Journal of Enzyme Inhibition and Medicinal Chemistry, 2002 Vol. 17 (3), pp. 197–202

Inhibition of Butyrylcholinesterase by Phenothiazine Derivatives

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(Received 20 March 2002)

The inhibition of horse serum butyrylcholinesterase (EC 3.1.1.8) by 10 phenothiazine or thioxanthene derivatives was studied with a purified enzyme. Most compounds were mixed inhibitors, but for some of them an apparent competitive inhibition was observed. The competitive inhibition constants (K_i) were in the range 0.05 to 5 μM. The structures of the inhibitors were modeled by geometry optimization with the AM1 semi-empirical molecular orbital method and octanol/water partition coefficients were estimated with the CLOGP software. Quantitative structure–activity relationships identified lipophilicity, molecular volume, and electronic energies as the main determinants of inhibition. This quantitative model suggested hydrophobic and charge–transfer interactions of the phenothiazine ring with a tryptophan residue at the "anionic" site of the enzyme, and a hydrophobic interaction of the lateral chain with non-polar amino acids.

Keywords: Butyrylcholinesterase; Phenothiazine; Lipophilicity; Molecular modeling; Structure–activity relationships

INTRODUCTION

Phenothiazine and thioxanthene derivatives are used mainly as neuroleptics. They have, however, many other interesting properties such as antibacterial or anticancer activities[1] or the ability to inhibit cholinesterases, with a specificity for butyrylcholinesterase.[2,3] It has been suggested that butyrylcholinesterase inhibitors could be helpful in the treatment of Alzheimer's disease.[4] Relationships between the electronic structure of the phenothiazines and their antibacterial and anticancer activities have been investigated.[1] Such studies, however, are lacking for the anticholinesterase activity, for which few inhibition constants have been reported. We have therefore measured the inhibition constants of a purified butyrylcholinesterase by a series of 10 phenothiazine and thioxanthene derivatives, and related them to the physico-chemical properties of the compounds by means of quantitative structure–activity relationships.

MATERIAL AND METHODS

Enzyme Inhibition

All reagents were from Sigma. The structure of the inhibitors is presented in Table I. Butyrylcholinesterase from horse serum had a specific activity of 580 U/mg. The enzymatic hydrolysis of butyrylthiocholine was measured at 37°C with a modified Ellman's method[5] using dithiobis-2-nitrobenzoate (DTNB) as chromogenic agent. The reaction mixture contained 2 mL of a solution of 0.4 mM DTNB in phosphate buffer (0.05 M, pH 7.5), 0.1 mL of a solution of inhibitor in methanol, and 0.2 mL of an enzyme solution (about 1 U/mL). This mixture was incubated at 37°C for 2 min and the reaction was started by adding 0.2 mL of a substrate solution. The final concentrations were: methanol 4%, butyrylthiocholine 0.05 to 0.25 mM. The progress curve

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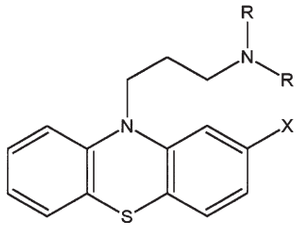
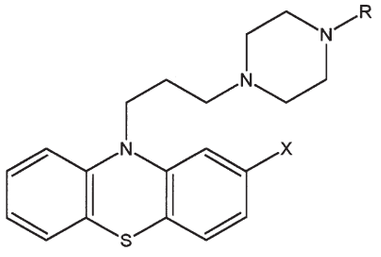
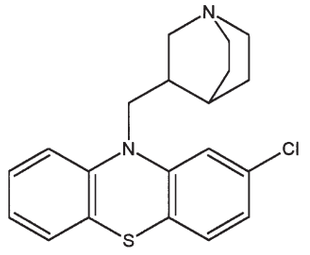
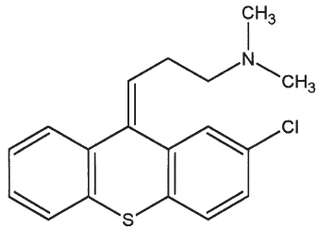
ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.1080/1475636021000003165

TABLE I Structure of inhibitors

Formula	No.	Name	X	R
	**1**	Promazine	H	CH_3
	2	Chlorpromazine	Cl	CH_3
	3	Triflupromazine	CF_3	CH_3
	4	Chlorproethazine	Cl	CH_2CH_3
	**5**	Perphenazine	Cl	CH_2CH_2OH
	6	Fluphenazine	CF_3	CH_2CH_2OH
	7	Prochlorperazine	Cl	CH_3
	8	Trifluoperazine	CF_3	CH_3
	**9**	Mequitazine		
	**10**	Chlorprothixene		

for the reaction was followed by monitoring the absorbance rise at 412 nm, due to the liberation of the thionitrobenzoate anion ($\varepsilon = 13,600\,\mathrm{L\,mol^{-1}\,cm^{-1}}$), in a Shimadzu UV-1205 spectrophotometer. Absorbance values were recorded every 0.5 s during 1 min and transferred to a microcomputer. Initial rates were computed by fitting a third-order polynomial to each progress curve. The competitive (K_i) and uncompetitive (K_i') inhibition constants were determined by unweighted non-linear regression, using our own program. Only the inhibition constants significant at the 5% level were retained.

Molecular Modeling

The compounds were modelized with HyperChem[6] or MOPAC[7] using the AM1 semi-empirical molecular orbital method with geometry optimization. The starting geometry of the compounds was chosen to be in agreement with previous molecular

modeling studies involving butyrylcholinesterase.[2] For all compounds, the following properties were computed on the refined structure:

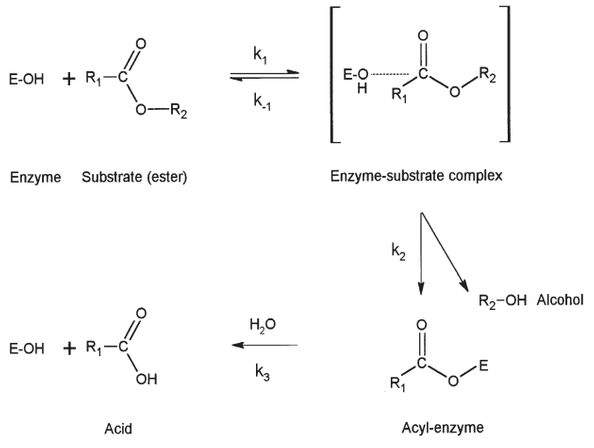
- Dipole moment (μ).
- Van der Waals volume (V).
- Energies of highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals.

In addition, the octanol/water partition coefficient ($\log P$), a common lipophilicity parameter, was estimated by Leo's CLOGP software.[8] Quantitative structure–activity relationships were computed by multiple linear regression, using our own software.[9]

RESULTS

Enzyme Inhibition

It is well known that the hydrolysis of an ester catalysed by a cholinesterase proceeds through an acyl-enzyme intermediate which reacts with water to regenerate the enzyme.[10]



The corresponding rate equation is:

$$v_0 = \frac{V_{\max}[S]_0}{K_m\left(1+\frac{[I]}{K_i}\right)+[S]_0\left(1+\frac{[I]}{K_i'}\right)}$$

where v_0 denotes the initial rate of hydrolysis, $[S]_0$ the initial substrate concentration, $[I]$ the inhibitor concentration, $V_{\max}$ the maximal velocity and K_m the Michaelis constant. The competitive inhibition constant (K_i) is the dissociation constant of the enzyme-inhibitor complex. It has been shown that for the hydrolysis of choline esters, $k_2 \gg k_3$, i.e. deacylation is rate-limiting, so that the enzyme-substrate complex does not accumulate. Hence, the uncompetitive inhibition constant (K_i') is usually regarded as reflecting the binding of the inhibitor to the acyl-enzyme only.[11]

Approximate values for the inhibition constants were obtained by graphical methods, using the Dixon plot for K_i and the Cornish–Bowden plot for K_i'. Representative plots for chlorproethazine are shown in Figure 1. The kinetic parameters ($V_{\max}$, K_m, K_i, K_i') were further refined by non-linear regression. Statistically significant K_i values were found for all phenothiazine derivatives; statistically significant K_i' values were found for 8 compounds out of 10. These inhibition constants are presented in Table II, where they are expressed as $pK_i = -\log K_i$ and $pK_i' = -\log K_i'$, where K_i and K_i' are the dissociation constants of the complexes, expressed in mol/L (this convention facilitates the comparison of compounds since pK_i and pK_i' increase when the inhibition increases). Most compounds were mixed inhibitors; however, in the case of prochlorperazine and chlorprothixene, the K_i' value could not be determined. The pK_i' value was almost always inferior to the pK_i value, showing that the inhibitors had less affinity for the acyl-enzyme than for the enzyme. This may be due to the steric hindrance of the butyryl group in the acyl-enzyme, and/or to a conformational change in the acyl-enzyme. In the case of chlorpromazine and mequitazine, pK_i and pK_i' were very close, resulting in an apparent non-competitive inhibition.

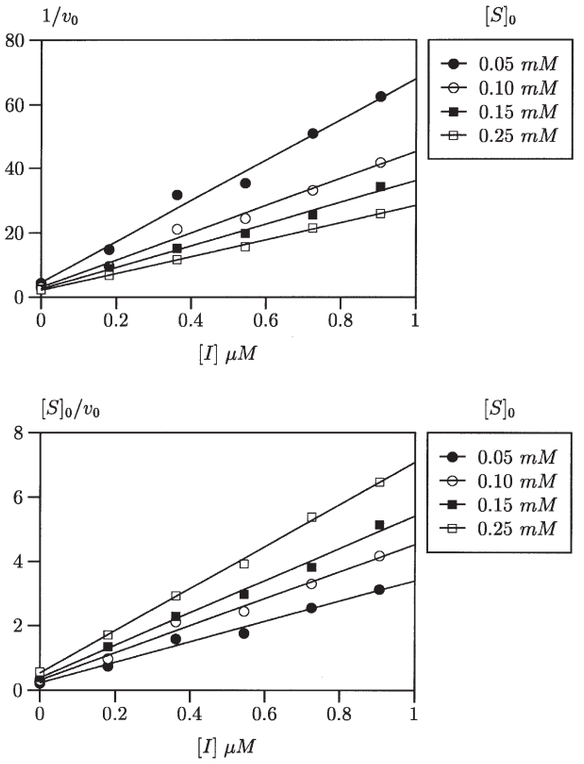


FIGURE 1 Dixon (upper) and Cornish–Bowden (lower) plots for the inhibition of butyrylcholinesterase by chlorproethazine. The substrate concentrations are shown in the legend. Initial rates (v_0) are expressed in U/(mL enzyme solution).

TABLE II Physico-chemical properties and cholinesterase-inhibiting activity of the phenothiazine derivatives*

Compound	E_{HOMO}	E_{LUMO}	μ	$V/100$	$\log P$	pK_i	pK_i'
Promazine	−7.62	−0.033	1.84	8.74	4.90	5.48	5.29
Chlorpromazine	−7.74	−0.280	1.43	9.16	5.80	5.67	5.7
Triflupromazine	−8.04	−0.644	3.16	9.52	6.11	5.62	5.34
Chlorproethazine	−7.77	−0.281	1.43	10.15	6.86	7.25	7.02
Perphenazine	−7.78	−0.299	1.74	11.41	4.32	5.60	5.26
Fluphenazine	−8.03	−0.662	3.31	11.77	4.63	5.28	5.46
Prochlorperazine	−7.77	−0.290	1.72	10.66	4.90	5.86	
Trifluoperazine	−8.02	−0.653	2.41	11.02	5.21	5.52	5.66
Mequitazine	−7.68	−0.088	0.40	9.10	5.21	6.08	6.01
Chlorprothixene	−7.84	−0.304	1.69	9.09	5.48	5.48	

* E_{HOMO}, E_{LUMO}: molecular orbital energies (eV); μ: dipole moment (D); V: molecular volume (Å^3); P: octanol/water partition coefficient (estimated with CLOGP 4.0); $pK_i = -\log K_i$, $pK_i' = -\log K_i'$ where K_i and K_i' are the competitive and uncompetitive inhibition constants expressed in mol/L.

Qualitative Structure–activity Relationships

The data of Table II allow a discussion of the influence of structure on the inhibiting properties of the compounds (in what follows, the numbers in boldface denote the compounds, and the corresponding pK_i values are shown in square brackets).

(1) Phenothiazine derivatives were active inhibitors of butyrylcholinesterase, mostly of the mixed type, although in some cases the difficulty of estimating the uncompetitive inhibition constant could result in an apparent competitive inhibitor.
(2) Ring substitution by Cl or CF_3 enhanced the binding affinity of the inhibitor (**1**/**2**/**3** [5.48/5.67/5.62]), but Cl was more efficient than CF_3 (**5**/**6** [5.60/5.28]; **7**/**8** [5.86/5.52]).
(3) Replacement of the N-dimethylamino group of chlorpromazine by diethylamino resulted in a strong increase in the binding affinity (e. g. **2**/**4** [5.67/7.25]). Chlorproethazine was indeed the strongest inhibitor in the series.
(4) Replacement of the terminal hydroxyethyl group of the piperazinyl phenothiazines by a methyl group resulted in an increased affinity (**5**/**7** [5.6/5.86], **6**/**8** [5.28/5.52]).
(5) The bulky group of mequitazine resulted in a strong inhibiting potency (**9** [6.08]).
(6) Replacement of the C–N bond of chlorpromazine by a C = C bond resulted in a slight decrease in the binding affinity (**2**/**10** [5.67/5.48]).

Quantitative Structure–activity Relationships

The best regression equations are presented in Table III. The results suggest that the inhibition depends mainly on the lipophilicity ($\log P$), molecular volume (V) and electronic properties (E_{HOMO}, E_{LUMO}) of the inhibitor. Using pK_i' instead of pK_i gave a similar relationship but the statistical significance was poorer, due to the reduced number of compounds for which pK_i' could be determined. The high correlation between E_{HOMO} and E_{LUMO} ($r = 0.987$) did not allow a distinction between the influences of these two parameters. We have therefore computed two separate relationships. Using E_{LUMO} had a slight statistical advantage but the interpretation was difficult since the coefficient of E_{LUMO} was positive, while a negative coefficient could be expected if the inhibition was dependent on the electron-accepting ability of the inhibitor (since E_{LUMO} decreases when this ability increases). On the other hand, the positive coefficient of E_{HOMO} suggested that the inhibition depends on the electron-donating potency of the inhibitor. So, the higher statistical significance of E_{LUMO} may be an artifact due to its correlation with E_{HOMO}. It seemed therefore justified to give the relationship with E_{HOMO}

TABLE III Structure–activity relationships of the phenothiazine derivatives*

Explained variable	$\log P$	$V/100$	E_{HOMO}	E_{LUMO}	c	r^2	s	F
pK_i	0.78 ± 0.11	0.39 ± 0.09	2.9 ± 0.6		20.3	0.90	0.21	19
	0.82 ± 0.09	0.45 ± 0.07		2.2 ± 0.3	−2.5	0.95	0.16	35
pK_i'	0.74 ± 0.19	0.37 ± 0.17	2.6 ± 1.1		18.3	0.85	0.35	5.6
	0.77 ± 0.16	0.41 ± 0.15		1.9 ± 0.7	−1.9	0.88	0.31	7.5

* The table gives the regression coefficients, with their standard deviations: c is the constant term, r^2 the coefficient of determination, s the residual standard deviation and F the variance ratio (ratio of explained variance to residual variance).

priority since its physical meaning was more straightforward. It was then possible to interpret our previous qualitative observations by computing the values of the three terms:

$$\Delta \mathrm{p}K_\mathrm{i}^{(\mathrm{hydrophob})} = 0.78 \log P;$$

$$\Delta \mathrm{p}K_\mathrm{i}^{(\mathrm{vol})} = 0.39(V/100);$$

$$\Delta \mathrm{p}K_\mathrm{i}^{(\mathrm{elec})} = 2.9\, \mathrm{E}_{\mathrm{HOMO}}$$

which represent, respectively, the contributions of hydrophobic interaction, volume effect and electronic interaction to the estimated inhibition constant ($\mathrm{p}K_\mathrm{i}$) of a given compound. The results showed that:

(1) Substitution of the phenothiazine ring by Cl increased the hydrophobic and volume effects but this was partly compensated by a decrease in the electronic interaction.
(2) Substitution by CF_3 resulted in a more important increase of the hydrophobic and volume effects, but this was almost completely compensated for by an important decrease in the electronic interaction. So, the overall effect of CF_3 substitution was weaker than the effect of Cl substitution.
(3) Replacement of the terminal dimethylamino group of chlorpromazine by a diethylamino group in chlorproethazine resulted in a marked increase in the hydrophobic and volume effects, without modification of the electronic term.
(4) Replacement of the terminal hydroxyethyl group (perphenazine, fluphenazine) by a methyl group (prochlorperazine, trifluoperazine) resulted in an important increase in the hydrophobic interaction, partly compensated for by the volume reduction, with no significant modification of the electronic interaction.
(5) In the case of mequitazine, both the hydrophobic and electronic interactions were facilitated, while the volume effect was slightly decreased.
(6) In the case of chlorprothixene, the hydrophobic interaction was facilitated, but at the expense of an important decrease in the electronic interaction, so that the overall effect was negative with respect to chlorpromazine.

DISCUSSION

Phenothiazines are known to be specific inhibitors of butyrylcholinesterase, but few inhibition constants have been reported so far.

Singh and Spassova[12] described a non-competitive inhibition of acetylcholinesterase by 4 phenothiazines including chlorpromazine and trifluoperazine. The inhibition constants were not reported. In our case, the inhibition was almost non-competitive since $\mathrm{p}K_\mathrm{i}$ and $\mathrm{p}K_\mathrm{i}'$ differed only slightly. This was in agreement with the classical view that phenothiazines bind mainly to the so-called "anionic" subsite of the enzyme, which is accessible in both the free enzyme and the acyl enzyme.[2,10] This subsite consists mainly of a tryptophan residue which can bind inhibitors by both hydrophobic and electronic (charge–transfer) interactions.

Radic *et al.*[2] used a cloned mouse butyrylcholinesterase to study a series of inhibitors including promazine and chlorpromazine, as well as two other phenothiazines, promethazine and ethopropazine, which were not included in our study. The measurements were carried out at pH 7.0 and 22°C. The authors found a mixed inhibition, and their K_i values agreed with ours, given the differences in enzyme sources and experimental conditions. These authors also found that ethopropazine (which has a N-diethylamino group) was more active than promethazine (which has a N-dimethylamino group); this is also in agreement with our results. Using a docking program, the same authors were able to build a molecular model of the ethopropazine–butyrylcholinesterase complex. This model suggested that the phenothiazine ring interacts with a tryptophan residue (at the so-called "anionic" subsite of the enzyme) and that the peripheral chain interacts with a phenylalanine residue. Our structure–activity relationships suggest that the phenothiazine ring interacts with the tryptophan by hydrophobic and electronic (charge–transfer) interactions, and that the peripheral chain interacts with a hydrophobic residue.

The binding of ethopropazine to butyrylcholinesterase was further studied by Saxena *et al.*,[3] using human butyrylcholinesterase and site-directed mutants. The authors showed that the inhibitor could bind in two different orientations, but that the binding energies were similar. This similarity justifies the approach taken in the present work, in which a single conformation was studied for each phenothiazine. Moreover, it has been shown by molecular dynamics simulation that the interconversion between the different conformers occurs spontaneously and rapidly in aqueous solution at room temperature.[13]

The structure–activity relationships developed in the present study contribute to the identification of the physico-chemical properties involved in the binding of phenothiazine to butyrylcholinesterase, and should be helpful to identify more active derivatives.

Acknowledgements

This work was supported in part by grants from the French *Ministère de la Santé* (Programme Hospitalier de Recherche Clinique, 2001) and from the *Fondation pour la Recherche Médicale*. The expert technical assistance of Jean-Hervé Comte is gratefully acknowledged.

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