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Differential binding of phenothiazine urea derivatives to wild-type human cholinesterases and butyrylcholinesterase mutants

Sultan Darvesh [a,b,c,d,*], Ian R. Pottie [d], Katherine V. Darvesh [d], Robert S. McDonald [d], Ryan Walsh [b], Sarah Conrad [d], Andrea Penwell [d], Diane Mataija [d], Earl Martin [d]

[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, Dalhousie University, Halifax, Nova Scotia, Canada
[d] Department of Chemistry, Mount Saint Vincent University, Halifax, Nova Scotia, Canada

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ABSTRACT

A series of *N*-10 urea derivatives of phenothiazine was synthesized and each compound was evaluated for its ability to inhibit human cholinesterases. Most were specific inhibitors of BuChE. However, the potent inhibitory effects on both cholinesterases of one sub-class, the cationic aminoureas, provide an additional binding mechanism to cholinesterases for these compounds. The comparative effects of aminoureas on wild-type BuChE and several BuChE mutants indicate a binding process involving salt linkage with the aspartate of the cholinesterase peripheral anionic site. The effect of such compounds on cholinesterase activity at high substrate concentration supports ionic interaction of aminoureas at the peripheral anionic site.

1. Introduction

Phenothiazines are heterocyclic molecules containing two benzene rings linked in a tricyclic system through nitrogen and sulfur atoms (Fig. 1). The first known member of this class of compounds, the purple dye thionine, was synthesized by Lauth in 1876, followed by the synthesis of another dye, methylene blue (Fig. 1), by Caro in 1877. The evidence that both thionine and methylene blue contained a phenothiazine tricycle was provided when the parent compound, phenothiazine, was prepared and characterized by Bernthsen in 1883.[1,2] Since the late 19th century, many phenothiazine derivatives have been synthesized and have proven to be highly eclectic in their biological activities.[1,2] Phenothiazines have been used as dyes, as biological stains and as pharmaceutical agents to treat a variety of diseases. Chlorpromazine (Fig. 1), for example, was one of the first compounds used as a neuroleptic to treat symptoms of psychosis.[1] The earlier phenothiazine derivative, methylene blue, in addition to many other applications,[3] has been used as a treatment for parasitic, bacterial and viral infections, for cancer treatment and, most recently, for the treatment of dementias, such as Alzheimer's disease (AD).[4]

The mechanism producing the positive effect of methylene blue in AD is thought to relate, at least in part, to its interference with the aggregation of β-amyloid and tau,[4] the neuropathological hallmarks of this disease. Another prominent feature of AD is cholinergic dysfunction.[5–7] In this regard, phenothiazine derivatives, in general, are known to inhibit cholinesterases.[8–13] Other drugs, including amitriptyline and morphine (Fig. 1) also inhibit cholinesterases,[14–18] but certain phenothiazines tend to be more selective inhibitors of butyrylcholinesterase (BuChE, EC 3.1.1.8) over acetylcholinesterase (AChE, EC 3.1.1.7).[8–13] In recent years, compounds with the ability to selectively inhibit BuChE have gained interest for the treatment of AD. It has been reported that selective BuChE inhibitors raise acetylcholine levels in the brains of animals.[19] Cholinesterase inhibition is deemed important because diminished brain acetylcholine appears to give rise to the dominant cognitive and behavioural symptoms of AD. Supporting this notion is the observation that treatment with cholinesterase inhibitors alleviates such symptoms.[20] The drugs of this type that are in current use for the treatment of AD, such as, donepezil, galantamine and rivastigmine, produce the inhibition of both AChE and BuChE, although to different degrees.[21] Since both hydrolytic enzymes are still present in AD brains, it is not clear whether the positive symptomatic relief produced by these drugs results mainly from AChE inhibition, BuChE inhibition or the combined inhibition of both enzymes. A comparative study of the effect of a highly selective inhibitor of BuChE, and one selective for AChE inhibition,

* Corresponding author. Address: Room 1308, Camp Hill Veterans' Memorial Building 1, 5955 Veterans' Memorial Lane, Halifax, Nova Scotia, Canada B3H 2E1. Tel.: +1 902 473 2490; fax: +1 902 473 7133.
E-mail address: sultan.darvesh@dal.ca (S. Darvesh).

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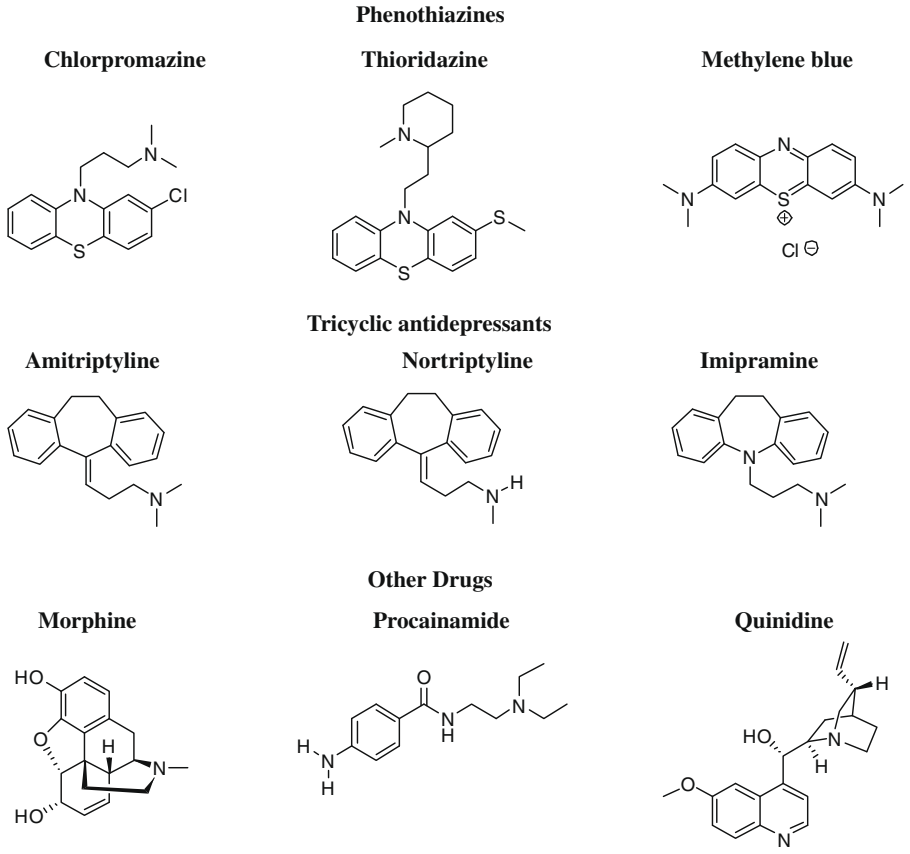


Figure 1. Structure of phenothiazines and other drugs that inhibit cholinesterases.

with those that inhibit both, may help solve this dilemma by demonstrating greater efficacy with one type of treatment. The observations that BuChE activity is elevated in AD, while AChE activity is diminished[22,23] and that BuChE activity predominates in regions of the brain involved in cognition and behaviour,[24,25] may favour the use of BuChE-specific inhibitors in treating AD.[26]

We have previously reported the synthesis of phenothiazine N-10-amide derivatives that are highly selective inhibitors of human BuChE[11,12] and a series of comparable phenothiazine carbamates[27] some of which inhibit only AChE.

Here we report the synthesis of a series of phenothiazine-N-10-urea derivatives which, for the most part, tend to be, like the corresponding amides, selective BuChE inhibitors. However, one sub-class of this series, the substituted aminoureas, is found to be able to produce potent inhibition of both BuChE and AChE. We provide evidence, through the direct use of BuChE mutant species and the effect of elevated substrate on wild-type human cholinesterases, that the aminoureas have a distinct mechanism for binding to cholinesterases, not evident with the other urea derivatives or the amide and carbamate derivatives of phenothiazine. Instead, these aminourea inhibitors have some features in common with cationic phenothiazines like methylene blue.[4,13,28]

2. Results and discussion

A total of 37 urea derivatives of phenothiazine were prepared from phenothiazine-10-carbonyl chloride and a variety of amines, as depicted in the reaction schemes in Figure 2. Of these, 22 compounds (see Section 4) have been synthesized previously. One amide derivative, 1-adamantylamide (compound **38**, see Section 4) was prepared earlier[11] but was not reported then, since it did not inhibit AChE or BuChE. That compound is reported here for structural comparison with the corresponding urea derivative (**14**), which is a robust inhibitor of BuChE (Table 1). Urea products were derived from alkyl, cycloalkyl and aryl amines, as well as certain substituted diamines, giving compounds that represent two general categories, ureas (Tables 1–3) and aminoureas (Table 4). Each compound was then tested for its ability to inhibit AChE and BuChE using a modification[21,27] of Ellman's spectrophotometric method.[29]

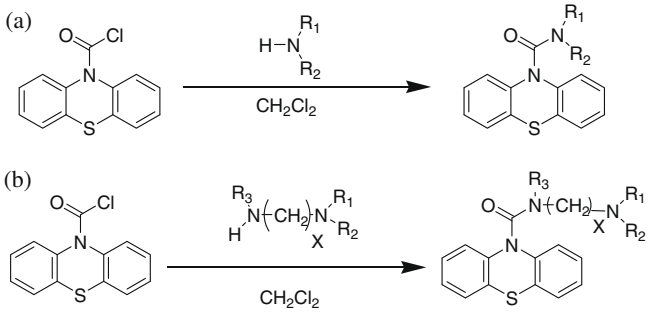


Figure 2. Synthetic schemes: (a) synthesis of phenothiazine ureas; (b) synthesis of phenothiazine aminoureas.

2.1. Synthetic chemistry

All the reactions were carried out by mixing phenothiazine-10-carbonyl chloride with an appropriate amine (Fig. 2a) or diamine (Fig. 2b), in dichloromethane. The mixture was stirred at room temperature and the reaction was monitored by thin layer chromatography until all the starting material was consumed or no change

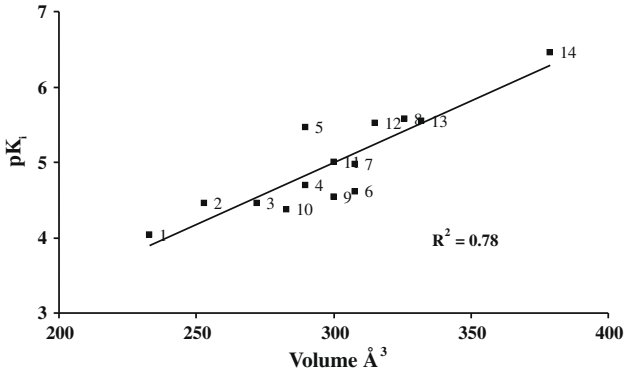


Figure 3. A plot of extent of BuChE inhibition (pK_i) versus molecular volume, of a series of phenothiazine alkyl ureas, demonstrating a linear relationship between potency of BuChE inhibition and molecular volume. Note that the best inhibitor of this series has the largest volume.

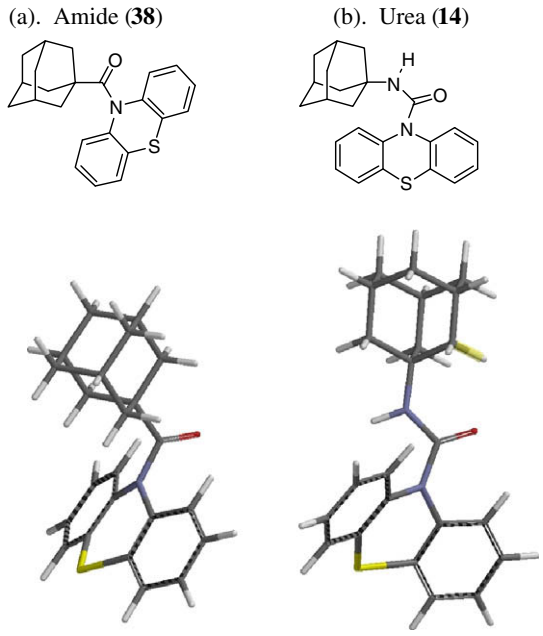


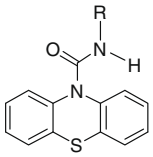
Figure 4. Structural and conformational differences between urea (**14**) and amide (**38**) phenothiazine adamantyl derivatives that determine the ability of these compounds to inhibit butyrylcholinesterase. The urea derivative (**14**) exhibits greater flexibility, permitting the phenothiazine tricycle to interact with the butyrylcholinesterase active site gorge, thus inhibiting the enzyme catalysis. The amide derivative (**38**) is more rigid and prevents the phenothiazine tricycle interaction with the enzyme. The lower figures representing the most stable conformers of these two compounds were generated using SPARTAN'06.[30]

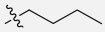
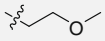
in the reaction mixture could be discerned. After workup, the products were purified by crystallization from petroleum ether/dichloromethane mixtures. All compounds were fully characterized by IR, ^{1}H and ^{13}C NMR spectroscopy, as well as low- and high-resolution (accurate mass) mass spectrometry. Physical data were consistent with the structure of each molecule synthesized. HPLC analysis of all purified compounds showed them to be more than 98% pure (see Supplementary data).

2.2. Enzyme kinetic studies

Each phenothiazine derivative was evaluated for its ability to inhibit purified human plasma BuChE and purified recombinant human AChE. Initially, each derivative was examined for cholinesterase inhibition at the highest inhibitor concentration, depending on solubility limits (up to 0.167 mM in 50% aqueous acetonitrile), and then through serial (1:10) dilutions in the same solvent to obtain an inhibition–concentration profile that indicated a range of inhibitor concentrations suitable for kinetic studies. Lineweaver–Burk plots were then generated for each compound in the absence of inhibitor and at two concentrations of inhibitor. A replot of slopes of these lines against inhibitor concentration gave the inhibition constant (K_i values, Tables 1–4) as the intercept on the *x*-axis. K_i represents the equilibrium constant for the dissociation of the enzyme–inhibitor complex (EI) into enzyme (E) and inhibitor (I) {EI → E + I, where $K_i = [E][I]/[EI]$}. A smaller K_i value indicates higher inhibitor potency. In certain of the data treatments, where indicated, the inhibitor affinities, instead of being expressed in exponential terms, are converted to the negative logarithm of the affinity constant ($-\log K_i$), or pK_i (as in Fig. 3). In this treatment a larger number represents a higher affinity. The inhibitor potency (K_i) was found to vary greatly throughout the series of ureas and

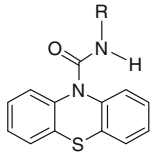
Table 1
Inhibition constants (K_i values), molecular volumes and Log *P* values for phenothiazine alkyl and cycloalkyl urea derivatives



#	R	K_i (μM) BuChE	K_i (μM) AChE	*V* (Å^3)	Log *P*
1	H	90.8 ± 11.8	89.3 ± 2.6	233	2.96
2	CH_3	18.3 ± 2.2	9.36 ± 3.51	253	3.20
3		35.0 ± 2.1	1450 ± 321	272	3.54
4		20.5 ± 5.0	None	290	4.02
5		3.41 ± 0.83	691 ± 39	290	3.86
6		24.7 ± 5.4	None	308	4.44
7		10.6 ± 1.6	None	308	3.97
8		2.66 ± 0.64	None	326	3.97
9		29.1 ± 7.5	None	300	3.04
10		42.5 ± 1.6	None	283	3.49
11		10.0 ± 1.7	None	300	3.91
12		2.99 ± 0.89	None	315	4.33
13		2.81 ± 0.33	None	332	4.75
14		0.35 ± 0.07	None	379	5.03

Log *P* indicates the relative solubility in *n*-octanol versus water, with the most hydrophobic molecules having the highest Log *P* value.

Table 2
Inhibition constants (K_i values), molecular volumes and Log *P* values for phenothiazine aryl and alkylaryl urea derivatives



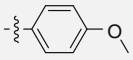
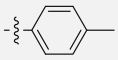
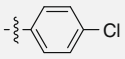
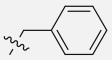
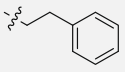
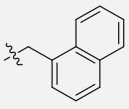
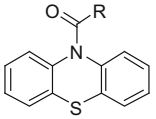
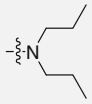
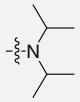
#	R	K_i (μM) BuChE	K_i (μM) AChE	*V* (Å^3)	Log *P*
15		0.31 ± 0.05	None	319	4.86
16		9.63 ± 1.67	None	365	4.74
17		12.1 ± 0.31	None	337	5.35
18		4.69 ± 0.04	None	332	5.42
19		0.71 ± 0.16	None	337	5.35
20		4.45 ± 1.27	None	337	4.93
21		0.19 ± 0.04	None	356	5.21
22		0.15 ± 0.02	None	388	5.93

Table 3
Inhibition constants (K_i values), molecular volumes and Log *P* values for phenothiazine disubstituted alkyl urea derivatives



#	R	K_i (μM) BuChE	K_i (μM) AChE	*V* (Å^3)	Log *P*
23		9.20 ± 1.74	400 ± 31	273	3.44
24		1.05 ± 0.13	None	310	3.11
25		0.43 ± 0.001	None	346	5.08
26		0.18 ± 0.08	None	346	4.75
27		0.56 ± 0.05	None	297	3.75
28		1.56 ± 0.43	None	314	4.17
29		7.10 ± 1.21	None	306	3.04

the factors contributing to this were explored by considering molecular parameters related to the structure of each compound and to that of the enzyme. The K_i values were independent of the pH between 6.8 and 8.0, as has been observed previously for other cholinesterase inhibitors.[23]

The inhibitory effect of compound **31** on wild-type BuChE and several BuChE mutants (D70G, F329A and Y332A) was examined at a single butyrylthiocholine concentration (1.6×10^{-4} M) using the modified Ellman method. Michaelis–Menten plots to examine aminourea inhibition were developed using fixed enzyme and inhibitor concentrations and wide range of substrate concentrations (3.5×10^{-5}–4.0×10^{-2} M) also using the modified Ellman method.

2.3. Molecular computational studies

Computational methods were used to determine certain molecular characteristics that could govern specificity and potency for cholinesterase inhibition.

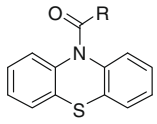
The total molecular volume was calculated, and is reported in Tables 1–4. Volumes were obtained by carrying out molecular mechanics calculations, using SPARTAN'06.[30] The Merck molecular force field (MMFF) was employed, selecting the most stable conformer for the volume calculation.[30]

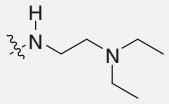
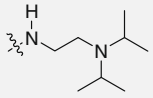
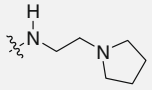
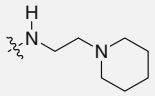
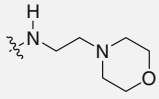
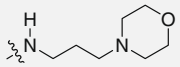
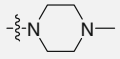
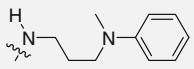
The log *P* values were calculated to predict the ability of the compound to cross the blood–brain barrier.[31] Experimentally, this is done by determining the concentration of the compound in *n*-octanol with that in water. The log of this partition value (*P*) provides an estimate of how efficiently the compound will cross the blood–brain barrier by diffusion. Often the solubility of a molecule in water is too low for the spectrophotometric analysis required in the experimental determination of log *P* values. In such cases, values can be obtained indirectly via methods such as the ALOGPS v 2.0 system[31] used herein. This method compares the structure of the molecule with a large database of known molecular partition coefficients. The larger the value of log *P*, the more hydrophobic the molecule and, hence, the greater the facility with which it can cross the blood–brain barrier. All the phenothiazine derivatives examined had calculated log *P* values greater than 2.5 (Tables 1–4). These partition coefficient values are all comparable to those calculated earlier for other phenothiazine derivatives.[12,27] In addition, although factors other than hydrophobicity may be involved in crossing the blood–brain barrier, the log *P* values in Tables 1–4 are comparable to those calculated earlier by the same method for drugs known to cross the blood–brain barrier.[12]

2.4. Butyrylcholinesterase inhibition by phenothiazine ureas

The inhibition of human BuChE by urea and aminourea derivatives of phenothiazine (Tables 1–4) was consistently of the reversible type. The relative potency of this inhibition was found to be directly related to the total molecular volume of the inhibitor. For example, a plot of pK_i ($-\log K_i$) versus molecular volume for the alkyl ureas shown in Table 1, produced a linear relationship, as depicted in Figure 3. This type of BuChE inhibition and the obvious relationship between potency and inhibitor size, are comparable to the inhibition of this enzyme by phenothiazine amides[11,12] and carbamates.[27] These similarities suggest a common mode for the binding of such phenothiazine derivatives to BuChE and, as described earlier,[9,27] this entails a highly favoured π–π association between the aromatic rings of the phenothiazine tricycle and the two aryl side chains of phenylalanine (F329) and tyrosine (Y332) on the E-helix[27] within the active site gorge of the enzyme. Thus,

Table 4
Inhibition constants (K_i values), molecular volumes, Log *P* values and pK_a values (distal nitrogen) of phenothiazine aminourea derivatives



#		K_i (μM) BuChE	K_i (μM) AChE	*V* (Å^3)	Log *P*	pK_a[a]
30		0.51 ± 0.06	16.3 ± 7.2	359	3.87	9.83
31		0.009 ± 0.0002	0.81 ± 0.062	395	4.51	10
32		0.21 ± 0.012	29.4 ± 7.6	347	3.51	9.12
33		0.02 ± 0.003	0.62 ± 0.13	364	3.93	9
34		6.91 ± 0.87	None	355	2.8	7.7
35		7.86 ± 0.52	None	373	2.9	7.7
36		17.1 ± 9.4	None	328	3.19	NC[b]
37		0.25 ± 0.08	None	405	5.38	6.0

[a] The pK_a values were obtained from Scifinder Scholar, published by the American Chemical Society. This software uses Advanced Chemistry Development (ACD/Labs) Software V8.19 for Solaris (© 1994–2009 ACD/Labs) calculate pK_a value.
[b] Program unable to calculate pK_a value.

as the size of the urea substituent on the phenothiazine scaffold is increased, the ability to block substrate access to the catalytic triad exhibits a concomitant increase. However, in spite of similarities between the inhibitory effects towards BuChE of phenothiazine ureas and their corresponding amides and carbamates, there were some notable exceptions. For example, the 1-adamantylcarbonyl amide derivative of phenothiazine (**38**) showed no inhibition of BuChE up to its solubility limit of approximately 20 μM. This has been attributed to the somewhat rigid nature of the amide bond (Fig. 4a) that, having the bulky adamantyl group attached, cannot be properly accommodated within the gorge to line up the phenothiazine moiety for binding to F329 and Y332. A similar phenomenon was earlier found to occur with the long 4-biphenylcarbonyl amide derivative[12] and carbamate derivative[27] that, likewise, showed no BuChE inhibition. In the case of 4-biphenylcarbonyl amide, BuChE inhibition was attained by the addition of a flexible methylene (CH_2) group into the system (4-biphenylacetylamide, $K_i = 1.7 \times 10^{-6}$ M).[12] The extra tetrahedral carbon atom permits a different 3-D geometry enabling the molecule to have increased molecular flexibility within the active site region. Similarly, this added flexibility, with concomitant BuChE inhibition, by the adamantyl urea derivative (Table 1) can also be attributed to the addition of a tetrahedral nitrogen atom to the adamantyl moiety in compound **14** (Fig. 4b), compared to the lack of BuChE inhibition observed with the adamantyl amide derivative (**38**) (Fig. 4a).

2.5. Acetylcholinesterase inhibition by phenothiazine ureas

As seen in Tables 1–3, urea derivatives of phenothiazine, like their amide counterparts,[11,12] showed little or no ability to inhibit AChE. The small volume of the AChE active site gorge (302 Å^3)[9] undoubtedly contributes to the inability of many of the larger ureas to gain access to this region of the enzyme for inhibition. Another factor thought to contribute to this absence of AChE inhibition is the presence of a tyrosine (Y337) residue[9,32] on the E-helix of human AChE.[27] This amino acid side chain interferes with the π–π interaction between the phenothiazine tricycle and residues F338 and Y341, that are equivalent to F329 and Y332 in BuChE, and that serve as the binding site for the phenothiazine moiety in BuChE.[9,11,12,27] The poor, but almost identical, AChE inhibitor affinity constants for small (molecular volume <310 Å^3) amide phenothiazines[11] appears also to operate with the alkyl ureas (Tables 1 and 3) and may result from a weak interaction of the carbonyl functionality with a hydrogen bonding group, such as that of Y124 or Y341, within the AChE active site region.[11]

2.6. Cholinesterase inhibition by phenothiazine aminoureas

In sharp contrast to the ureas, a number of the substituted aminoureas, such as compound **31** (Table 4), were found to be potent inhibitors of both AChE and BuChE (Fig. 5). There is little doubt that

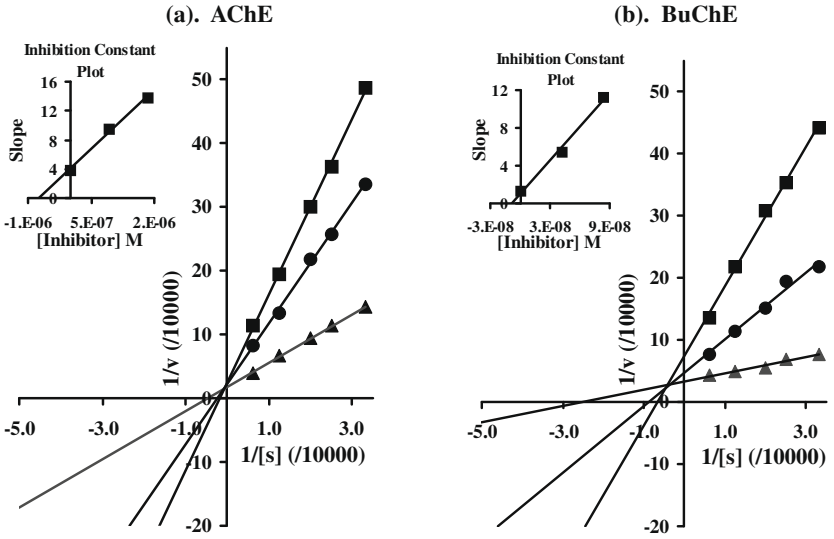


Figure 5. Lineweaver–Burk plots of; (a) acetylcholinesterase hydrolysis of acetylthiocholine in the absence (▲) and presence of (● = 9.3×10^{-7} M and ■ = 1.9×10^{-6} M) of *N*-[2-(*N*′,*N*′-diisopropylamino)ethyl]-10*H*-phenothiazine-10-carboxamide (**31**) and (b) butyrylcholinesterase hydrolysis of butyrylthiocholine in the absence (▲) and presence (● = 4.2×10^{-8} M and ■ = 8.3×10^{-8} M) of (**31**). Inhibition constants were derived from the plot in the inset, where the K_i for acetylcholinesterase is 8.1×10^{-7} M and the K_i for butyrylcholinesterase is 9.0×10^{-9} M.

the BuChE inhibition involves the usual π–π interaction between F329 and Y332 of the enzyme and the phenothiazine moiety.[11,12,27] However, the K_i value for compound **31,** being in the nanomolar range for BuChE, implies that other factors may be influencing greater binding of this type of molecule to the enzyme. This additional binding site may also provide the unusually high AChE inhibition (10^{-7} M range) by the same compound (Table 4). This robust AChE inhibition occurs in spite of the large molecular volume (395 Å^3) of this inhibitor compared to the active site gorge volume (302 Å^3). This implies binding of the compound at the periphery of the AChE gorge.

One factor that could contribute to the ability of a substituted aminourea, such as compound **31**, to provide robust inhibition of both cholinesterases (Fig. 5) is the potential of the substituent distal amino group to be positively charged under the conditions (pH 8.0) of the Ellman essay. To test the importance of a potential positive charge in binding substituted aminoureas to cholinesterases, calculated approximate pK_a values of the distal substituent tertiary amino groups (Table 4) were considered with respect to the ability to produce potent AChE inhibition. As can be seen in that table, only aminoureas that would be predominantly cationic under the reaction conditions (pH 8.0 or below) (e.g., compounds **30–33**, but not **34–37**) show the inhibition of AChE. That is, only those substituents with pK_a values well above the assay pH of 8.0 inhibit AChE. The same reasoning could be invoked to explain why BuChE showed unusually high sensitivity to inhibition by compounds such as **31** (Table 4). That is, in addition to the novel cationic binding with the substituent nitrogen, BuChE should still be capable of forming π–π interaction with the phenothiazine moiety to provide inhibition constant values in the nanomolar range (compound **31**, $K_i = 9.0 \times 10^{-9}$ M), comparable to that produced by the large 9-anthrylcarbonyl amide.[12]

2.7. Inhibition of butyrylcholinesterase mutants

To further address the question of a common feature of both AChE and BuChE that would facilitate inhibition through binding a cationic nitrogen atom, the common anionic aspartic acid residue (D74 in human AChE and D70 in human BuChE) was considered. This common component of the peripheral anionic site (PAS) of cholinesterases was tested as a major contributor to cholinesterase inhibition by aminoureas with the use of mutant BuChE species. Thus, a comparative study was made of the inhibition of wild-type BuChE and the BuChE mutants D70G, F329A and Y332A. The effect of 2-(*N*,*N*-diisopropylamino ethyl) phenothiazine urea (**31**) on these four BuChE enzyme species is summarized in Figure 6. As can be seen in this representation, at an inhibitor concentration of 50 nM, wild-type BuChE was inhibited by approximately 50%, with no inhibitory effect on the D70G mutant enzyme at that concentration. A 10-fold increase in inhibitor concentration inhibited wild-type BuChE almost completely, while D70G BuChE was still at over 80% of its original activity with no inhibitor (Fig. 6). The importance of Y332 in binding this inhibitor is also evident in the retention of nearly 50% activity at 500 nM inhibitor with Y332A BuChE. F329A BuChE also exhibits greater resistance to

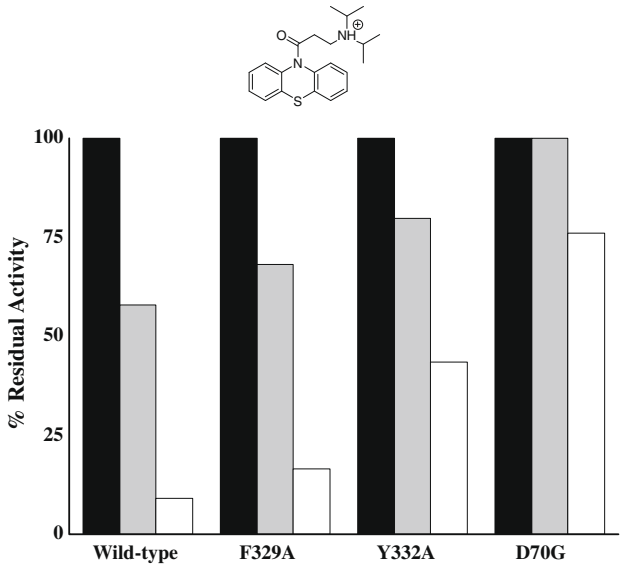


Figure 6. Comparison of residual enzymatic activity of wild-type BuChE and mutant enzyme species, F329A, Y332A and D70G, in the absence (black bar) and presence of two concentrations (grey bar = 5×10^{-8} M and white bar = 5×10^{-7} M) of *N*-[2-(*N*′,*N*′-diisopropylamino)ethyl]-10*H*-phenothiazine-10-carboxamide (**31**).

inhibition by compound **31** than wild-type BuChE, but the effect is less pronounced than for Y332A BuChE. Thus, robust inhibition of BuChE by aminoureas, such as compound **31**, can be attributed to the additional salt linkage of this cationic urea with D70 of the peripheral anionic site, in addition to the usual π–π interaction between the phenothiazine tricycle and F329 and Y332 (Fig. 7b). It may therefore be reasonable to assume that the corresponding anionic group in human AChE (D74) is largely responsible for the strong inhibition of that enzyme by compound **31** (Fig. 7a).

2.8. Effect of substrate concentration on aminourea inhibition

One of the most important differences between AChE and BuChE is the effect on enzymatic activity at high substrate concentration. At elevated substrate concentrations, AChE exhibits substrate inhibition while BuChE exhibits substrate activation under these conditions.[33–35] This effect is known to result as a consequence of conformational changes to the enzymes because of the interaction between D74 in AChE and D70 in BuChE, with cationic substrates such as acetylcholine.[35] Thus, the interaction between the cationic substrate and the anionic amino acid residue at the PAS leads to conformational changes that produce the opposite effects in AChE and BuChE. Experiments with an aminourea (**31**) and the BuChE D70G mutant suggest that the cationic amionoureas form both a salt bridge between D70 and an electrostatic interaction with aromatic amino acids F329 and Y332 of the enzyme. This effect is lost when the D70 is mutated to the neutral amino acid glycine. Based on these observations, AChE was assumed to interact with aminoureas, like compound **31**, solely through the ionic linkage with D74.

To further explore the involvement of the PAS of both cholinesterases in binding cationic aminoureas, the known effect of substrate binding to the anionic aspartate residues was exploited. As can be seen in Figure 8, at substrate concentration above 1 mM

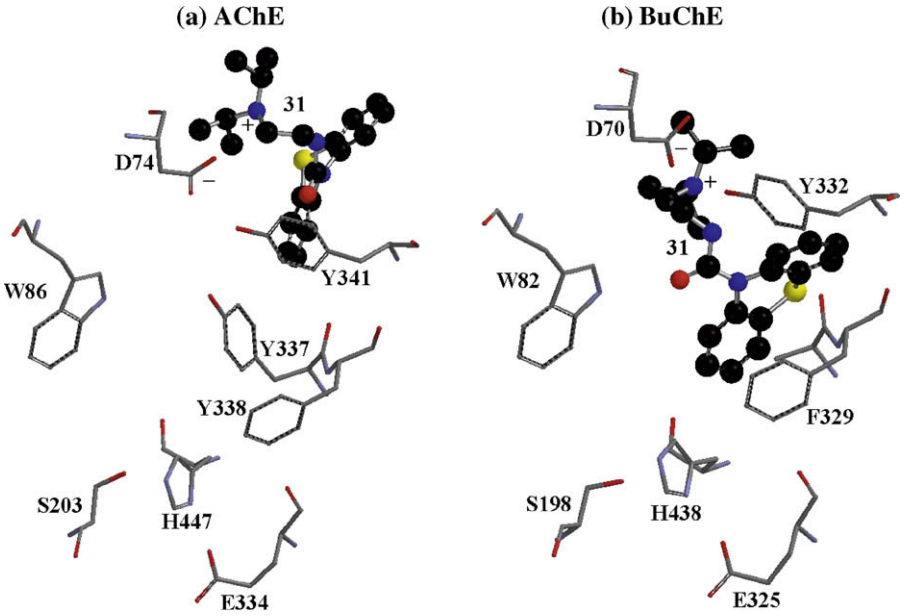


Figure 7. Acetylcholinesterase and butyrylcholineterase active site gorges showing ionic bonding between the cationic disubstituted aminourea, *N*-[2-(*N*′,*N*′–diisopropylamino)ethyl]-10*H*-phenothiazine-10-carboxamide (**31**) and (a) D74 of the human acetylcholinesterase and (b) D70 human butyrylcholinesterase peripheral anionic sites. With this enzyme π–π interaction between F329 and Y332 and the phenothiazine rings can also occur; in AChE this is prevented due to the presence of Y337. The figure was generated using the SPARTAN′06 program.[30] First, the crystal structure of human acetylcholinesterase,[32] (PDB ID: 1B41) and human butyrylcholinesterase (PDB ID: 1POI)[52] was obtained from the protein databank[53] and PyMol was employed to delete all amino acids save for those selected residues found in the active site.[54] Compound **31** was optimized separately, using molecular mechanics and the MMFF force field, within SPARTAN′06 to obtain the preferred conformer.[30] Using SPARTAN′06, the optimized compound **31** was manually fitted into the active site gorge to represent simulated binding.

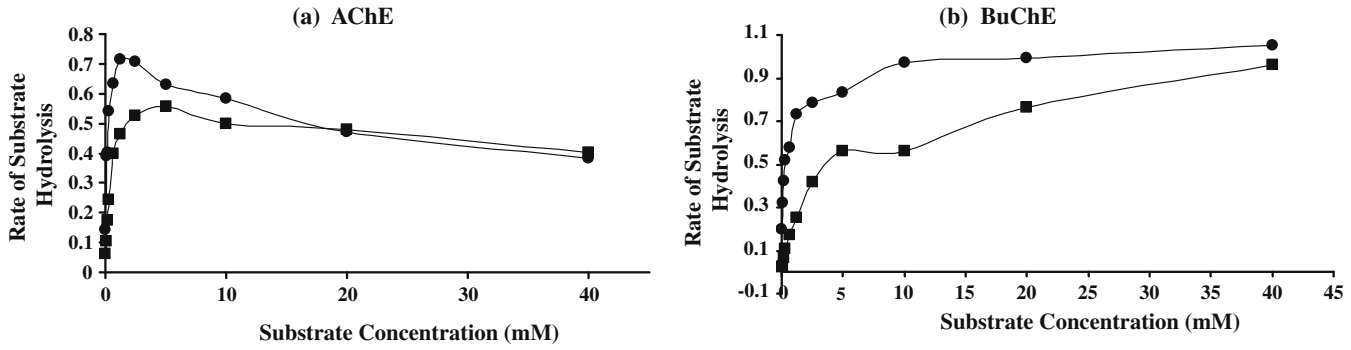


Figure 8. Michaelis–Menten plots of the effect on cholinesterase hydrolysis of increasing concentration of acetylthiocholine (1.95×10^{-5}–4.0×10^{-2} M) in the absence (●) and presence (■) of *N*-[2-(*N*′,*N*′-diisopropylamino)ethyl]-10*H*-phenothiazine-10-carboxamide (**31**) on; (a) acetylcholinesterase, indicating inhibition of the enzyme at high substrate concentration. Note that in the presence of **31** (1.8×10^{-6} M) there is no effect of this compound at substrate concentration greater that 20 mM and on (b) butyrylcholinesterase, showing the activation of the enzyme at high substrate concentration. Note that in the presence of **31** (1.8×10^{-7} M) the inhibition by this compound is not completely overcome even at 40 mM acetylthiocholine.

there is the expected diminished AChE activity (Fig. 8a) and elevated BuChE activity (Fig. 8b). In the presence of the aminourea (**31**), there is a marked inhibition of both enzymes at lower substrate concentrations (up to 10 mM). However, for AChE, the inhibition of the enzyme by **31** is eliminated at very high substrate concentration (20 mM). This can be interpreted to be a consequence of the constant occupation of D74 by the cationic acetylthiocholine substrate at elevated concentration, preventing binding of the cationic aminourea at this site to exert its inhibitory effect. BuChE also shows a marked inhibition by **31** at lower substrate concentration but this inhibition shows a trend, like AChE, to be overcome at higher acetylthiocholine concentration (>10 mM). Unlike the loss of aminourea inhibition at 20 mM for AChE, the inhibition of BuChE by **31** is not completely lost, even at 40 mM, in spite of the fact that the inhibitor concentration is one tenth that used for AChE. This is attributed to the fact that, in addition to being able to form a salt bridge with D70 in BuChE, the aminourea (**31**) is also able to bind (Fig. 7b), through π–π interaction, between the phenothiazine tricycle and F329 and Y332 in BuChE.[11,12,27] Because of the presence of Y337 in AChE,[9] this additional π–π binding cannot occur with AChE (Fig. 7a).

3. Conclusions

Urea derivatives of phenothiazine inhibit human BuChE to an extent that can be directly related to the molecular volume of the compound. These compounds show little or no inhibition of AChE because of active site volume restrictions and because of interference with binding of the phenothiazine tricycle to the E-helix,[12] due to the presence of Y337 in AChE (see Fig. 7a). Substituted aminourea derivatives inhibit the activity of both cholinesterases through ionic bonding between a cationic distal nitrogen on the substituent attached to phenothiazine and the aspartate residue of the peripheral anionic site. These aminoureas provide a unique class of phenothiazine derivatives that can be potent inhibitors of both cholinesterases. Such inhibitors could prove useful for comparison with phenothiazine derivatives that are selective inhibitors of AChE or BuChE in the quest for the most effective treatment of cognitive impairment.

Currently, drugs used for the treatment of symptoms of AD are thought to exert their effects by inhibiting the enzymatic activity of cholinesterases, prolonging the action of acetylcholine in cholinergic neurotransmission.[20] In addition, long-term use of certain cholinesterase inhibitors has been shown to lead to overexpression of cholinesterases, AChE in particular.[36,37] Whether compounds described herein are also capable of inducing changes in cholinesterase expression will require further studies. It has also been shown that the peripheral anionic site of AChE promotes amyloid fibril formation.[38] Since phenothiazine aminoureas, such as **31,** appear to affect the peripheral anionic site of AChE, they should interfere with AChE promoting amyloid fibril formation. It has also been reported that the 40 amino acid residue segment at the carboxy terminal of BuChE attenuates the process of amyloid fibril formation.[39] Since aminoureas like **31** appear to bind to the peripheral anionic site of BuChE, well away from the carboxy terminal, they should not interfere with this enzyme's ability to attenuate amyloid fibril formation.

4. Experimental

4.1. Materials

Purified human plasma butyrylcholinesterase (BuChE, EC 3.1.1.8) and recombinant mutant BuChE species (D70G, F329A and Y332A) were from Dr. Oksana Lockridge (University of Nebraska Medical Center).[35] Purified recombinant human acetylcholinesterase (AChE, EC 3.1.1.7), acetylthiocholine, butyrylthiocholine and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma (St. Louis, MO). Phenothiazine-10-carbonyl chloride was purchased from Acros Chemicals. Commercial reagents/solvents were purchased from Fischer Scientific, Caledon Laboratories Inc. or Aldrich Chemical Co. Inc., and were used as received, except for the liquid amines, which were distilled prior to use.

4.2. Organic synthesis and purification

Of the total of 37 urea derivatives of phenothiazine prepared, 22 compounds (**1**,[40] **2**,[41] **3**,[41] **4**,[42] **5**,[42] **6**,[42] **7**,[42] **15**,[41,43,44] **20**,[41,45] **21**,[46] **23**,[47] **24**,[48] **25**,[42] **26**,[42] **27**,[48] **28**,[48] **29**,[49] **30**,[48] **32**,[50] **33**,[50] **34**,[46] **36**[51]) have been synthesized previously.

For the procedure for synthesis of phenothiazine ureas, typically, a mixture of phenothiazine-10-carbonyl chloride (1.00 g, 3.82 mmol), and amine (9.55 mmol) in CH_2Cl_2 (40 mL) was stirred at room temperature until analysis by thin layer chromatography revealed no unreacted phenothiazine-10-carbonyl chloride remained, or no further change was observed. All reactions were monitored using thin layer chromatography (TLC) which was performed on Macherey Nagel Polygram® Sil G/UV_{254} precoated silica plates (0.20 mm thickness). The developing solvent was dichloromethane or dichoromethane/ethyl acetate mixtures. TLC plates were visualized using short wave (254 nm) UV lamp, then an I_2 chamber. Reaction periods were typically in the range of 1−3 h. The reaction mixture was then washed with 0.1 M HCl (3 × 30 mL), 0.1 M NaOH (2 × 30 mL), and water (1 × 30 mL) then dried over $MgSO_4$. The solvent was removed by reduced pressure to afford the crude urea. This material was dissolved in the minimum amount of petroleum ether, and precipitated by the addition of CH_2Cl_2. All solids were recrystallized using a mixed solvent technique with petroleum ether/CH_2Cl_2 mixtures, except for compound **38** which was recrystallized from petroleum ether.

4.3. Analysis of synthesized compounds

Melting points were measured using a Mel-Temp II apparatus from Laboratory Services Inc. and are uncorrected. Infrared spectra were recorded as Nujol mulls between sodium chloride plates on a Nicolet model 205 or a Nicolet Avatar 330 FT-IR spectrometer. Peak positions were reproducible within 1−2 cm^{-1}. Nuclear magnetic resonance spectra were recorded at the Atlantic Region Magnetic Resonance Centre, Dalhousie University, on a Bruker AC-250F operating at 250.1 MHz for proton and 62.9 MHz for carbon or a Bruker AVANCE 500 operating at 500.13 MHz for proton and 125.76 MHz for carbon-13. Chemical shifts are reported in ppm relative to TMS, in $CDCl_3$ solution. Reported multiplicities are apparent. 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. The purity of the synthesized compounds was also determined using an Agilent Technologies 1200 series HPLC system with either a C18 reverse phase column with methanol as the mobile phase or a silica gel column with 5% methanol in dichloromethane as the mobile phase.

4.4. Analytical data

4.4.1. Phenothiazine-10-carboxamide[40] (1)

Compound **1** 55%; colourless crystals, mp 191–194 °C. IR (Nujol): 3451, 3286, 3183, 1655, 1591, 1566, 1308, 1268, 1240, 1093,

1033, 755 cm^{-1}. ^1H NMR: 5.19(s, 2H), 7.17–7.23(m, 2H), 7.28–7.35(m, 2H), 7.40(dd, J = 7.6, 1.2 Hz, 2H), 7.59(dd, J = 7.9, 0.9 Hz, 2H). ^{13}C NMR: 126.7, 126.9, 127.3, 128.1, 133.4, 138.7, 155.5. EI-MS (m/z): 242(M^+), 200, 199(base), 198, 197, 167, 166, 154. HRMS (El) M^+(found): 242.0505; calcd for $C_{13}H_{10}N_2OS$: 242.0514.

4.4.2. *N*-Methyl-10*H*-phenothiazine-10-carboxamide[41] (2)

Compound **2** 68%; colourless crystals; mp 168–169 °C. IR: 3311, 1660, 1519, 1409, 1313, 1299, 1286, 1253, 1156, 760, 747, 734 cm^{-1}. ^1H NMR: 2.82(d, J = 4.6 Hz, 3H), 4.95(broad s, 1H), 7.16–7.22(m, 2H), 7.28–7.34(m, 2H), 7.39(dd, J = 7.6, 1.5 Hz, 2H), 7.57(dd, J = 7.9, 1.2 Hz, 2H). ^{13}C NMR: 27.6, 126.5, 127.1, 127.2, 128.0, 133.4, 138.8, 155.3. EI-MS (m/z): 256(M^+; base), 200, 199, 198, 167, 166, 154. HRMS (EI) M^+(found): 256.0685; calcd: 256.0670 (for $C_{14}H_{12}N_2OS$).

4.4.3. *N*-Ethyl-10*H*-phenothiazine-10-carboxamide[41] (3)

Compound **3** 42%; colourless crystals; mp 114–116 °C. IR: 3439, 3310, 1645, 1589, 1518, 1320, 1300, 1281, 1249, 1236, 757 cm^{-1}. ^1H NMR: 1.10(t, J = 7.2 Hz, 3H), 3.22–3.32(m, 2H), 5.00(broad s, 1H), 7.13–7.20(m, 2H), 7.26–7.32(m, 2H), 7.32–7.38(m, 2H), 7.54–7.57(m, 2H). ^{13}C NMR: 15.2, 35.8, 126.5, 127.1, 127.2, 128.1, 133.4, 138.8, 154.5. EI-MS (m/z): 270(M^+), 201, 200, 199(base), 198, 167, 166, 154, 140, 127. HRMS (EI) M^+(found): 270.0832; calcd: 270.0827 (for $C_{15}H_{14}N_2OS$).

4.4.4. *N*-Propyl-10*H*-phenothiazine-10-carboxamide[42] (4)

Compound **4** 71%; colourless crystals; mp 105–107 °C. IR: 3384, 3317, 1660, 1590, 1504, 1317, 1299, 1280, 1258, 1233, 1154, 762, 750 cm^{-1}. ^1H NMR: 0.88(t, J = 7.5 Hz, 3H), 1.51(sextet, J = 7.3 Hz, 2H), 3.17–3.25(m, 2H), 5.00(broad s, 1H), 7.15–7.23(m, 2H), 7.27–7.35(m, 2H), 7.39(dd, J = 7.9, 1.8 Hz, 2H), 7.57(dd, J = 7.9, 1.2 Hz, 2H). ^{13}C NMR: 11.3, 23.0, 42.6, 126.4, 127.0, 127.1, 128.0, 133.4, 138.8, 154.5. EI-MS (m/z): 284(M^+), 201, 200, 199(base), 198, 167, 166, 154, 140, 84, 48. HRMS (EI) M^+(found): 284.0992; calcd: 284.0983 (for $C_{16}H_{16}N_2OS$).

4.4.5. *N*-Isopropyl-10*H*-phenothiazine-10-carboxamide[42] (5)

Compound **5** 68%; colourless crystals, mp 116–118 °C. IR: 3308, 1654, 1515, 1326, 1311, 1280, 1250, 1233, 1167, 760, 749 cm^{-1}. ^1H NMR: 1.12(d, J = 6.7 Hz, 6H), 3.94–4.10(m, 1H), 4.75–4.79(d, J = 7.3 Hz, 1H), 7.14–7.21(m, 2H), 7.26–7.33(m, 2H), 7.38(dd, J = 7.6, 1.5 Hz, 2H), 7.56(dd, J = 7.6, 1.5 Hz, 2H). ^{13}C NMR: 23.0, 43.0, 126.4, 127.0, 127.1, 128.1, 133.4, 138.8, 153.8. EI-MS (m/z): 284(M^+), 200, 199(base), 198, 197, 196, 171, 167, 166. HRMS (EI) M^+(found): 284.0984; calcd: 284.0983 (for $C_{16}H_{16}N_2OS$).

4.4.6. *N*-Butyl-10*H*-phenothiazine-10-carboxamide[42] (6)

Compound **6** 72%; colourless crystals, mp 111–114 °C. IR: 3334, 1659, 1531, 1317, 1301, 1283, 1253, 1232, 1123, 756 cm^{-1}. ^1H NMR: 0.91(t, J = 7.5 Hz, 3H), 1.26–1.36(m, 2H), 1.44–1.52(m, 2H), 3.22–3.27(m, 2H), 4.99(broad s, 1H), 7.17–7.22(m, 2H), 7.29–7.34(m, 2H), 7.39(dd, J = 7.7, 1.5 Hz, 2H), 7.58(dd, J = 7.9, 1.3 Hz, 2H). ^{13}C NMR: 13.7, 20.0, 31.9, 40.6, 126.4, 127.0, 127.1, 128.0, 133.4, 138.9, 154.5. EI-MS (m/z): 298(M^+), 201, 200, 199(base), 198, 197, 171, 167, 166, 154. HRMS (EI) M^+(found): 298.1136; calcd: 298.1140 (for $C_{17}H_{18}N_2OS$).

4.4.7. *N*-*tert*-Butyl-10*H*-phenothiazine-10-carboxamide[42] (7)

Compound **7** 99%; colourless crystals, mp 115–118 °C. IR: 3436, 1679, 1505, 1264, 1315, 1283, 1255, 1203, 756, 746 cm^{-1}. ^1H NMR: 1.34(s, 9H), 4.90(broad s, 1H), 7.16–7.21(m, 2H), 7.28–7.33(m, 2H), 7.40(dd, J = 7.8, 1.4 Hz, 2H), 7.57(dd, J = 7.8, 1.2 Hz, 2H). ^{13}C NMR: 29.1, 51.2, 126.3, 127.0, 127.1, 128.0, 133.4, 139.0, 153.4. EI-MS (m/z): 298(M^+), 201, 200, 199(base), 198, 197, 167, 166, 57. HRMS (EI) M^+(found): 298.1140; calcd: 298.1140 (for $C_{17}H_{18}N_2OS$).

4.4.8. *N*-Neopentyl-10*H*-phenothiazine-10-carboxamide (8)

Compound **8** 66%, colourless crystals, mp 80.4–82 °C. IR: 3467, 1688, 1512, 1315, 1279, 1250, 1224, 767, 760 cm^{-1}. ^1H NMR: 0.87(s, 9H), 3.07(d, J = 6.2 Hz, 2H), 5.03–5.05(m, 1H), 7.19–7.24(m, 2H), 7.30–7.35(m, 2H), 7.41(dd, J = 7.7, 1.3 Hz, 2H), 7.59(dd, J = 7.9, 1.1 Hz, 2H). ^{13}C NMR: 27.1, 32.1, 51.9, 126.5, 127.1, 127.2, 128.0, 133.5, 138.8, 154.8. EI-MS (m/z): 312(M^+), 200, 199(base), 198, 197, 167, 166, 154, 114, 95, 71, 69. HRMS (EI) M^+(found): 312.1294; calcd: 312.1296 (for $C_{18}H_{20}N_2OS$).

4.4.9. *N*-(2-Methoxyethyl)-10*H*-phenothiazine-10-carboxamide (9)

Compound **9** 81%; colourless crystals, mp 118–120 °C. IR: 3380, 1666, 1500, 1314, 1299, 1276, 1255, 1231, 1196, 1100, 1026, 998, 775, 750 cm^{-1}. ^1H NMR: 3.31(s, 3H), 3.41–3.48 (m, 4H), 5.39(broad s, 1H), 7.17–7.22(m, 2H), 7.28–7.33(m, 2H), 7.39(dd, J = 7.7, 1.3 Hz, 2H), 7.58(dd, J = 7.9, 1.1 Hz, 2H). ^{13}C NMR: 40.7, 58.7, 71.3, 126.4, 126.9, 127.1, 1278.0, 133.3, 138.7, 154.6. EI-MS (m/z): 300(M^+), 201, 200, 199(base), 198, 167, 166. HRMS (EI) M^+(found): 300.0934; calcd: 300.0932 (for $C_{16}H_{16}N_2O_2S$).

4.4.10. *N*-Cyclopropyl-10*H*-phenothiazine-10-carboxamide (10)

Compound **10** 58%; colourless crystals, mp 132–133.5 °C. IR: 3533, 3464, 3325, 1679, 1645, 15889, 1509, 1314, 1299, 1284, 1261, 1249, 778, 738 cm^{-1}. ^1H NMR: 0.45–0.51(m, 2H), 0.68–0.76(m, 2H), 2.61–2.70(m, 1H), 5.18(broad s, 1H), 7.15–7.21(m, 2H), 7.26–7.33(m, 2H), 7.38(dd, J = 7.6, 1.5 Hz, 2H), 7.54(dd, J = 7.9, 1.2 Hz, 2H). ^{13}C NMR: 7.1, 23.8, 126.8, 127.3, 127.5, 128.3, 133.6, 138.9, 155.7. EI-MS (m/z): 282(M^+), 201, 200, 199(base), 198, 167, 166, 154. HRMS (EI) M^+(found): 282.0846; calcd: 282.0827 (for $C_{16}H_{14}N_2OS$).

4.4.11. *N*-Cyclobutyl-10*H*-phenothiazine-10-carboxamide (11)

Compound **11** 61%, colourless crystals, mp 133–135.5 °C. IR: 3319, 1673, 1655, 1589, 1567, 1514, 1315, 1300, 1284, 1253, 1206, 1032, 754 cm^{-1}. ^1H NMR: 1.57–1.85(m, 4H), 2.23–2.38(m, 2H), 4.22–4.38(m, 1H), 5.10(d, J = 7.3 Hz, 1H), 7.13–7.20(m, 2H), 7.25–7.33(m, 2H), 7.37(dd, J = 7.6, 1.5 Hz, 2H), 7.56(dd, J = 7.6, 1.2 Hz, 2H). ^{13}C NMR: 15.0, 31.4, 46.2, 126.5, 127.0, 127.2, 128.1, 133.4, 138.7, 153.5. EI-MS (m/z): 296(M^+), 201, 200, 199(base), 198, 167, 166, 154, 55. HRMS (EI) M^+(found): 296.0986; calcd: 296.0983 (for $C_{17}H_{16}N_2OS$).

4.4.12. *N*-Cyclopentyl-10*H*-phenothiazine-10-carboxamide (12)

Compound **12** 52%; colourless crystals, mp 125–126 °C. IR: 3412, 1677, 1503, 1316, 1303, 1252, 1124, 1030, 763 cm^{-1}. ^1H NMR: 1.25–1.38(m, 2H), 1.52–1.63(m, 4H), 1.91–2.03(m, 2H), 4.06–4.20(m, 1H), 4.89(d, J = 7.0 Hz, 1H), 7.15–7.21(m, 2H), 7.27–7.33(m, 2H), 7.38(dd, J = 7.6, 1.5 Hz, 2H), 7.56(dd, J = 7.9, 1.2 Hz, 2H). ^{13}C NMR: 23.6, 33.1, 52.7, 126.4, 126.9, 127.1, 128.0, 133.3, 138.8, 154.1. EI-MS (m/z): 310(M^+), 200, 199(base), 198, 167, 166, 154, 127, 88, 86, 84. HRMS (EI) M^+(found): 310.1140; calcd: 310.1140.0827 (for $C_{18}H_{18}N_2$0S).

4.4.13. *N*-Cyclohexyl-10*H*-phenothiazine-10-carboxamide (13)

Compound **13** 88%; colourless crystals, mp 159–160 °C. IR: 3416, 1676, 1507, 1321, 1256, 1215, 1030, 765 cm^{-1}. ^1H NMR: 1.30–1.15(m, 3H), 1.30–1.38(M, 2H), 1.54–1.63(m, 3H), 1.90–1.93(m, 2H), 3.65–3.72(m, 1H), 4.85(d, J = 7.5 Hz, 1H), 7.15–7.18(m, 2H), 7.27–7.30(m, 2H), 7.37(dd, J = 7.7, 1.1 Hz, 2H), 7.56(dd, J = 7.9, 0.9 Hz, 2H). ^{13}C NMR: 24.7, 25.5, 33.2, 49.7, 126.3, 126.9, 127.0, 128.0, 133.3, 138.8, 153.7. EI-MS (m/z): 324(M^+), 201, 200, 199(base), 198, 167, 166, 154, 128. HRMS (EI) M^+(found): 324.1290; calcd: 324.1296 (for $C_{19}H_{20}N_2OS$).

4.4.14. *N*-(Adamant-1-yl)-10*H*-phenothiazine-10-carboxamide (14)

Compound **14** 75%, colourless crystals, mp 167–167.5 °C. IR: 3426, 1692, 1512, 1359, 1343, 1319, 1311, 1298, 1250, 1219, 1085, 767, 7.38 cm^{-1}. ^1H NMR: 1.61–1.67(m, 6H), 1.87(d, 1H), 1.93–1.97(m, 6H), 2.02–2.10(m, 3H), 4.79(broad s, 1H), 7.16–7.19(m, 2H), 7.25–7.35(m, 2H), 7.37(dd, *J* = 7.9, 1.2 Hz, 2H), 7.58(dd, *J* = 7.9, 1.2 Hz, 2H). ^{13}C NMR ($CDCl_3$): 29.6, 36.5, 42.0, 51.9, 126.4, 127.1, 127.2, 128.1, 133.4, 139.0, 153.1. EI-MS (*m/z*): 376(M^+), 200, 199(base), 198, 167, 166, 154, 135, 120, 93, 79, 77. HRMS (EI) M^+(found): 376.1607; calcd: 376.1609 (for $C_{23}H_{24}N_2OS$).

4.4.15. *N*-Phenyl-10*H*-phenothiazine-10-carboxamide[41,43,44] (15)

Compound **15** 68%; colourless crystals, mp 175.9–176.8 °C (lit. mp 180 °C).[43] IR: 3314, 3272, 1674, 1660, 1597, 1525, 1440, 1324, 1236, 757 cm^{-1}. ^1H NMR: 6.91(broad s, 1H), 7.02–7.09(m, 1H), 7.22–7.41(m, 8H), 7.45(dd, *J* = 7.6, 1.5 Hz, 2H), 7.67(dd, *J* = 7.9, 1.2 Hz, 2H). ^{13}C NMR: 119.6, 123.6, 126.9, 127.0, 127.4, 128.3, 128.9, 133.7, 138.0, 138.3, 151.8. EI-MS (*m/z*): 318(M^+), 201, 200, 199(base), 198, 167, 166, 154, 119, 84, 77. HRMS (EI) M^+(found): 318.0827; calcd: 318.0827 (for $C_{19}H_{14}N_2OS$).

4.4.16. *N*-(4-Methoxyphenyl)-10*H*-phenothiazine-10-carboxamide (16)

Compound **16** 77%; colourless crystals, mp 149–151.5 °C. IR (Nujol): 3297, 1670, 1600, 1529, 1516, 1319, 1257, 1226, 1178, 1034, 829, 752 cm^{-1}. ^1H NMR ($CDCl_3$): 3.77(s, 3H), 6.75–6.87(m, 3H), 7.21–7.48(m, 8H), 7.66(d, *J* = 7.3 Hz, 2H). ^{13}C NMR ($CDCl_3$): 55.5, 114.1, 121.9, 126.8, 127.0, 127.4, 128.3, 131.1, 133.7, 138.4, 152.3, 156.2. EI-MS (*m/z*): 348(M^+), 201, 200, 199(base), 198, 167, 166. HRMS (EI) M^+(found): 348.0935; calcd: 348.0932 (for $C_{20}H_{16}N_2O_2S$).

4.4.17. *N*-*p*-Tolyl-10*H*-phenothiazine-10-carboxamide (17)

Compound **17** 62%; colourless crystals, mp 174.3–175 °C. IR (Nujol): 3408, 1686, 1592, 1515, 1403, 1316, 1296, 1276, 1258, 1226, 1193, 813, 762, 755 cm^{-1}. ^1H NMR ($CDCl_3$): 2.31(s, 3H), 6.85(broad s, 1H), 7.08–7.12(m, 2H), 7.24–7.30(m, 4H), 7.36–7.41(m, 2H), 7.46(d, *J* = 7.6 Hz, 2H), 7.69(d, *J* = 7.9 Hz, 2H). ^{13}C NMR ($CDCl_3$): 20.8, 119.8, 126.9, 127.1, 127.4, 128.3, 129.5, 133.2, 133.7, 135.5, 138.4, 152.0. EI-MS (*m/z*): 332(M^+; base), 201, 200, 199, 198, 167, 166, 154, 133, 132. HRMS (EI) M^+(found): 332.0984; calcd: 332.0983 (for $C_{20}H_{16}N_2OS$).

4.4.18. *N*-(4-Chlorophenyl)-10*H*-phenothiazine-10-carboxamide (18)

Compound **18** 85%; colourless crystals, mp 171.6–172.9 °C. IR (Nujol): 3418, 1694, 1591, 1518, 1398, 1308, 1232, 1194, 1088, 1012, 824, 766, 758 cm^{-1}. ^1H NMR ($CDCl_3$): 6.89(broad s, 1 H), 7.20–7.42(m, 8H), 7.46(dd, *J* = 7.6, 1.5 Hz, 2H), 7.65(dd, *J* = 7.9, 1.5 hz, 2H). ^{13}C NMR ($CDCl_3$): 120.9, 127.0, 127.1, 127.5, 128.4, 128.6, 128.9, 133.7, 136.8, 138.1, 151.7. EI-MS (*m/z*): 354(M^{+2}), 352(M^+), 200, 199(base), 198, 167, 166, 154, 153, 125. HRMS (EI) M^+(found): 352.0422; calcd: 352.0437 (for $C_{19}H_{13}N_2OSCl$).

4.4.19. *N*-*o*-Tolyl-10*H*-phenothiazine-10-carboxamide (19)

Compound **19** 69%; colourless crystals, mp 151–153.5 °C. IR (Nujol): 3435, 3417, 1687, 1588, 1529, 1312, 1243, 1198, 760 cm^{-1}. ^1H NMR ($CDCl_3$): 2.02 (s, 3H), 6.81 (broad s, 1H), 6.95–7.02 (m, 1H), 7.07–7.30 (m, 4H), 7.34–7.38 (m, 2H), 7.46 (dd, *J* = 7.6, 1.5 Hz, 2H), 7.68 (dd, *J* = 7.9, 1.2 Hz, 2H), 7.93 (d, *J* = 7.9 Hz, 1H). ^{13}C NMR ($CDCl_3$): 17.7, 121.7, 124.1, 126.9, 127.0, 127.3, 127.4, 127.8, 128.3, 130.3, 133.8, 136.3, 138.4, 152.1. EI-MS (*m/z*): 33(M^+), 200, 199(base), 198, 167, 154, 133. HRMS (EI) M^+(found): 332.0975; calcd: 332.0983 (for $C_{20}H_{16}N_2OS$).

4.4.20. *N*-Benzyl-10*H*-phenothiazine-10-carboxamide[41,45] (20)

Compound **20** 94%; colourless crystals, mp 135–136.8 °C (lit. mp 137–140 °C).[44] IR (Nujol): 3355, 1663, 1508, 1313, 1283, 1259, 1230, 1028, 754, 699 cm^{-1}. ^1H NMR ($CDCl_3$): 4.52 (d, *J* = 5.7 Hz, 2H), 5.38–5.39 (m, 1H), 7.22–7.26 (m, 2H), 7.28–7.38 (m, 7H), 7.44 (dd, *J* = 7.8, 1.1 Hz, 2H), 7.64 (d, *J* = 8.1 Hz, 2H). ^{13}C NMR ($CDCl_3$): 44.9, 126.7, 127.2, 127.3, 127.4, 127.5, 128.2, 128.7, 133.6, 38.7, 138.9, 154.7. EI-MS (*m/z*): 332 (M^+), 201, 200, 199(base), 198, 167, 154, 91, 77. HRMS (EI) M^+(found): 332.0982; calcd: 332.0983 (for $C_{20}H_{16}N_2OS$).

4.4.21. *N*-(2-Phenylethyl)-10*H*-phenothiazine-10-carboxamide[46] (21)

Compound **21** 18%, Colourless crystals, mp 111.5–113 °C. IR (Nujol): 3419, 1677, 1515, 1306, 1272, 1251, 1031, 773, 757, 750, 706 cm^{-1}. ^1H NMR ($CDCl_3$): 2.78(t, *J* = 6.7 Hz, 2H), 3.43–3.51(m, 2H), 4.96–5.00(m, 1H), 7.09–7.43(m, 13H). ^{13}C NMR ($CDCl_3$): 35.7, 42.1, 126.4, 126.5, 127.0, 127.1, 127.9, 128.54, 128.8, 133.4, 138.6, 139.0, 154.5. EI-MS (*m/z*): 346(M^+), 201, 200, 199(base), 198, 167, 166, 154. HRMS (EI) M^+(found): 346.1129; calcd: 346.1140 (for $C_{21}H_{18}N_2OS$).

4.4.22. *N*-(Naphthalen-1-ylmethyl)-10*H*-phenothiazine-10-carboxamide (22)

Compound **22** 69%; colourless crystals, mp 182–184.5 °C. IR (Nujol): 3338, 1650, 1527, 1255, 1230, 805, 776, 753 cm^{-1}. ^1H NMR ($CDCl_3$): 4.92(d, *J* = 5.2 Hz, 2H), 5.28–5.32(m, 1H), 7.12–8.07(m, 15H). ^{13}C NMR ($CDCl_3$): 43.2, 123.5, 125.4, 126.0, 126.2, 126.55, 126.63, 127.0, 127.3, 128.1, 128.5, 128.8, 131.4, 133.5, 133.8, 138.6, 154.4. EI-MS (*m/z*): 382(M^+), 200, 199(base), 198, 183, 167, 166, 154, 141, 139, 128, 127. HRMS (EI) M^+(found): 382.1128; calcd: 382.1140 (for $C_{24}H_{18}N_2OS$).

4.4.23. *N*,*N*-Dimethyl-10*H*-phenothiazine-10-carboxamide (23)

Compound **23** 50%; colourless crystals, mp 99–101 °C (lit. mp 94–96 °C).[47] IR (Nujol): 1668, 1587, 1281, 1247, 1171, 1087, 1066, 1029, 1021, 945, 760, 750 cm^{-1}. ^1H NMR ($CDCl_3$): 2.86(s, 6H), 7.05–7.10(m, 2H), 7.19–7.24(m, 2H), 7.28(dd, *J* = 7.6, 1.4 Hz, 2H), 7.61(dd, *J* = 8.1, 1.1 Hz, 2H). ^{13}C NMR ($CDCl_3$): 37.7, 121.1, 124.7, 127.5, 127.5, 128.0, 141.5, 157.9. EI-MS (*m/z*): 270(M^+), 200, 199(base), 198, 197, 154, 72. HRMS (EI) M^+(found): 270.0834; calcd: 270.0827 (for $C_{15}H_{14}N_2OS$).

4.4.24. *N*,*N*-Diethyl-10*H*-phenothiazine-10-carboxamide (24)

Compound **24** 84%; colourless crystals, mp 86.7–88 °C (lit. mp 91–93 °C).[48] IR (Nujol): 1676, 1415, 1311, 1289, 1269, 1237, 1215, 1157, 1091, 1036, 847, 752 cm^{-1}. ^1H NMR ($CDCl_3$): 1.03(t, *J* = 7.1 Hz, 6H), 3.37(q, *J* = 7.1 Hz, 4H), 6.99–7.03(m, 2H), 7.13–7.16(m, 2H), 7.21(dd, *J* = 7.7, 1.2 Hz, 2H), 7.43(d, *J* = 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$): 12.9, 42.4, 119.3, 124.3, 125.9, 127.3, 127.4, 141.6, 156.9. EI-MS (*m/z*): 298(M^+), 199, 198(base), 197, 196, 167, 166, 101, 100, 72. HRMS (EI) M^+(found): 298.1142; calcd: 298.1140 (for $C_{17}H_{18}N_2OS$).

4.4.25. *N*,*N*-Dipropyl-10*H*-phenothiazine-10-carboxamide[42] (25)

Compound **25** 51%; colourless crystals, mp 84.2–85.8 °C. IR (Nujol): 1671, 1591, 1570, 1415, 1304, 1237, 1206, 1163, 1135, 1102, 1038, 931, 755, 741 cm^{-1}. ^1H NMR ($CDCl_3$): 0.82(t, *J* = 7.6 Hz, 6H), 1.43–1.59(m, 4H), 3.21–3.25(m, 4H), 6.98–7.06(m, 2H), 7.11–7.19(m, 2H), 7.22(dd, *J* = 7.7, 1.5 Hz, 2H), 7.41(dd, *J* = 7.9, 1.3 Hz, 2H). ^{13}C NMR ($CDCl_3$): 11.4, 21.2, 50.1, 119.1, 124.4, 125.8, 127.4, 127.5, 141.7, 157.2. EI-MS (*m/z*): 326(M^+; base), 200, 199, 198, 197, 167, 166, 154, 129, 128, 86. HRMS (EI) M^+(found): 326.1457; calcd: 326.1453 (for $C_{19}H_{22}N_2OS$).

4.4.26. *N*,*N*-Diisopropyl-10*H*-phenothiazine-10-carboxamide[42] (26)

Compound **26** 22%; colourless crystals, mp 163–165 °C. IR (Nujol): 1675, 1592, 1570, 1432, 1314, 1285, 1243, 1225, 1205, 1155, 1089, 1041, 818, 748, 740 cm^{-1}. ^1H NMR ($CDCl_3$): 1.23(d, J = 6.7 Hz, 12H), 4.02 (m, 2H), 6.94–7.01(m, 2H), 7.08–7.19(m, 4H), 7.30(dd, J = 7.9, 1.2 Hz, 2H). ^{13}C NMR ($CDCl_3$): 20.4, 48.3, 117.9, 124.0, 124.4, 127.3, 127.4, 141.7, 155.1. EI-MS (*m/z*): 326(M^+), 200, 199, 198, 197, 167, 166, 154, 129, 128, 87, 86, 69. HRMS (EI) M^+(found): 326.1462; calcd: 326.1453 (for $C_{19}H_{22}N_2OS$).

4.4.27. (10*H*-Phenothiazin-10-yl)(pyrrolidin-1-yl)methanone (27)

Compound **27** 74%; colourless crystals, mp 136.2–137.5 °C (lit. mp 137–139 °C).[48] IR (Nujol): 1653, 1588, 1567, 1308, 1244, 1233, 1033, 760 cm^{-1}. ^1H NMR ($CDCl_3$): 1.72–1.80(m, 4H), 3.19(m, 4H), 7.05–7.08(m, 2H), 7.19–7.23(m, 2H), 7.27(dd, J = 7.7, 1.2 Hz, 2H), 7.69(d, J = 8.1 Hz, 2H). ^{13}C NMR ($CDCl_3$): 25.3, 47.7, 122.5, 124.9, 127.4, 127.5, 129.4, 141.4, 156.4. EI-MS (*m/z*): 296(M^+), 199, 198, 197, 171, 154, 98. HRMS (EI) M^+(found): 296.0997; calcd: 296.0983 (for $C_{17}H_{16}N_2OS$).

4.4.28. (10*H*-Phenothiazin-10-yl)(piperidin-1-yl)methanone (28)

Compound **28** 82%; colourless crystals, mp 118–121 °C (lit. mp 118–120 °C).[48] IR (Nujol): 1651, 1589, 1567, 1481, 1404, 1307, 1277, 1246, 1219, 1133, 1028, 988, 939, 771, 762, 648 cm^{-1}. ^1H NMR ($CDCl_3$): 1.41–1.48(m, 4H), 1.51–1.56(m, 2H), 3.38–3.43(m, 4H), 7.08–7.12(m, 2H), 7.22–7.25(m, 2H), 7.29(d, J = 7.6 Hz, 2H), 7.63(d, J = 8.1 Hz, 2H). ^{13}C NMR ($CDCl_3$): 24.3, 25.3, 46.7, 121.1, 124.7, 127.38, 127.44, 127.7, 141.6, 156.9. EI-MS (*m/z*): 310 (M^+), 199, 198, 113, 112, 69, 56. HRMS (EI) M^+(found): 310.1128; calcd: 310.1140 (for $C_{18}H_{18}N_2OS$).

4.4.29. Morpholino(10*H*-phenothiazin-10-yl)methanone[49] (29)

Compound **29** 95%; colourless crystals, mp 135.2–137.2 °C. IR (Nujol): 1653, 1409, 1357, 1315, 1301, 1283, 1267, 1249, 1226, 1117, 998, 939, 757 cm^{-1}. ^1H NMR ($CDCl_3$): 3.36–3.41(m, 4H), 3.55–3.59(m, 4H), 7.12–7.15(m, 2H), 7.26–7.30(m, 2H), 7.33–7.35(m, 2H), 7.71(d, J = 8.1 Hz, 2H). ^{13}C NMR ($CDCl_3$): 46.1, 66.2, 122.1, 125.2, 127.5, 127.6, 128.9, 141.2, 157.1. EI-MS (*m/z*): 312(M^+; base), 199, 198, 171, 154, 113, 112, 69. HRMS (EI) M^+(found): 312.0925; calcd: 312.0932 (for $C_{17}H_{16}N_2O_2S$).

4.4.30. *N*-[2-(*N*′,*N*′–Diethylamino)ethyl]-10*H*-phenothiazine-10-carboxamide hydrochloride salt (30)

Compound **30** 43%; colourless crystals, mp 184–186 °$C_{(dec)}$ (lit. mp 184–186 °$C_{(dec)}$).[48] IR (Nujol): 3366, 1658, 1512, 1319, 1256, 771 cm^{-1}. ^1H NMR (DMSO-d_6): 1.20(t, J = 7.2 Hz, 6H), 3.07–3.11(m, 6H), 7.21–7.28(m, 2H), 7.32–7.39(m, 2H), 7.47(dd, J = 7.6, 1.5 Hz, 2H), 7.60(dd, J = 7.9, 0.9 Hz, 2H), 10.72(broad s, 1H). ^{13}C NMR (DMSO-d_6): 8.5, 35.4, 46.9, 49.2, 126.6, 127.4, 127.6, 127.8, 132.3, 138.6, 154.3. EI-MS (*m/z*): 342(M^++H^+), 269, 201, 200, 199, 198, 167, 166, 154, 143, 100, 86. HRMS (EI) M^+(found): 342.1418; calcd: 342.1640 (for $C_{19}H_{24}N_3OS^+$).

4.4.31. *N*-[2-(*N*′,*N*′–Diisopropylamino)ethyl]-10*H*-phenothiazine-10-carboxamide (31)

Compound **31** 54%; colourless crystals, mp 109–111.5 °C. IR (Nujol): 3336, 1657, 1511, 1284, 1177, 756 cm^{-1}. ^1H NMR (DMSO-d_6): 0.81(d, J = 6.6 Hz, 12H), 2.51(t, J = 6.0 Hz, 2H), 2.85(septet, J = 6.6 Hz, 2H), 3.22–3.26(m, 2H), 7.15–7.19(m, 2H), 7.26–7.30(m, 2H), 7.36(dd, J = 7.8, 1.2 Hz, 2H), 7.56(dd, J = 7.9, 1.0 Hz, 2H). ^{13}C NMR (DMSO-d_6): 20.6, 38.9, 42.6, 47.1, 126.4, 127.2, 127.5, 128.0, 133.6, 139.1, 154.9. EI-MS (*m/z*): 369 (M^+), 368, 354, 269, 199, 198, 172, 171(base), 167, 157, 129, 114, 72. HRMS (EI) M^+(found): 369.1875; calcd: 369.1875 (for $C_{21}H_{27}N_3OS$).

4.4.32. *N*-[2-(Pyrrolidinyl)ethyl]-10*H*-phenothiazine-10-carboxamide[50] (32)

Compound **32** 28%, colourless crystals, mp 94–95.5 °C. IR (Nujol): 3373, 3331, 1673, 1536, 1494, 1309, 1277, 1247, 1153, 755 cm^{-1}. ^1H NMR: 1.65–1.71(m, 4H), 2.42–2.46(m, 4H), 2.57(t, J = 6.3 Hz, 2H), 3.32–3.37(m, 2H), 5.71(t, J = 5.8 Hz, 1H), 7.15–7.19(m, 2H), 7.26–7.30(m, 2H), 7.37(dd, J = 7.7, 1.3 Hz, 2H), 7.56(dd, J = 8.0, 1.0 Hz, 2H). ^{13}C NMR: 23.7, 39.6, 53.7, 54.3, 126.5, 127.1, 127.2, 128.0, 133.4, 139.0, 154.8. EI-MS (*m/z*): 339(M), 269, 201, 200, 199(base), 198, 167, 166, 142, 141, 84. HRMS (EI) M^+ (found): 339.1400; calcd: 339.1405 (for $C_{19}H_{21}N_3OS$).

4.4.33. *N*-[2-(Piperidinyl)ethyl]-10*H*-phenothiazine-10-carboxamide[50] (33)

Compound **33** 63%, colourless crystals, mp 67–70 °C. IR (Nujol): 3427, 1685, 1505, 1316, 1298, 1282, 1250, 1219, 773, 752 cm^{-1}. ^1H NMR: 1.33–1.45(overlapping m, 6H), 2.23–2.34(m, 4H), 2.38(t, J = 6.0 Hz, 2H), 3.28–3.32(m, 2H), 5.87(t, J = 5.6 Hz, 1H), 7.16–7.19(m, 2H), 7.28–7.31(m, 2H), 7.37(dd, J = 7.7, 1.1 Hz, 2H), 7.56(dd, J = 7.9, 0.9 Hz, 2H). ^{13}C NMR: 24.3, 26.0, 37.4, 53.9, 56.6, 126.3, 127.0, 127.1, 127.8, 133.2, 138.8, 154.5. EI-MS (*m/z*): 340(M+H^+), 353($M-^+$), 269, 200, 199, 198, 167, 156, 155(base), 99, 98. HRMS (EI) M^+ (found): 353.1559; calcd: 353.1562 (for $C_{20}H_{23}N_3OS$).

4.4.34. *N*-(2-Morpholinoethyl)-10*H*-phenothiazine-10-carboxamide[46] (34)

Compound **34** 67%, colourless crystals, mp 106.5–108 °C. IR (Nujol): 3440, 1675, 1510, 1143, 1113, 936, 913, 850, 769 cm^{-1}. ^1H NMR: 2.31–2.39(m, 4H), 2.42(t, J = 6.4 Hz, 2H), 3.28–3.35(m, 2H), 3.48–3.60(m, 4H), 5.75(broad s, 1H), 7.14–7.22(m, 2H), 7.26–7.33(m, 2H), 7.38(dd, J = 7.6, 1.5 Hz, 2H), 7.54 (dd, J = 7.9, 1.2 Hz, 2H). ^{13}C NMR: 37.0, 53.0, 56.5, 67.0, 126.5, 127.1, 127.2, 128.0, 133.4, 138.8, 154.6. EI-MS (*m/z*): 355(M^+), 200, 199(base), 198, 167, 157, 100. HRMS (EI) M^+ (found): 355.1367; calcd: 355.1354 (for $C_{19}H_{21}N_3OS$).

4.4.35. *N*-(3-Morpholinopropyl)-10*H*-phenothiazine-10-carboxamide (35)

Compound **35** 62%, colourless crystals, mp 112–114 °C. IR (Nujol): 3348, 1656, 1516, 1319, 1278, 1254, 1119, 767, 759 cm^{-1}. ^1H NMR: 1.60–1.70(m, 2H), 2.27–2.38 (m, 6H), 3.34–3.44 (m, 6H), 5.79(t, J = 5.0 Hz, 1H), 7.15–7.22(m, 2H), 7.27–7.34(m, 2H), 7.39(dd, J = 7.6 Hz, 2H), 7.61 (dd, J = 7.9, 1.3 Hz, 2H). ^{13}C NMR: 25.4, 40.3, 53.7, 57.4, 66.6, 126.3, 126.9, 127.1, 128.0, 133.1, 139.0, 154.7. EI-MS (*m/z*): 369(M^+), 201, 200, 199, 198, 172, 171(base), 167, 166, 100. HRMS (EI) M^+ (found): 369.1506; calcd: 369.1511 (for $C_{20}H_{23}N_3O_2S$).

4.4.36. (4-Methylpiperazin-1-yl)(10H-phenothiazin-10-yl)methanone (36)

Compound **36** 26%, colourless crystals, mp 237–238 °$C_{(dec)}$ (lit mp: 228–230 °C).[51] IR (Nujol): 1673, 1415, 1300, 1280, 1247, 975, 753, 742 cm^{-1}. ^1H NMR: 2.68–2.83(m, 5H), 3.27–3.38(m, 2H), 3.45–3.60(m, 2H), 3.80–3.91(m, 2H), 7.14–7.21(m, 2H), 7.26–7.34(m, 2H), 7.37 (dd, J = 7.6, 1.5 Hz, 2H), 7.69(dd, J = 7.9, 1.2 Hz, 2H), 12.97(broad s, 1H). ^{13}C NMR: 42.6, 43.2, 52.5, 123.6, 126.0, 127.6, 127.8, 130.4, 140.1, 156.7. EI-MS (*m/z*): 325(M^+, base peak), 200, 199, 198, 167, 154, 128, 127, 85, 70. HRMS (EI) M^+ (found): 325.1241; calcd: 325.1249 (for $C_{18}H_{19}N_3OS$).

4.4.37. *N*-(3-(Methyl(phenyl)amino)propyl)-10*H*-phenothiazine-10-carboxamide (37)

Compound **37** 26%, colourless crystals, mp 110–111.5 °C. IR (Nujol): 3441, 1675, 1598, 1506, 1316, 1251, 1214, 1072, 990, 769, 756 cm^{-1}. ^1H NMR: 1.77–1.84(m, 2H), 2.82(s, 3H), 3.31–3.38(m, 4H), 5.33(broad t, J = 5.3 Hz, 1H), 6.60–6.69(m, 2H), 6.75(t, J = 7.4 Hz, 1H), 7.18–7.25(m, 4H), 7.26–7.30(m, 2H), 7.40(dd, J = 7.7, 1.3 Hz, 2H), 7.55(dd, J = 7.9, 1.3 Hz, 2H). ^{13}C NMR: 27.3, 38.4, 39.7, 51.5, 113.2, 117.1, 126.8, 127.4, 127.4, 128.3, 129.4, 133.7, 139.0, 149.8, 155.0. EI-MS on salt (*m/z*): 390(M^+), 199(base), 191, 167, 166, 154, 104, 91, 77. HRMS on salt (EI) M^+ (found): 390.1632; calcd: 390.1640 (for $C_{23}H_{24}N_3OS^+$).

4.4.38. (Adamant-1-yl)(10*H*-phenothiazin-10-yl)methanone (38)

Compound **38** 10%, dark greenish-brown crystals, mp 177–178.1 °C. IR (Nujol): 1655, 1585, 1318, 1284, 1257, 1234, 1174, 763, 740 cm^{-1}. ^1H NMR: 1.49–1.62(m, 6H), 1.82–1.88(m, 9H), 7.19(td, J = 7.6, 1.5 Hz, 2H), 7.29(td, J = 7.7, 1.5 Hz, 2H), 7.45(dd, J = 7.6, 1.5 Hz, 2H), 7.62(dd, J = 7.6, 1.2 Hz, 2H). ^{13}C NMR: 28.4, 36.3, 39.7, 44.9, 126.4, 126.7, 127.4, 128.1, 134.6, 141.0, 178.0. EI-MS (*m/z*): 361(M^+), 198, 136, 135(base), 107, 93, 79. HRMS (EI) M^+ (found): 361.1480; calcd: 361.1500 (for $C_{23}H_{23}NOS$).

4.5. Enzyme kinetic studies

The esterase activity of AChE and BuChE was determined by a modification[21] of the Ellman[29] spectrophotometric method. Briefly, 1.35 mL of buffered DTNB solution (usually pH 8.0), 0.05 mL of human recombinant AChE (~0.03 units) or purified human serum BuChE (~0.05 units) in 0.1% aqueous gelatin and 0.05 mL of 50% aqueous acetonitrile or one of the phenothiazine derivatives dissolved in this solvent, in a glass, or quartz, stoppered cuvette of 1 cm path-length. Serial dilutions of (1:10) of each inhibitor 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 0.05 mL acetylthiocholine (for AChE) or butyrylthiocholine, except where acetylthiocholine is indicated (for BuChE) in an aqueous solution at a final concentration of 1.6×10^{-4} M. Final volume was always 1.5 mL. 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–vis spectrophotometer (Milton-Roy, Ivyland, PA) set at λ = 412 nm. This provided a rate versus [I] profile for the selection of suitable inhibitor concentrations for further analysis. These experiments were generally done at least in triplicate and the values averaged. The final concentration of acetonitrile (1.67% v/v), in the Ellman assays, was found to have no measurable effect on the activity of AChE or BuChE. 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. Inhibitor concentrations to be used were based on the previously generated inhibitor concentration/rate profile. The replot 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. Kinetics were generally carried out at pH 8.0 because of the faster rate of hydrolysis of choline esters,[23] which allows for more efficient kinetic analysis. Furthermore, it has been shown previously[23] that K_i values are independent of pH between 6.8 and 8.0. To confirm this in the present study, the K_i value for compound 31 with BuChE and butyrylthiocholine as substrate was 6.6×10^{-9} M at pH 7.4 which was comparable to the value determined at pH 8.0 (9.0×10^{-9} M, Table 4). As a consequence, all the K_i values reported in Tables 1–4 were determined at pH 8.0 for comparison of relative inhibitor potency under the same conditions.

To examine the effect of high substrate concentration on the inhibition of cholinetsrases by aminoureas, such as compound **31**, Michaelis–Menten plots ([substrate] vs rate) were developed over a wide range of substrate concentrations (0.039–40 mM of acetylthiocholine) that would ensure substrate inhibition of AChE and substrate activation of BuChE. These observations were made in the absence and presence of an appropriate concentration of compound **31**.

4.6. Calculation of molecular parameters

Computational chemistry studies were carried out using molecular mechanics methods, employing SPARTAN'06.[30] The Merck molecular force field (MMFF) was used, selecting the most stable conformer for the calculation of molecular volume.[30] Figure 7 was generated using the SPARTAN'06 program.[30] First, the crystal structure of human acetylcholinesterase,[32](PDB ID: 1B41) and human butyrylcholinesterase (PDB ID: 1POI)[52] was obtained from the protein databank[53] and PyMol was employed to delete all amino acids save for those selected residues found in the active site.[54] Compound **31** was optimized separately, using molecular mechanics and the MMFF force field, within SPARTAN'06 to obtain the preferred conformer.[30] Using SPARTAN'06, the optimized compound **31** was manually fitted into the active site gorge to represent simulated binding.

4.7. Calculation of log *P* values

In order to assess the ability of the compound to cross the hydrophobic blood–brain barrier, log *P* values were obtained using the ALOGPS method.[31]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.066.

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