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The Mechanism for the Inhibition of Acetylcholinesterases by Irinotecan (CPT-11)[1]

MOLECULAR PHARMACOLOGY

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ABSTRACT

Irinotecan (CPT-11) is an anticancer drug that occasionally produces acute cholinergic side effects. Preliminary findings suggest that these are mediated through the inhibition of acetylcholinesterase (AChE). In this study, the inhibition of various AChEs by CPT-11 was studied. The lactone form of CPT-11 resulted in apparent noncompetitive inhibition of electric eel and both human recombinant and erythrocyte AChE with K_i values of 0.065, 0.19, and 0.29 μM, respectively. The carboxylate form of CPT-11 was approximately 10 times less potent. Apparent noncompetitive inhibition of AChE may arise through several mechanisms, and those relevant to CPT-11 were identified from key experimental findings. First, the inhibition by CPT-11 was found to be instantly reversible in dilution studies. Second, incubation of the enzyme with CPT-11 before the introduction of neostigmine protected the enzyme from inactivation. Third, regeneration of the active enzyme after preincubation with neostigmine was totally suppressed by the addition of 2 μM CPT-11, indicating that CPT-11 is a potent inhibitor of decarbamoylation and, by inference, deacylation. Additional experiments with tacrine revealed functional differences between these two inhibitors. Also, preliminary molecular modeling of the interaction between AChE and CPT-11 indicated that the latter does not bind at the same site as tacrine. Displacement studies with the peripheral site-specific ligand, propidium, confirmed that CPT-11 binds at this site. The rapid reversibility of the inhibition of AChE by CPT-11 and the lower activity of the carboxylate form are likely reasons for the transient nature of the cholinergic toxicity observed clinically.

Irinotecan (CPT-11; see Fig. 1) is an anticancer alkaloid currently registered for the treatment of metastatic colorectal adenocarcinoma (Rothenberg, 1996). One of the acute side effects observed during therapy with CPT-11 is a syndrome consisting of vomiting and diarrhea accompanied by abdominal cramps, diaphoresis, and accommodation disturbances (Gandia et al., 1993). The syndrome is usually transient, and its ready reversal by atropine (Rothenberg et al., 1993) indicates a cholinergic mechanism.

Despite the demonstration that CPT-11 is a potent inhibitor of dog and electric eel acetylcholinesterase (AChE; Kawato et al., 1993; Rivory et al., 1996b), the mechanism of this syndrome has been ascribed to ganglionic stimulation (Gandia et al., 1993). This finding is based on the structural similarity between the bipiperidino side chain of CPT-11 and a known stimulant of nicotinic receptors of autonomic ganglia, dimethylphenylpiperazinium iodide. No significant inhibition of AChE from washed red blood cells of patients treated with CPT-11 was observed in early clinical studies,

and Gandia et al. (1993) considered this observation to be supportive evidence for a non-AChE-mediated mechanism.

As mentioned above, CPT-11 has been shown to be an inhibitor of AChE. Interestingly, a broad range of xenobiotics share this property through binding to human AChE at a number of sites, including the catalytic triad (Ser[203], His[447], Glu[334]), neighboring hydrophobic residues (Trp[86], Tyr[133], Tyr[337], and Phe[338]), the acyl pocket (Phe[295] and Phe[297]), or a peripheral binding site (Tyr[72], Tyr[124], Glu[285], Trp[286], Tyr[341], and Asp[74]), as reviewed by Ariel et al. (1998). The best known inhibitors of AChE are those belonging to the carbamate and organophosphate classes. Whereas inhibition with organophosphates is irreversible, carbamates (e.g., physostigmine and neostigmine) inhibit AChE transiently through the hydrolysis of the ester and the transfer of the carbamate moiety to Ser[203] at the active site of the enzyme. This carbamoylated enzyme intermediate is inactive but can be reconverted slowly through the loss of the corresponding carbamic acid. For this reason they are sometimes referred to as being "reversible" inhibitors (Taylor, 1980).

There is also a large number of ligands that interact in a reversible manner with the aromatic side chains of the hy-

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ABBREVIATIONS: CPT-11, irinotecan; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ATChI, acetylthiocholine iodide; BTChI, butyryl-thiocholine iodide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid).

drophobic residues that line the active-center gorge (Quinn, 1987; Ariel et al., 1998). One of these, tacrine (see Fig. 1), has been extensively investigated over the last few years for the possible palliation of Alzheimer's disease (Gracon et al., 1998). Tacrine has also been reported to have a protective effect against organophosphates (Galli et al., 1992).

The aim of the current study was 2-fold. Primarily, we wanted to investigate the kinetics of the interaction between AChE and CPT-11 to define the mechanism of the inhibition and its likely clinical significance in terms of the acute side effects observed in patients. Second, because of the great interest in the therapy of Alzheimer's dementia with AChE-targeted drugs, we compared the mechanistic properties of CPT-11 with those of tacrine, relative to the inhibition of AChE.

Experimental Procedures

Materials. CPT-11 was supplied by Rhône-Poulenc Rorer (Neuilly, France). Acetylthiocholine iodide (ATChI), butyrylthiocholine iodide (BTChI), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), propidium iodide, benzoylcholine chloride, and decamethonium bromide were all purchased from Sigma Chemical Co. (St. Louis, MO). Human recombinant and electric eel (type III) AChE (EC 3.1.1.7) were obtained from Sigma, as were human and horse serum butyrylcholinesterase (BChE, EC 3.1.1.8). Tacrine was kindly provided by Professor Mac Christie (University of Sydney, Sydney, Australia). All other reagents were of the highest grade commercially available.

Enzyme Preparation from Human Erythrocytes. Human erythrocyte AChE was prepared from blood collected from a hemochromatosis patient, essentially as described by Al-Jafari et al. (1995). Erythrocytes were separated by centrifugation at $2000g$ (15 min, ambient temperature) and washed three times with PBS (pH 7.4). They were lysed by the addition of 5 volumes of distilled, deionized H_2O at ambient temperature for 90 min. The lysate was centrifuged at $12000g$ for 25 min at 4°C, and the pellet was washed three times with H_2O. The final pellet was reconstituted in 2 ml of 0.1 M potassium phosphate buffer (pH 7.2 with 1 mM EDTA) and solubilized with 1% Triton X-100. The aliquot was centrifuged ($20,800g$, 5 min), and the pellet was discarded. The preparation was stored at $-20°C$.

Assays of Enzyme Activity. The enzyme assays of both AChE and BChE were performed spectrophotometrically according to the method of Ellman et al. (1961) at 30°C, using ATChI and BTChI as substrates, respectively. The formation of thionitrobenzene was followed spectrophotometrically at 412 nm ($\Delta\epsilon_{412 \text{ nm}} = 14150 \text{ M}^{-1} \text{ cm}^{-1}$; Riddles et al., 1979) at 30°C in 0.1 M phosphate buffer (pH 7.3; 0.3 mM DTNB) with a Uvikon 810 spectrophotometer (Kontron, Schliesen, Switzerland) after the addition of respective substrates and approximately 0.05 U of the required esterase, except for horse serum BChE, where 10 U was used. The total assay volume was 3.2 ml, unless indicated otherwise.

CPT-11 exists in vivo in equilibrium between a lactone and a carboxylate form (Rivory et al., 1994), and we performed studies on both. These were generated as described previously using acidic and basic solutions, respectively (Rivory and Robert, 1994).

The effect of preincubation of AChE or BChE (0.05 U) with CPT-11 lactone (0.08 and 3.85 μM) was studied over three time periods: 10, 20, and 30 min. At the end of each incubation, the enzyme assay was initiated by the addition of the appropriate substrate at a concentration equal to the K_m of the enzyme as determined in initial experiments.

The inhibition of horse serum BChE by CPT-11 was also investigated using a second substrate, benzoylcholine. It has been established that the debenzoylation step is rate limiting in the hydrolysis of this substrate (Xie et al., 1999). Hydrolysis of benzoylcholine was recorded spectrophotometrically ($\lambda = 240$ nm) at 25°C in 0.1 M phosphate buffer (pH 7.3) with a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) after the addition of substrate (equal to a reported K_m of 20 μM, Xie et al., 1999), a range of CPT-11 concentrations, and approximately 1 U of horse serum esterase. Hydrolytic activity was calculated from the difference in molar absorptivity of benzoylcholine and benzoic acid, $\Delta\epsilon_{240 \text{ nm}} = 6700 \text{ M}^{-1} \text{ cm}^{-1}$.

To carry out some of the experiments described below, it was first necessary to establish concentration-response relationships for the inhibition of AChE. Inhibitors were added to a cuvette already containing DTNB (0.3 mM) as well as ATChI at a concentration equal to the predetermined K_m. The reaction was initiated with the addition of AChE (0.05 U). The final concentrations of CPT-11 investigated ranged from 0.0078 to 7.81 μM, whereas they were 0.0008 to 0.78 μM for tacrine. The concentration resulting in a 90% reduction of enzyme activity (IC_{90}) was estimated from the resulting plots.

Dilution Studies. The reversibility of the inhibition of AChE by CPT-11 (both species) was determined by first incubating the enzyme with CPT-11 (both species) for 5 min at 30°C in 0.1 M potassium phosphate buffer (pH 7.3) containing concentrated AChE (5 U/ml), CPT-11 (at IC_{90} for both species), and ATChI (absent or at $3 \times K_m$). An aliquot (10 μl) from each incubation was then added to a cuvette containing 3.1 ml of DTNB (0.3 mM) and ATChI (K_m). The resulting dilution yielded a final concentration of CPT-11 lactone and carboxylate of 6.4 and 25.7 nM, respectively. The activity of AChE was expressed as a percentage of that observed with a corresponding control (no CPT-11) incubation. An identical set of experiments was performed with tacrine instead, and, in this instance, the final concentration of inhibitor postdilution was 0.3 nM.

Neostigmine Protection Studies. The ability of CPT-11 to protect AChE from deactivation by neostigmine, a carbamate inhibitor of AChE, was also investigated. Preincubation experiments were performed exactly as described above except that neostigmine was included in the appropriate preincubations at a concentration equivalent to its IC_{90}. ATChI was not present in this initial incubation phase. The activity of AChE was expressed as a percentage of that observed postdilution into the reaction mixture with a corresponding control (no neostigmine) incubation.

Inhibition of Deacylation. As mentioned previously, the incubation of AChE with the carbamate esters physostigmine and neostigmine leads to the formation of carbamoylated enzyme complexes that are hydrolyzed back to active enzyme with half-lives of the order of 15 to 30 min (Wilson and Harrison, 1961). To establish whether CPT-11 and tacrine are inhibitors of deacylation, we used the carbamoylated AChE species produced by incubating electric eel AChE (250 U/ml) with neostigmine (1 μM) at 30°C for 5 min as a surrogate of the acyl-enzyme complex (Froede and Wilson, 1984). Aliquots of these incubations were then added to Eppendorf tubes
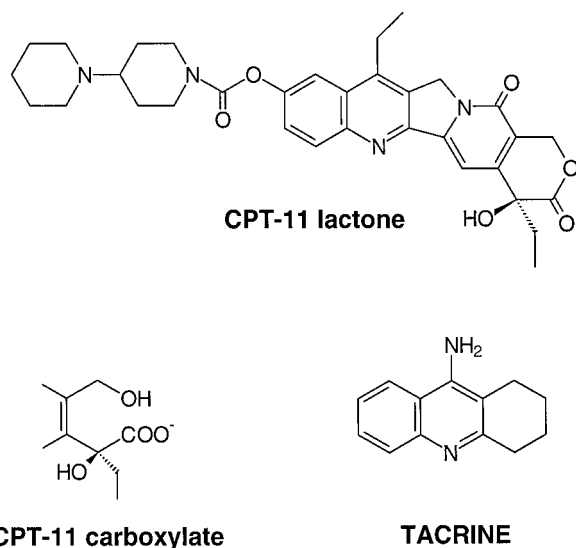

Fig. 1. Structural formula of CPT-11 (lactone and carboxylate) and tacrine.

CPT-11 lactone

CPT-11 carboxylate

TACRINE

containing either CPT-11 (2 μM as lactone or carboxylate), tacrine (0.5 μM), or the corresponding vehicle. This incubate was kept at 30°C, and aliquots (10 μl) were removed at regular intervals (0, 5, 10, 15, 20, 25, and 30 min) and transferred to a cuvette already containing DTNB (0.3 mM) and ATChI (at K_m). The final concentrations of AChE, neostigmine, CPT-11, and tacrine in each of the final reaction cuvettes were 0.016 U and 0.6, 6.4, and 1.6 nM, respectively.

Hydrolysis of CPT-11 by AChE and BChE. In vivo, CPT-11 is converted to the active metabolite SN-38 by carboxylesterases (Kaneda and Yokokura, 1990; Rivory et al., 1996a). This is achieved through the hydrolysis of the carbamate side chain in an analogous fashion to the hydrolysis of physostigmine and neostigmine by AChE. Preliminary analysis of the kinetics of inhibition revealed that CPT-11 is mostly a noncompetitive inhibitor of AChE (Dodds and Rivory, 1998) and, therefore, unlikely to be a substrate of this enzyme. Nevertheless, we wanted to rule out this possibility, and AChE (electric eel, 10 U) was incubated in the presence of 10 μM CPT-11 in 0.1 M potassium phosphate buffer at 30°C. Experiments were carried out in an identical manner for BChE (horse serum, 10 U). SN-38 is the highly fluorescent first product of the reaction (P_1 in Fig. 2), and its production was monitored with HPLC using a modification of the method described by Rivory and Robert (1994). Aliquots (20 μl) were removed at selected time points (0, 10, 20, 30, and 60 min) and mixed immediately with 50 μl of ACN/0.05 N HCl (33:66, v/v). Separation was carried out using a C_8 symmetry column (3.9 × 150 mm, Waters, Millipore Corp., Milford, MA) at ambient temperature and with a solvent flow of 1.0 ml/min. The mobile phase consisted of acetonitrile and 0.075 M ammonium acetate buffer (pH 4.5) mixed with the solvent selection valve in the proportion of 26:74 (v/v). Fluorescence detection (RF-10AXL, Shimadzu, Sydney, Australia) was optimized for SN-38 detection with excitation and emission wavelengths set at 380 and 540 nm, respectively. Data were collected and analyzed using CLASS VP software (version 4.2, Shimadzu). Standard samples were prepared from a 5 mg/ml stock solution of SN-38 in dimethyl sulfoxide diluted serially into 0.1 M potassium phosphate buffer, pH 7.3. Using this assay, the lower limit of quantitation of SN-38 was 5 nM, representing a 0.05% conversion of substrate (10 μM CPT-11).

Ligand Displacement Measurements. The affinity of CPT-11 for the peripheral binding site of electric eel AChE was tested using a known peripheral site-specific ligand, propidium iodide, essentially as per Taylor et al. (1974). A shift in the excitation wavelength follows the complexation of propidium iodide and AChE (Taylor et al., 1974. Fluorescence intensity was monitored in a Perkin-Elmer LS-50 fluorimeter (Perkin-Elmer, Silverwater, Australia) using 10-mm path length cuvettes at 25°C. AChE (2 μM) was incubated with 1 μM propidium iodide in 1 mM Tris-HCl, pH 8.0. Aliquots of CPT-11 lactone (0.0001–10.0 μM) were added successively, and emission was monitored at 595 nm upon excitation at 535 nm. The experiments were repeated using decamethonium bromide (0.0001–10.0 μM) as a positive control of propidium displacement in an identical manner to that described above.

All experiments were performed at least in triplicate and are quoted as mean ± S.D. The Michaelis-Menten parameters (V_{max}, K_m) for the AChE- and BChE-catalyzed hydrolysis of ATChI and BTChI,

respectively, were obtained by nonlinear regression of the velocity data using Sigmaplot (Jandel Scientific, Corte Madera, CA).

Determination of Inhibitor Constants. The inhibition of AChE was investigated using a wide substrate concentration range (7–220 μM) and three concentrations of CPT-11 as lactone or carboxylate. Initially, Dixon plots were used to identify the type of inhibition involved (competitive, noncompetitive, etc.). Estimates of the Michaelis-Menten parameters were obtained by nonlinear regression (Sigmaplot; Jandel Scientific). Preliminary data indicated that CPT-11 acted mostly as a noncompetitive inhibitor of AChE, and initial estimates of the inhibitor constant (K_i) were obtained with the nonlinear regression of the observed $V_{max(i)}$ using:

$$V_{max(i)} = \frac{V_{max(0)}}{\left(1 + \dfrac{i}{K_i}\right)} \tag{1}$$

However, plotting the data in Eadie-Hofstee format revealed some inconsistencies with a "pure" noncompetitive scheme, and a more detailed kinetic evaluation was carried out as outlined below.

The general scheme illustrating the appropriate equilibrium model is depicted in Fig. 2. The hydrolysis of acetylcholine catalyzed by AChE involves an acyl-enzyme intermediate (EC). Because k_2/k_3 is of the order of 1.5 to 6 (Wilson and Cabib, 1956; Froede and Wilson, 1984), enzyme deacylation is at least partially rate limiting, and apparent noncompetitive inhibition occurs if the inhibitor is capable of binding to the enzyme and to the acyl-enzyme complex. In short, apparent noncompetitive behavior arises as a mixture of competitive and uncompetitive mechanisms. Binding to the Michaelis-Menten complex (ES) may also occur but is less consequential. This model was also selected on the basis of the results of the other experiments performed, which revealed that CPT-11 is an inhibitor of decarbamoylation and that inhibition is instantly reversible.

From the scheme represented in Fig. 2, it can be shown that the velocity of the reaction (v) in the presence of inhibitor at concentration i is related to the maximal velocity in the absence of inhibitor (V_{max}) by:

$$v = \frac{V_{max}S}{K_{m'}\left(\dfrac{i}{K_E} + 1\right) + S\left(\dfrac{i}{K_{EC}} + \dfrac{i}{K_{ES}}\dfrac{k_3}{k_2} + \dfrac{k_3}{k_2} + 1\right)} \tag{2}$$

where S is the substrate concentration and $K_{m'}$ is the apparent Michaelis-Menten constant as given by:

$$\frac{(k_2 + k_{-1})}{k_1}\frac{k_3}{k_2} \tag{3}$$

For apparent noncompetitive kinetics to occur, it has been shown that $K_E \approx K_{ES} \approx K_{EC}$. Furthermore, because $k_2 > k_3$, the velocity equation will be approximated by:

$$v = \frac{V_{max}S}{K_{m'}\left(\dfrac{i}{K_E} + 1\right) + S\left(\dfrac{i}{K_{EC}} + 1\right)} \tag{4}$$

Plotting v versus v/S will yield lines with y-intercepts of $V_{max(i)}$ equal to:

$$\frac{V_{max(0)}}{\left(\dfrac{i}{K_{EC}} + 1\right)} \tag{5}$$

and a slope of:

$$-\frac{K_{m'}\left(\dfrac{i}{K_E} + 1\right)}{\left(\dfrac{i}{K_{EC}} + 1\right)} \tag{6}$$

Fig. 2. An equilibrium model for the proposed reaction scheme involving two enzyme-substrate intermediates for CPT-11 (CPT-11 was found to be an inhibitor of deacylation).

As mentioned previously, when the inhibitor binds with equal affinity to both the free enzyme and the acyl-enzyme complex, the slope will simply be $K_{m'}$ and the kinetics will take on the appearance of noncompetitive behavior. If several concentrations of inhibitor are tested, a series of parallel lines will be generated. If a slight inequivalence of the affinity for the two enzyme intermediates is present, some nonparallelism will become apparent. An investigation into the relationship between the values yielded by eqs. 5 and 6 and i can be used to estimate K_E and K_{EC}.

Results

Inhibition Kinetics of AChE and BChE by CPT-11. CPT-11 was found to be a potent inhibitor of the AChE hydrolysis of ATChI. This behavior was observed for each AChE source and for both CPT-11 species (lactone and carboxylate) and was not increased with preincubation before assay (data not shown). In fact, there was a slight reduction in the inhibition of AChE by CPT-11 lactone with increased incubation times. In contrast, CPT-11 (3.85 μM) had no effect on human BChE hydrolysis of BTChI even when preincubated with CPT-11 lactone for up to 30 min (data not shown). Similar results were observed for horse serum BChE. Indeed, the highest concentrations of CPT-11 lactone and carboxylate (15.6 μM) only achieved 14.5 $\pm$ 0.002% and 3.8 $\pm$ 1.063% inhibition of this enzyme, respectively. We also examined the effect of CPT-11 on the hydrolysis of benzoylcholine by horse serum BChE. Interestingly, the same concentrations of CPT-11 lactone and carboxylate (i.e., 15.6 μM) inhibited the hydrolyis of benzoylcholine to a much greater extent than that of BTChI with corresponding IC$_{50}$ values of 3.96 and 37.3 μM for CPT-11 lactone and carboxylate, respectively.

The kinetics of AChE in the presence of CPT-11 displayed typical noncompetitive behavior in a Dixon plot format as represented by a series of straight lines with a common x-axis intercept (Fig. 3). When presented as an Eadie-Hofstee plot,

pure noncompetitive inhibition results in a series of parallel lines. However, with CPT-11, the lines were not strictly parallel and displayed some convergence, particularly for the carboxylate form of CPT-11 (Fig. 4B). Similar observations were made with the other sources of AChE (data not shown). Tacrine also displayed noncompetitive inhibition of AChE, but in this case, slight divergence was evident in the Eadie-Hofstee plot (Fig. 4C).

Assuming steady-state conditions, the inhibition constants K_E and K_{EC} were determined for CPT-11 using the equations relating the intercepts and slopes of the Eadie-Hofstee plots as a function of i for each source of AChE. These results are shown in Table 1. The values for tacrine, observed with electric eel AChE, are also included in Table 1 for comparison. The inhibitor constants indicate that the lactone form of CPT-11 is approximately 10 times more potent than its corresponding carboxylate, although this varies according to the
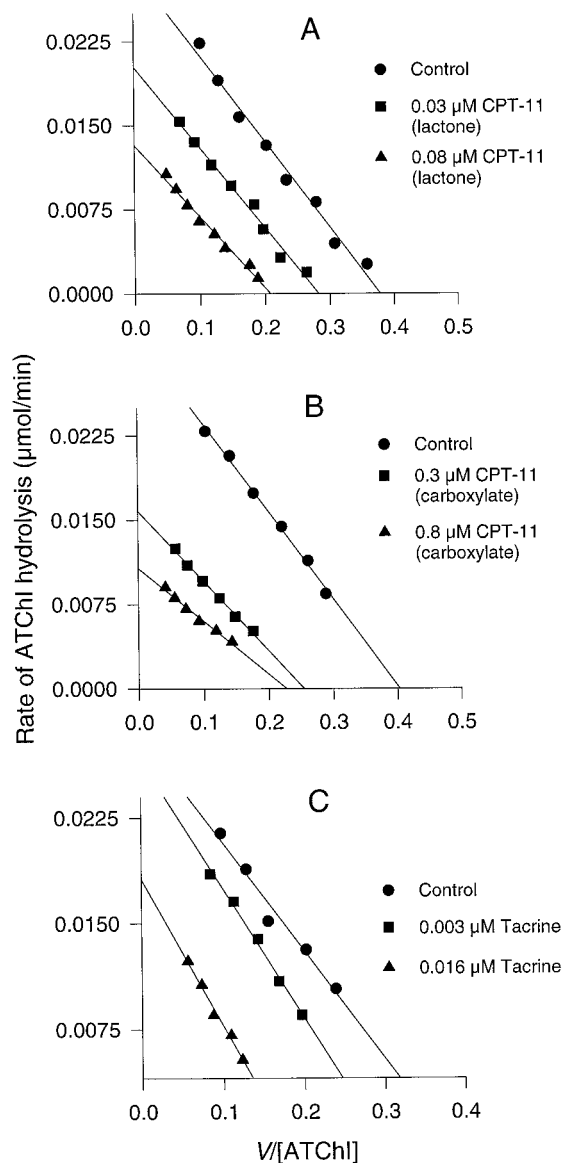
Fig. 4. Eadie-Hofstee plots for inhibition by CPT-11 lactone (A), CPT-11 carboxylate (B), and tacrine (C) of AChE (electric eel) hydrolysis of ATChI. The plotted lines for CPT-11 display some convergence, which suggests $K_E > K_{EC}$. The opposite is true for tacrine, where the lines diverge slightly.

Fig. 3. Double-reciprocal plot for inhibition by CPT-11 (0, 0.03, and 0.08 μM lactone) of AChE hydrolysis of ATChI. The concentration of enzyme was 0.016 U in a reaction mixture of 0.1 M potassium phosphate buffer, pH 7.3, containing 0.3 mM DTNB and varying ATChI concentrations.

source of AChE. As expected on the basis of the slight convergence of the Eadie-Hofstee plots of CPT-11, the K_{EC} values observed are lower in comparison to K_E in each case (Table 1). The opposite is true for tacrine, where the lines diverge slightly (Fig. 4C).

Dose-Response Studies. To ascertain the inhibition of AChE likely to be encountered with clinically relevant concentrations of CPT-11, a series of concentration-response studies was carried out, and the results (in terms of IC_{50} and IC_{90}) are also included in Table 1. The IC_{50} values were in concert with the K_{EC} values in the sense that the potency of CPT-11 was greatest for electric eel AChE and then human recombinant and erythrocyte preparations, respectively.

Reversible Inhibition of AChE Hydrolysis. The concentration-response studies also served to determine the IC_{90} values of CPT-11, as required for the dilution experiments. The latter revealed that the inhibition of all AChE preparations by both CPT-11 and tacrine (electric eel AChE) are instantly and completely reversible within the limitations of the experiment. These limitations are, first, that the dilution and assay of activity required several seconds to achieve, precluding an analysis of the kinetics of reversal over a shorter time scale. Second, although the magnitude of this dilution was greater than 300-fold (see *Experimental Procedures*), the final concentration of CPT-11 (6.4 nM) resulted in some residual inhibition (5–10%) at the time of spectrophotometric analysis. The extent of inhibition as a result of this low concentration of inhibitor was confirmed for each AChE source using both CPT-11 species.

Protective Effect of CPT-11 against AChE Inactivation by Neostigmine. Additional dilution studies revealed that CPT-11 affords protection of all AChE preparations from the carbamate neostigmine (Fig. 5). With 2 μM CPT-11 (lactone), electric eel AChE was essentially totally protected from 0.5 μM neostigmine.

Potent Inhibition of Deacylation by CPT-11 But Not Tacrine. In separate experiments, electric eel AChE was preincubated with neostigmine to yield the carbamoylated intermediate. This intermediate was then incubated in the presence of CPT-11 or its vehicle for set periods of time after which the reaction mix was again diluted and AChE activity was monitored to measure recovery of the enzyme. When CPT-11 (2 μM) was added at the point of the first dilution, it resulted in the potent inhibition of enzyme regeneration (Fig. 6A) with the greatest effect observed for the lactone form. In the absence of CPT-11 and over a period of 30 min, AChE activity rose to a maximum of 4.0 ± 0.01 nmol/min, which is essentially identical with the activity of the enzyme alone when incubated for the same period of time (i.e., total recov-

ery of enzyme). The CPT-11 concentration remaining during assay, 6.4 nM, yielded only 5 to 10% inhibition under identical conditions and was, therefore, insufficient to explain the observed inhibition. In contrast, tacrine (0.5 μM) did not significantly modify the rate of recovery of the enzyme (Fig. 6B).

CPT-11 Is a Substrate for BChE But Not AChE. Incubations of CPT-11 (10 μM) with electric eel AChE (10 U) for up to 60 min at 30°C yielded concentrations of SN-38 that did not increase significantly with incubation ($p = .45$). A similar result was observed for the control incubations ($p = .73$). A small quantity of SN-38 was observed in both groups of samples (mean = 13.2 nM) as a result of its being a minor contaminant of CPT-11. However, as depicted in Fig. 7, the incubations containing CPT-11 (10 μM) and horse BChE (10 U) revealed a significant production of SN-38 as a function of time ($p = .00025$), indicating the ability of BChE to convert CPT-11 to SN-38.

Displacement of Bound Propidium by CPT-11. The titrations of AChE-propidium iodide in the presence of CPT-11 (lactone; 0.0001–10.0 μM) were shown to decrease the fluorescence intensity associated with propidium iodide binding to AChE. Figure 8 reveals that CPT-11 is effective in dissociating propidium (1 μM) from electric eel AChE (2 μM) in 1 mM Tris-HCl (pH 8.0, 25°C) to a greater extent than the bisquaternary ligand, decamethonium.

Discussion

In this study, we have investigated the inhibition of AChE by CPT-11 to further examine the mechanism for the cholinergic toxicity observed in some patients. Preliminary studies suggested that CPT-11 is a noncompetitive inhibitor of AChE (Dodds and Rivory, 1998).

Apparent noncompetitive inhibition may arise through several mechanisms. First, mechanism-based inhibitors such as organophosphates yield noncompetitive kinetics because the loss of active enzyme is a function of the concentration of inhibitor (Dixon and Webb, 1979). Although inactivation through carbamoylation of AChE could be proposed on the basis of structural homology between CPT-11 and carbamate inhibitors of AChE, this is not consistent with the lack of production of SN-38. Even if stoichiometric production of SN-38 had occurred (as with organophosphates), we would have expected an increase in the SN-38 concentration of approximately 20 nM (i.e., 4 times the limit of quantitation of SN-38). In any case, the dilution experiments indicate that inhibition by CPT-11 is instantly reversible, and this mechanism can be rejected.

TABLE 1
Interaction of CPT-11 (lactone and carboxylate) and tacrine with various preparations of acetylcholinesterase

	Electric Eel			Human Erythrocyte		Human Recombinant	
	CPT-11		Tacrine	CPT-11		CPT-11	
	Lactone	Carboxylate		Lactone	Carboxylate	Lactone	Carboxylate
μM							
$K_E{}^a$	0.098	1.10	0.008	0.77	3.85	0.26	2.97
$K_{EC}{}^a$	0.067	0.40	0.027	0.30	2.34	0.14	1.76
$IC_{50}{}^b$	0.080	0.47	0.030	0.53	2.84	0.45	2.28
$IC_{90}{}^b$	1.17	7.80	0.39	6.12	>10	3.91	>10

[a] Values determined with the equilibrium model (Fig. 2), which may not be applicable to tacrine.
[b] Model-independent analysis from inhibitor concentration-effect experiments.

Second, noncompetitive inhibition of esterases may arise through binding of inhibitor to several of the intermediates of the catalytic cycle. We found CPT-11 to be a potent inhibitor of decarbamoylation and, by inference, deacylation. Therefore, the equilibrium model of Fig. 2 was sufficient to explain the noncompetitive behavior of CPT-11. Tacrine, on the other hand, did not inhibit deacylation, and noncompetitive behavior would require the invocation of disequilibrium effects at the level of the acyl-enzyme intermediate.

The K_E value of 8 nM obtained for tacrine is similar to that of 9.5 nM reported by Wu and Yang (1988). In general, the inhibition of AChE by tacrine has been variously reported to be noncompetitive or nearly noncompetitive, with dissociation constants varying from 5 nM to 2 μM (as reviewed by Wu and Yang, 1988).

CPT-11, the carboxylate in particular, led to some significant convergence of the Eadie-Hofstee plots (Fig. 4B). Departure from pure noncompetitive inhibition may arise from the fact that the assumption that $k_2 \gg k_3$ may not hold. The presence of a non-negligible k_3/k_2 term in eq. 2 would lead to the overestimation of K_{EC} relative to K_E (as seen, for example, with tacrine) and the intercept from eq. 4 would be >1. However, with CPT-11, we found $K_{EC} < K_E$ and the intercept ≈ 1 in all cases. This would indicate that the affinity of CPT-11 is greater for the acyl-enzyme intermediate rather than the free enzyme. This slight difference is, if anything, underestimated by the assumption of $k_2 \gg k_3$.

Dilution studies with tacrine demonstrated that, as for CPT-11, binding is instantly reversible. This observation confirms the findings of Wu and Yang (1988) and those of Dawson (1990) but is not in agreement with Heilbronn (1961), who reported only a partial reduction in the extent of tacrine inhibition after 48 h of dialysis.

In this study, CPT-11 was found to be relatively specific

toward AChE with no significant inhibition of the hydrolysis of BTChI by human BChE, even at concentrations as high as 3.85 μM CPT-11 (lactone). In comparison, concentrations of this magnitude resulted in >95% inhibition of AChE in dose-concentration studies. The incubation experiments showed that AChE activity recovered slightly when incubated with CPT-11 lactone over a period of 30 min. Spontaneous conversion of CPT-11 to its carboxylate form occurs rapidly in aqueous solutions with a half-life of ~30 min (Akimoto et al., 1994). Therefore, it is likely that the recovery was due to the conversion of the lactone to the less potent carboxylate form.

Interestingly, CPT-11 inhibited the horse BChE hydrolysis of benzoylcholine to a much greater extent than the hydrolysis of BTChI. This difference may be attributable to the fact that the hydrolysis of benzoylcholine is deacylation limited and that CPT-11 may inhibit deacylation by several esterases, including human liver microsomal carboxylesterase (Rivory et al., 1996a).

Recent studies suggest that conformationally constrained compounds appear to have greater affinity for AChE than for BChE (Galli et al., 1996), possibly because these interact with the six conserved aromatic residues that line the active site gorge of *Torpedo* AChE but that are absent in BChE (Harel et al., 1992). However, camptothecin itself and SN-38 do not have
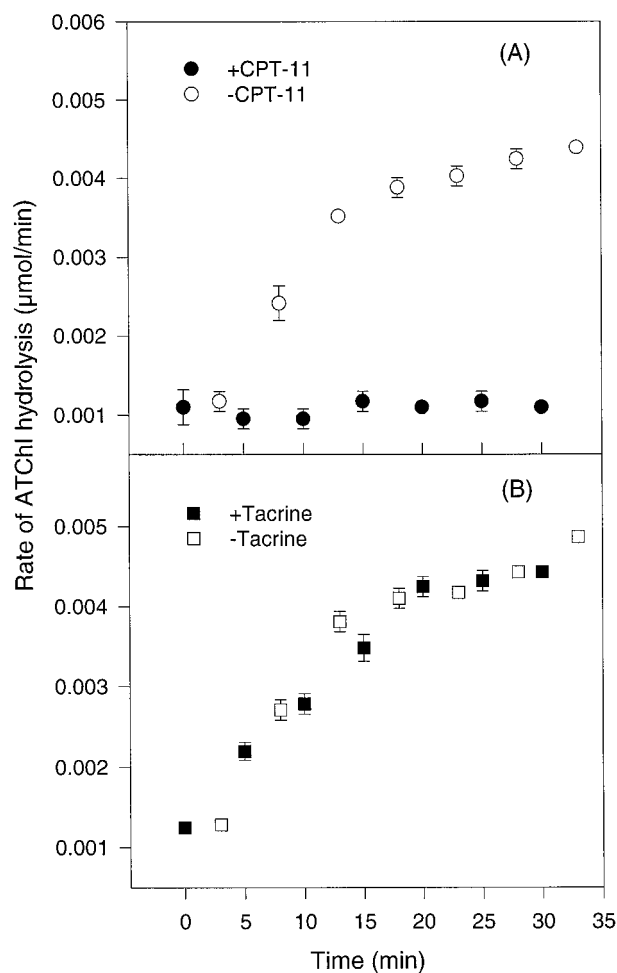

Fig. 5. Protection of AChE from neostigmine carbamoylation by CPT-11 lactone (2 μM). Enzyme activity was measured after dilution of incubates in 0.1 M potassium phosphate buffer, pH 7.3, containing AChE (electric eel; 5 U/ml) in the presence of 0.5 μM neostigmine (A), buffer (B), 2.0 μM CPT-11 and 0.5 μM neostigmine (C), and 2.0 μM CPT-11 (D). Upon dilution, the final concentrations of AChE, neostigmine, and CPT-11 were equal to 0.016 U and 1.6 and 6.4 nM, respectively.

Fig. 6. Inactivation and regeneration of ATChI hydrolysis from carbamoylated AChE (electric eel) in (A) the absence (○) and presence (●) of 2.0 μM CPT-11 lactone and (B) in the absence (□) and presence (■) of 0.5 μM tacrine.

significant activity against AChE (Kawato et al., 1993; Rivory et al., 1996b). Indeed, derivatives with nitrogenous substitutions at the camptothecin C-10 carbon have been shown to be inhibitors of AChE (Kawato et al., 1993), suggesting that interaction with at least one anionic site is required.

Although there are similarities between tacrine and CPT-11 with regard to the kinetics of inhibition and protection of AChE against carbamates, there appear to be substantial differences in the manner in which these ligands interact with AChE. For example, tacrine is a potent inhibitor of both AChE and BChE (Berman and Leonard, 1992; Galli et al., 1992). Also, tacrine binds to both the choline subsite and hydrophobic residues within the active center of the enzyme (Ariel et al., 1998). Indeed, molecular modeling studies are in support of the positioning of tacrine within the catalytic region of AChE (Sussman et al., 1993). In contrast, our attempts to model the situation with CPT-11 revealed that it is too large to bind exclusively to the active center. Furthermore, our propidium ligand studies support the involvement of the peripheral anionic site in the interaction. It would be of some interest to pursue studies of AChE mutants to reveal the residues involved in this interaction.

Cloning, sequencing, and site-directed mutagenesis studies have revealed subtle but important differences between the structures of AChE and BChE (Harel et al., 1992). Six conserved aromatic residues, which line the active site gorge of *Torpedo* AChE, are absent in BChE. Furthermore, replacement of AChE Phe[288] and Phe[290] by leucine and valine, respectively, produce an esterase that hydrolyses BTChI, presumably by allowing the butyryl group to be accommodated into the larger esteratic pocket (Harel et al., 1992). Thus, it is possible that CPT-11 can position itself farther into the active site of BChE and be hydrolyzed to SN-38.

Small ligands that bind to the peripheral binding site may affect enzyme activity. This may be due to "cross talk" between the peripheral and active centers (Shafferman et al., 1992; Ordentlich et al., 1993), although the use of nonequilibrium kinetics may also account for such an effect (Szegletes et al., 1998). However, the relatively large size of CPT-11, the hydrolysis of CPT-11 by BChE, and the protection of AChE from neostigmine together suggest that CPT-11 is unlikely to bind exclusively to the peripheral site of AChE.

In this investigation, we found significant differences in the inhibition by the two forms of CPT-11, with the lactone approximately 10-fold more potent than the carboxylate. Greater affinity of the lactone most likely occurs due to the net negative charge carried by the corresponding carboxylate form. Studies of the ligand charge effect on AChE inhibition have shown a consistent requirement for cationic or uncharged species irrespective of the precise location of binding at the AChE site (Hasan et al., 1981).

Importantly, the concentrations of CPT-11 that inhibit human AChE are well within the range observed in the plasma of patients toward the end of infusion of CPT-11 (de Forni et al., 1994). It is not surprising that Gandia et al. (1993) did not observe significant inhibition of AChE from washed red blood cells of treated patients, given its rapid reversibility upon dilution. Although CPT-11 is administered in the lactone form, the apparent interconversion of CPT-11 lactone to the less potent carboxylate form in patients is rapid, with a mean half-life of 9.5 min (Rivory et al., 1994). This, together with the potential for rapid reversibility of the inhibition, most likely explains the transient nature of the toxicity.

In conclusion, CPT-11 is a relatively potent, reversible, and selective inhibitor of AChE that has properties consistent with the acute cholinergic toxicity observed in some patients. In addition, CPT-11 may provide an interesting lead compound for the investigation of the inhibition of deacylation by AChE.
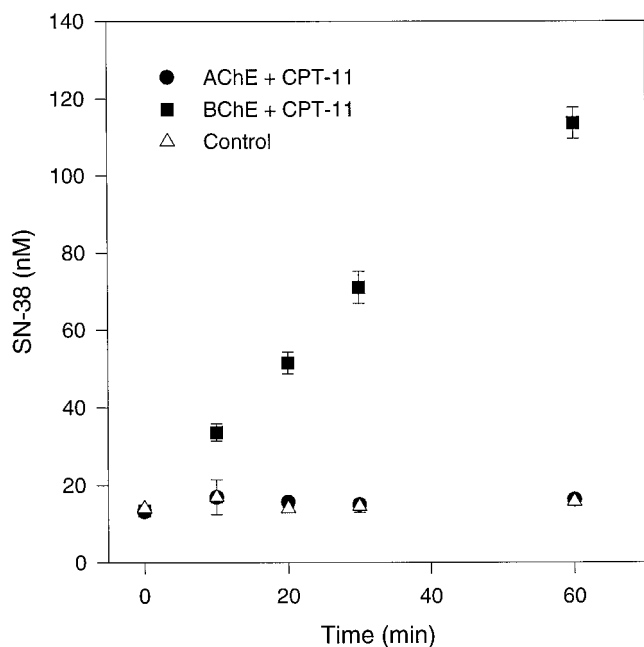
Fig. 7. Conversion of 10 μM CPT-11 to SN-38 by electric eel AChE ($\bullet$), horse BChE ($\blacksquare$), and control ($\triangle$) as a function of time. Incubations were carried out in 0.1 M potassium phosphate buffer, pH 7.3, at 37°C.
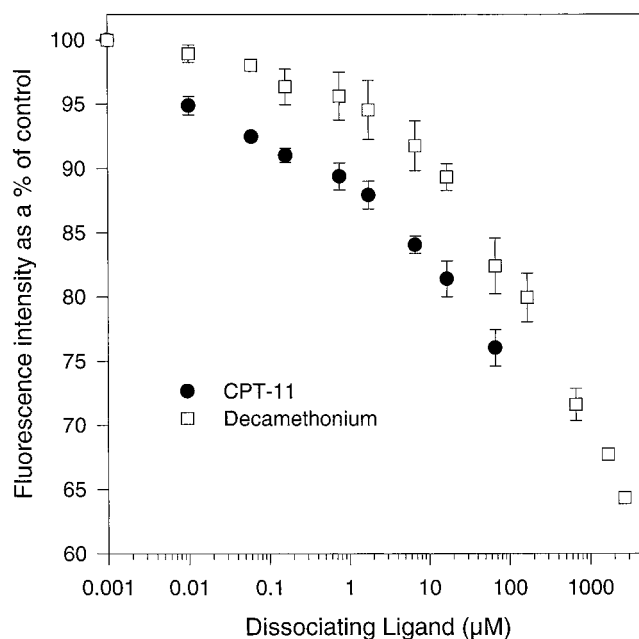

Fig. 8. Back-titration of the propidium-AChE complex (1.0 μM propidium, 2.0 μM electric eel AChE) with CPT-11 ($\bullet$) and decamethonium ($\square$) in 1 mM Tris-HCl, pH 8.0, at 25°C.

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