 has demonstrated remarkable antitumor activity in human tumor xenograft models and is currently undergoing both Phase II and Phase III clinical trials in adults and children (2–4). Additionally, it has recently been approved as second-line therapy for patients with advanced colorectal cancer. Initial results indicate that CPT-11 and other drugs that stimulate topoisomerase I-mediated DNA cleavage will be effective agents for chemotherapy. CPT-11 activation has been thought to occur in the liver due to the preponderance of carboxylesterases present in this tissue and the production of SN-38 following incubation of CPT-11 with liver microsomal fractions in vitro (5–7).

Esterases are a ubiquitous family of enzymes that have been classified based upon the metabolism of simple substrates. Included within this family are the AcChEs, which degrade the neurotransmitter molecule acetylcholine to acetate and choline (8). Specific inhibitors of these proteins are highly toxic, resulting in a cholinergic syndrome (salivation, bradycardia, and visual disturbances) that, if left untreated, can lead to death. The related BuChEs are present in large amounts in plasma, although their natural substrate and, hence, their biological significance are unknown (8, 9). Because these proteins are involved in the metabolism of the muscle relaxant succinylcholine (10, 11), patients undergoing surgery are usually monitored for plasma BuChE activity levels to avoid unexpected post surgical apnea.

Patients treated with high doses of CPT-11 rapidly develop a cholinergic syndrome that can be ameliorated by administration of the anticholinergic agent atropine (12). These data suggest that CPT-11 or a metabolite may directly inhibit AcChE function. Therefore, we examined the potential of CPT-11, SN-38, and APC, a nontoxic metabolite of CPT-11 (13), to inhibit AcChE activity in vitro. In addition, we have examined the activation of CPT-11 by BuChE and identified a novel route of catalysis of the drug.

MATERIALS AND METHODS

Enzymes and Drugs. All enzymes, inhibitors, and substrates were purchased from Sigma Biochemicals (St. Louis, MO) with the exception of human serum BuChE (Biogenesis Inc., Sandown, NH). One unit of BuChE enzyme activity was defined as the amount of enzyme responsible for the conversion of 1 \( \mu \)mol of butyrylcholine to choline and butyrate per min at 37°C. CPT-11, SN-38, and APC were gifts kindly provided by Dr. J. P. McGovren (Pharmacia Upjohn, Piscataway, NJ).

Molecular Modeling of Cholinesterase-CPT-11 Complexes. The crystal structure of Torpedo californica AcChE (Protein Data Bank entry no. 2ACE; Ref. 14) was used as a basis for constructing models of CPT-11 interaction with human AcChE and BuChE. BuChE was modeled by replacing active site residues Trp-279, Phe-288, and Phe-290 of AcChE with alanine, leucine, and valine, respectively. Modifications were made using the Biopolymer package of the Insight II/Discover molecular modeling software from MSI, Inc. (San Diego, CA). Because residues 279, 288, and 290 are identical in human and Torpedo AcChE, the AcChE-CPT-11 model was constructed using the 2ACE sequence, as described below.

The CPT-11 model was constructed using the Builder package of Insight II/Discover and atomic charges were generated using the moderate neglect of differential overlap method (15). The amino acid residues within a 10–Å radius of all atoms of the CPT-11 were made flexible, whereas those at further distances were fixed during the modeling process (16, 17). To model CPT-11 in the AcChE active site, residues 279, 288, and 290 in the final BuChE-CPT-11 model were transformed back into those of AcChE (i.e., Trp, Phe, and Phe, respectively). The AcChE model was then minimized as described for the BuChE model.

Esterase Assay. Esterase activity was measured as described previously using \( \beta \)-NA as a substrate (18, 19). IC\(_{50}\)s were determined using at least six concentrations of inhibitor.

Cholinesterase Assay. Cholinesterase activity was measured based upon the conversion of PTC to thiocholine, which reacts with 5,5’-dithiobis-2-nitrobenzoic acid to generate 5-thio-2-nitrobenzoate (20, 21).

AcChE Assay. AcChE activity was determined as described previously (22) using acetylthiocholine as a substrate using modifications as reported by Doctor et al. (23). All kinetic data points were performed in quadruplicate. \( K_s \)s were determined using Dixon plots of \( [I] \) versus 1/v using at least six different inhibitor concentrations.

Quantitation of CPT-11 and SN-38. Separation and detection of CPT-11 and SN-38 were performed by HPLC, as described previously (24).

Kinetcs of CPT-11 Metabolism. To determine \( K_{m,s} \) and \( V_{max,s} \) for BuChEs, enzyme was incubated with CPT-11 for appropriate time intervals (see “Results”) at 37°C in 200 \( \mu \)l of 50 mM Hepes (pH 7.4). The amounts of SN-38 in the reaction were quantitated by HPLC. Data were fitted to a one site binding hyperbolic function using the GraphPad Prism software, and \( K_{m,s} \) and \( V_{max,s} \) were determined from the equation describing the curve fit.

Transient Transfection of COS7 Cells. COS7 cells were transfected with mammalian expression vectors as described previously (19, 25). After 48 h, cells were harvested by trypsinization, and extracts were prepared by sonication in minimal volumes of 50 mM Hepes (pH 7.4) on ice.
RESULTS

Modeling of CPT-11 in the Active Site of AcChE and BuChE. The crystal structure of AcChE has been solved (14), and the coordinates have been used to model CPT-11 within the active site of the protein. However, because the crystal structure of the BuChEs has not been determined, we performed computer modeling using the protein data bank coordinates of the electric eel AcChE (14) to assess the binding of CPT-11 within the active sites of these proteins. Replacement of amino acid residues Trp-279, Phe-288, and Phe-290 with alanine, leucine, and valine, respectively, has been shown to convert AcChE into enzymes that can hydrolyze butyrylthiocholine (26).

We, therefore, modeled CPT-11 into a AcChE/BuChE chimera containing the above mutations. The CPT-11 was positioned into the active site of the model BuChE, such that the carbonyl of the carbamate moiety of CPT-11 was as close as possible to the active site His-440 and Ser-200, while avoiding direct overlap of the CPT-11 atoms with any atom from the BuChE. The piperidinopiperidine moiety of CPT-11 was oriented toward Trp-84, as suggested in the acetylcholine-AcChE and -BuChE models of Sussman and colleagues (14, 26). However, due to the distance from the carbamate moiety to the piperidinopiperidine rings, these latter portions of CPT-11 were placed behind the Trp-84 residue (see Fig. 1C) into the open gorge in the surrounding active site.

The minimized structure of the CPT-11 chimeric BuChE model is shown in Fig. 1. A and B, with BuChE represented by an α-carbon ribbon trace. The deep active site gorge was large enough to allow

![Fig. 1. Molecular model of the BuChE-CPT-11 complex. A, stereo pair of BuChE (represented by α-carbon ribbon; green) with CPT-11 (gray) positioned in the active site. B, stereo pair close-up view of A. C, stereo pair close-up view of CPT-11 in the active site with the relevant neighboring amino acid residues.](image-url)
positioning of the piperidinopiperidine group within the protein. With minimal perturbation to the active site, His-440 Ne2 and Ser-200 Oγ (the catalytic residues) are within 3.4 Å and 3.7 Å of the carbamate ester oxygen and carbonyl carbon, respectively (Fig. 1C). Thus, CPT-11 could readily fit within the active site of the BuChE model.

As shown in Fig. 1C, the amino acid residues Ala-279, Leu-288, and Val-290 surround the A–E rings of CPT-11 and were positioned to allow the large ring system to insert into the active site. When these residues were replaced by Trp, Phe, and Phe, respectively, as in AcChE, these bulkier amino acids were predicted to displace the drug, such that the Ser-200 and His-440 were unable to react with the carbamate moiety. Indeed, when we model CPT-11 with AcChE, the distance between the active site His-440 Ne2 and the carbamate ether oxygen increases from 3.4 to 3.7 Å. The distance from Ser-200 Oγ to the carbamate carbonyl carbon of CPT-11, however, is the same (3.7 Å) for both enzyme models.

Inhibition of AcChE by CPT-11. Because the modeling studies indicated that CPT-11 could fit within the active site of AcChEs and BuChEs but that interactions with individual amino acids were different, we assessed the metabolism of drug by a series of cholinesterases. Initially, we incubated electric eel and human AcChE with 25 μM CPT-11 for 22 h at 37°C in 50 mM Hepes (pH 7.4) and analyzed reaction products by HPLC. Under these conditions, no SN-38 was detected (data not shown). Fig. 2, however, demonstrates that CPT-11 does inhibit the catalysis of 3 mM o-NPA and 4 mM PTC by AcChE. Fifty % inhibition of o-NPA metabolism was observed with 0.31 and 0.14 μM CPT-11 for the human and eel AcChE, respectively. With PTC as a substrate, 50% enzyme inhibition was seen with 1.3 and 2.0 μM CPT-11 for the human and eel AcChEs, respectively. Because these concentrations of CPT-11 are readily achievable in the plasma of patients undergoing chemotherapy (3), we determined the Ks of CPT-11 for the human and eel AcChE using the surrogate AcChE substrate, acetylthiocholine.

Table 1 indicates the apparent Ks for CPT-11 with purified amphiphilic human and eel AcChE. The two enzymes had similar Ks in the nanomolar range (Table 1). Because CPT-11 is metabolized to both APC and SN-38 in patients, we also assessed the inhibition of AcChE activity by these two compounds. Although both metabolites could inhibit the metabolism of acetylthiocholine by AcChE, the Ks were 70–95-fold greater than that for CPT-11 (Table 1). Because these concentrations have not been reported in patient plasma, it is likely that CPT-11 is responsible for AcChE inhibition in vivo.

CPT-11 Is a Substrate for BuChE. Similar to the above studies with AcChEs, we also examined the ability of BuChEs to convert CPT-11 to SN-38. We incubated 25 μM CPT-11 with 800, 80, or 8 units of equine BuChE for 22 h at 37°C in 50 mM Hepes (pH 7.4) and analyzed the reaction products by HPLC. We detected SN-38 with as little as 8 units of BuChE (Fig. 3A). With 800 units of enzyme, >98% of the CPT-11 was converted to SN-38. Similarly, with 5 units of human BuChE, drug activation was observed; however, the reactions were less efficient than those with the equine protein.

Kinetics of CPT-11 Conversion by BuChEs. To compare the efficiency of CPT-11 catalysis by human and horse BuChEs, we incubated each enzyme with concentrations of drug, ranging from 0.1 to 125 μM. For the equine enzyme, 100 units were used, and reactions were allowed to proceed for 5 min at 37°C. For reactions with the human BuChE, 5 units of enzyme were incubated with substrate overnight at 37°C.

Table 2 indicates that, although similar Kmax were observed for each enzyme, the human BuChE is ~250-fold less efficient than the equine protein at drug activation, as indicated by the Vmax.

Inhibition of BuChE-mediated CPT-11 Metabolism by Tacrine. Tacrine specifically inhibits cholinesterases because it forms a covalent bond with the active site serine residue. This reagent, therefore, should prevent activation of CPT-11 by BuChEs. Therefore, we incubated 10 units of equine BuChE with 25 μM CPT-11 in the presence of 1 mM tacrine. After incubation at 37°C for 22 h, >99% inhibition of the conversion of CPT-11 to SN-38 was observed (Fig. 4B). These data support the conclusion that BuChEs metabolize CPT-11.

Activation of CPT-11 by Esterases in Mouse Plasma. CPT-11 is converted to SN-38 by mouse plasma. However, because both carboxylesterases and cholinesterases are present in plasma, the relative contribution of each enzyme to drug activation is unknown. To assess the contribution of each enzyme, we incubated 5 μM CPT-11 with 50

<table>
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<tr>
<th>Ks (μM)</th>
<th>Human</th>
<th>Electric eel</th>
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<tbody>
<tr>
<td>CPT-11</td>
<td>0.415 ± 0.099</td>
<td>0.194 ± 0.019</td>
</tr>
<tr>
<td>SN-38</td>
<td>39.4 ± 3.6</td>
<td>ND†</td>
</tr>
<tr>
<td>APC</td>
<td>28.8 ± 3.8</td>
<td>ND†</td>
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*Values were determined from Dixon plots of kinetic data.
†ND, not determined.
of mouse plasma in a total volume of 200 μl of 50 mM Hepes (pH 7.4). Reactions were also performed containing 1 mM of either the cholinesterase inhibitor tacrine or 1 mM total esterase inhibitor, BNPP. Table 3 indicates the amount of SN-38 produced following a 3-h incubation with CPT-11 as well as the amounts of both carboxylesterase and cholinesterase activity in the samples. Incubation of plasma with tacrine completely inhibited cholinesterase activity and reduced the amount of SN-38 produced by ~70%, suggesting that cholinesterases were responsible for the majority of CPT-11 metabolism. Because tacrine does not inhibit carboxylesterases, this inhibitor had no effect on the conversion of o-NPA. In contrast, BNPP reduced both carboxylesterase and cholinesterase activity and inhibited virtually all conversion of CPT-11 to SN-38. Although complete inhibition of CPT-11 metabolism was observed, only partial inhibition (75%) of o-NPA catalysis was seen. This difference can be ascribed to the different concentrations of substrate, 5 μM CPT-11 and 3 mM o-NPA present in the assays. These data suggest that at least 70% of the conversion of CPT-11 to SN-38 in mouse plasma is mediated by cholinesterases.

Activation of CPT-11 by Mouse BuChE Expressed in COS7 Cells. To confirm the hypothesis that mouse BuChE could activate CPT-11, we isolated the cDNA encoding this protein by PCR and transiently expressed the enzyme in COS7 cells (19, 27). Forty-eight h following transfection, cells sonicates were incubated with 25 μM CPT-11. Fig. 5 shows that, although cell extracts expressing the mouse BuChE activated CPT-11, sonicates from vector transfected cells did not. These data confirm that mouse BuChEs can convert CPT-11 to SN-38 in vitro.

DISCUSSION

i.v. infusion of CPT-11 can result in drug plasma concentrations of >17 μM (3). Because we have determined that the $K_i$ for human AcChE with CPT-11 is 415 nM, significant inhibition of AcChE function would be expected in vivo following administration of the drug. Indeed, the side effects that occur within the first 2 h following CPT-11 administration, including abdominal cramping, lacrimation,
salivation, and visual disturbances, are all consistent with inhibition of AcChE activity (12). Additionally, these side effects can be alleviated by administering atropine, a potent AcChE antagonist. Recent data have indicated that repeated low-dose CPT-11 treatment is more effective in the treatment of human tumor xenografts in immunodeprived mice than single high-dose administration (2, 4, 28). Even effective in the treatment of human tumor xenografts in immune-deficient mice, the same dose of CPT-11 is less effective in the treatment of CPT-11-deprived mice than single high-dose administration (2, 4, 28). Even effective in the treatment of human tumor xenografts in immune-deficient mice, the same dose of CPT-11 is less effective in the treatment of CPT-11-deprived mice than single high-dose administration (2, 4, 28). Even effective in the treatment of human tumor xenografts in immune-deficient mice, the same dose of CPT-11 is less effective in the treatment of CPT-11-deprived mice than single high-dose administration (2, 4, 28). Even effective in the treatment of human tumor xenografts in immune-deficient mice, the same dose of CPT-11 is less effective in the treatment of CPT-11-deprived mice than single high-dose administration (2, 4, 28). Even effective in the treatment of human tumor xenografts in immune-deficient mice, the same dose of CPT-11 is less effective in the treatment of CPT-11-deprived mice than single high-dose administration (2, 4, 28). Even effective in the treatment of human tumor xenografts in immune-deficient mice, the same dose of CPT-11 is less effective in the treatment of CPT-11-deprived mice than single high-dose administration (2, 4, 28). Even effective in the treatment of human tumor xenografts in immune-deficient mice, the same dose of CPT-11 is less effective in the treatment of CPT-11-deprived mice than single high-dose administration (2, 4, 28).

In vivo, the exact concentration of CPT-11 that induces a cholinergic effect will depend on several factors, including drug metabolism and clearance, abundance of AcChE, and rate and mode of drug administration.

The active site of AcChE is present at the bottom of a gorge that is lined with hydrophobic amino acid residues (14). Hence, hydrophobic molecules are attracted into the gorge where catalysis occurs. Because CPT-11 has a very hydrophobic planar aromatic ring structure, the drug would be expected to localize within the active site gorge. However, modeling studies confirm the displacement of the catalytic amino acid residues from the ester linkage in CPT-11 in AcChE and indicate that CPT-11 is unlikely to be a substrate for this enzyme. These results suggest that the cholinergic diarrhea observed in patients following CPT-11 administration is due to the direct inhibition of AcChE by the drug. Similarly, modeling studies with BuChEs indicate that the active site cleft is sufficiently large to accommodate drug access and binding, and this prediction is consistent with the observed results.

To assess the relative contribution of both carboxylesterases and cholinesterases in the activation of CPT-11 in mouse plasma, we incubated plasma with CPT-11 in the presence of the esterase inhibitors, tacrine, and BNPP. Because tacrine reduced the amount of SN-38 produced by plasma by 70%, we conclude that the majority of CPT-11 activation by mouse plasma is due to cholinesterases. Confirmation of the metabolism of CPT-11 by BuChEs was demonstrated following the transient expression mouse enzyme in COS7 cells. Cell extracts derived from these transfections converted CPT-11 to SN-38, consistent with the data generated by incubation of drug with mouse plasma. These results indicate that the activation of CPT-11 may not be mediated entirely by carboxylesterases.

Kinetic studies with the human and equine BuChEs demonstrated that the human BuChE is much less efficient than the horse BuChE at CPT-11 activation. A comparison of the hydrolysis of CPT-11 by a rabbit and human carboxylesterase has yielded similar observations, the human enzyme being ~150-fold less efficient at drug activation than the rabbit counterpart. Indeed, the activation of CPT-11 by human esterases is the least efficient of several that have been analyzed (6).

Because BuChEs represent a significant proportion of the total cholinesterases activity detectable in human plasma (9, 29), the activation of CPT-11 by these enzymes may significantly contribute to the amount of SN-38 formed in patients. It has been proposed that CPT-11 is converted to SN-38 in the liver and that any antitumor effect is a result of the diffusion of SN-38 into the bloodstream (6, 7).

Because our data indicate that direct drug activation by plasma enzymes may contribute to the generation of SN-38, we speculate that this route of drug activation may be important in humans. We are currently developing assays that will assess the contribution of BuChE-mediated CPT-11 activation in vivo.

Fig. 5. HPLC chromatograms demonstrating the activation of CPT-11 by COS7 cell extracts expressing mouse BuChE.

REFERENCES


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