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Original article

Quinolizidinyl derivatives of bi- and tricyclic systems as potent inhibitors of acetyl- and butyrylcholinesterase with potential in Alzheimer's disease

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ABSTRACT

On the pattern of the potent and selective butyrylcholinesterase (BChE) inhibitors ethopropazine and Astra1397, sets of quinolizidinyl derivatives of bi- and tricyclic (hetero)aromatic systems were studied as dual, or BChE-selective inhibitors. All compounds exhibited activity against both cholinesterases, but inhibition of BChE was generally stronger, with submicromolar IC_{50} values for most of them (e.g. **15**: IC_{50} *versus* BChE = 0.15 μM; SI = 47). However, in a subset of quinolizidinyl derivatives of 6-hydroxycoumarin an inverted selectivity for acetylcholinesterase (AChE) was observed (e.g. **46**: IC_{50} *versus* AChE = 0.35 μM; SI = 0.06). Docking studies furnished a sound interpretation of the observed different enzyme activity. Several of the studied compounds have shown, in the past, additional pharmacological properties (as antagonism on presynaptic muscarinic autoreceptor; inhibition of enkephaline aminopeptidase and antipsychotic activity) of some relevance in Alzheimer's disease, and may, therefore, represent hits for the development of interesting single-entity multi-target drugs.

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by a gradual decline of cognitive processes, later associated with behavioral and psychiatric symptoms. The multifactorial pathogenesis of AD includes accumulation of aggregates of β-amyloid (Aβ) and tau protein and loss of cholinergic neurons, with consequent deficit of the neurotransmitter acetylcholine (ACh) [1].

The inhibition of acetylcholinesterase (AChE, EC 3.1.1.7), that is responsible for the breakdown of ACh, has proven a successful approach to relieve some cognitive and behavioral symptoms of AD [2,3]. In advancing AD, AChE levels in the brain are declining, but a progressive increase (up to 90%) of butyrylcholinesterase (BChE, EC. 3.1.1.8) is observed, which too is able, even if at lower rate, to hydrolyze Ach [4]. Selective BChE inhibitors have already been reported to increase the ACh levels in the brain, and, very interestingly, to also reduce the formation of abnormal amyloid [5–7]. Therefore the discovery of potent and highly selective BChE inhibitors and/or of dual AChE–BChE inhibitors, is an actively pursued goal in AD. Indeed, many research projects in the field have been focused on the identification of new ligands addressing multiple key targets through the so-called "single entity-multitarget ligand" or "multi-target directed ligand" approaches [8].

At present, cholinesterase inhibitors (Fig. 1) are the most commonly used drugs for the treatment of mild and moderate AD, despite no long-term efficacy has been proved. Donepezil [9] and galantamine [10] are highly active and specific AChE inhibitors, while rivastigmine [11] is a dual (and long lasting) inhibitor, which has been reported to co-inhibit AChE and BChE in human brain with equal potency [11c], in contrast with results on human erythrocytes and plasma enzymes [7] (Fig. 1). The first approved drug, tacrine [12], was recently withdrawn because of high incidence of hepatotoxicity, while clinical trials with eptastigmine [13] have been suspended due to adverse hematological effects.

Abbreviations: Aβ, β-amyloid; ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; ADME, absorption, distribution, metabolism and excretion; BChE, butyrylcholinesterase; ChE, cholinesterase; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); HB, hydrogen bond; NMDA, N-methyl-d-aspartate; PAS, peripheral anionic site; SI, selectivity index; TcAChE, torpedo californica acetylcholinesterase.

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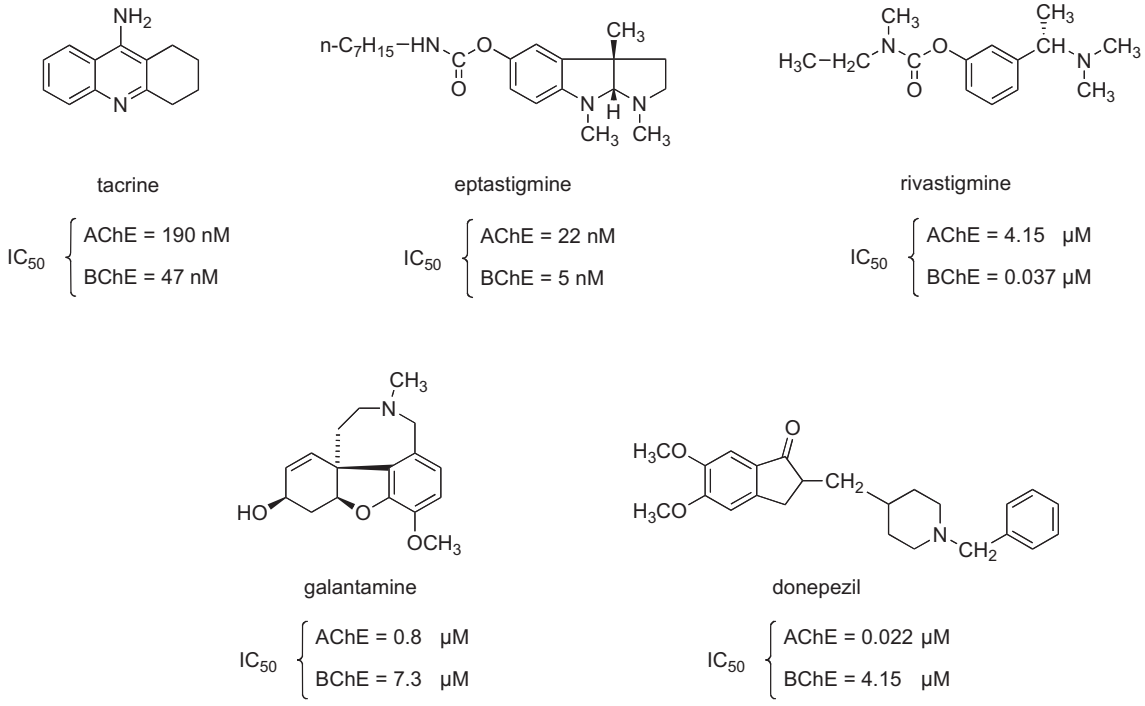


Fig. 1. Cholinesterase inhibitors used in AD therapy. IC_{50} for human erythrocyte and plasma enzymes.

An alternative symptomatic treatment of moderate-to-advanced AD may be made with memantine [14], an N-methyl-D-aspartate (NMDA) receptor antagonist, while there is no approved treatment with proven disease-modifying effects [2b].

Potent and highly selective BChE inhibitors are represented by some physostigmine derivatives and analogs, as phenethylcymserine [15] and [4-(4-morpholinyl)butyl]carbamic acid 3-(1,1-dimethyl-2-dimethylaminoethyl)phenyl ester [16] (MF 8622), and also by some phenothiazine derivatives as ethopropazine [17], N-[(2-diethylamino)propionyl]phenothiazine [18] (ASTRA1397; **28**) and 10-(9-anthrylcarbonyl)phenothiazine [19] (Fig. 2). The last compound is the most potent (K_i = 3.5 nM) among a large set of 10-aroyl-, 10-(ω-aryl)alkanoyl- and 10-alkanoylphenothiazines studied by Darvesh and coll. [19,20] and, interestingly, is devoid of any basic group and exhibits a log P value as high as 6.31. According to the authors, enzyme selectivity and inhibitory potency might be related to the larger active site gorge of BChE in respect to AChE, while the two aromatic rings of phenothiazine provided the binding to BChE through π–π interactions with two aromatic side chains of residues F329 and Y332.

Thus, in order to achieve novel cholinesterase inhibitors, either dual or, even better, selective for BChE and to gain insight on the structural determinants of high BChE affinity and selectivity we have investigated a number of derivatives of phenothiazine and other related tricyclic systems. Moreover, to better exploit the key role of the tricyclic systems in ligand binding, some additional compounds derived from diphenylamine and diphenylmethane and from bicyclic heterocycles as coumarin, were also considered. Indeed coumarin moiety has been demonstrated to be compatible with high anticholinesterase potency [21,22].

Most of the tested compounds are characterized by the presence of a quinolizidine ring, a basic moiety that is simultaneously bulky and high lipophilic, linked to the aromatic moieties through spacers of different length and flexibility. It is worth noting that even simple derivatives of quinolizidine nucleus, as the *epi*-lupinine esters of 4-hydroxycinnamic and ferulic acids [23] and dicarboxylic acid esters of lupinine [24], are endowed with some anticholinesterase activity. Glutaric acid ester of lupinine exhibited K_i = 199 and 14800 nM *versus* AChE and BChE, respectively; quaternarization reduced the potency *versus* AChE while increased that for BChE, thus shifting the ratio of the corresponding K_i values from 74 to 4.3.

Some well known tricyclic drugs, as the antiparkinson ethopropazine [17,25] and methixene [26], the antipsychotic triflupromazine [27] and periciazine [28] as well as **28** [18,29] and the antimalarial quinacrine [30], have been included in our biological screening. Three of them which have been already proven to be endowed with anticholinesterase action (i.e., ethopropazine, **28** and quinacrine) served also as reference compounds.

On the whole 48 compounds were evaluated for their inhibitory activity on AChE and BChE from bovine erythrocytes and equine serum, respectively. Their structures and inhibition data are collected in Table 1.

2. Chemistry

Most of the studied compounds have been already described by some of us [31–42] and investigated for different pharmacological aims, and were now purposely reprepared according to the references cited for each of them in Table 1. Ethopropazine (**1**), periciazine (**3**) and methixene (**14**) were recovered and thoroughly purified from commercially available drugs (Parsidol®, Neuleptil® and Tremaril®, respectively), while quinacrine (**40**) was purchased from Sigma–Aldrich. The remaining compounds **4**, **16**, **21**, **24**, **25**, **33**, **42** and **45**–**48**, in Table 1 were obtained as follows.

Compound **16** was obtained by reacting the previously described **15** [36] with methyl iodide.

The quinuclidinyl phenothiazine **4**, though described in a patent [43a], has been now prepared with minor modifications by reacting phenothiazine with 3-quinuclidinyl tosylate [43b] obtaining a product with a higher melting point. The phenothiazine derivative **33** was prepared by reacting phenothiazine with 2-bromopropionyl bromide, followed by thiolupinine [(1*R*,9a*R*)

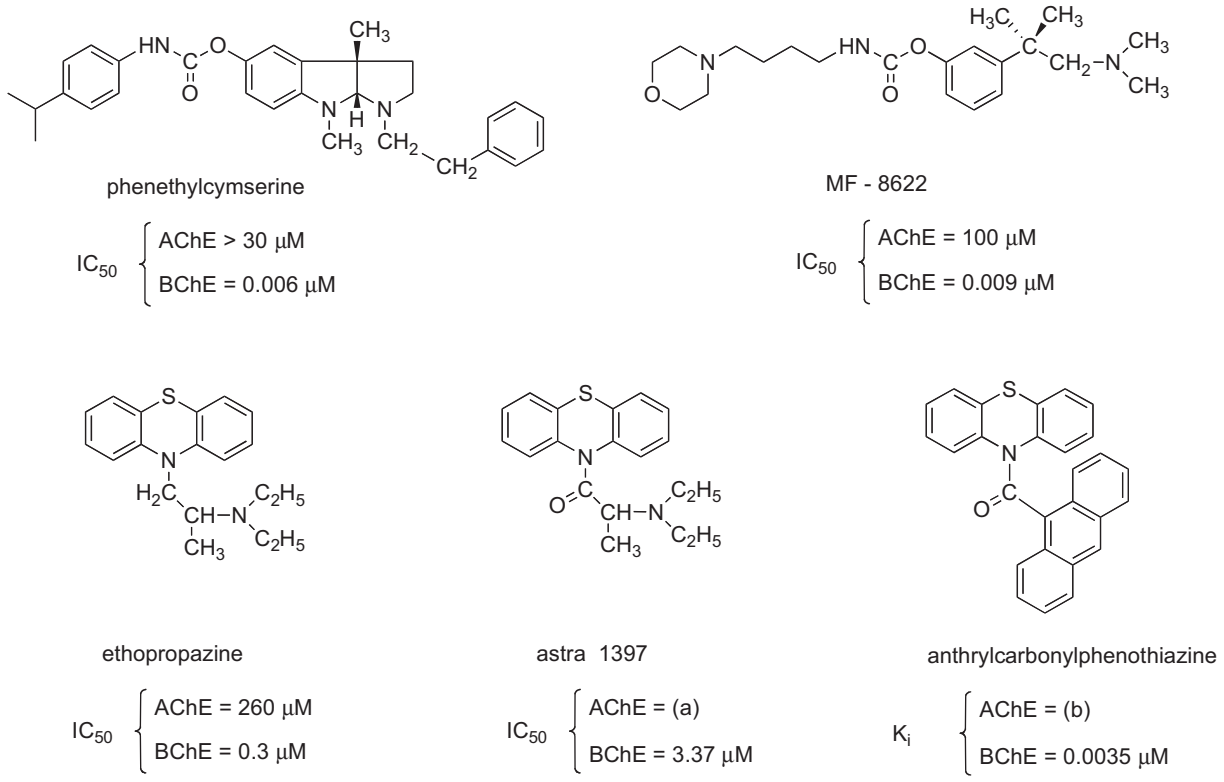


Fig. 2. Investigational cholinesterase inhibitors. Ic_{50} for human enzymes. (a) Activity undetected up to 100 μM. (b) Activity undetected up to solubility limit.

octahydro-2*H*-quinolizine-1-methanethiol] [44], (Scheme 1). The intermediate 2-bromopropionylphenothiazine has been already described [18a,d].

Reacting chlorolupinane [(1*R*,9a*R*)-1-(chloromethyl)-octahydro-2*H*-quinolizine] [45] with, respectively, carbazole and diphenylamine, the lupinyl derivatives **21** and **24** were obtained. Lupinyl diphenylmethane **25** was obtained by treating the previously described 1,1-diphenyl-2-(octahydroquinolizin-1-yl)ethanol [33] with red phosphorus and hydriodic acid (Scheme 2).

To synthesize the quinacrine analog **42** it was preliminarily necessary to prepare the 2-(lupinylthio)ethylamine by $LiAlH_4$ reduction of lupinylthioacetamide, in turn obtained by reacting thiolupinine [44] with iodoacetamide. Finally the aminocompound was reacted with 6,9-dichloro-2-methoxyacridine (Scheme 3). The quinacrine analog **43** [42] has been reprepared according to the described procedure [42], but it was obtained as crystals instead of an oil and in quite higher yield.

Finally to synthesize the coumarin derivatives **45**–**48**, the required 6-(ω-bromo-alkyl)hydroxycoumarin intermediates (**c**, **d**, **e**) were prepared and then reacted with aminolupinane [(1*S*,9a*R*)-1-(aminomethyl)-(octahydro-2*H*-quinolizine)] [46] or thiolupinine [44] (Scheme 4).

3. Biological assays

Compounds in Table 1 were tested for their inhibitory activity toward AChE and BChE by the classical spectrophotometric Ellman's assay [47]. IC_{50} values were determined only for compounds showing an inhibition >60% at 10 μM concentration. Inhibition data of low-active compounds are expressed as % of inhibition at the concentration of 10 μM. The enzyme selectivity was assessed by the selectivity index SI, that is the IC_{50} AChE/IC_{50} BChE affinity ratio.

4. Molecular modeling

The program GOLD [48] was used in all the docking simulations. In the present investigation, GOLD returned a confident reliability as it was capable to dock donepezil (Fig. 1) only at the expense of a low rmsd value (0.99 Å) compared to the binding conformation determined by X-ray crystallography (PDB entry 1EVE).

5. Results and discussion

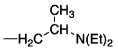
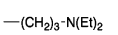
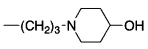
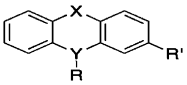
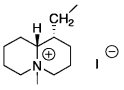
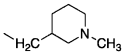
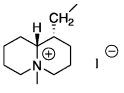
The inhibitory activities of compounds **1**–**48** toward AChE and BChE are collected in Table 1.

First of all it was observed that inhibition data of reference compounds ethopropazine (**1**) and **28** within the limits of the different enzyme source and assay experimental conditions, are in good agreement with literature data [17,18d].

All tested compounds exhibited some degree of activity on both AChE and BChE and their IC_{50} values were in the low micromolar or submicromolar range for at least one enzyme, with the exception of compounds **38** and **39**, that exhibited only a moderate activity on both enzymes. Thus the bulky and highly lipophilic quinolizidine moiety, that characterized the majority of the studied compounds, appeared as well suited for the expression of a good enzyme inhibitory activity. When comparing the lupinyl (quinolizidin-1α-ylmethyl) derivatives of the diverse tricyclic systems with the corresponding non-quinolizidine derivatives, a higher potency on BChE was generally observed (compare **5**, **7**, **15**, and **41** with **4**, **2**, **14**, and **40**, respectively). Two exceptions were represented by ethopropazine (**1**) and **28** that are more potent than lupinylphenothiazine (**5**) and homolupinanoylphenothiazine (**29**), respectively.

Generally, the quinolizidine derivatives inhibited BChE quite more strongly than AChE, with the only exception of four coumarin (**45**–**48**) and one acridine (**43**) derivatives which exhibited an

Table 1
Structure and ChEs inhibitory activity of compounds **1**–**48**.

General structure	X	Y	R	R′	Nr[a]	IC_{50} (μM) or % inhibition at 10 μM[b]		SI[c]
						AChE	BChE	
	S	N		H	**1**[d]	(*34* ± *4*)	0.72	
	S	N		CF_3	**2** [27,32]	(*45* ± *4*)	7.4	
	S	N		CN	**3**[e]	7.0	0.23	30
	S	N		H	**4**	(*41* ± *2*)	3.0	
	S	N		H	**5** [32]	(*39* ± *1*)	2.0	
	S	N		H	**6** [36]	(*52* ± *4*)	0.51	
	S	N		CF_3	**7** [32]	(*59* ± *1*)	4.0	
	O	N		H	**8** [32]	(*37* ± *3*)	2.2	
	Se	N		H	**9** [32]	(*24* ± *2*)	1.1	
	Se	N		H	**10** [34]	(*20* ± *2*)	0.87	
	CH_2	N		H	**11** [32]	(*50* ± *1*)	1.4	
	CH_2–CH_2	N		H	**12** [32]	(*55* ± *3*)	2.2	
	CH=CH	N		H	**13** [32]	8.3	2.2	3.8
	S	CH		H	**14**[f]	(*43* ± *4*)	1.6	
	S	CH		H	**15** [36]	7.0	0.15	47
	S	CH		H	**16**	(*44* ± *3*)	0.41	
	S	C–OH		H	**17** [33]	(*42* ± *4*)	0.95	
	S	C		H	**18** [33]	(*58* ± *3*)	0.93	

(*continued on next page*)

Table 1 (*continued*)

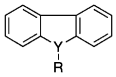
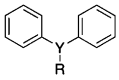
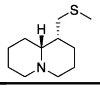
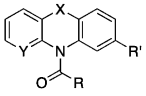
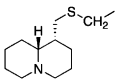
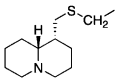
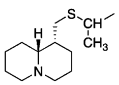
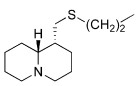
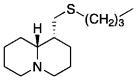
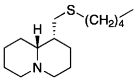
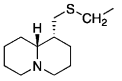
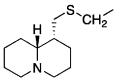
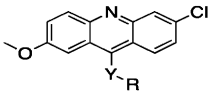
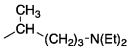
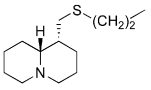
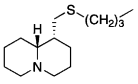
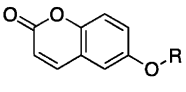
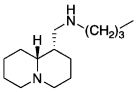
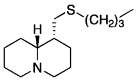
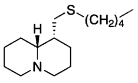
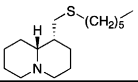
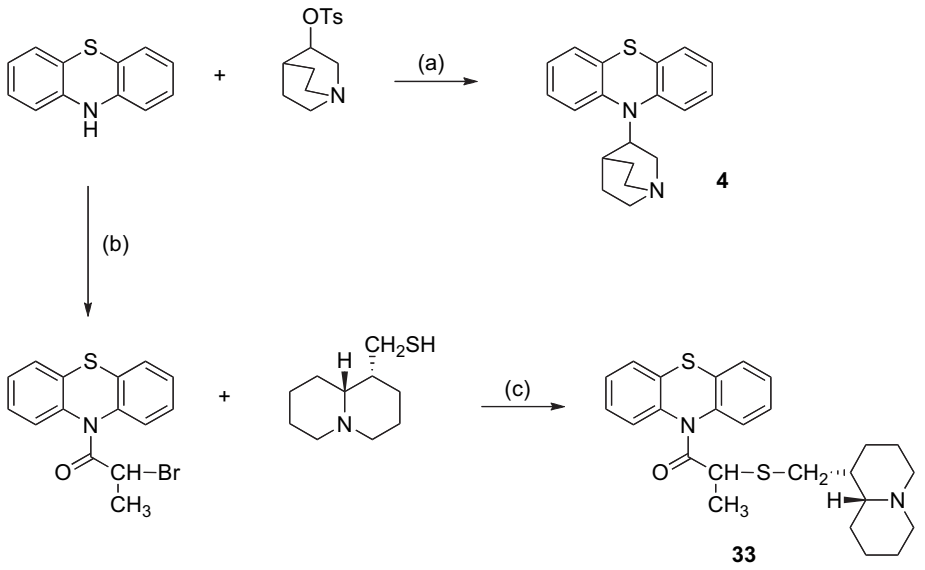
General structure	X	Y	R	R′	Nr[a]	IC_{50} (μM) or % inhibition at 10 μM[b]		SI[c]
						AChE	BChE	
	CH_2–CH_2	C		H	**19** [33]	(*46 ± 2*)	4.2	
	CH=CH	C		H	**20** [33]	8.3	2.0	4.1
		N			**21**	(*41 ± 5*)	2.0	
		CH			**22** [35]	(*45 ± 4*)	0.63	
		CH			**23** [38]	(*48 ± 4*)	1.1	
		N			**24**	8.9	8.1	1.1
		CH			**25**	6.7	5.5	1.2
		C			**26** [33]	(*35 ± 4*)	3.8	
		CH			**27** [39]	(*45 ± 2*)	3.3	
	S	CH		H	**28** [18],[g]	(*45 ± 1*)	0.84	
	S	CH		H	**29** [36]	(*49 ± 1*)	3.0	
	S	CH		H	**30** [41]	(*30 ± 1*)	4.8	
	S	CH		H	**31** [41]	(*32 ± 2*)	0.43	
	S	CH		OCH_3	**32** [41]	(*47 ± 2*)	1.2	
	S	CH		H	**33**	(*40 ± 5*)	0.47	
	S	CH		H	**34** [41]	(*47 ± 2*)	0.75	

Table 1 (*continued*)

General structure	X	Y	R	R′	Nr[a]	IC_{50} (μM) or % inhibition at 10 μM[b]		SI[c]
						AChE	BChE	
	S	CH		H	**35** [41]	6.8	0.74	9.2
	S	CH		H	**36** [41]	5.7	0.89	6.4
	CH_2–CH_2	CH		H	**37** [41]	(*34* ± *4*)	0.86	
	NH–CO	N		H	**38** [40]	(*42* ± *5*)	(*28* ± *3*)	
	NH–CO	N		H	**39** [40]	(*29* ± *3*)	(*34* ± *4*)	
		NH			**40**[h]	(*49* ± *2*)	4.5	
		NH			**41** [37]	7.3	0.87	8.4
		NH			**42**	0.84	0.34	2.5
		NH			**43** [42]	0.22	0.69	0.32
		S			**44** [42]	(*57* ± *3*)	4.7	
					**45**	6.6	(*40* ± *4*)	
					**46**	0.35	5.4	0.06
					**47**	1.2	4.3	0.28
					**48**	0.68	5.8	0.12

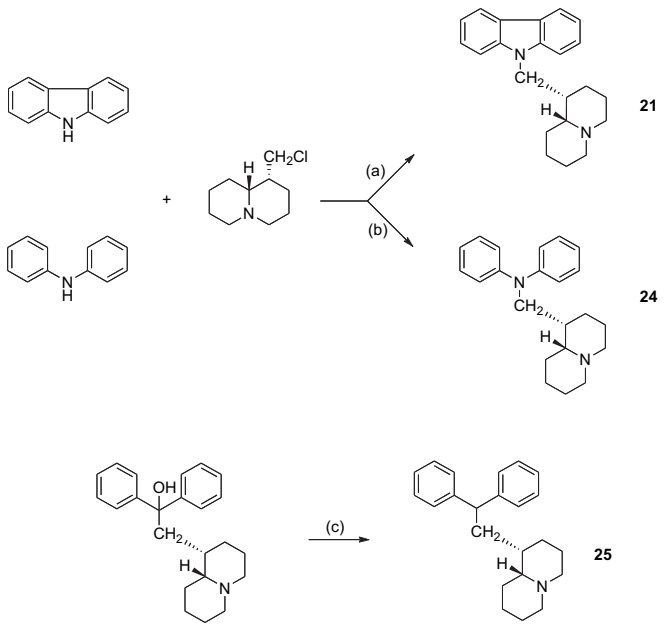
[a] Notes: Superscript numbers refer to bibliographic notes.
[b] inhibition data from 2 or 3 different experiments are expressed as IC_{50} (μM; relative SEM < 10%) or percent of inhibition at 10 μM ± SEM, italics, (in parentheses).
[c] SI is the selectivity index defined as IC_{50} AChE/IC_{50} BChE affinity ratio.
[d] Ethopropazine.
[e] Periciazine.
[f] Methyxene.
[g] ASTRA1397.
[h] Quinacrine.



Scheme 1. Reagents and conditions: (a) $NaNH_2$, dry xylene, 4h, reflux; b) 2-bromopropionyl bromide, toluene, 3h, reflux; (c) abs. EtOH, 7 h, reflux.

inverted selectivity. Thus the claimed importance of a bulky tricyclic system for the selectivity for BChE inhibition was further supported.

Moreover, in many cases (**6**, **15**–**18**, **31**, **33**–**37** and **41**) their BChE inhibitory potency was higher than or comparable to that of the potent reference drugs ethopropazine (**1,** $IC_{50} = 0.72$ μM) and **28** ($IC_{50} = 0.84$ μM). Within these inhibitors, compound **15** exhibited the highest BChE affinity ($IC_{50} = 0.15$ μM) and selectivity (SI = 47). The high potency on BChE may be associated with a potency on AChE which can be either rather poor, confirming the high selectivity previously observed for ethopropazine, **28** and other phenothiazine derivatives [49], or even quite good, giving rise to dual, but generally BChE preferring, inhibitors. The latter case was observed in compounds **3**, **15**, **35**, **36** and **41**, whose particular interest will be discussed later. Other dual inhibitors, still BChE preferring, but characterized by a more balanced potency in the micromolar range for both enzymes, were represented by compounds **13**, **20**, **24** and **25**. Quinacrine analogs **42** and **43** were worth of note for the inhibition of both enzymes with submicromolar IC_{50} values and an opposite selectivity.



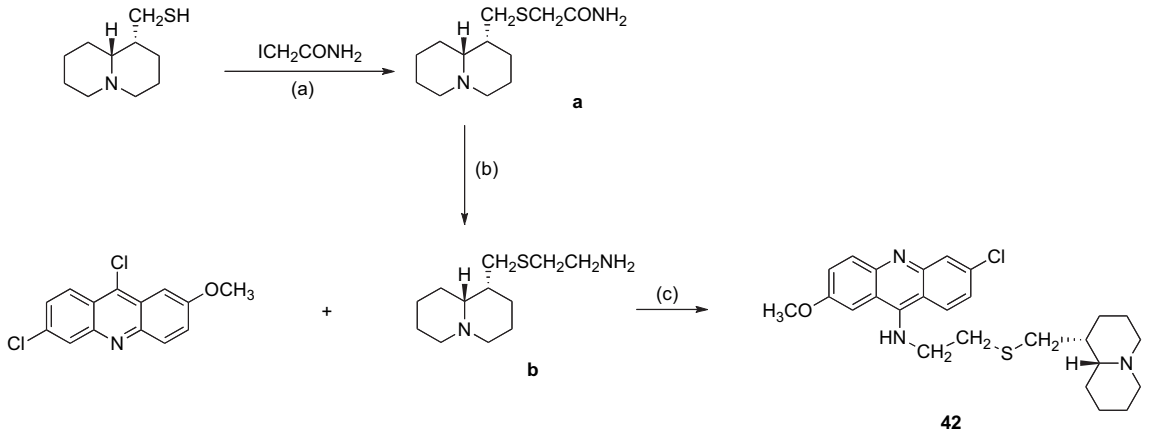
Scheme 2. Reagents and conditions: (a) K_2CO_3, toluene, 18 h, reflux; (b) K_2CO_3/KOH, DMSO, 110 °C, 30 h; (c) HI, red phosphorus, glacial CH_3COOH, 3 h, reflux.

Comparing the activities of N- and C-lupinyl derivatives of the phenothiazine and of the other bioisosteric tricyclic systems (**5**–**13**, **15**–**17** and **21**–**23**), it was observed that the IC_{50} values for BChE inhibition were distributed in the rather large range from 0.15 μM to 4.0 μM. Similarly the IC_{50} values for the C-lupinylidene derivatives **18**–**20** were spread in the range from 0.93 μM to 4.2 μM. The observed differences of potency may be related to a number of structural features as the size of the central ring (from 5- to 7-membered), the dihedral angle formed by the two benzene rings and the more or less rigid arrangement of the basic side chain in respect to the tricyclic system, as well as to the presence of substituents on the aromatic nuclei.

These structural features played different degrees of importance, when more homogeneous subsets of compounds were considered. Thus, the replacement of the sulfur bridge of phenothiazine with an oxygen or selenium atom, one or two methylene groups, a vinylene, or with a direct link between the benzene rings (with the consequent variation of ring size and dihedral angle) produced only minor modifications of the IC_{50} values, that remained in the range from 1.0 μM to 2.2 μM (O, CH=CH and CH_2–CH_2 < S < – < CH_2 < Se).

A clear increase of potency was observed when the lupinyl moiety is linked to a carbon atom instead than a nitrogen, independently from the size (6- or 5-membered) of the central ring (**15** and **22** *versus* **5** and **21**, respectively). On the contrary, the size of the central ring (and the corresponding different dihedral angle of the benzene rings attached to it) became important in the case of C-lupinylidene derivatives (**18**–**20**).

Moreover, the same structural modification may produce different results when effected on different ring systems. Thus the quaternarization of the quinolizidine nitrogen produced an increase or decrease of potency if effected on lupinylphenothiazine (**5**→**6**) or on lupinylthioxanthene (**15**→**16**), with a consequent leveling of potency in the final compounds. Similarly, the replacement of the lupinyl moiety with the *epi*-lupinyl gave rise to more or



Scheme 3. Reagents and conditions: (a) EtOH, 1 h; (b) $LiAlH_4$, dry THF, 18 h, reflux; (c) phenol, 110 °C, 5 h.

less potent compounds when applied, respectively, on the phenselenazine (**9**→**10**) or on the fluorene (**22**→**23**) derivatives.

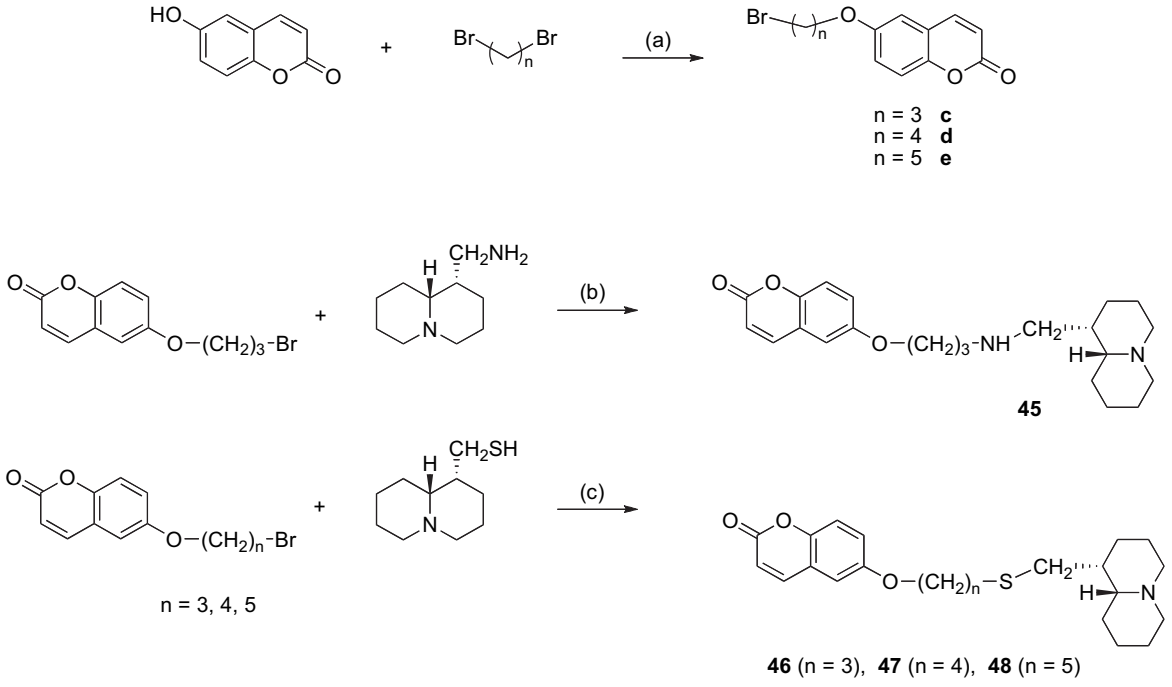
The introduction on the aromatic moiety of substituents, even so different as CF_3 and CH_3O, produced a reduction of potency in the two cases so far considered (**5**→**7** and **31**→**32**). It is possible that, beside the opposite influence exerted by the two groups on both electronic distribution and lipophilicity, the increase of the molecular volume, that could exceed the optimal one, might play a more important role [49].

The suppression of the sulfur bridge in compounds **5** and **15**, and, therefore, of any constraint to the movement of the two phenyl residues, produced a clear reduction of BChE inhibitory activity, associated with some improvement of AChE inhibition. The corresponding open compounds **24** and **25**, while no more so able to fill up the large gorge of BChE, may better accommodate in the narrower one of AChE, thus resulting in a comparable activity on both enzymes. On the other hand, the opening of the central ring in the lupinylidene derivatives, probably due to some residual rigidity related to the double bond, produced a reduction of activity on both enzymes in the case of compounds **18** and **20**, but even a slight increase of BChE inhibition for compound **19**.

Concerning the acyl derivatives of phenothiazine and related tricyclic systems, it is worth noting that compounds **29**–**37** exhibited a clear selectivity for BChE as it is known for the reference compound **28**. In our hands **28** resulted somewhat more potent than previously described by Elsinghorst and coll. [18d] (IC_{50} = 0.84 μM, instead of 3.37 μM), and this may be accounted for by the differences in enzyme source and assay experimental conditions.

The homolupinanoylphenothiazine **29** (the closer quinolizidinyl analog of **28**) displayed a little lower potency than the reference drug, and the epimeric compound **30** exhibited an even lower potency. However, the progressive elongation of the linker between phenothiazine and quinolizidine nuclei produced at first a strong increase of potency, that was, somewhat attenuated afterward, giving place to compounds that were comparable or a little superior to **28**.

Interestingly, the inhibitory activity on AChE, which was very modest in compounds **29**–**34**, became significant in compounds **35**



Scheme 4. Reagents and conditions: (a) $CsCO_3$, KI, dry CH_3CN, 160 °C, MW, 1 h. (b) DMF, 120 °C, 20 h; (c) DMF, 140 °C, 20 h.

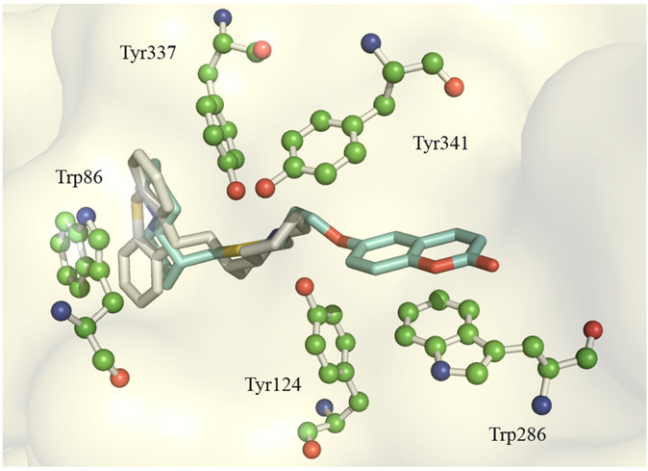


Fig. 3. Top-scored docking pose of inhibitors **46** and **15** rendered in cyan and gray capped stick models, respectively. The surface of AChE is displayed in background, relevant amino acid residues are represented in ball and stick models colored according to the atom code and numbered on the basis of AChE (PDB entry: 1B41).

and **36**, whose linker is formed by a heteroalkyl chain of 6 and 7 members respectively, in good agreement with literature data from tacrine [50] and edrophonium homo- and hetero-dimers [21c,d].

When the phenothiazine and the iminodibenzyl ring systems of compounds **29**–**37** were replaced by the dihydropyridobenzodiazepinone nucleus (**38** and **39**), the inhibitory activity on both enzymes became very poor, independently from the linker length (compare **38** with **29**, and **39** with **37** and **31**). Since the molecular shape and size of these compounds were not much different, the observed drop of the inhibitory activity should be related to the reduced lipophilicity or to the enhanced hydrogen-bonding capability of the pyridobenzodiazepinone ring which could be blocked in an inappropriate position on the enzyme gorge rim.

Besides the valuable inhibitory activity on cholinesterases, the studied derivatives of phenothiazine and related tricyclic systems deserve some further comments for their additional pharmacological properties that were disclosed in the past and which could lead to the development of interesting single entity multi-target compounds.

Compounds **29**–**32** and **34**–**39**, together with other analogs, have been studied in the past [41] as ligands for muscarinic M_1 and M_2 receptor subtypes, and most of them have been found to be endowed with nanomolar affinity for both subtypes, but selectivity for one or the other was only moderate. Preliminary functional studies indicated that these compounds acted as muscarinic antagonists. Moreover, also compounds **4**, **5** and particularly **15** have been found [51] to antagonize ACh activity on the presynaptic muscarinic autoreceptor in rat brain synaptosomes, with IC_{50} around 0.1 μM, while ethopropazine (**1**) did not antagonize ACh effect up to a concentration of 1 μM. Since the blockade of presynaptic M_2 subtype enhances the ACh release in the brain it may be of utility in the treatment of AD, even with some advantage over the use of AChE inhibitors, whose beneficial effects at the postsynaptic receptors are hampered by the ACh inhibition of its own release. Indeed selective muscarinic M_2 antagonists, as otenzepad (AT-DX 116), 8-chloro-11-[{4-[3-[(2,2-dimethylbutyryl (ethyl)amino)ethylamino]butyl]-1-piperidinyl}acetyl]-6*H*-pyrido[2,3-b] [1,4]benzodiazepin-6-one (BIBN 99) and others have been shown to facilitate memory storage in mice and rats [52]. Interestingly, low doses, otherwise sub-effective, of otenzepad and physostigmine acted synergistically. As a consequence, it would be particularly attractive to study more in depth compounds like **15** and **29**–**39** that combining muscarinic antagonism and anticholinesterase activity may represent another class of multi-target ligands which are particularly pursued in AD [8]. Moreover, these compounds are characterized by high lipophilicity (and, hence, by a likely good brain penetration), and it would be of interest to investigate their effects *in vivo* on animal models of AD by selecting compounds overlapping ACh autoreceptor antagonism and cholinesterase inhibitory activity, either dual (**15, 35** and **36**), or BChE selective (**34** and **37**).

The search of novel phenothiazine and related tricyclic systems derivatives that could provide cholinesterase inhibition combined with more selective M_2 receptor antagonism surely deserves further efforts.

The concomitant antimuscarinic and anticholinesterase activities of these compounds might also be of interest in the prophylaxis of poisoning by irreversible cholinesterase inhibitors, used as insecticides or as chemical warfare agents, as already pointed out by Dahlbom in 1962 [29,53] for **28** and other dialkylaminoacylphenothiazines. The preventive reversible block of cholinesterases may protect from irreversible inhibitors which can undergo to hydrolysis by paraoxonases and other detoxifying enzymes. Indeed pyridostigmine, eventually associated with atropine, has been used as prophylactic agent during the first Persian Gulf War by the U.S. Army.

On the other hand, it is remarkable the discovery of the high and selective BChE inhibitory activity of periciazine (**3**; $IC_{50} = 0.23$ μM; SI = 30), that resulted 32-fold more potent than triflupromazine (**2**; $IC_{50} = 7.4$ μM), which, in turn, exhibited comparable potency with the most used anti-psychotics (i. e., perphenazine, fluphenazine, etc) as found by Debord and coll. [49b]. Periciazine [28] is an old, somewhat neglected antipsychotic which is enjoying a renewed interest as sedative for agitated or aggressive patients, and which, on the base of our preliminary results, should deserve a more appropriate investigation for the treatment of AD patients with both cognitive and psychotic disorders. The molecular framework and side chain of periciazine should also be used as starting point to design novel compounds with improved cholinesterase inhibitory activity.

Finally, it has been recently shown [54] that **28** and few other phenothiazine derived drugs, but not ethopropazine (**1**) (differing from the former for a methylene in place of a carbonyl group in the side chain), are able to inhibit human plasmatic aminopeptidase splitting leucine^5-enkephalin. Therefore it would be of interest to investigate if other aminoacylphenothiazines, like compounds **29**–**36** of the present study, share the capability of **28** to inhibit the aminopeptidase. Indeed the protection of leucine^5-enkephalin which is involved in the striatal indirect pathway neurons, could be important for delaying neurodegeneration in Parkinson's disease.

To evaluate the influence on ChE inhibitory potency and selectivity of the planarity and size of the tricyclic systems attached, by linkers of different length, to the basic quinolizidine moiety, two additional series of compounds were evaluated, which, differently from those analyzed so far, were characterized by a fully planar tri- or bicyclic systems. A first group of compounds (**41**–**44**), are structurally related to quinacrine (**40**) whose inhibitory activity on cholinesterases is known since 1943 [30], and to which it may be related the development of tacrine [12].

In line with the dual binding site AChE inhibition and single entity-multitarget ligand approaches, a number of homo- and hetero-dimers of tacrine have been developed [50]. Depending on the linker length and nature, and on the peculiar substitution pattern and structural properties of the generally polycyclic (hetero)aromatic moieties tethered to the tacrine amino group, inhibitors with a great variety of ChE inhibitory potency and selectivity have been obtained [18d,50].

On the other hand, even if various aminoacridines have been shown to inhibit cholinesterases [12a,b], and the simple 9-aminoacridine [49a] has been found as potent as tacrine on AChE and BChE, a systematic structural modification of quinacrine itself in order to improve its inhibitory activity has not been carried yet. This prompted us to investigate the effect of replacing the basic, aliphatic novoldiamine moiety of quinacrine with a quinolizidinylalkylamino residue of increasing length (**41**–**43**).

Compounds **41**–**43** exhibited a low-micromolar or submicromolar inhibitory potency on both ChEs, resulting, in particular, from 3 to 13-fold more potent than quinacrine on BChE. The inhibition of both ChEs was at first improved with the elongation of the linker, however, when the spacer was formed by a 6-membered heteroalkyl chain (**43**), the inhibitory potency on BChE started to decline (IC_{50} = 0.69 μM), while that on AChE was still increasing (IC_{50} = 0.22 μM). Thus compound **43** showed even an inverted, albeit small, AChE selectivity (SI = 0.32).

Spacers of similar length did not produce the inversion of selectivity among the phenothiazine derivatives, thus the basic nature of acridine may contribute to a different positioning of compounds on the enzyme gorge rim. Moreover, because of the presence of two basic centers, the possibility of two different binding modes should be considered for these acridine derivatives, as in the case of tacrine homodimers, whose acridine ring may interact either with the catalytic or the peripheral site of the enzyme.

It is worth noting the quite lower potency of compound **44** in respect to the isosteric **41**, suggesting the importance of the 9-NH group (lacking in **44** where it is replaced by an S atom) for the reinforcement of the binding to the enzyme. The potential alternative binding of acridine derivatives and their binding interactions at the two ChE binding sites have been studied by docking simulations and will be discussed later.

Finally, the last subset of compounds studied in the present study was characterized by the presence of the bicyclic coumarin moiety, whose size is definitely smaller, than that of the tricyclic systems previously discussed.

In the last ten years a variety of coumarin derivatives have been investigated as inhibitors of AChE [21,22]. The coumarin unit was linked, directly or by means of a spacer, either to a moiety itself endowed with cholinesterase inhibitory property (like a phenolic carbamate or a edrophonium-like unit), or to a bulky tertiary benzylamino group, like that of compounds 3-(4-{[benzyl(methyl)amino]methyl}phenyl)-6,7-dimethoxy-2*H*-2-chromenone (AP 2238) and 3-(4-{[benzyl(ethyl)amino]methyl}phenyl)-6,7-dimethoxy-2*H*-2-chromenone (AP 2243) [22a]. These generally basic, molecular moieties might bind to the catalytic site, while the coumarin residues could occupy the PAS of the enzyme.

As it has been recently shown [21d], the anchoring position of the linker connecting a edrophonium-like moiety to the coumarin ring plays an important role in binding affinity, being the 3-, 6- and 7-substituted derivatives more potent than the corresponding 4-, 5- and 8-substituted regioisomers. For this reason and after a preliminary docking run on both ChEs of the diverse coumarin regioisomers, derivatives **45**–**48** with the linker anchored at position 6 of the coumarin ring, were designed as likely potent AChE inhibitors with a reversed enzyme selectivity compared to the other examined compounds. Very gratifyingly, all the designed compounds exhibited good selectivity for AChE over BChE, being the highest potency and selectivity observed for compound **46** (IC_{50} = 0.35 μM; SI = 0.06) with a spacer formed by a 6-membered heteroalkyl chain, thus further supporting the trend observed with other dual binding site coumarin derivatives [21c,d].

The replacement of a basic NH group for the sulfur, within the 6-membered heteroalkyl spacer that provide the maximal potency, produced a net decrease of potency *versus* both AChE and BChE (compare **45** *versus* **46**), in full agreement with previous findings [21c,d].

6. Molecular modeling studies

A further and deeper computational study was undertaken to highlight how the structural variations of the examined inhibitors might be related with changes in AChE and BChE affinities, and more importantly, to interpret the inversion of selectivity from BChE to AChE observed by replacing the phenothiazine (or related tricyclic systems) with a coumarin ring. To this end, molecular docking simulations were carried out on two selected inhibitors, **15** and **46,** that showed the highest selectivity toward BChE and AChE being the SI equal to 47 and 0.06, respectively.

Inhibitor **15** was docked into both the BChE and AChE binding sites. Satisfactorily, a higher score was observed for the top-scored solution occurring in BChE (−17.37 kJ/mol) compared to AChE (−13.88 kJ/mol). The most striking difference was related to the adoption of two quite diverse binding modes within the two enzymatic active sites. In fact, docking simulations within BChE larger gorge showed that the charged quinolizidine ring was engaged in a cation−π interaction with Trp82 (1P0I numbering) at the primary binding site while the thioxanthene moiety established hydrophobic and/or π−π interactions with Phe118 and, to a lesser extent, Trp231 (1P0I numbering). Conversely, the characteristic presence of the PAS in the narrower gorge of AChE was likely responsible of both the different binding mode and the decremented score occurring for all docking poses of inhibitor **15** that actually disclosed a weaker affinity. As shown in Fig. 3, the charged quinolizidine ring was in fact embedded into a network of tyrosine residues of the narrow gorge (i.e., Tyr124, Tyr337, Tyr341 − 1B41 numbering) while the butterfly conformation of the thioxanthene ring enwrapped the Trp86 by establishing hydrophobic and/or π−stacking interactions.

Comparative docking studies conducted on the strongest AChE selective inhibitor, that is the coumarin derivative **46**, resulted a top-scored solution with a far more favorable fitness energy for AChE (i.e., −57.11 kJ/mol) than for BChE (i.e., −41.60 kJ/mol). The visual inspection of the top-scored solution from the docking on AChE illustrated in Fig. 3 disclosed the flat coumarin ring trapped into a hydrophobic slot formed by Trp286 and Tyr341 at the PAS and the quinolizidine moiety contacting, via a cation−π interaction, the Trp86 in the primary binding site. Such findings agreed with those coming from our investigations on coumarin−edrophonium hetero-dimers [21d]. On the other hand, molecular docking of **46** on BChE revealed that the face-to-face interaction between the charged quinolizidine ring and the Trp86 at the primary binding site was well conserved while the lack of PAS prevented an efficient interaction with the coumarin ring. On this basis, the decreased affinity toward BChE may be nicely interpreted and taken into account for the rational design of new selective AChE inhibitors.

Eventually, the binding mode of **43**, bearing the acridine planar tricyclic system, and exhibiting high affinity and slight selectivity toward AChE (IC_{50} = 0.22 μM and SI = 0.32, respectively), was also investigated via molecular docking. As the pK_a estimation for compound **43** by ACD-Labs. (vers. 7.0, ACD, Toronto, Ontario, Canada) and MoKa (vers. 1.1.0, Molecular Discovery, Perugia, Italy) programs indicated the possible existence of an equilibrium between the mono-protonated (at the quinolizidine nitrogen atom) and the di-protonated (at the quinolizidine and acridine nitrogen atoms) species, docking simulations were carried out with both the two differently charged forms of the inhibitor. Docking simulation of the mono-protonated form pointed out that the charged quinolizidine moiety was preferentially involved in cation-π

interactions with the tryptophan residues of PAS and of catalytic binding site in the case of AChE and BChE, respectively. Similar binding modes were also experienced by the di-protonated species. More importantly, it is worth saying that docking scores were in good agreement with the experimental IC_{50} for both mono-protonated (i.e., −49.98 kJ/mol for AChE *versus* −36.38 kJ/mol for BChE) and di-protonated (i.e., −54.54 kJ/mol for AChE *versus* −40.79 kJ/mol for BChE) species of compound **43**.

7. Conclusion and perspectives

In order to achieve novel cholinesterase inhibitors, either dual, or, even better, selective for BChE, we have prepared and tested a number of derivatives of phenothiazine and other tricyclic systems bearing a bulky and strongly basic quinolizidine ring linked through different kinds of spacers.

All compounds exhibited activity against both ChEs, but inhibition of BChE was generally stronger, with submicromolar IC_{50} values for most of them. BChE affinity and selectivity were maximal for lupinylthioxanthene (**15,** $IC_{50} = 0.15$ μM, SI = 47). The elongation of the spacer improved the inhibition of AChE, but this became prevailing over BChE inhibition only for one acridine derivative (**43**; SI = 0.32).

On the other hand, in a subset of compounds in which the quinolizidine ring is tethered to the 6-hydroxycoumarin, a general selectivity for AChE was observed. This was maximal in compound **46** ($IC_{50} = 0.36$ μM, SI = 0.06) containing a 6-atoms spacer, further supporting the relation of potency with spacer length, made with other kinds of coumarin derivatives [21c,d].

In the case of phenothiazine and related non planar tricyclic systems derivatives, BChE selectivity and inhibitory potency could be related to a fundamental π−π interaction between the aromatic rings of the inhibitor and the aromatic side chains of the amino acids of the enzyme gorge rim. The protonated quinolizidine residue should bind preferentially at the catalytic site through a cation−π interaction. These π interactions may be modulated by steric and electronic factors, in relation to the peculiar nature of the tricyclic system and eventual substituents. Molecular docking furnished a sound interpretation of the observed molecular selectivity and inhibitor potency by disclosing the existence of viable but alternative binding modes whose occurrence was primarily determined by different interactions at the primary and/or peripheral binding sites of BChE and AChE.

For acridine derivatives (**40**–**44**), an equilibrium between the mono- and di-protonated species was reasonably supposed on the basis of the pK_a estimation of the two protonatable nitrogen atoms. In the case of AChE, molecular docking of compound **43** revealed that the expected occupancy of the primary binding site by the acridine moiety was prevalently observed for both the mono- and di-protonated species while a reverse binding mode resulted prevalent, again for both the differently protonated species, in the case of BChE.

On the other hand, the planar and smaller sized coumarin ring of compounds **46**–**48** could fit better the PAS of AChE than the corresponding, dissimilar region of BChE in agreement with the experimental inhibition data.

Several of the presently studied compounds, besides the valuable inhibitory activity on cholinesterases, have shown in the past additional pharmacological properties and therefore, represent hits which could lead to the development of interesting single-entity multi-target drugs.

Thus selected compounds, combining cholinesterase inhibition with either M_1/M_2 muscarinic subtype antagonism (as, particularly, **15** and **36**, among several others), or with antipsychotic action (as periciazine **3**), or with inhibition of leucine^5−enkephaline aminopeptidase (as **28**, and maybe some of its analogs **29**–**36**), surely deserve deeper evaluations, to substantiate a possible reinforced central cholinergic activity, or the capability to improve the cognitive and psychotic facets of AD, or even to protect from intoxications related to the agricultural or terroristic use of irreversible ChEIs. Particularly, for periciazine a prompt clinical trial on AD patients should be fostered to observe the improvement of cognitive disorders besides the eventual psychotic symptoms. Indeed, this drug displayed a 30-fold higher BChE inhibition ($IC_{50} = 0.23$ μM), compared with the most potent anti-psychotics and its ADME and toxicological characteristics are well documented through a 40 years long therapeutic use.

Eventually, it must be emphasized the character of phenothiazine and related tricyclic systems as privileged substructures from which novel interesting multipotent compounds could still be obtained with the use of appropriate substituents and linkers to other suitably chosen molecular moieties.

8. Experimental section

8.1. Chemistry

Melting points were taken in open glass capillaries on a Büchi apparatus and were uncorrected. ^{1}H NMR spectra were recorded in $CDCl_3$ on Varian Gemini spectrometer; chemical shifts (d) are reported in ppm from internal Me_4Si: coupling constants (*J*) are reported in Hz; Q = octahydroquinolizine ring. Column chromatography (CC) was effected using alumina (Merck). Elemental analyses were performed on Carlo Erba EA 1110 CHNS-0 instrument in the Microanalysis Laboratory of the Department of Pharmaceutical Sciences of Genoa University. The analytical results are within ±0.3% of calculated values (see Supporting Information). The results of NMR spectra and elemental analyses indicated that the purity of all compounds was higher than 95%.

8.1.1. 10-(1-Azabicyclo[2,2,2]oct-3-yl)phenothiazine (*4*)

a) Quinuclidinyl tosylate hydrochloride, prepared according to Grob et al. [43b], was dissolved in ice-cold water and treated with 2N NaOH. The ester was extracted with ether, the ether solution was dried over Na_2SO_4, and evaporated to dryness. The oily residue was heated to 50 °C under vacuum till constant weight.
b) Phenothiazine (2.27 g, 11.4 mmol) was dissolved in warm dry xylene (30 mL) and added with finely ground sodium amide (0.63 g, ∼16 mmol). The mixture was refluxed for 1.5 h and after adding quinuclidinyl tosylate (2.74 g, 9.91 mmol) refluxed for further 4 h. The xylene was evaporated under vacuum and the residue was taken up with 0.5N HCl filtering some insoluble tarry material. The acid solution was refluxed for 1 h to hydrolyze some unreacted quinuclidinyl tosylate and after cooling was extracted with ether. Finally the solution was basified with 6N KOH and extracted with ether. The organic phase was washed several times with water to remove some quinuclidinol, dried over Na_2SO_4 and evaporated to dryness. The residue was crystallized from dry ether/pentane giving 0.6 g of product with m.p. 168.5–169.5 °C (Lit. [43a] m.p. 160–162 °C). Yield 19.6%. Anal. calcd for $C_{19}H_{20}N_2S$: C 73.93, H 6.54, N 9.08, found: C 73.69, H 6.53, N 9.14.

8.1.2. 5-Methyl-1-(9H-thioxanthen-9-ylmethyl)-(1S,9aR)-octahydroquinolizinium iodide (*16*)

A mixture of 1-(9*H*-thioxanthen-9-ylmethyl)-(1*S*,9a*R*)-octahydroquinolizine **15** [36] (0.021 g, 0.06 mmol) and 0.1 mL of

iodomethane (0.23 g, 1.6 mmol) was stirred for 30 min at r.t.. After adding some dry ether, the precipitate was filtered and washed with dry ether, leaving 0.025 g of white amorphous solid with m.p. 195–198 °C (swelling at 115 °C). Yield 84.8%. Anal. calcd for $C_{24}H_{30}NSI$: C 58.65, H 6.15, N 2.85, S 6.52, found: C 58.54, H 5.90, N 2.53, S 6.80.

*8.1.3. 9-[(1S,9aR)-(Octahydro-1H-quinolizin-1-yl)methyl]-9H-carbazole (**21**)*

In a 100 mL two-neck flask equipped with nitrogen inlet, carbazole (0.67 g, 4 mmol), powdered anhydrous potassium carbonate (0.33 g, 2.4 mmol), powdered potassium hydroxide (0.29 g, 4.4 mmol) and 18-crown-6 (0.1 g, 0.4 mmol) were suspended in toluene (30 mL).

The mixture was stirred under nitrogen at room temperature for 1 h, a solution of chlorolupinane [45] (0.82 g, 4.4 mmol) in toluene (5 mL) was added dropwise and the mixture was further stirred at reflux for 18 h. After cooling, the inorganic salts were filtered and washed with toluene. The toluene solution was washed with saturated potassium chloride solution to remove the catalyst and then extracted with 15% hydrochloric acid. The acidic solution was basified (pH = 9) cautiously with saturated sodium carbonate solution. The oily product was extracted with dichloromethane and the solution was dried over anhydrous sodium sulfate. After removing the solvent, the oily residue was distilled in vacuo to remove the unreacted chlorolupinane and then chromatographed on alumina (1:25), eluting with dichloromethane. The oily product crystallyzed by rinsing with a little dry ether, leaving 0.22 g of white crystals with m.p. = 109–110 °C. Yield 17.3%. ^{1}H NMR ($CDCl_3$): δ 1.07–2.48 (m, 14H of *Q*); 2.82–3.06 (m, $2H_\alpha$ near N of *Q*); 4.40–4.77 (m, 2H, CH_2N); 7.12–7.34 (m, 2 arom. H); 7.48 (d, J = 3.6, 4 arom. H); 8.14 (d, J = 7.8, 2 arom. H). Anal. calcd for $C_{22}H_{26}N_2$: C 82.97, H 8.23, N 8.80, found: C 83.08, H 8.52, N 9.01.

*8.1.4. N-[(1S,9aR)-(Octahydro-1H-quinolizin-1-yl)methyl]-N-phenylaniline (**24**)*

In a 100 mL two-neck flask equipped with nitrogen inlet, diphenylamine (0.68 g, 4 mmol), powdered anhydrous potassium carbonate (0.33 g, 2.4 mmol) and powdered sodium hydroxide (0.18 g, 4.4 mmol) were suspended in dimethyl sulfoxide (30 mL).

The mixture was stirred under nitrogen at room temperature for 1 h, a solution of chlorolupinane [45] (0.82 g, 4.4 mmol) in dimethyl sulfoxide (3 mL) was added dropwise, and the mixture was then stirred at 110 °C for 30 h. After cooling, the mixture was poured into water (150 mL) and extracted with toluene. The organic phase, after washing with water to remove any residual dimethyl sulfoxide, was extracted with 15% hydrochloric acid. The acidic phase was basified (pH = 8) cautiously with saturated sodium carbonate solution and extracted with dichloromethane. The dichloromethane solution was dried over anhydrous sodium sulfate and the solvent removed. The oily residue was distilled in vacuo to remove the unreacted chlorolupinane and then chromatographed on alumina (1:25), eluting with dichloromethane. A yellow oil was obtained (0.38 g). Yield 29.7%. ^{1}H NMR ($CDCl_3$): δ 1.04–2.23 (m, 14H of *Q*); 2.71–2.98 (m, $2H_\alpha$ near N of *Q*); 3.76–4.13 (m, 2H, CH_2N); 6.75–7.12 (m, 6 arom. H,); 7.16–7.42 (m, 4 arom. H). Anal. calcd for $C_{22}H_{28}N_2$: C 82.45, H 8.81, N 8.74, found: C 82.19, H 8.98, N 8.97.

*8.1.5. 1,1-Diphenyl-2-[(1S,9aR)-(octahydro-1H-quinolizin-1-yl)] ethane (**25**)*

To a solution of 1,1-diphenyl-2-(octahydroquinolizin-1-yl) ethanol [33] (1 g, 3 mmol) in glacial acetic acid (10 mL) were added hydriodic acid (3 mL) and red phosphorus (0.8 g) and the reaction was refluxed for 3 h. After cooling water was added, the precipitate was filtered and washed with water. The solid was boiled with ethanol (40 mL), then filtering the unreacted phosphorus. The organic solution was basified with NaOH 6N and evaporated under vacuum. The residue was distributed between water and ether. The ether solution was evaporated to dryness, leaving a yellow oil (0.84 g). Yield: 85%. Oil. B.p. (p = 0.1 torr) 175 °C, air bath. TLC: R_f = 0.75 (Al_2O_3, Et_2O). ^{1}H NMR ($CDCl_3$): δ 1.04–2.05 (m, 14H of *Q*); 2.10–2.42 (m, 2H of CH_2-Q); 2.75–2.97 (m, $2H_\alpha$ near N of *Q*); 3.87–4.03 (m, 1H, $CHCH_2$-Q); 7.02–7.43 (m, 10 arom. H). ESI-MS m/z 320 $[M + H]^+$. Anal. calcd for $C_{23}H_{29}N$: C 86.47, H 9.15, N 4.38, found: C 86.53, H 9.13, N 4.66.

*8.1.6. 2-[(1R,9aR)-(Octahydro-1H-quinolizin-1-yl)methylthio]-1-(10H-phenothiazin-10-yl)propan-1-one (**33**)*

A solution of 0.46 g (2.5 mmol) of thiolupinine [44] in 5 mL of absolute ethanol was rapidly added to a solution of 0.84 g (2.5 mmol) of N-(2-bromo-2-propionyl)phenothiazine in 30 mL of absolute ethanol. The solution was refluxed under nitrogen for 7 h and then evaporated under reduced pressure. The residue was dissolved in acidic water and extracted with ether to remove the unreacted bromoalkanoylphenothiazine. The acid solution was basified with 2N NaOH solution and extracted with dichloromethane. The organic solution was washed with water, dried and evaporated to dryness. The residue was chromatographed on neutral alumina, eluting with ether. An oil was obtained (0.73 g). Yield 66.4%. ^{1}H NMR ($CDCl_3$): δ 1.09–2.15 (m, 14H of *Q*, with superimposed d, J = 7.1, 3H of CH_3); 2.52–2.96 (m, 4H, $2H_\alpha$ near N of *Q* and 2H of CH_2S); 3.84 (q, 1H, CHC(O)); 7.03–7.88 (m, 8 arom. H). Anal. calcd for $C_{25}H_{30}N_2OS_2$: C 68.45, H 6.89, N 6.39, S 14.62, found: C 68.67, H 7.17, N 6.13, S 14.77.

*8.1.7. 2-{[(1S,9aR)-(Octahydro-2H-quinolizin-1-yl)methyl]thio} acetamide (**a**)*

A solution of thiolupinine [44] (2.0 g, 10.8 mmol) and iodoacetamide (2.06 g, 10.8 mmol) in ethanol (10 mL) was stirred for 1 h under nitrogen. The ethanol was removed and the residue was partitioned between ether and acidic water. The acid solution was basified with 2N NaOH and extracted with dichloromethane. The organic phase was dried (Na_2SO_4) and evaporated, leaving 2.43 g of lupinylthioacetamide, that was crystallized from dry ether. M.p. = 110–111 °C. Yield 92.7%. Anal. calcd for $C_{12}H_{22}N_2OS$: C 59.46, H 9.15, N 11.56, found: C 59.47, H 9.21, N 11.76.

*8.1.8. 2-{[(1S,9aR)-(Octahydro-2H-quinolizin-1-yl)methyl]thio} ethan-1-amine (**b**)*

To a suspension of $LiAlH_4$ (1.55 g, 41 mmol) in anhydrous THF (20 mL), a solution of 2 g (8.3 mmol) of lupinylthioacetamide in THF (70 mL) was added dropwise. The mixture was stirred for 30 min at r.t. and then refluxed for 18 h. After cooling, the reaction mixture was treated dropwise, and in the order, with 3 mL of H_2O, 3 mL of 15% NaOH and 3 mL of H_2O. The precipitate was filtered and washed thoroughly with ether. The organic solution was dried over KOH pellets and the solvent removed. The oily residue was distilled under vacuum (130 °C, air bath, at 0.05 torr) to give 0.88 g of colorless oil. Yield 46.8%. Anal. calcd for $C_{12}H_{24}N_2S$: C 63.10, H 10.58, N 12.27, S 14.05, found: C 63.46, H 10.79, N 12.04, S 13.71.

*8.1.9. 6-Chloro-2-methoxy-9-{N-[2-(1S,9aR)-(octahydro-2H-quinolizin-1-yl)methylthio]ethylamino} acridine (**42**)*

A mixture of 6,9-dichloro-2-methoxyacridine (0.49 g, 1.75 mmol), the above amine (0.40 g, 1.75 mmol) and phenol (1.10 g) was heated for 5 h at 110 °C. After cooling the mixture was treated with 2N NaOH till strong alkalinity and extracted with ether. The organic phase was washed with 2N NaOH, then with H_2O and, finally, extracted with 5% acetic acid. The acid solution was alkalinized with 2N NH_3 and extracted with dichloromethane.

The organic solution was dried over anhydrous sodium sulfate and, after removing the solvent, the oily residue was chromatographed on alumina (1:30), eluting with dichloromethane. A yellow solid with m.p. = 88–91 °C was obtained (0.48 g). Yield 58.5%. ^{1}H NMR ($CDCl_3$): δ 1.08–2.21 (m, 14H of *Q*); 2.58–3.04 (m, 6H, 2H$_\alpha$ near N of *Q*, 2H of *CH$_2$*-S and 2H of S*CH$_2$*); 3.86 (t, *J* = 5.6, 2H of NH*CH$_2$*); 4.02 (s, 3H, OCH_3); 5.48 (s, 1H, NH collapses with D_2O); 7.22–7.54 (m, 3 arom. H); 7.95–8.24 (m, 3 arom. H). Anal. calcd for $C_{26}H_{32}ClN_3OS$: C 66.43, H 6.86, N 8.94, S 6.82, found: C 66.15, H 6.88, N 8.89, S 6.53.

*8.1.10. 6-Chloro-2-methoxy-9-{N-[3-(1S,9aR)-(octahydro-2H-quinolizin-1-yl)methylthio]propyl amino}acridine (**43**)*

According to the procedure already described [42], the title compound was obtained as a yellow solid with m.p. = 78–80 °C (yield 61.8%), instead of an oil (yield 22%). Therefore new ^{1}H-NMR data and elemental analysis are reported. ^{1}H NMR ($CDCl_3$): δ 1.12–2.18 (m, 16H, 14H of *Q* and 2H of $CH_2CH_2CH_2$); 2.53–2.86 (m, 6H, 2H$_\alpha$ near N of *Q*, 2H of *CH$_2$*-S and 2H of S*CH$_2$*); 3.82 (t, *J* = 7.2, 2H of NH*CH$_2$*); 3.98 (s, 3H, OCH_3); 5.03 (s, 1H, NH collapses with D_2O); 7.18–7.58 (m, 3 arom. H); 7.92–8.12 (m, 3 arom. H). Anal. calcd for $C_{27}H_{34}ClN_3OS$: C 66.98, H 7.08, N 8.68, S 6.62, found: C 66.74, H 6.99, N 8.42, S 6.33.

*8.1.11. General procedure for the preparation of 6-[(ω-Bromoalkyl) oxy]-2H-chromen-2-ones (**c, d, e**)*

A Pyrex© vessel was charged with a magnetic stirring bar and a Weflon™ heating bar. 6-hydroxycoumarin (0.65 g, 4.0 mmol) was added and dissolved in anhydrous acetonitrile (20 mL). Then the suitable dibromoalkane (24.0 mmol), cesium carbonate (1.3 g, 4.0 mmol) and potassium iodide (0.066 g, 0.4 mmol) were added and the vessel mixture was placed in the microwave reactor and irradiated at 160 °C for 1 h. After cooling, the reaction mixture was concentrated to dryness and the resulting residue was purified by flash chromatography (purity always > 96%) by eluting with different gradient mixtures of ethyl acetate in *n*-hexane. The isolated solid compounds were used for the subsequent reaction without any further purification.

*8.1.11.1. 6-(3-Bromopropoxy)-2H-chromen-2-one (**c**).* Purified by flash chromatography (gradient eluent: ethyl acetate in *n*-hexane 0→20%). Yield: 67%. ^{1}H NMR ($CDCl_3$): δ 2.34 (q, *J* = 6.1, 2H); 3.62 (t, *J* = 6.1, 2H); 4.14 (t, *J* = 6.1, 2H); 6.43 (d, *J* = 9.6, 1H); 6.94 (d, *J* = 3.0, 1H); 7.11 (dd, *J* = 3.0, 9.1, 1H); 7.27 (d, *J* = 9.1, 1H); 7.65 (d, *J* = 9.6, 1H). IR (cm^{-1}): 1113, 1275, 1568, 1710.

*8.1.11.2. 6-(4-Bromobutoxy)-2H-chromen-2-one (**d**).* Purified by flash chromatography (gradient eluent: ethyl acetate in *n*-hexane 0→25%). Yield: 48%. ^{1}H NMR (DMSO-d_6): δ 1.76–1.90 (m, 2H); 1.92–2.01 (m, 2H); 3.60 (t, *J* = 6.6, 2H); 4.03 (t, *J* = 6.3, 2H); 6.47 (d, *J* = 9.6, 1H); 7.18 (dd, *J* = 2.8, 9.1, 1H); 7.27 (d, *J* = 2.8, 1H); 7.32 (d, *J* = 9.1, 1H); 7.96 (d, *J* = 9.6, 1H). IR (cm^{-1}): 1106, 1279, 1567, 1712.

*8.1.11.3. 6-[(5-Bromopentyl)oxy]-2H-chromen-2-one (**e**).* Purified by flash chromatography (gradient eluent: ethyl acetate in *n*-hexane 0→25%). Yield: 84%. ^{1}H NMR (DMSO-d_6): δ 1.46–1.59 (m, 2H); 1.74 (q, *J* = 6.6, 2H); 1.85 (q, *J* = 6.6, 2H); 3.55 (t, *J* = 6.6, 2H); 3.99 (t, *J* = 6.3, 2H); 6.47 (d, *J* = 9.6, 1H); 7.17 (dd, *J* = 3.0, 8.8, 1H); 7.27 (d, *J* = 3.0, 1H); 7.31 (d, *J* = 9.1, 1H); 7.98 (d, *J* = 9.6, 1H). IR (cm^{-1}): 1108, 1279, 1569, 1723.

*8.1.12. 6-{3-[N-(1S,9aR)-(Octahydro-2H-quinolizin-1-yl)methyl] aminopropoxy}-2H-chromen-2-one (**45**)*

A solution of aminolupinane [46] (10 mmol) and 6-(3-bromopropoxy)-2*H*-chromen-2-one (5 mmol) in 6 mL of DMF was heated with stirring (120 °C, 20 h) in a Aldrich pressure tube. The solvent was removed under reduced pressure and the residue was taken up with water, alkalinized with 2N NaOH and extracted with Et_2O. The organic layer was dried (Na_2SO_4) and evaporated to afford an oily residue which was crystallized from dry Et_2O.

Yield: 35%. M.p. = 80–83 °C (Et_2O). ^{1}H NMR ($CDCl_3$): δ 1.06–2.15 (m, 16H, 14H of Q and 2H of $NHCH_2CH_2CH_2O$); 2.71–3.94 (m, 6H, 2H$_\alpha$ near N of *Q*, 2H of *CH$_2$*-Q and 2H of NH*CH$_2$*CH_2CH_2O); 4.08 (t, *J* = 7.0, 2H of $NHCH_2CH_2CH_2O$); 4.95 (s, 1H, NH collapses with D_2O); 6.44 (d, *J* = 9.2, 1 arom. H); 6.96–7.35 (m, 3 arom. H); 7.68 (d, *J* = 9.6, 1 arom. H). Anal. calcd for $C_{22}H_{30}N_2O_3$: C 71.32, H 8.16, N 7.56, found: C 71.52, H 8.44, N 7.84.

*8.1.13. 6-{ω-[(1R,9aR)-(Octahydro-1H-quinolizin-yl)methylthio] alkoxy}-2H-chromen-2-ones (**46**–**48**). General method*

In a Aldrich pressure tube, to a solution of thiolupinine [44] (0.17–0.53 mmol) in DMF (1–2 mL) the proper ω-bromoalkoxy-2*H*-chromen-2-one [21c,d] was added. The tube was flushed with N_2, closed and heated to 140 °C for 20 h. After cooling the DMF was removed under vacuum. The residue was taken up in acidic H_2O, extracted with ether and, after alkalinization, extracted with CH_2Cl_2.

The organic phase was dried (Na_2SO_4) and the solvent was removed under vacuum; the residue was purified as indicated for each compound.

*8.1.13.1. **46**.* M.p. = 104–105 °C (Et_2O). Yield: 56%. ^{1}H NMR ($CDCl_3$): δ 1.12–2.25 (m, 16H, 14H of *Q* and 2H of $CH_2CH_2CH_2$); 2.62–2.96 (m, 6H, 2H$_\alpha$ near N of *Q*, 2H of *CH$_2$*-S and 2H of S*CH$_2$*); 4.12 (t, *J* = 6.2, 2H of *CH$_2$*O); 6.46 (d, *J* = 9.6, 1 arom. H); 6.95 (d, *J* = 2.8, 1 arom. H); 7.14 (dd, *J* = 2.8, 8.6, 1 arom. H); 7.29 (d, *J* = 8.6, 1 arom. H); 7.68 (d, *J* = 9.6, 1 arom. H). Anal. calcd for $C_{22}H_{29}NO_3S$: C 68.18, H 7.54, N 3.61, S 8.27, found: C 68.13, H 7.73, N 3.41, S 7.95.

*8.1.13.2. **47**.* M.p. = 83–84 °C (CC, Al_2O_3, CH_2Cl_2). Yield 48%. ^{1}H NMR ($CDCl_3$): δ 1.06–2.16 (m, 18H, 14H of *Q* and 4H, $CH_2CH_2CH_2CH_2$); 2.46–3.08 (m, 6H, 2H$_\alpha$ near N of *Q*, 2H of *CH$_2$*-S and 2H of S*CH$_2$*); 4.02 (t, *J* = 6.2, 2H of *CH$_2$*O); 6.46 (d, *J* = 9.6, 1 arom. H); 6.95 (d, *J* = 2.8, 1 arom. H); 7.12 (dd, *J* = 2.8, 8.8, 1 arom. H); 7.29 (d, *J* = 8.8, 1 arom. H); 7.68 (d, *J* = 9.6, 1 arom. H). Anal. calcd for $C_{23}H_{31}NO_3S$: C 68.79, H 7.78, N 3.49, S 7.99, found: C 68.68, H 7.66, N 3.22, S 7.92.

*8.1.13.3. **48**.* M.p. = 59–60 °C (CC, Al_2O_3, CH_2Cl_2). Yield 55%. ^{1}H NMR ($CDCl_3$): δ 1.08–2.12 (m, 20H, 14H of *Q* and 6H of $CH_2CH_2CH_2CH_2CH_2$); 2.52–3.02 (m, 6H, 2H$_\alpha$ near N of *Q*, 2H of *CH$_2$*-S and 2H of S*CH$_2$*); 4.03 (t, *J* = 6.0, 2H of *CH$_2$*O); 6.45 (d, *J* = 9.6, 1 arom. H); 6.94 (d, *J* = 2.8, 1 arom. H); 7.13 (dd, *J* = 2.8, 8.7, 1 arom. H); 7.28 (d, *J* = 8.7, 1 arom. H); 7.68 (d, *J* = 9.6, 1 arom. H). Anal. calcd for $C_{24}H_{33}NO_3S$: C 69.36, H 8.00, N 3.37, S 7.72, found: C 69.11, H 8.31, N 3.43, S 7.44.

8.2. Molecular modeling

GOLD (version 4.1.2), a genetic algorithm based software, was used for the docking study and GoldScore was chosen as a fitness function. GoldScore is made up of four components that account for protein-ligand binding energy: protein-ligand hydrogen bond energy (external H-bond), protein-ligand van der Waals energy (external vdw), ligand internal van der Waals energy (internal vdw), and ligand torsional strain energy (internal torsion). Parameters used in the fitness function (hydrogen bond energies, atom radii and polarizabilities, torsion potentials, hydrogen bond directionalities, and so forth) were taken from the GOLD parameter file. In the present study, the 3D coordinates of hAChE (PDB code

1B41) and hBChE (PDB code 1P0I) were retrieved from the Protein Data Bank. The target proteins were prepared by adding hydrogen atoms, completing and optimizing missing residues, removing water and the cocrystallized molecules. As well known [55], the histidine side chains cannot normally be placed into the electron density map unambiguously. As a result, the protonation state of such residues was adjusted according to the formation of HB networks that was further confirmed upon visual inspection. The basic amino functional groups were protonated, aromatic amino functional groups were left uncharged and carboxylic groups were considered to be deprotonated. Molecular docking resulted 10 poses for inhibitor in a sphere of 10Å radius centered on the centroid atom of E2020 cocrystallized with TcAChE (PDB code 1EVE) previously aligned to hAChE.

8.3. ChEs inhibition assays

The inhibition assays of AChE, from bovine erythrocytes (0.36 U/mg) and BChE, from equine serum (13 U/mg), were run in phosphate buffer 0.1 M, at pH 8.0. Acetyl- and butyrylthiocoline iodides were used as substrates and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as the chromophoric reagent [47]. Inhibition assays were carried out on an Agilent 8453E UV–visible spectrophotometer equipped with a cell changer. Solutions of tested compounds were prepared starting from 10 mM stock solutions in DMSO, that were diluted with aqueous assay medium to a final content of organic solvent always lower than 1%. AChE inhibitory activity was determined in a reaction cuvette containing 200 μL of a solution of AChE (0.415 U/mL in 0.1 M phosphate buffer, pH 8.0), 100 μL of a 3.3 mM solution of DTNB in 0.1 M phosphate buffer (pH 7.0) containing 6 mM $NaHCO_3$, 100 μL of a solution of the inhibitor (six-seven concentrations ranging from 1×10^{-8} to 1×10^{-4} M), and 500 μL of phosphate buffer, pH 8.0. After incubation for 20 min at 25 °C, acetylthiocholine iodide (100 μL of 5 mM aqueous solution) was added as the substrate, and AChE-catalyzed hydrolysis was followed by measuring the increase of absorbance at 412 nm for 3.0 min at 25 °C. The concentration of compound which determined 50% inhibition of the AChE activity (IC_{50}) was calculated by non-linear regression of the response–log(concentration) curve, using GraphPad Prism® v. 5. BChE inhibitory activity was assessed similarly using butyrylthiocholine iodide as the substrate.

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