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Inhibition of acetylcholinesterase by the anticancer prodrug CPT-11

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Abstract

CPT-11 (irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) is an anticancer prodrug that has been approved for the treatment of colon cancer. It is a member of the camptothecin class of drugs and activation to the active metabolite SN-38, is mediated by carboxylesterases (CE). SN-38 is a potent topoisomerase I poison and is highly effective at killing human tumor cells, with IC_{50} values in the low nM range. However, upon high dose administration of CPT-11 to cancer patients, a cholinergic syndrome is observed, that can be rapidly ameliorated by atropine. This suggests a direct interaction of the drug or its metabolites with acetylcholinesterase (AChE). Kinetic studies indicated that CPT-11 was primarily responsible for AChE inhibition with the 4-piperidinopiperidine moiety, the major determinant in the loss of enzyme activity. Structural analogs of 4-piperidinopiperidine however, did not inhibit AChE, including a benzyl piperazine derivate of CPT-11. These results suggest that novel anticancer drugs could be synthesized that do not inhibit AChE, or alternatively, that novel AChE inhibitors could be designed based around the camptothecin scaffold.

Keywords: CPT-11; Acetylcholinesterase; Inhibition; Butyrylcholinesterase

1. Introduction

The development of effective anticancer agents in recent years has been limited, in part, due to the failure to identify agents whose spectrum of activity, and/or antitumor efficacy is any better than agents that are currently available. With the notable exception of Gleevec, virtually all chemotherapeutic agents in clinical use are non-selective poisons, resulting in toxicity to normal tissues as well as to tumor cells. However, within these classes of compounds, there are some very effective drugs whose toxicity can be managed by extensive supportive care. This includes the camptothecins, of which the parent compound (camptothecin (CPT)) was originally identified as potential antitumor agent in 1966 [1]. Subsequent drug development has produced two highly effective CPT analogs, topotecan and CPT-11 that are currently used for the treatment of ovarian and colon cancer, respectively [2–5]. These drugs have shown dramatic responses in the treatment of a variety of solid

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tumors. For example in a Phase I trial with CPT-11 in pediatric patients with a spectrum of tumor histiotypes, responses were observed in 21 out of 23 individuals [6]. Such efficacy in these small scale trials, that a designed to assess safety and toxicity, are very uncommon.

As with all cytotoxic chemotherapeutic agents, side effects occur following CPT-11 administration. These include a cholinergic syndrome that occurs within 1 h of drug infusion resulting in lacrimation, miosis, increased salivation diaphoresis, flushing, rhinitis and intestinal hyperperistalsis that usually leads to diarrhea [7–10]. These toxicities can be rapidly alleviated with atropine. The dose limiting toxicity for CPT-11 however is delayed diarrhea that occurs 48–96 h following drug administration. This is thought to arise from two different mechanisms. Firstly, since CPT-11 is eliminated via the bile into the duodenum, and this region of the gut has high levels of carboxylesterase activity, direct conversion of the drug to SN-38 will result in direct injury to the gut epithelia and hence diarrhea [11,12]. Secondly, the SN-38 is subject to glucuronidation in the liver and also expelled into the intestine though the bile. Glucuronidases present within the bacterial flora of the gut can the convert the glucuronide back to SN-38, resulting in direct toxicity to dividing cells within the crypts and villi [13–15]. Measures to prevent both of these mechanisms and hence reduce the delayed diarrhea associated with CPT-11 are currently being explored [12,13].

Since a cholinergic syndrome rapidly occurs following high dose i.v. CPT-11 administration that can be ameliorated by atropine, this suggests that the drug directly interacts with AChE. To confirm if this was the case, and to identify the domains within the drug that might be responsible for AChE inhibition, we have performed a series of detailed kinetic analyses with CPT-11 and a variety of analogs. These studies demonstrate that the 4-piperidinopiperidino moiety present at the 10-position of the molecule (Fig. 1) is primarily responsible for enzyme inhibition. In addition, we demonstrate that butyrylcholinesterase (BChE) can convert CPT-11 to SN-38,

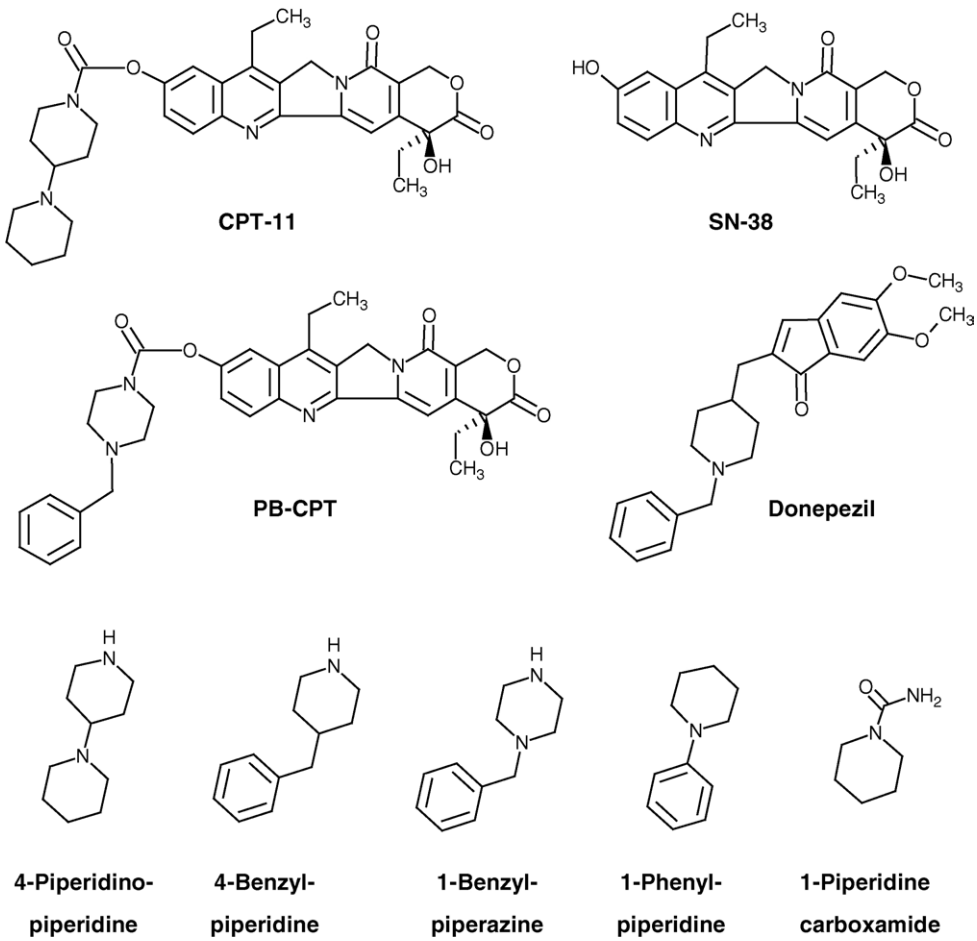

Fig. 1. Chemical structures of CPT-11, SN-38, PB-CPT, donepezil and small molecules tested for AChE inhibition.

and may play a role in the production of the active metabolite in vivo.

2. Materials and methods

2.1. Enzymes and drugs

hAChE, *Electrophorus electricus* AChE (*Ee*AChE) and hBChE were all obtained from Sigma Biochemicals (St. Louis, MO). *Torpedo californica* AChE (*Tc*AChE) was prepared as previously described [16–18]. CPT-11 and SN-38 were gifts kindly provided by Dr. J.P. McGovren (Pfizer, New York, NY). Donepezil was obtained from the St. Jude Children's Research Hospital pharmacy as Aricept tablets. An analog of CPT-11 that contains a piperidinobenzyl moiety at the 10-position of the molecule (PB-CPT, 7-ethyl-10-[4-(1-piperazino)-1-benzyl]carbonyloxycamptothecin; see Fig. 1) was synthesized as described by Yoon et al. [19].

2.2. Chemicals

4-Piperidinopiperidine (4-PP), 1-phenylpiperidine, 1-benzylpiperazine, 1-piperidine carboxamide, 4-benzylpiperidine, acetylthiocholine chloride and 5,5′-dithiobis-2-nitrobenzoic acid were all obtained from Sigma Biochemicals.

2.3. Acetylcholinesterase inhibition assay

AChE inhibition was determined using a spectrophotometric microtiter plate assay using 1 mM acetylthiocholine as a substrate, as described by Ellman et al. [20] and Doctor et al. [21]. Routinely, AChE at a concentration of 0.3 unit (U) per millilitre was used, where 1 U is the amount of enzyme required to hydrolyze 1 μmol of acetylthiocholine iodide at pH 7.4 at 37 °C. For inhibitor studies, compounds were dissolved in DMSO and kinetic data points were performed in quadruplicate with at least 8 inhibitor concentrations per assay. DMSO concentrations never exceeded 1% in the reaction. Data were plotted using the GraphPad Prism program, fitted to a one site binding hyperbolic function and IC_{50} (the concentration of inhibitor that reduced enzyme activity by 50%) was determined.

2.4. CPT-11 activation assays

To monitor the ability of enzymes to convert CPT-11 to SN-38, samples were incubated with 25 μM CPT-11 for up to 24 h in 50 mM Hepes pH 7.4 at 37 °C. Reactions were terminated by the addition of an equal volume of cold acidified methanol and samples were centrifuged at 14,000 × *g* for 10 mins to remove any precipitated protein. The levels of CPT-11 and SN-38 in the supernatants were then quantitated using reverse phase HPLC.

2.5. Separation and determination of CPT-11 and SN-38

CPT-11 and SN-38 present in methanolic supernatants were separated, and the concentrations determined, using reverse phase HPLC [22]. Briefly samples were applied to a 300 × 3.9 mm 4 μM Nova-Pak C_{18} column equilibrated in 75 mM ammonium acetate, 25% acetonitrile, pH 4.0, at a flow rate of 1 ml/min. Under these conditions, CPT-11, and SN-38 (all lactones) elute at 5.2 and 7.5 min, respectively. Drug levels were quantitated using a Jasco FP-2020 fluorescent detector with excitation and emission wavelengths of 375 and 550 nm, respectively. CPT-11 and SN-38 concentrations were then calculated using Beckman 32 Karat Software.

3. Results

3.1. Activation of CPT-11 by acetylcholinesterase and butyrylcholinesterase

As AChE is a member of the esterase class of proteins, potentially AChE could convert CPT-11 to SN-38. To assess whether these enzymes can activate the drug, we incubated either 20 U or 200 U of hAChE or hBChE with 25 μM CPT-11 for up to 24 h, and determined the amounts of SN-38 produced. As indicated in Table 1, no SN-38 was seen in samples incubated with hAChE. In contrast, readily detectable levels of the active metabolite were present following incubation with hBChE. Therefore, hBChE can activate CPT-11, whereas hAChE cannot. Since large amounts of BChE are present in human plasma, potentially this enzyme might contribute to drug activation, and presumably antitumor activity in cancer patients.

Table 1
Activation of CPT-11 by human AChE and BChE

Enzyme	SN-38 (pg)		
	1 h	4 h	24 h
hAChE (20U)	0	0	0
hAChE (200U)	0	0	0
hBChE (20U)	206	958	4380
hBChE (200U)	6520	9840	47100

Table 2
Inhibition of AChE by CPT-11 and structurally related compounds

Compound	IC_{50} (μM) with indicated enzyme (95% CI)		
	hAChE	*Ee*AChE	*Tc*AChE
CPT-11	0.97 (0.83–1.14)	0.35 (0.21–0.58)	0.87 (0.80–0.94)
SN-38	>100	>100	>100
PB-CPT	>100	ND[a]	>100
4-Piperidinopiperidine	61.4 (42.4–89.0)	>100	7.6 (6.0–9.6)
1-phenylpiperidine	>100	>100	>100
1-Benzylpiperazine	>100	>100	>100
1-Piperidine carboxamide	>100	>100	>100
4-Benzylpiperidine	>100	>100	>100
Donepezil	0.015 (0.0085–0.029)	0.026 (0.18–0.38)	0.032 (0.27–0.38)

[a] ND: not determined.

3.2. Inhibition of acetylcholinesterase by CPT-11

Since a cholinergic syndrome is associated with high dose CPT-11 administration, we hypothesized that the drug might interact with and inhibit AChE. Therefore, we assessed the ability of CPT-11 and several small molecules demonstrating structural similarity to the drug (Fig. 1) to inhibit AChE derived from humans, *Electrophorus electricus* and *Torpedo californica*.

As can be seen in Table 2, CPT-11 was a potent inhibitor of all three enzymes, with IC_{50} values of 970, 350 and 870 nM, respectively. This is consistent with our previously published preliminary results [23]. As expected donepezil was a highly effective inhibitor of all of the AChEs tested.

To determine whether the SN-38 or the 4-dipiperidino moiety was primarily responsible for enzyme inhibition, we assessed the ability of various molecules that form the components of the drug to inhibit AChE. Hence, 4-PP and SN-38 were monitored for AChE inhibition. As indicated in Table 2, 4-PP was a weak inhibitor of all three AChEs, suggesting that this compound contributes to enzyme inhibition by CPT-11. In contrast, no inhibition was observed with 1-phenylpiperidine, 1-benzylpiperazine, 1-piperidine carboxamide or 4-benzylpiperidine. Similarly, SN-38 was a very poor inhibitor of the three enzymes.

3.3. Lack of AChE inhibition by PB-CPT

Since the 4-dipiperidino moiety demonstrates similarity to the benzyl piperidine chemotype present within donepezil, we hypothesized that elimination of this structure in a CPT-11 analog might reduce or eliminate AChE inhibition.

We therefore synthesized a 4-piperazinobenzyl analog (Fig. 1; [19]), which we predicted would be a much weaker inhibitor of the enzyme. Enzymatic assays confirmed our hypothesis with IC_{50} values of greater than 100 μM for hAChE and *Tc*AChE (Table 2). These results indicate that the piperidino rings are a major factor in AChE enzyme inhibition. Interestingly, PB-CPT had a lower Km for the human carboxylesterase that activates CPT-11, but this compound is less cytotoxic due to the lower production of SN-38 [19]. However, the design of novel CPT-11 analogs based upon the analysis of the inhibition of AChE of the small molecule substituents might be possible.

4. Discussion

CPT-11 is probably the best new broad spectrum anticancer drug to be identified in the last 30 years. It demonstrates remarkable antitumor activity in animal models [24,25] and these results have been translated into effective responses in patients diagnosed with a variety of solid tumors [6]. However, as with all chemotherapeutic agents, severe toxicity is frequently seen with CPT-11 administration. While the cholinergic syndrome is not life threatening, it is very unpleasant for the individual and the patient requires constant monitoring during drug infusion [9]. These symptoms can be alleviated by administering atropine, consistent with the notion that CPT-11 and/or its metabolites directly interact with AChE.

Here, we provide evidence that the parent drug CPT-11, is a potent AChE inhibitor and is likely responsible, at least in part, for the cholinergic syndrome that is observed following i.v. infusion. Our results indicate that both CPT-11 and the dipiperidino moiety at the 10-position of the molecule, 4-PP, can inhibit AChE. Hence, it is probable that this domain contributes to the binding and inhibition of the enzyme. Since this moiety is similar to the benzylpiperidino group present in donepezil,

potentially these groups could interact with the same residues within the AChE active site. Crystallographic studies with CPT-11 and AChE to determine if this is the case are currently underway.

Confirmation that the dipiperidino group was in part responsible for AChE inhibition was demonstrated by the development of an analog of CPT-11, PB-CPT. This compound was a very poor inhibitor of all enzymes tested (Table 1), suggesting that the camptothecin ring structure does not play a major role in enzyme inhibition. This was also confirmed by data demonstrating that SN-38, the hydrolysis product of CPT-11, was a poor inhibitor of AChE (Table 2). However, PB-CPT was not as potent as CPT-11 at inducing cytotoxicity in human tumor cell lines, presumably due to lower levels of carboxylesterase-mediated drug activation [19]. We are currently undertaking structural studies with CPT-11 in complex with AChE to identify the interactions that occur between the drug and the amino acids present within the protein. We anticipate that CPT-11 will localize within the active site as has been predicted from molecular modeling studies [23].

While CPT-11 is not a substrate for AChE, we also assessed the ability of BChE to convert the drug to SN-38. These studies demonstrate that the enzyme is relatively inefficient at drug metabolism, however due to the abundance of BChE in human plasma, potentially the metabolic activation of CPT-11 seen in cancer patients is in part due to hydrolysis by this enzyme. Also, since several variants of BChE have been identified in humans that significantly affect substrate hydrolysis [26–28], individuals carrying such polymorphisms might demonstrate alterations in CPT-11 hydrolysis. Since any effect on drug metabolism would not be observed until the patient receives CPT-11, correlation of any increased toxicity or decreased efficacy observed after drug administration might not be readily apparent. A detailed analysis of the ability of the different BChE variants to metabolize CPT-11 should therefore be performed.

Overall, our studies indicate that CPT-11 is a potent inhibitor of AChE, and this probably accounts for the cholinergic syndrome that is observed in cancer patients following high dose i.v. infusion. Therefore, alternate drug scheduling might be effective in ameliorating this toxicity. In addition, our data suggests that modification of the CPT-11 structure to remove the 4-piperidinopiperidine moiety at the 10-position of the molecule, might yield anticancer drugs with reduced ability to inhibit AChE. Furthermore we have demonstrated that BChE can activate CPT-11, and may influence drug hydrolysis in vivo. Finally, these studies suggest that novel AChE inhibitors based upon the camptothecin scaffold could be designed. Such agents might be efficacious in the treatment of Alzheimer's disease. We are currently pursuing such avenues of research.

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