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Structure–activity relationships for inhibition of human cholinesterases by alkyl amide phenothiazine derivatives

Sultan Darvesh,[a,b,c,*] Robert S. McDonald,[c] Andrea Penwell,[c] Sarah Conrad,[c] Katherine V. Darvesh,[c] Diane Mataija,[c] Geraldine Gomez,[c] Angela Caines,[c] Ryan Walsh[b] and Earl Martin[c]

[a]*Department of Medicine (Neurology), Dalhousie University, Halifax, Nova Scotia, Canada*
[b]*Department of Anatomy and Neurobiology, Dalhousie University, Halifax, Nova Scotia, Canada*
[c]*Department of Chemistry, Mount Saint Vincent University, Halifax, Nova Scotia, Canada*

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Abstract—Several lines of evidence indicate that inhibition of butyrylcholinesterase (BuChE) is important in the treatment of certain dementias. Further testing of this concept requires inhibitors that are both BuChE-selective and robust.

N-alkyl derivatives (**2**, **3**, **4**) of phenothiazine (**1**) have previously been found to inhibit only BuChE in a mechanism involving π–π interaction between the phenothiazine tricyclic ring system and aromatic residues in the active site gorge. To explore features of phenothiazines that affect the selectivity and potency of BuChE inhibition, a series of N-carbonyl derivatives (**5**–**25**) was synthesized and examined for the ability to inhibit cholinesterases.

Some of the synthesized derivatives also inhibited AChE through a different mechanism involving carbonyl interaction within the active site gorge. Binding of these derivatives takes place within the gorge, since this inhibition disappears when the molecular volume of the derivative exceeds the estimated active site gorge volume of this enzyme. In contrast, BuChE, with a much larger active site gorge, exhibited inhibition that increased directly with the molecular volumes of the derivatives. This study describes two distinct mechanisms for binding phenothiazine amide derivatives to BuChE and AChE. Molecular volume was found to be an important parameter for BuChE-specific inhibition.

1. Introduction

In Alzheimer's disease (AD), loss of cholinergic neurotransmission in the brain contributes to the salient cognitive and behavioral disturbances.[1–3] Loss of cholinergic cells is accompanied by reduced concentration of the neurotransmitter acetylcholine (ACh), and of one of its hydrolyzing enzymes, acetylcholinesterase (AChE EC 3.1.1.7).[1,3] In contrast, the level of the related enzyme, butyrylcholinesterase (BuChE EC 3.1.1.8), is elevated.[4] The use of cholinesterase inhibitors has been shown to be effective for the treatment of AD.[5,6] Clinical efficacy of these drugs is thought to result from inhibition of AChE, thus prolonging the availability of ACh for neurotransmission.[5,6] However, several lines of evidence suggest that inhibition of BuChE may also be important in the treatment of AD.[7–11] For example, inhibition of BuChE leads to an increased level of brain ACh.[12] Furthermore, high levels of BuChE are found associated with all the neuropathological lesions in AD.[13–15] In addition, BuChE is expressed in neurons in areas of the brain involved in cognition and behavior.[16,17] Also, AChE knockout mice are viable, indicating that BuChE is able to compensate for the absence of AChE.[18] Finally, all of the drugs that have shown efficacy in the treatment of AD inhibit both AChE and BuChE, albeit to different degrees.[19] For example, the K_i value for inhibition of AChE by donepezil is 0.024 μM while that for inhibition of BuChE is 2.21 μM. On the other hand, the K_i value for inhibition of AChE by tetrahydroaminoacridine is 0.136 μM, while that for inhibition of BuChE is 0.00528 μM.[19]

In order to further examine the importance of BuChE inhibition in the treatment of AD, highly selective BuChE inhibitors are being developed.[8,20–22] A number of phenothiazine derivatives have been shown to inhibit

* Corresponding author. Address: Room 1308, Camp Hill Veterans' Memorial Building 1, 5955 Veterans' Memorial Lane, Halifax, Nova Scotia, Canada B3H 2E1; e-mail: sultan.darvesh@dal.ca

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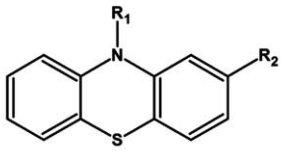
cholinesterases, especially BuChE (Fig. 1).[23,24] Furthermore, many phenothiazine derivatives, such as chlorpromazine (**3**) and ethopropazine (**4**) have been used in clinical practice to treat several diseases of the central nervous system, including schizophrenia,[25] and tremor.[26–28] This implies that a phenothiazine derivative that is a potent and highly selective BuChE inhibitor would be expected to be tolerated as a therapeutic agent.

In an attempt to develop phenothiazine derivatives that are selective inhibitors of BuChE, we sought to examine structure–activity relationships of inhibition of cholinesterases by phenothiazine derivatives. A series of N-carbonyl derivatives of phenothiazine were, therefore, synthesized. In this study, these N-10 alkyl amides were examined with respect to how structural variations of the derivatives affected potency and selectivity toward cholinesterase inhibition. Inhibitory potency was evaluated on the basis of inhibitor constants (K_i values), and the trends observed were considered with respect to calculated molecular parameters, namely, molecular volume and the angle of fold of the tricyclic ring system. Replacement of the hydrogen atom on the nitrogen of phenothiazine by a carbonyl led to AChE and BuChE inhibition. The AChE inhibition is observed to be restricted by the molecular volume of the alkyl amide phenothiazine and this restriction can be related to the smaller volume of the AChE active site gorge. On the other hand, the potency of BuChE inhibition exhibited a direct relationship with increasing molecular volumes of the phenothiazine amide derivatives. This work explores the structural features of N-10 amide derivatives of phenothiazine that influence the selectivity and potency of such compounds toward BuChE in particular, and toward human cholinesterases in general.

2. Results

2.1. Synthetic chemistry

The N-substituted phenothiazine amides were prepared by refluxing phenothiazine with the appropriate acid chloride (Fig. 2) and an equivalent amount of triethylamine in dichloromethane solution. Purification of the amides was performed by sequential chemically active extraction, silica gel column chromatography, and crystallization. Twenty phenothiazine derivatives were synthesized. Some of the derivatives synthesized (Fig. 2) have been described elsewhere.[29] In the present study, all the compounds were fully characterized. All purified compounds proved to be homogeneous by thin layer chromatographic analysis, and ^{1}H NMR revealed all of them to be more than 98% pure. All compounds were fully characterized by IR, and ^{1}H and ^{13}C NMR spectroscopy, as well as low and high-resolution mass spectrometry. Newly synthesized molecules were also further characterized by elemental analysis.



	R_1	R_2
1 Phenothiazine	H	H
2 N-methylphenothiazine	CH_3	H
3 Chlorpromazine	$CH_2CH_2CH_2N(CH_3)_2$	Cl
4 Ethopropazine	$CH_2CH(CH_3)N(CH_2CH_3)_2$	H

Figure 1. Structure of phenothiazine and several N-10-alkyl derivatives.

2.2. Biological evaluations

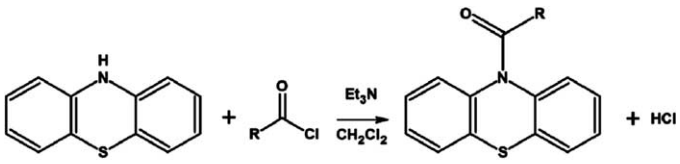
Each phenothiazine derivative was evaluated for its ability to inhibit BuChE and/or AChE using a modification[30] of Ellman's spectrophotometric method.[31] Initially, each derivative was examined at the highest concentration, depending on solubility limits (1–5 mM in 50% aqueous acetonitrile), to determine whether a compound inhibited BuChE, AChE, or both. Subsequently, serial dilution (1:10) profiles were generated for each compound to determine a range of concentrations suitable for kinetic studies. Lineweaver–Burk plots were then generated for each compound. A replot of slopes of these lines versus inhibitor concentration gave the inhibition constants (K_i; Table 1).

The enzyme kinetic studies with phenothiazine (**1**), N-methyl phenothiazine (**2**), chlorpromazine (**3**), and ethopropazine (**4**) (Fig. 1) showed that they inhibited BuChE, without any effect on AChE, at concentrations up to 0.167 mM (Table 1). The presence of larger N-alkyl amino groups, as in chlorpromazine (**3**) and ethopropazine (**4**), substantially improved inhibition of BuChE, over that of (**1**) or (**2**), as reflected by smaller K_i values (Table 1).

Of the 13 noncyclic amide derivatives synthesized (**5**–**17**), seven derivatives, (**5**–**8**, **11**, **16**, **17**) inhibited both BuChE and AChE (Table 1). Notably, the N-acetyl derivative (**5**) had an inhibition potency toward BuChE comparable to the parent, phenothiazine (**1**). However, in contrast to the parent phenothiazine (**1**), derivative **5** inhibited AChE as well, with a K_i value similar to that for inhibition of BuChE (Table 1). When the number of carbon atoms attached to the carbonyl group was increased, for example, acetyl (**5**) to propanoyl (**6**) to butanoyl (**7**), BuChE inhibition was increased, while inhibition toward AChE was diminished (Table 1). Derivatives with five or more carbon atoms attached to the N-position of phenothiazine showed marked improvements in BuChE inhibition but did not inhibit AChE, with the exception of derivative **11** (Table 1).

Of the seven derivatives with cyclic side chains, two (**18** and **19**), inhibited both AChE and BuChE (Table 1). Those analogs of this group with six or more carbon atoms attached to the N-position of phenothiazine inhibited BuChE only (Table 1).

The inhibition of BuChE by all the phenothiazine alkyl amides tested was consistently mixed noncompetitive in nature. In contrast, those derivatives that affected AChE displayed competitive inhibition of this enzyme.



	Derivative	R
5	Acetyl	CH_3
6	Propanoyl	CH_2CH_3
7	Butanoyl	$(CH_2)_2CH_3$
8	Methylpropanoyl	$CH(CH_3)_2$
9	Pentanoyl	$(CH_2)_3CH_3$
10	3-Methylbutanoyl	$CH_2CH(CH_3)_2$
11	Dimethylpropanoyl	$C(CH_3)_3$
12	Hexanoyl	$(CH_2)_4CH_3$
13	2-ethylbutanoyl	$CH(CH_2CH_3)_2$
14	3,3-Dimethylbutanoyl	$CH_2C(CH_3)_3$
15	Heptanoyl	$(CH_2)_5CH_3$
16	Chloroacetyl	CH_2Cl
17	Methoxyacetyl	CH_2OCH_3
18	Cyclopropyl carbonyl	
19	Cyclobutyl carbonyl	
20	Cyclopentyl carbonyl	
21	Cyclohexyl carbonyl	
22	Cycloheptyl carbonyl	
23	Cyclopentylacetyl	
24	Cyclohexylacetyl	

Figure 2. Synthetic scheme for the preparation of the phenothiazine derivatives and the structures of compounds synthesized.

Table 1. Inhibition constants (K_i values) and molecular properties of phenothiazine and derivatives

Compounds (see Fig. 1)	BuChE (K_i μM)	[a]AChE (K_i μM)	Relative potency BuChE:AChE	Molecular volume (Å^3)	'Butterfly' angle (°)
Phenothiazine (**1**)[b]	31.8 ± 10.4	None		212 ± 8	180
N-Methyl phenothiazine (**2**)[b]	36.9 ± 2.7	None		223 ± 3	160
Chlorpromazine (**3**)[b]	3.22 ± 1.82	None		340 ± 15	162
Ethopropazine (**4**)[b]	0.166 ± 0.05	None		363 ± 17	161
5	35.8 ± 2.5	38.9 ± 3.5	1.1	244 ± 9	159
6	25.6 ± 5.4	46.7 ± 10.3	1.8	271 ± 17	160
7	16.2 ± 3.5	30.4 ± 5.9	1.9	296 ± 9	157
8	13.2 ± 2.7	36.2 ± 4.9	2.7	299 ± 23	161
9	10.2 ± 1.5	None		315 ± 12	158
10	6.46 ± 1.11	None		318 ± 17	157
11	6.33 ± 0.18	29.5 ± 4.5	4.7	308 ± 7	154
12	6.37 ± 0.44	None		327 ± 11	160
13	6.43 ± 1.22	None		342 ± 17	157
14	3.59 ± 0.47	None		323 ± 17	155
15	8.31 ± 1.07	None		358 ± 13	160
16	10.9 ± 3.9	79.8 ± 10.9	7.3	275 ± 8	165
17	29.6 ± 6.3	24.1 ± 2.4	0.8	281 ± 13	157
18	23.1 ± 0.8	44.8 ± 4.1	1.9	296 ± 9	147
19	4.8 ± 0.56	55.6 ± 24.7	11.6	309 ± 23	165
20	4.21 ± 0.4	None		315 ± 10	162
21	1.26 ± 0.22	None		343 ± 11	159
22	0.86 ± 0.16	None		362 ± 18	163
23	2.22 ± 0.40	None		334 ± 22	160
24	1.68 ± 0.23	None		365 ± 27	159

[a] For all the compounds for which the K_i value is listed as 'none', there was no detectable inhibition at concentrations of the phenothiazine derivative up to 1.67×10^{-4} M, or to the solubility limit of the compound.
[b] Reference compounds.

In summary, phenothiazine and derivatives with an alkyl side chain attached directly to the nitrogen of the ring inhibited BuChE only. Addition of a carbonyl carbon at this position of the ring led to inhibition of both BuChE and AChE, as long as the substituent linked to the carbonyl was small.

The K_i values for the inhibition of BuChE ranged from $0.166 \pm 0.05\,\mu M$ to $36.9 \pm 2.7\,\mu M$. For those derivatives that also inhibited AChE, the K_i values ranged from $24.1 \pm 2.4\,\mu M$ to $79.8 \pm 10.9\,\mu M$. The BuChE-specific K_i values of some of the synthesized derivatives, such as cyclopeptyl carbonyl (**22**) and cyclopeptyl acetyl (**24**) (Table 1) compared favorably with drugs used to treat Alzheimer's disease (donepezil; AChE = $0.024 \pm 0.0071\,\mu M$ and BuChE = $2.21 \pm 0.37\,\mu M$), galantamine; AChE = $0.520 \pm 0.031\,\mu M$ and BuChE = $2.09 \pm 0.78\,\mu M$ and tetrahydroaminoacridine; AChE = $0.136 \pm 0.027\,\mu M$ and BuChE = $0.00528 \pm 0.00037\,\mu M$).[19]

2.3. Calculations of molecular parameters

Previous observations[24] have suggested that the large molecular volume of the phenothiazine derivative ethopropazine (**4**) is a significant factor in determining its BuChE-specificity. That is, the active site gorge of BuChE, but not of AChE, can accommodate this molecule. On the other hand, the nonplanar nature of the phenothiazine ring system, which has been described as having a 'butterfly' shape[32] may also be important. Variations in the angle between the two aromatic rings, an angle which will be referred to as the 'butterfly' angle, could also play a role in determining inhibitor specificity and potency. The 'butterfly' angle and total molecular volume of each derivative were calculated to determine whether either or both parameters were related to selectivity and inhibition potency. When calculating molecular parameters such as the 'butterfly' angle and the molecular volume, there are a number of possible methods available. One such method that is suited for a series of mid-sized molecules such as ours, is molecular mechanics. In this method, the energy of the compound is expressed as a sum of contributions involving the geometrical parameters of bond-stretching, bond-bending, torsion, and nonbonded interactions. The specific function chosen, along with the choice of parameters used, together describe the force field. The conformer having the lowest energy was obtained using the MMFF94 force field (PC Spartan Pro, Wavefunction, Inc., 18401 Von Karman, Suite 370, Irvine, California, 92612).

2.4. 'Butterfly' angles of the phenothiazine derivatives

Before calculating molecular parameters for all the derivatives, the molecular mechanics calculation method we chose was compared to other available methods, namely, Hartree-Fock/STO-3G and B3LYP/6-31G(d)[33–35] using two simpler compounds, namely, phenothiazine (**1**) and acetyl phenothiazine (**5**) (Fig. 3), employing the Gaussian 98 suite of programs.[36] 'Butterfly' angles and computed energies of the derivatives, whose geometries were optimized in the above manner, are presented in Table 2.

The B3LYP/6-31G(d) results obtained for phenothiazine (**1**) in the present study (Table 1) were in accord with the work at the B3LYP level carried out by Palafox et al.[37] They reported a calculated 'butterfly' angle of 152.2° for phenothiazine (**1**), in comparison with crystal

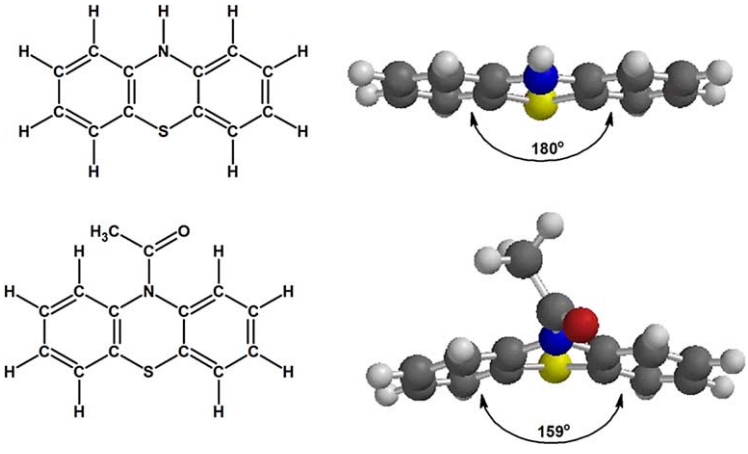


Figure 3. 'Butterfly' angles for phenothiazine (**1**) and acetyl phenothiazine (**5**).

Table 2. Comparison of calculation methods for 'butterfly' angles

Method	Energy (hartree) Phenothiazine **1**	Energy (hartree) Acetylphenothiazine **5**	'Butterfly' angle (°) Phenothiazine **1**	'Butterfly' angle (°) Acetylphenothiazine **5**
MM	—		180	159
HF/STO-3G	−901.0197	−1050.8336	165	147
B3LYP/6-31G(d)	−915.6436	−1068.2925	150	135

structure results of 158.5° for the orthorhombic form[38] and 153.3° for the monoclinic form.[39] As indicated in Table 2, while molecular mechanics provided 'butterfly' angles that were closer to planar than other methods, it did reproduce the trend of 'butterfly' angle decrease upon N-substitution. For this reason, in all subsequent calculations on the phenothiazine derivatives, geometry optimizations based on the best conformer were carried out at the molecular mechanics level. 'Butterfly' angles for phenothiazine (**1**) and the analogs considered in this study varied from approximately 180° for phenothiazine (**1**) itself to approximately 160° for most compounds, to a low value of 145° for the cyclopropyl carbonyl derivative (**18**) (Table 1). The nature of the molecular mechanics method is such that the same calculated 'butterfly' angle will be obtained every time calculations on a given molecule are carried out. In this sense, precision is much higher than the significant figures of the reported value would suggest (Table 1). However, as is noted above, there are variations of up to 17% between calculated results and crystallographic data. No consistent pattern of 'butterfly' angle trends emerged that would explain the observed variations in inhibitor potency and selectivity for these derivatives.

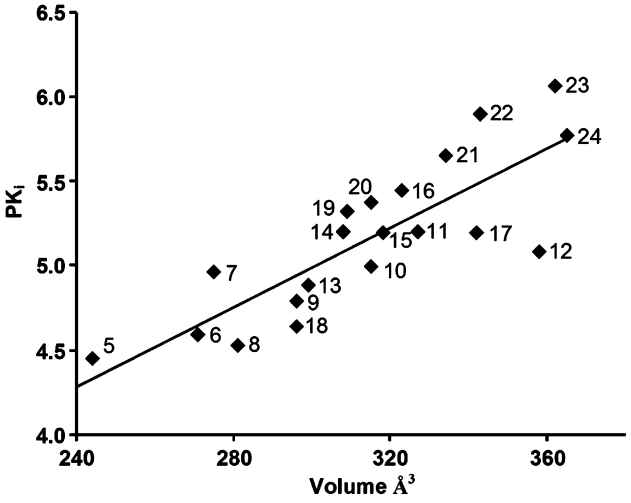


Figure 4. Relationship between molecular volume and potency of inhibition of butyrylcholinesterase by N-carbonyl phenothiazine derivatives. $-\log K_I$ (pK_I) is plotted against calculated molecular volumes. The linear regression equation was applied to determine the line of best fit and follows the equation $pK_I = 0.0118 \times \text{volume} + 1.4602$ ($R^2 = 0.68$).

2.5. Molecular volumes of the phenothiazine derivatives

Molecular volume was expected to play a key role in the inhibitory properties of each molecule as has been suggested for ethopropazine (**4**).[23] One way to represent the molecular volume is to select a surface of fixed electron density (0.001 e/bohr^3, in this case) and to compute the volume within that isodensity surface.[40] Ab initio methods were required in order to obtain an electron density surface. Accordingly, single-point Hartree-Fock/STO-3G calculations were carried out starting from the atomic coordinates obtained at the molecular mechanics level. Molecular volumes computed in this manner, based on an average of five separate calculations, are summarized in Table 1 and range from 212 Å^3 for phenothiazine (**1**) itself to 365 Å^3 for the cyclohexylacetyl derivative (**24**). This parameter was found to be the major determinant for BuChE-specificity and inhibitor potency for the N-carbonyl amides studied here (Fig. 4).

3. Discussion

Molecular modeling, site-directed mutagenesis, and X-ray crystallographic studies have revealed a number of distinct domains associated with the active sites of cholinesterases.[23,41–46] Within each of these domains, there are amino acid side chain clusters that control substrate specificity and inhibitor binding.[23,44]

In both cholinesterases, a serine–histidine–glutamate catalytic triad is located near the bottom of a 20 Å deep gorge (Fig. 5).[41,46–49] Another domain is the 'acyl pocket' that holds the alkyl substituent attached to the carbonyl of the substrate. In AChE, this pocket has two bulky phenylalanine residues, F295 and F297, and thus is only able to accommodate the small methyl moiety of acetylcholine. In BuChE, however, these residues are replaced by two smaller amino acid residues, L286 and V288, allowing for accommodation of bigger substituent groups, such as those in butyrylcholine, succinylcholine, and benzoylcholine, to be held in place for catalysis. A further important domain is found at the lip of the active site gorge and is referred to as the peripheral anionic site (Fig. 5). The amino acid side chain cluster of this domain in AChE consists of Y72, D74, Y124, W286, and Y341.[43] This domain is involved in substrate inhibition in AChE.[42] In BuChE, the amino acid residues at the equivalent positions are N68, D70, Q119, A277, and Y332 and are involved in substrate activation in this enzyme.[50] These substitutions at the periphery of the gorge in BuChE provide a larger opening for substrate and inhibitor molecules to enter the active site gorge.

Another domain located within the active site gorge is the cation-π domain (Fig. 5). This is made up of two amino acid residues, W86 and Y337, in AChE, with Y337 being replaced by A328 in BuChE. These residues, especially the conserved W86 (W82 in BuChE), have been shown to be important in stabilizing the quaternary ammonium moiety of choline esters.[41,42] As shown previously,[23] ethopropazine is able to bind to BuChE by means of π–π interaction between the heterocyclic ring system and amino acid residues Y332 and F329 in the active site gorge. The amino acid residue Y337 in AChE has been shown to interfere with this π–π binding.[23] Replacement of this amino acid residue by A328 in BuChE alleviates the interference with π–π interactions for phenothiazine and its derivatives. Further stabilization of the interaction of BuChE and ethopropazine (**4**) appears to involve an electrostatic attraction between the anionic side chain of D70 in the enzyme and the cationic side chain nitrogen of the inhibitor.[23] These combined ligands provide a robust inhibitor constant for ethopropazine (**4**) (Table 1). It is clear that the amide

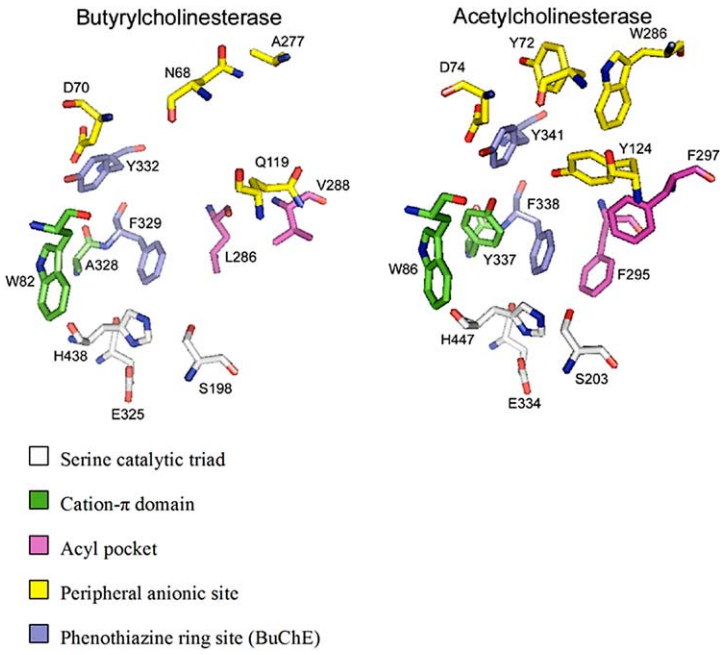


Figure 5. Active-site gorge of cholinesterases. The figure was generated using the Pymol program and crystal structures available in the protein database.[45–48]

derivatives do not have the side chain nitrogen as in ethopropazine (**4**) to provide additional binding at D70. This gives some of the amide derivatives inhibitor properties less robust than ethopropazine (**4**). On the other hand, in spite of the cationic side chain nitrogen being absent, compound (**22**) which has a molecular volume comparable to ethopropazine (**4**) (Table 1) has a K_i value that is not very different from that of ethopropazine (**4**) (Table 1).

The inability of many phenothiazine derivatives to inhibit AChE is also related to the small size of the AChE active site gorge volume (302.31 Å^3) compared to that of BuChE (501.91 Å^3).[22] This undoubtedly plays a key role in determining specificity toward BuChE inhibition by certain phenothiazine derivatives such as ethopropazine (**4**) whose van der Waals volume, calculated to be 317.6 Å^3, is larger than the volume of the AChE active site gorge.[23] It should be noted that our calculated volume of ethopropazine (**4**) differs somewhat from the volume reported by Saxena et al.[23] (363 Å^3 vs 317.6 Å^3, respectively). This discrepancy is due to the different methods used in obtaining the volumes, but does not alter the principle that the relative volumes of inhibitor and active site gorge are important.

In the present study, molecular volume is also observed to be a key factor in determining potency of BuChE inhibition and the selectivity toward BuChE and AChE.

3.1. Butyrylcholinesterase inhibition

All of the phenothiazine amides reported here inhibit BuChE, as do the parent (**1**) and the N-alkyl substituted phenothiazines (**2**, **3**, **4**) (Table 1). Direct comparison between the 10-alkyl and 10-acyl derivatives is problematic, however, since several molecular parameters (electron density in the tricyclic ring system, presence of a polar carbonyl group, molecular volume) are changing synchronously. Within the amide series only, it is clear that BuChE inhibition in general becomes more potent as the size of the alkyl group or cycloalkyl ring increases. That the calculated molecular volume is a good predictor of potency of BuChE inhibition is borne out by the graph of pK_i versus molecular volume (Fig. 4). The reasonably linear correlation between these parameters illustrates that an increase in the molecular volume by about 50% (**5**–**22**) causes a decrease in K_i value by about 40-fold. It should be noted that the volumes of all our derivatives are well below the estimated active site gorge volume for BuChE (502 Å^3).[23] Two observations provide compelling evidence that phenothiazine derivative enter the active site gorge, namely, a linear correlation between increasing volume and inhibitor potency, and the earlier observation of π–π interaction between the phenothiazine moiety and aromatic amino acid residues within the active site gorge.[23]

All of our amide derivatives show mixed noncompetitive inhibition with BuChE, implying that both substrate and inhibitor are able to bind to BuChE simultaneously.

There are several noteworthy differences in the specificity and potency of inhibition of AChE by the phenothiazine amides, which point toward a fundamental difference in the inhibition of this enzyme.

3.2. Acetylcholinesterase inhibition

Perhaps the most significant aspect of the present study, in terms of understanding different modes of interaction with inhibitors, is the observation of AChE inhibition by the phenothiazine amides with small alkyl or cycloalkyl groups attached to the N-carbonyl. To our knowledge,

this represents the first report of human AChE inhibition by phenothiazine derivatives. However, this observation is not due solely to the small molecular volume of these derivatives since neither the parent phenothiazine (**1**) nor its N-methyl derivative (**2**), both of which are smaller than the N-acetyl derivative (Table 1), inhibit AChE. Clearly, AChE inhibition must be a result of the N-carbonyl moiety, either a direct result of the carbonyl group acting as a hydrogen bond acceptor or the indirect influence of the electron-withdrawing carbonyl on the electron density in the tricyclic ring system (vide infra). In contrast to the BuChE inhibition results, and with the exception of the 10-chloroacetyl derivative (**16**), there appears to be no systematic trend in K_i (AChE) with structure of the alkyl or cycloalkyl group since all K_i values fall within the range $40 \pm 16\,\mu M$. The 10-chloroacetyl derivative (**16**), has a K_i value twice that of the average above. Examination of the spectral data for this compound (see Experimental) clearly reveals that the electron-withdrawing nature of the chlorine has a much larger effect on the proximate carbonyl group than it does on the more remote phenothiazine ring. The carbonyl thus becomes a much poorer hydrogen bond acceptor and this leads to weaker AChE inhibition. Both kinetic analysis and spectral studies point to the 10-carbonyl group as the significant new factor in these amide derivatives that permit AChE inhibition, possibly through hydrogen bonding at a side chain group such as that of Y124, and this, in conjunction with the molecular volume limit for inhibition (vide infra) as well as the competitive nature of that inhibition, indicates that these derivatives enter the active site gorge for binding.

Careful examination of the AChE inhibition results in Table 1 reveals that inhibition disappears dramatically when the molecular volume of the derivative increases above a certain critical volume. A fascinating illustration of this fact occurs with the series of derivatives with five-carbon side chains. The straight-chain pentanoyl derivative (**9**) has a molecular volume of $315\,Å^3$ while the branched-chain derivatives, 3-methylbutanoyl (**10**) and dimethylpropanoyl (**11**), have molecular volumes of 318 and $308\,Å^3$, respectively. Of these three, only the dimethylpropanoyl derivative (**11**) was found to inhibit AChE. Thus, there appears to be a critical volume limit of about $310\,Å^3$ above which no AChE inhibition is observed, presumably because the derivatives will not fit into the active site gorge. It is intriguing that this limit is remarkably close to the AChE gorge volume ($302\,Å^3$), estimated from X-ray crystallographic data.[23]

One other molecular parameter, the 'butterfly' angle, was investigated regarding its role in determining the presence of AChE inhibition. The fact that both the N-methyl (**2**) and the N-acetyl derivatives have the same 'butterfly' angle (~160°) while only the latter derivative inhibits AChE appears to discount the significance of the 'butterfly' angle in determining the presence of AChE inhibition.

In contrast to the noncompetitive inhibition of BuChE by these phenothiazine amides, those that inhibit AChE do so competitively, which implies mutually exclusive interactions. This indicates that the active site gorge of AChE is blocked to the substrate when the enzyme–inhibitor complex is formed, and vice versa.

It is clear from this study that phenothiazine amide derivatives do enter the active site gorge of human cholinesterases and that binding to BuChE involves a distinct mechanism from that involved for inhibition of AChE. In the former case the phenothiazine tricyclic ring system provides the major ligand while for AChE the amide carbonyl provides the point of interaction. Molecular volume is an important factor in both cases and provides a limiting parameter for exclusion of AChE inhibition. Thus, molecular volume of the phenothiazine amide derivatives provides a simple parameter for obtaining BuChE-specific inhibition and increasing the potency of inhibition toward this enzyme.

Phenothiazine derivatives are a class of heterocyclic synthetic compounds, many of which, such as chlorpromazine (**3**) and ethopropazine (**4**) have been primarily used for treatment of neurological disorders.[25–28] Interestingly, ethopropazine (**4**) has also been associated with improved cognitive function.[51] It is known that phenothiazine derivatives are well tolerated as pharmaceutical agents. Thus, derivatives of phenothiazine that are inhibitors of cholinesterases may have a potential for use in the treatment of dementias. This may be especially true of many compounds that are both specific and potent inhibitors of BuChE. Furthermore, these compounds have the potential to clarify further the biological functions of BuChE.

4. Experimental section

4.1. Materials

Butyrylcholinesterase (BuChE, EC 3.1.1.8) from human plasma was obtained from Roche. Recombinant human acetylcholinesterase (AChE, EC 3.1.1.7), acetylthiocholine, butyrylthiocholine, chloropromazine, 5,5′-dithiobis(2-nitrobenzoic acid)(DTNB), ethopropazine, phenothiazine were purchased from Sigma (St. Louis, MO). Acid chlorides were purchased from Aldrich Chemical Co. Inc. (Milwaukee, Wi) and used without further purification. For those unavailable commercially, acid chlorides were prepared from the corresponding acids using oxalyl chloride according to the procedure below.

4.2. Chemistry

4.2.1. General. Melting points were determined on a Mel-Temp II apparatus and are uncorrected. Thin layer chromatography was carried out using silica gel sheets with fluorescent indicator (0.20 mm thickness; Macherey–Nagel) and dichloromethane or a dichloromethane/ethyl acetate mixture as developing solvent. Plates were visualized using a short wavelength UV lamp. Infrared spectra were recorded as Nujol mulls between sodium chloride plates on a Nicolet Model 205 FT-IR spectrometer. Peak positions were obtained in the 'Peak

Pick' mode, and were reproducible within 1–2 cm^{-1}. Nuclear magnetic resonance spectra were recorded at the Atlantic Region Magnetic Resonance Centre at Dalhousie University on a Bruker AC-250F spectrometer, operating at 250.1 MHz for proton and 62.9 MHz for carbon. Chemical shifts are reported in ppm relative to TMS, in $CDCl_3$ solution. Mass spectra were recorded at Dalhousie University on a CEC 21-110B spectrometer using electron ionization at 70 V and an appropriate source temperature with samples being introduced by means of a heatable port probe. Accurate mass measurements were also made on this machine operated at a mass resolution of 8000 by computer controlled peak matching to appropriate PFK reference ions. Mass measurements were routinely within 3 ppm of the calculated value. Elemental microanalysis of all new compounds was performed by Guelph Chemical Laboratories Ltd, Guelph, ON, Canada.

4.3. Synthesis

4.3.1. General method for N-substituted phenothiazine amides. A solution containing phenothiazine (5.1 mmol), acid chloride (12.5–25 mmol), and triethylamine (5.0 mmol) in dichloromethane (50 mL) was refluxed with stirring until TLC analysis revealed that all phenothiazine was consumed. Reaction periods ranged from 1 h to 19 days. The cooled reaction mixture was then washed successively with 5% aqueous sodium bicarbonate (3 × 40 mL), 5% hydrochloric acid (3 × 40 mL) followed by water (40 mL). The solution was dried ($MgSO_4$), filtered, and solvent removed under vacuum. The crude solid product was then routinely purified by column chromatography using silica gel 60 (63–200 μ) (Caledon Laboratories Ltd) as adsorbant and dichloromethane or a dichloromethane/ethyl acetate mixture as eluent. Fractions containing only the desired product were combined, the solvent evaporated, and the solid recrystallized from petroleum ether/dichloromethane (2:1).

4.3.2. General method for acid chloride preparation. To a solution of carboxylic acids (12.5–25 mmol) in benzene (40 mL) was added dropwise a 2–3-fold excess of oxalyl chloride in benzene (10 mL). The reaction mixture was stirred for 1 day at room temperature at which time the solvent and excess oxalyl chloride were removed on the rotary evaporator. The residual acid chloride was used directly in the phenothiazine amide synthesis (see above).

4.4. Analytical data

4.4.1. Acetyl phenothiazine (5). Using 3 equiv of acid chloride, after a 1 day reflux period, workup, followed by column chromatography, and recrystallization afforded a 57.6% yield of colorless crystals. Mp 202–203 °C (lit. mp 204 °C[52]). IR (Nujol): 1671, 1318, 1259, 1237, 1029, 1012, 765, 729 cm^{-1}. ^1H NMR ($CDCl_3$) 2.20 (s, 3H), 7.22 (d of t, J = 7.6, 1.5 Hz, 2H), 7.32 (d of t, J = 7.6, 1.5 Hz, 2H), 7.43 (d of d, J = 7.6, 1.5 Hz, 2H), 7.50 (broad d, $J \sim 7.6$ Hz, 2H). ^{13}C NMR ($CDCl_3$): 23.13, 126.91, 127.08, 127.28, 128.04, 133.10, 139.02, 169.46. EIMS (low res): 241 (M^+), 200, 199 (base), 198, 167, 166, 154, 127, 69, 43. EIMS (high res): M^+ (obs) 241.0560; calcd for $C_{14}H_{11}NOS$ = 241.0561.

4.4.2. Propanoyl phenothiazine (6). Using 2 equiv of acid chloride, after 18 h of reflux, workup, followed by column chromatography, and recrystallization afforded a 49.6% yield of colorless crystals. Mp 85–87 °C (lit. mp 87–88 °C[53]). IR (Nujol): 1677, 1254, 1237, 1181, 1126, 965, 733, 725 cm^{-1}. ^1H NMR ($CDCl_3$): 1.10 (t, J = 7.5 Hz, 3H), 2.46 (q, J = 7.5 Hz, 2H), 7.19 (d of t, J = 7.6, 1.5 Hz, 2H), 7.30 (d of t, J = 7.6, 1.5 Hz, 2H), 7.41 (d of d, J = 7.6, 1.5 Hz, 2H), 7.49 (d of d, J = 7.6, 1.5 Hz, 2H). ^{13}C NMR ($CDCl_3$): 9.57, 27.96, 126.79, 126.98, 127.34, 127.99, 133.28, 138.91, 173.02. EIMS (low res): 255 (M^+), 200, 199 (base), 198, 167, 166, 154, 127, 69, 57, 29. EIMS (high res): M^+ (obs) 255.0705; calcd for $C_{15}H_{13}NOS$ = 255.0718.

4.4.3. Butanoyl phenothiazine (7). Using 2.5 equiv of acid chloride, after a 3 h reflux period, workup, followed by column chromatography, and recrystallization afforded a 49.4% yield of colorless crystals. Mp 87.5–90.5 °C (lit. mp 94 °C[54]). IR (Nujol): 1678, 1298, 1283, 1249, 1180, 768, 755 cm^{-1}. ^1H NMR ($CDCl_3$): 0.86 (t, J = 7.3 Hz, 3H), 1.62 (sextet, J = 7.3 Hz, 2H), 2.43 (t, J = 7.3 Hz, 2H), 7.20 (d of t, J = 7.6, 1.5 Hz, 2H), 7.30 (d of t, J = 7.6, 1.5 Hz, 2H), 7.43 (d of d, J = 7.6, 1.5 Hz, 2H), 7.50 (broad d, J = 7.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): 13.98, 19.03, 36.53, 127.02, 127.22, 127.61, 128.25, 133.57, 139.18, 172.37. EIMS (low res): 269 (M^+), 200, 199, 198 (base), 154, 71, 69, 50, 42. EIMS (high res): M^+ (obs) = 269.0871; calcd for $C_{16}H_{15}NOS$ = 269.0874.

4.4.4. Methylpropanoyl phenothiazine (8). Using 2.5 equiv of acid chloride, after a 3 h reflux period, workup, followed by column chromatography, and recrystallization produced a 42.9% yield of colorless crystals. Mp 147–149 °C (lit. mp 143–144 °C[29]). IR (Nujol): 1674, 1458, 1388, 1272, 1251, 769, 756, 734 cm^{-1}. ^1H NMR ($CDCl_3$): 1.08 (d, J = 6.7 Hz, 6H), 3.05 (septet, J = 6.7 Hz, 1H), 7.20 (d of t, J = 7.6, 1.5 Hz, 2H), 7.30 (d of t, J = 7.6, 1.5 Hz, 2H), 7.43 (d of d, J = 7.6, 1.5 Hz, 2H), 7.50 (d of d, J = 7.6, 1.5 Hz, 2H). ^{13}C NMR ($CDCl_3$): 19.64, 30.95, 126.73, 126.97, 127.21, 128.02, 133.55, 139.00, 176.59. EIMS (low res): 269 (M^+), 200, 199, 198, 154, 127, 71, 69, 45, 43 (base), 41, 39, 27. EIMS (high res): M^+ (obs) 269.0874; calcd for $C_{16}H_{15}NOS$ = 269.0874.

4.4.5. Pentanoyl phenothiazine (9). Using 3 equiv of acid chloride, after 4 days of reflux, workup followed by column chromatography, and recrystallization afforded a 19.2% yield of colorless crystals. Mp 87.5–91.5 °C (lit. mp 93 °C[55]). IR (Nujol): 1673, 1279, 1254, 1178, 1105, 769, 755, 732 cm^{-1}. ^1H NMR ($CDCl_3$): 0.81 (t, J = 7.2 Hz, 3H), 1.26 (m, 2H), 1.57 (m, 2H), 2.46 (t, J = 7.5 Hz, 2H), 7.22 (d of t, J = 7.6, 1.5 Hz, 2H), 7.32 (d of t, J = 7.6, 1.5 Hz, 2H), 7.44 (d of d, J = 7.6, 1.5 Hz, 2H), 7.50 (br d, $J \sim 7.6$ Hz, 2H). ^{13}C NMR ($CDCl_3$): 13.75, 22.18, 27.43, 33.97, 126.73, 126.93, 127.33, 127.96, 133.32, 138.96, 172.29. EIMS (low res):

283 (M^+), 200, 199 (base), 198, 167, 166, 154, 85. EIMS (high res): M^+ (obs) 283.1037; calcd for $C_{17}H_{17}NOS$ = 283.1031. Anal. ($C_{17}H_{17}NOS$): calcd, C 72.05, H 6.05, N 4.94, S 11.32; found, C 72.49, H 6.37, N 4.98, S 11.24.

4.4.6. 3-Methylbutanoyl phenothiazine (10). Using 2.5 equiv of acid chloride, after a 2.5 h reflux period, workup, followed by column chromatography, and recrystallization gave a 67.8% yield of slightly off-white crystals. Mp 157.5–160 °C. IR (Nujol): 1676, 1310, 1250, 767, 755 cm^{-1}. ^1H NMR ($CDCl_3$): 0.87 (d, J = 6.7 Hz, 6H), 2.10 (monet, J = 6.7 Hz, 1H), 2.38 (d, J = 6.7 Hz, 2H), 7.21 (d of t, J = 7.6, and 1.5 Hz, 2H), 7.32 (d of t, J = 7.6, 1.5 Hz, 2H), 7.44 (d of d, J = 7.6, 1.5 Hz, 2H), 7.51 (broad d, J = 7.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): 22.53, 25.91, 43.07, 126.79, 126.98, 127.47, 128.04, 133.47, 139.04, 171.63. EIMS (low res): 283 (M^+), 238, 200, 199 (base), 198, 167, 166, 154, 127, 85, 84, 87, 69, 57. EIMS (high res): M^+ (obs) 283.0999; calcd for $C_{17}H_{17}NOS$ = 283.1031. Anal. ($C_{17}H_{17}NOS$): calcd, C 72.05, H 6.05, N 4.94, S 11.32; found, C 71.71, H 6.25, N 4.62, S 11.15.

4.4.7. Dimethylpropanoyl phenothiazine (11). Using 5 equiv of acid chloride, after 19 days of reflux, a small quantity of unreacted phenothiazine remained. Workup followed by column chromatography, and recrystallization afforded 18.1% yield of colorless crystals. Mp 119–120 °C (lit. mp 150–152 °C[29]). IR (Nujol): 1670, 1297, 1285, 1257, 1223, 1152, 1126, 1029, 771, 754, 729, 667 cm^{-1}. ^1H NMR ($CDCl_3$): 1.13 (S, 9H), 7.20 (d of t, J = 7.6, 1.5 Hz, 2H), 7.30 (d of t, J = 7.6, 1.5 Hz, 2H), 7.45 (d of d, J = 7.6, 1.5 Hz, 2H), 7.60 (d of d, J = 7.6, 1.5 Hz, 2H). ^{13}C NMR ($CDCl_3$): 29.16, 41.64, 126.56, 126.77, 127.76, 128.06, 134.83, 140.78, 178.10. EIMS (low res): 283 (M^+, base), 200, 199, 198, 167, 166, 85, 57, 40, 29. EIMS (high res): M^+ (obs) 283.1025; calcd for $C_{17}H_{17}NOS$ = 283.1031.

4.4.8. Hexanoyl phenothiazine (12). Using 2.1 equiv of acid chloride, after a 3 h reflux period, workup, followed by column chromatography, and recrystallization afforded a 33.4% yield of colorless crystals. Mp 124–126 °C. IR (Nujol): 1682, 1322, 1260, 1170, 1125, 764, 758, 724 cm^{-1}. ^1H NMR ($CDCl_3$): 0.78–0.85 (m, 3H), 1.17–1.27 (m, 4H), 1.53–1.65 (m, 2H), 2.45 (t, J = 7.5 Hz, 2H), 7.20 (d of t, J = 7.6, 1.5 Hz, 2H), 7.31 (d of t, J = 7.6, 1.5 Hz, 2H), 7.42 (d of d, J = 7.6, 1.5 Hz, 2H), 7.50 (br d, J = 7.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): 14.18, 22.64, 25.31, 31.49, 34.53, 127.02, 127.22, 127.61, 128.25, 133.60, 139.21, 172.60. EIMS (low res): 297 (M^+), 200, 199 (base), 198, 167, 166, 154, 42. EIMS (high res): M^+ (obs) 297.1197; calcd for $C_{18}H_{19}NOS$ = 297.11187. Anal. ($C_{18}H_{19}NOS$): calcd, C 72.69, H 6.44, N 4.71, S 10.78; found, C 72.26, H 6.81, N 4.40, S 10.53.

4.4.9. 2-Ethylbutanoyl phenothiazine (13). Using 2 equiv of acid chloride, after a 1 day reflux period, workup, followed by column chromatography, and recrystallization afforded a 50.7% yield of colorless crystals. Mp 79.5–81 °C (lit. mp 85–86 °C[29]). IR (Nujol): 1665, 1328, 1307, 1282, 1246, 1179, 1148, 1127, 1029, 768, 753, 653 cm^{-1}. ^1H NMR ($CDCl_3$): 0.88 (br m, 6H), 1.3–1.8 (br m, 4H), 2.7–2.8 (m, 1H), 7.20 (d of t, J = 7.6, 1.5 Hz, 2H), 7.31 (d of t, J = 7.6, 1.5 Hz, 2H), 7.43 (d of d, J = 7.6, 1.5 Hz, 2H), 7.50 (br d, J = 7.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): 12.15, 26.03, 44.90, 126.88, 127.05, 127.84, 128.21, 133.95, 139.18, 175.69. EIMS (low res): 297 (M^+), 200, 199, 198 (base), 171, 166, 154, 127, 99, 71, 69, 55. EIMS (high res): M^+ (obs) 297.1196; calcd for $C_{18}H_{19}NOS$ = 297.1187.

4.4.10. 3,3-Dimethylbutanoyl phenothiazine (14). Using 5 equiv of acid chloride, after a 1 h reflux period, workup, followed by column chromatography, and recrystallization afforded a 45.6% yield of pale green crystals. Mp 125–128 °C. IR (Nujol): 1670, 1252, 1233, 1194, 1125, 1029, 761, 751, 728 cm^{-1}. ^1H NMR ($CDCl_3$): 0.95 (s, 9H), 2.45 (s, 2H), 7.21 (d of t, J = 7.6, 1.5 Hz, 2H), 7.32 (d of t, J = 7.6, 1.5 Hz, 2H), 7.40 (d of d, J = 7.6, 1.5 Hz, 2H), 7.50 (br d, J = 7.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): 29.72, 31.56, 45.41, 126.65, 126.90, 127.56, 128.06, 133.60, 139.13, 170.76. EIMS (low res): 297 (M^+), 200, 199 (base), 198, 167, 166, 154, 99, 71, 57. EIMS (high res): M^+ (obs) 297.1186; calcd for $C_{18}H_{19}NOS$ = 297.1187. Anal. ($C_{18}H_{19}NOS$): calcd, C 72.69, H 6.44, N 4.71, S 10.78; found, C 72.31, H 6.88, N 4.46, S 10.36.

4.4.11. Heptanoyl phenothiazine (15). Using 2 equiv of acid chloride, after a 5 day reflux period, workup, followed by flash chromatography, and recrystallization afforded a 47.8% yield of colorless crystals. Mp 110.5–112 °C. IR (Nujol): 1682, 1311, 1303, 1256, 1172, 763, 724 cm^{-1}. ^1H NMR ($CDCl_3$): 0.82 (t, J = 6.9 Hz, 3H), 1.1–1.3 (m, 6H), 1.58 (m, 2H), 2.45 (t, J = 7.5 Hz, 2H), 7.20 (d of t, J = 7.6, 1.4 Hz, 2H), 7.31 (d of t, J = 7.6, 1.4 Hz, 2H), 7.43 (d of d, J = 7.6, 1.4 Hz, 2H), 7.50 (br d, J = 7.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): 14.13, 22.54, 25.40, 28.82, 31.57, 34.36, 126.81, 127.01, 127.42, 128.04, 133.41, 139.04, 172.40. EIMS (low res): 311 (M^+), 200, 199 (base), 198, 167, 166, 154, 42. EIMS (high res): M^+ (obs) 311.1332; calcd for $C_{19}H_{21}NOS$ = 311.1344. Anal. ($C_{19}H_{21}NOS$): calcd, C 73.27, H 6.80, N 4.50, S 10.30; found, C 72.99, H 7.19, N 4.17, S 9.96.

4.4.12. Chloroacetyl phenothiazine (16). Using 2.5 equiv of acid chloride, after a 2 h reflux period, workup, column chromatography, and recrystallization gave a 49.5% yield of off-white crystals. Mp 115–117 °C (lit. mp 113.5–114.5 °C[56]). IR (Nujol): 1693, 1672, 1348, 1336, 1249, 1170, 1126, 1031, 761, 650 cm^{-1}. ^1H NMR ($CDCl_3$): 4.19 (s, 2H), 7.27 (d of t, J = 7.6, 1.5 Hz, 2H), 7.36 (d of t, J = 7.6, 1.5 Hz, 2H), 7.47 (d of d, J = 7.6, 1.5 Hz, 2H), 7.59 (d of d, J = 7.6, 1.5 Hz, 2H). ^{13}C NMR ($CDCl_3$): 41.97, 126.59, 127.37, 127.45, 128.16, 133.17, 137.88, 165.51. EIMS (low res): 277 M^+ (^{37}Cl), 275 M^+ (^{35}Cl), 200, 199, 198 (base), 171, 166, 165, 154, 127, 84, 77, 69, 49. EIMS (high res): M^+ (obs) 275.0178; calcd for $C_{14}H_{10}NOS^{35}Cl$ = 275.0172.

4.4.13. Methoxyacetyl phenothiazine (17). Using 2.2 equiv of acid chloride, after a 21 h reflux period,

workup, column chromatography, and recrystallization gave rise to a 56.2% yield of slightly off-white crystals. Mp 135–136.5 °C. IR (Nujol): 1685, 1677, 1315, 1301, 1285, 1257, 1124, 938, 769, 753, 724, 654 cm^{-1}. ^1H NMR ($CDCl_3$): 3.40 (s, 3H), 4.15 (s, 2H), 7.24 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.33 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.45 (d of d, $J = 7.6$, 1.5 Hz, 2H), 7.53 (br d, $J = 7.6$ Hz, 2H). ^{13}C NMR ($CDCl_3$): 59.52, 71.19, 126.95, 127.43 (two peaks), 128.28, 133.27, 138.22, 168.32. EIMS (low res): 271 (M^+), 199, 198 (base), 154, 127, 69, 45. EIMS (high res): M^+ (obs) 271.0668; calcd for $C_{15}H_{13}NO_2S = 271.0667$. Anal. ($C_{15}H_{13}NO_2S$): calcd, C 66.40, H 4.83, N 5.16, S 11.82; found, C 65.91, H 4.73, N 4.72, S 11.66.

4.4.14. Cyclopropyl carbonyl phenothiazine (18). Using 2 equiv of acid chloride, after a 3.5 day reflux period, workup, followed by column chromatography, and recrystallization afforded a 72.2% yield of off-white crystals. Mp 117–119 °C (lit. mp 112–114 °C[29]). IR (Nujol): 1672, 1395, 1256, 1173, 762, 752 cm^{-1}. ^1H NMR ($CDCl_3$): 0.77–0.89 (m, 2H), 1.16–1.25 (m, 2H), 1.78–1.91 (m, 1H), 7.21 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.32 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.44 (d of d, $J = 7.6$, 1.5 Hz, 2H), 7.62 (d of d, $J = 7.6$, 1.5 Hz, 2H). ^{13}C NMR ($CDCl_3$): 9.77, 12.93, 126.59, 126.84, 127.26, 127.92, 132.90, 138.88, 172.31. EIMS (low res): 267 (M^+), 200, 199 (base), 198, 154, 69, 40. EIMS (high res): M^+ (obs) 267.0710; calcd for $C_{16}H_{13}NOS = 267.0718$.

4.4.15. Cyclobutyl carbonyl phenothiazine (19). Using 2.2 equiv of acid chloride, after a 1 day reflux period, workup, followed by column chromatography, and recrystallization afforded a 68.5% yield of off-white crystals. Mp 145–147.5 °C. IR (Nujol): 1670, 1275, 1254, 1170, 765 cm^{-1}. ^1H NMR ($CDCl_3$): 1.7–1.9 (m, 4H), 2.2–2.4 (m, 2H), 3.48 (br pentet, $J = 8$ Hz, 1H), 7.19 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.30 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.40 (d of d, $J = 7.6$, 1.5 Hz, 2H), 7.48 (br d, $J = 7.6$ Hz, 2H). ^{13}C NMR ($CDCl_3$): 17.55, 25.57, 38.17, 126.72, 126.97 (two peaks), 127.79, 133.18, 138.76, 174.24. EIMS (low res): 281 (M^+), 200, 199 (base), 198, 197, 167, 154, 127, 83, 69, 54, 38. EIMS (high res): M^+ (obs) 281.0871; calcd for $C_{17}H_{15}NOS = 281.0874$. Anal. ($C_{17}H_{15}NOS$): calcd, C 72.57, H 5.37, N 4.98, S 11.40; found, C 72.10, H 5.49, N 4.75, S 11.12.

4.4.16. Cyclopentyl carbonyl phenothiazine (20). Using 2.6 equiv of acid chloride, after a 1 day reflux period, workup, followed by column chromatography, and recrystallization afforded a 65.5% yield of very pale green crystals. Mp 141–142.5 °C. IR (Nujol): 1670, 1583, 1309, 1250, 764, 751 cm^{-1}. ^1H NMR ($CDCl_3$): 1.4–1.9 (m, 8H), 3.13 (pentet, $J = 7.7$ Hz, 1H), 7.22 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.30 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.44 (d of d, $J = 7.6$, 1.5 Hz, 2H), 7.53 (d of d, $J = 7.6$, 1.5 Hz, 2H). ^{13}C NMR ($CDCl_3$): 26.35, 30.96, 41.73, 126.67, 126.93, 127.30, 127.94, 133.59, 139.12, 176.07. EIMS (low res): 295 (M^+), 200, 199 (base), 198, 166, 154, 69. EIMS (high res): M^+ (obs) 295.1021; calcd for $C_{18}H_{17}NOS = 295.1031$. Anal. ($C_{18}H_{17}NOS$): calcd, C 73.19, H 5.80, N 4.74, S 10.85; found, C 72.80, H 5.86, N 4.33, S 10.72.

4.4.17. Cyclohexyl carbonyl phenothiazine (21). Using 3 equiv of acid chloride, after a 1 h reflux period, workup, followed by column chromatography, and recrystallization afforded a 29.4% yield of off-white crystals. Mp 157–158.5 °C (lit. mp 148–150 °C[29]). IR (Nujol): 1677, 1316, 1272, 1252, 1231, 1165, 1122, 1029, 969, 767, 753, 658 cm^{-1}. ^1H NMR ($CDCl_3$): 1.0–1.8 (overlapping multitriplets, 10H), 2.72 (t of t, $J = 11.3$, 3.3 Hz, 1H), 7.21 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.31 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.43 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.50 (d of t, $J = 7.6$, 1.5 Hz, 2H). ^{13}C NMR ($CDCl_3$): 25.45, 25.53, 29.44, 40.95, 126.60, 126.81, 127.08, 127.94, 133.42, 138.92, 175.34. EIMS (low res): 309 (M^+), 200, 199 (base), 198, 171, 167, 166, 154, 127, 111, 83, 69, 55. EIMS (high res): M^+ (obs) 309.1202; calcd for $C_{19}H_{19}NOS = 309.1187$.

4.4.18. Cycloheptyl carbonyl phenothiazine (22). Using 3 equiv of acid chloride, after a 5 day reflux period, workup, followed by column chromatography, and recrystallization afforded a 44.8% yield of pale green crystals. Mp 100–102.5 °C. IR (Nujol): 1674, 1308, 1242, 1152, 769, 759, 658 cm^{-1}. ^1H NMR ($CDCl_3$): 1.2–2.0 (overlapping multiplets, 12H), 2.91 (m, 1H), 7.20 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.30 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.42 (d of d, $J = 7.6$, 1.5 Hz, 2H), 7.49 (br d, $J = 7.6$ Hz, 2H). ^{13}C NMR ($CDCl_3$): 26.55, 28.35, 31.51, 41.66, 126.73, 126.95, 127.39, 128.05, 133.65, 139.08, 176.73. EIMS (low res): 323 (M^+), 199 (base), 198, 171, 166, 154, 127, 97, 69, 55, 41. EIMS (high res): M^+ (obs) 323.1339; calcd for $C_{20}H_{21}NOS = 323.1344$. Anal. ($C_{20}H_{21}NOS$): calcd, C 74.27, H 6.54, N 4.33, S 9.91; found, C 73.82, H 6.53, N 4.37, S 9.75.

4.4.19. Cyclopentylacetyl phenothiazine (23). Using 3 equiv of acid chloride, after a 2 h reflux period, workup, followed by column chromatography, and recrystallization afforded a 50.8% yield of colorless crystals. Mp 152–154 °C. IR (Nujol): 1668, 1334, 1315, 1259, 1116, 1029, 765, 730 cm^{-1}. ^1H NMR ($CDCl_3$): 0.9–1.2 (m, 2H), 1.4–1.6 (m, 4H), 1.7–1.9 (m, 2H), 2.22 (m, 1H), 2.50 (d, $J = 7.0$ Hz, 2H), 7.21 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.32 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.44 (d of d, $J = 7.6$, 1.5 Hz, 2H), 7.50 (br d, $J = 7.6$ Hz, 2H). ^{13}C NMR ($CDCl_3$): 24.98, 32.51, 36.86, 40.28, 126.80, 127.01, 127.48, 128.05, 133.46, 139.05, 171.94. EIMS (low res): 309 (M^+), 200, 199 (base), 198, 171, 166, 154, 127, 83, 69, 67, 55, 41. EIMS (high res): M^+ (obs) = 309.1193; calcd for $C_{19}H_{19}NOS = 309.1187$. Anal. ($C_{19}H_{19}NOS$): calcd, C 73.75, H 6.19, N 4.53, S 10.36; found, C 73.27, H 6.22, N 4.05, S 10.28.

4.4.20. Cyclohexylacetyl phenothiazine (24). Using 3 equiv of acid chloride, after a 1 h reflux period, workup, followed by column chromatography, and recrystallization afforded a 64.5% yield of colorless crystals. Mp 173–174 °C. IR (Nujol): 1688, 1326, 1259, 1100, 770, 729 cm^{-1}. ^1H NMR ($CDCl_3$): 0.7–2.0 (overlapping

multiplets, 11H), 2.36 (d, $J = 7.0$ Hz, 2H), 7.21 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.32 (d of t, $J = 7.6$, 1.5 Hz, 2H), 7.43 (d of d, $J = 7.6$, 1.5 Hz, 2H), 7.51 (broad d, $J = 7.6$ Hz, 2H). ^{13}C NMR ($CDCl_3$): 26.08, 26.19, 33.03, 35.26, 41.58, 126.91, 127.41, 127.95, 133.37, 139.00, 171.57. EIMS (low res): 323 (M^+), 200, 199 (base), 198, 167, 166, 154, 97, 83, 54. EIMS (high res): M^+ (obs) 323.1334; calcd for $C_{20}H_{21}NOS$ = 323.1344. Anal. ($C_{20}H_{21}NOS$): calcd, C 74.27, H 6.54, N 4.33, S 9.91; found, C 73.98, H 6.62, N 3.90, S 9.74.

4.5. Biological evaluation

4.5.1. Enzyme kinetic studies. The esterase activity of AChE and BuChE was determined by a modification[30] of the method described by Ellman et al.[31] Briefly, 2.7 mL of buffered DTNB solution (pH 8.0), 0.1 mL of AChE (0.03 units) or BuChE (0.05 units) in 0.005% aqueous gelatin and 0.1 mL of 50% aqueous acetonitrile or one of the phenothiazine derivative dissolved in this solvent, in a quartz cuvette of 1 cm path length. Serial dilutions of each compound in 50% acetonitrile were tested for the ability to inhibit either AChE or BuChE. The mixture was zeroed at 412 nm, and the reaction was initiated by the addition of acetylthiocholine or butyrylthiocholine in an aqueous solution at a final concentration of 1.6×10^{-4} M. The reactions were performed at 23 °C. The rate of change of absorbance (ΔA/min), reflecting the rate of hydrolysis of acetylthiocholine or butyrylthiocholine, was recorded every 5 s for 1 min, using a Milton-Roy 1201 UV–visible spectrophotometer (Milton-Roy, Ivyland, PA) set at $\lambda = 412$ nm. These experiments were generally done at least in triplicate and the values averaged. Lineweaver–Burk plots were generated by using a fixed amount of cholinesterase and varying amounts of substrate (3×10^{-5}–1.6×10^{-4} M) in the presence or absence of the inhibitors. The re-plot of the slopes of the above double reciprocal plots against inhibitor concentration gave the inhibitor constant (K_i) as the intercept on the x-axis.

4.6. Calculation of molecular parameters

Molecular mechanics calculations were carried out using the MMFF94 force field (PC Spartan Pro, Wavefunction, Inc., 18401 Von Karman, Suite 370, Irvine, California, 92612). Hartree-Fock and density functional theory calculations were carried out using the Gaussian 98 suite of programs.[36]

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