Rational Design of Reversible Acetylcholinesterase Inhibitors

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Abstract: A large amount of structural information on AChE and AChE-inhibitor complexes is currently available. Based on that, molecular modeling studies can be intensively used to gain insight into the mechanism of action of the enzyme and the molecular determinants that modulate the potency of inhibitors. In turn, this knowledge can be exploited to design new compounds leading to more effective cholinergic strategies. This manuscript reviews recent developments in the design of reversible acetylcholinesterase inhibitors.

INTRODUCTION

Alzheimer's disease (AD) is the fourth leading cause of death in people over 65 years old in western industrialized countries [1]. In spite of the multifactorial nature of AD [2,3], most treatment strategies have been directed to two main targets: i) the β-amyloid (Aβ) peptide and ii) the cholinergic transmission. There is an evidence that aggregation and deposition of (Aβ) are major events in the appearance and development of the disease [4-6]. Nevertheless, the most successful approach to date for the (symptomatic) treatment of AD is the cholinergic hypotesis [7-9], which postulates that at least some of the cognitive decline experienced by AD patients results from a deficiency in the neurotransmitter acetylcholine (ACh) in the central nervous system.

Cholinergic neurotransmission can be enhanced by drugs acting at both pre-synaptic and post-synaptic (choline precursors, ACh releasers, M_2 muscarinic antagonists, M_1 muscarinic agonists, nicotinic agonists) levels [10]. However, the only drugs currently approved for the treatment of the cognitive deficit in AD act at the synaptic level by inhibiting acetylcholinesterase (AChE), thus increasing the bioavailability of ACh at the synaptic cleft and improving cholinergic neurotransmission.

ACETYLCHOLINESTERASE

The main function of AChE (EC 3.1.1.7) is the hydrolysis of ACh at the cholinergic synapses [11]. The hydrolysis reaction proceeds by nucleophilic attack to the carbonyl carbon, acylating the enzyme and liberating choline, followed by a rapid hydrolysis of the acylated enzyme yielding acetic acid, and the restoration of the enzyme.

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The 3D structure of *Torpedo californica* AChE was initially solved at 2.8 Å resolution [12], and later refined to 2.5 Å resolution [13]. The enzyme monomer is an α/β protein (537 residues) that contains a 12-stranded mixed β sheet surrounded by 14 α helices. The most remarkable feature is a deep and narrow gorge (around 20 Å long) that penetrates halfway into the enzyme and widens out close to its base. Fourteen highly conserved aromatic residues line a substantial portion of the surface of the gorge. The active site has a catalytic triad (Ser200-His440-Glu327) similar to that in serine proteases, but with the replacement of the aspartic acid by a glutamic (Glu327). Glu199 is also believed to be the key for orienting the imidazole of His440 according to the steric and electrostatic features of the ligand bound to Ser200 [14,15]. The π electron distribution of Trp84 is the binding pocket for the quaternary moiety of choline, the interaction being stabilized by strong cation-π contacts [16-18]. Another triptophane (Trp279) defines a peripheral anionic site, which might act as an initial binding site for substrate entry, thus accelerating the hydrolysis of ACh at low substrate concentration [19], and might also modulate cation clearance and product release [20].

The long and narrow active site gorge seems inconsistent with the enzyme's high catalytic rate, as underlined by the k_{cat}/K_m value of ~10^9 $M^{-1}.s^{-1}$ [21,22]. Such a catalytic efficiency is likely related to the existence of a strong electrostatic field that directs cations into the active site gorge [23-28], hindering the diffusion of the substrate out of the active site. The role of the specific charged residues (particularly Asp72) in the electrostatic attraction of ligands has been extensively studied by McCammon and co-workers [29-31].

CHOLINERGIC DRUGS

Owing to the structural complexity of the active site gorge in AChE, the large diversity of AChE inhibitors with different mechanisms of inhibition and selectivity is not surprising. In fact, AChE inhibitors can be broadly classified in four categories [see Ref. 32 for review]:

i) *pseudo-irreversible* inhibitors, such as physostigmine, which form a carbamoylated complex with the serine residue of the active site,

ii) *irreversible* inhibitors, including organophosphates, such as metrifonate, that form a stable phosphorylated complex with the catalytic serine residue,

iii) *transition state* analogues, such as trifluoromethylketones, that form with the serine residue at the catalytic triad a tetrahedral hemiketal adduct, and

iv) *reversible* inhibitors, including a variety of compounds interacting with either the binding site near the catalytic triad, the peripheric binding site or both.

REVERSIBLE AChE INHIBITORS

Tacrine and Aminoacridine Analogs

The first drug approved by the FDA for the palliative treatment of mild to moderate AD was tacrine (Cognex®; Fig. **1**; [33,34]). Harel *et al.* [35] reported a detailed analysis of the 3D crystallographic structure of the *Tc*AChE-tacrine (PDB entry 1ACJ) complex and compared it with the structures of the AChE complexes with decamethonium and edrophonium (1ACL and 2ACK). As noted in this study, tacrine is stacked against the rings of Trp-84 and Phe330, the ring nitrogen is hydrogen-bonded to the main-chain carbonyl oxygen of His440, and the amino nitrogen forms a hydrogen bond to a water molecule (Fig. **2**). Decamethonium is oriented along the narrow gorge leading to the active site, with one of its quaternary groups opposed to the ring of Trp84, and the other to that of Trp279. Finally, the quaternary nitrogen of edrophonium interacts with the ring of Trp84, and its *m*-hydroxyl displays bifurcated hydrogen bonding to two members of the catalytic triad, Ser200 and His440. The benzene ring of Phe330 adopts a different orientation for the three inhibitors: whereas it is stacked against the quinoline ring of tacrine, it lies parallel to the methylene chain of decamethonium or contacts the ethyl substituent of edrophonium.

Owing to the severe side-effects of tacrine, efforts have been conducted to develop tacrine-related analogues with improved pharmacological properties (Fig. **1**), like amiridine (NIK-247) [36], velnacrine [37,38], 1,4-methylenetacrine [39], dihydroquinazoline-based compounds (I; [40]), and different tetracyclic derivatives [41,42].

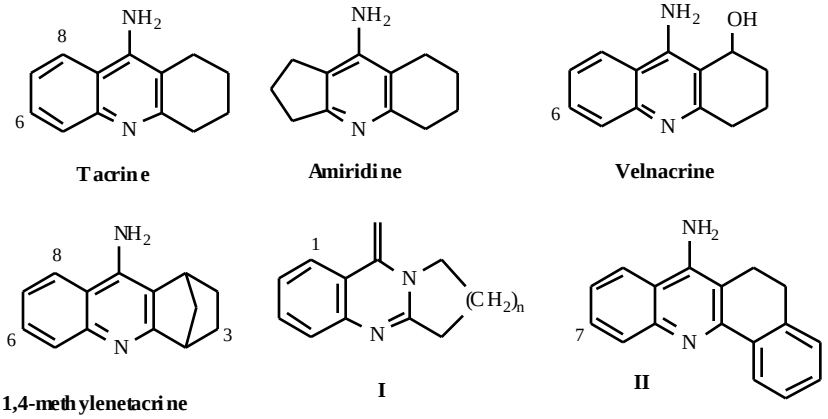


Fig. (1). Schematic representation of aminoacridine-like AChE inhibitors.

SAR studies demonstrated that inclusion of halogen atoms at positions 6 or 8 of tacrine has a positive effect on the AChE inhibitory activity (Table 1). Wlodek *et al.* [43] compared the effect of introducing a chloro substituent at

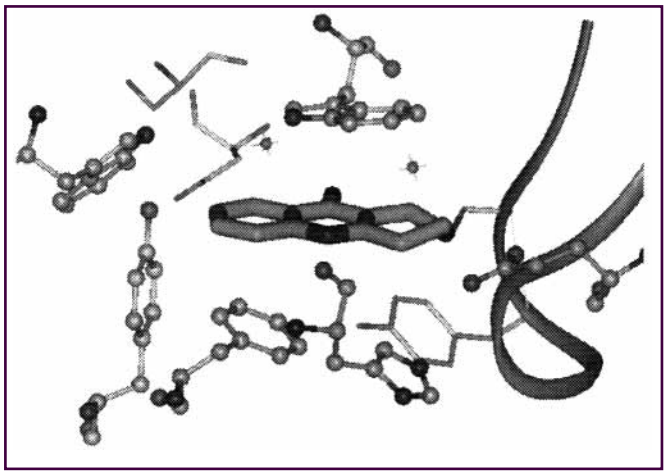


Fig. (2). Details of the 3D structure of AChE complex with tacrine.

position 6 of tacrine by means of free energy and Poisson-Boltzmann calculations. Both techniques predicted that 6-chlorotacrine binds stronger than tacrine to the enzyme, in agreement with the experimental data, and suggested that the increased affinity is related to the electron density redistribution upon chlorination of tacrine.

Table 1. AChE Inhibitory Activity of Selected Aminoacridine Compounds and their Haloge-nated Derivatives

Compound	IC_{50} (nM) [a]	IC_{50} (nM) [b]	IC_{50} (μM) [c]
Tacrine [d]	33.5	9.33	
6-Cl	1.8	2.87	
8-Cl	9.4	4.43	
1,4-methylenetacrine [d]	31.5	11.67	
6-Cl	4.5	2.33	
8-Cl	42.2	6.52	
Velnacrine [e]			4.0
6-F			0.292
6-Cl			0.0117
I (n=3) [f]	3760 [b]		15.0 [f]
3-Cl	158		1.9
1-Cl	1700		4.75

a Human red blood cell. b Electric eel. c Rat brain d [39]. e [37]. f [40].

Recanatini *et al*. have examined a series of 9-amino-1,2,3,4-tetrahydroacridine and 11*H*-indeno[1,2-b]quinolin-10-ylamine (II; Fig. **1**) compounds [42,44]. Whereas the inclusion of halogens (or even substituents like CH_3 or NO_2) increases the inhibitory potency of tacrine, halogenation at position 7 of the indenoquinoline derivatives has a detrimental effect on the inhibitory activity (Table 2). Based both on classical SAR analysis and CoMFA studies [44], they identified two features that influence the activity of 9-amino-1,2,3,4-tetrahydroacridine: i) the negative steric contribution of substituents in position 7, and ii) a favorable hydrophobic contribution exerted by substituents at position 6. The reverse effect of substituents at position 7 of 11*H*-indeno[1,2-b]quinolin-10-ylamine was related to the different binding mode of this latter compound compared to tacrine [42]: the indenoquinoline unit stacks between Trp84 and Phe 330, but it is rotated around the axis perpendicular to the ring relative to tacrine, thus changing the pattern of contacts with the binding site. Moreover, owing to the fact that near all substitutions in the tetracyclic nucleus decrease the AChE inhibitory activity, they suggested that compound II is likely the maximum sizable inhibitor that can bind near the active site of the enzyme.

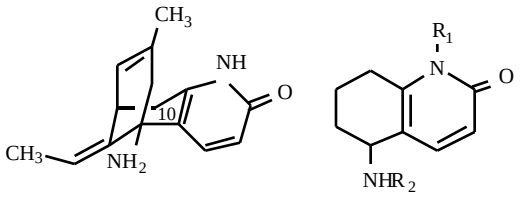
Table 2. AChE Inhibitory Activity of Selected of 9-Amino-1,2,3,4-Tetrahydroacridine and 11*H*-Indeno[1,2-b]quinolin-10-ylamine Compounds

Compound	IC50 (μM) [a]
Tacrine [b]	0.25
6-F	0.087
6-Cl	0.0099
6-CH_3	0.1
6-NO_2	0.028
II [c]	0.68
7-F	1.2
7-Cl	6.5

a Human erythrocyte. b [44]. c[42].

Huperzine

This compound (Fig. **3**) is an alkaloid isolated from *Huperzia serrata* used in the Chinese traditional medicine [45,46]. The structure of the AChE complex with (-)-huperzine A, which is the most active enantiomer [47,48] (PDB entry 1VOT; Fig. **4**) revealed that the amino group of this inhibitor interacts with the aromatic rings of Trp84 and Phe330 [13]. Interestingly, the conformation of the phenyl ring of Phe330 (torsional angle $\chi 1 \sim -170$ degrees) is similar to that found in the native enzyme or in the complex with edrophonium, but differs from that found in the complex with tacrine ($\chi 1 \sim 160$ degrees). There is also a hydrogen-bond between the carbonyl group of (-)-huperzine A and the hydroxyl oxygen of Tyr130, and an unusually short contact between the ethylidene methyl group and the main oxygen of His440. Finally, several water molecules link the inhibitor to different residues in the binding pocket.



(-)-huperzine A **III**

Fig. (3). Schematic representation of (-)-huperzine A and a tetrahydroquinolinone analogue.

The positioning of (-)-huperzine A in the binding site appears to be almost orthogonal to the plausible orientation of Ach within the active site [12,13]. This may explain why several orientations predicted by modeling studies were erroneous [49,50]. In a docking study three possible orientations of (-)-huperzine A in the binding site were identified [51]. One of the three candidate orientations differs only slightly from the binding mode in the X-ray structure, the main difference being that the pyridone oxygen should be bonded to the main-chain nitrogen of Ser124 rather than to the hydroxyl group of Tyr130. Molecular dynamics simulations suggest that the binding of (-)-huperzine A to AchE is mediated by weak hydrogen bonds between the methyl group at the three carbon bridge of (-)-huperzine A and the phenol hydroxyl oxygen of Tyr121 and the main-chain oxygen of Gly118.

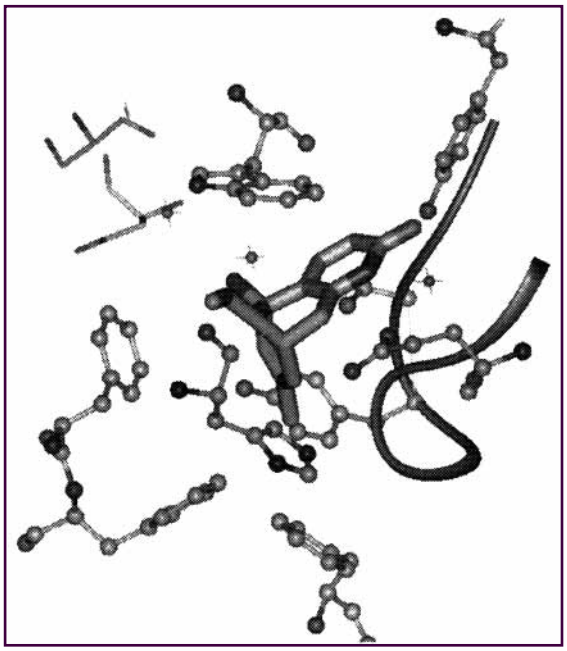


Fig. (4). Detail of the 3D structure of the AChE complex with (-)-huperzine A.

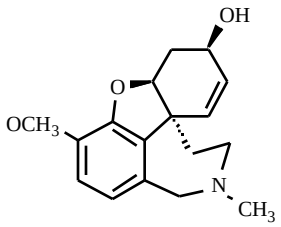
Much effort has been addressed to the synthesis of huperzine A analogues [53-61] as putative new lead. Nevertheless, few modifications have yielded inhibitory activities comparable to that of the parent compound. Particularly, introduction of an axial methyl group into position 10 of huperzine A increases the potency for AChE inhibition 8-fold, while the equatorial form is about 1.5-fold less active than huperzine A (Table 3). Molecular modeling of these analogues revealed that hydrophobic interactions with the active site are responsible for the favorable contribution of the C-10 axial methyl group [57]. The attachment of substituents larger than methyl resulted in a drop in inhibitory activity due to steric hindrance.

Table 3. K_I Values for the Inhibition of AchE for Selected Analogues of Huperzine A

Compound [a]	K_I (μM) [b]
(±)-huperzine A [b]	0.0236
10-methyl (axial)	0.003
10-methyl (equatorial)	0.035
(±)-10,10-dimethylhuperzine A	0.0167

a [57,58]. b Fetal bovine serum.

Structurally related analogues of huperzine A have been examined as potential AchE inhibitors, as in the case of tetrahydroquinolinones (III; Fig. **3**) [62]. The most active compounds were those having R_1= prenyl and R_2= 2-ClPh$(CH_2)_2$, 3-ClPh$(CH_2)_2$, 3,4-Cl_2Ph$(CH_2)_2$, and 4-(HO)Ph$(CH_2)_2$, they being in all cases 6-fold less potent than (±)-huperzine A as AchE inhibitors. Although these



Galanthamine

Fig. (5). Schematic representation of galanthamine.

compounds were designed as partial structures of huperzine A, they must bind to the enzyme in a different way, as both the quinoline nitrogen and the amino group must be sustituted in order to obtain good enzyme affinity.

Galanthamine

This compound (Nivalin®) is a tertiary amine alkaloyd (Fig. **5**) extracted from several species of *Amaryllidacae* recently approved in several countries for the treatment of AD. The orientation and conformation of galanthamine in the active site of AChE has been examined by docking studies [63] and the predicted model closely agrees with the binding mode found in the two X-ray structures of the *Tc*AChE-galanthamine complex (PDB entries 1QTI and 1DX6; [64,65]). Galanthamine binds at the base of the active site gorge interacting with both the acyl-binding pocket and the quaternary ammonium binding site. However, the tertiary amine group of galanthamine interacts by a water bridge with Trp 84 and Phe 330. This later residue shows an orientation of its side chain (χ1 ~ 125 degrees) different from that found in the AChE complexes with tacrine and (-)-huperzine. Additional anchoring points are formed by hydrogen-bonding between the methoxy oxygen and the hydroxyl group of Ser200, and between the hydroxyl group of the ciclohexenol ring with the carboxyl group of Glu199.

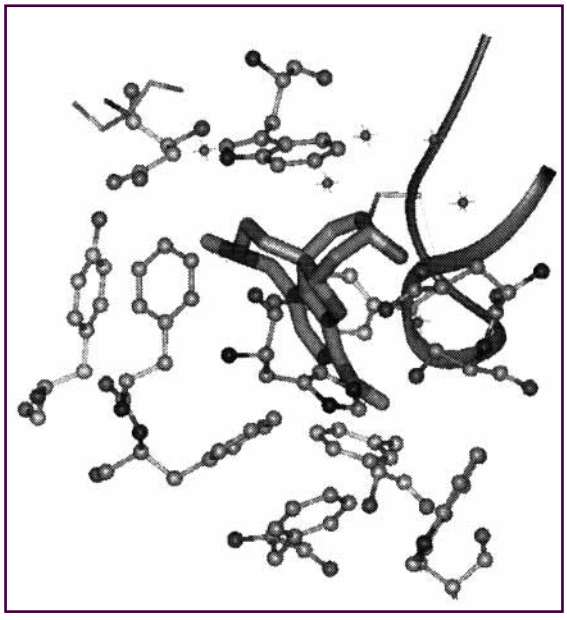


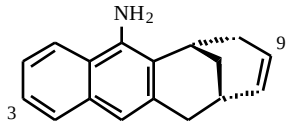
Fig. (6). Detail of the 3D structure of the AChE complex with galanthamine.

Different water-mediated contacts between the protein and the drug are also found. Finally, the ciclohexene ring and the two methylene groups in the tetrahydroazepine ring face towards the ring of Trp84. Based on this stuctural information, the differences in inhibitory activity of several galanthamine derivatives have been rationalized [64].

Huprines

This class of AChE inhibitors (Fig. **7**) were conceived as hybrids between tacrine and (-)-huperzine A [66], as they combine the 4-aminoquinoline unit of tacrine and the carbobicyclic unit of (-)-huperzine A. The underlying assumption was that the heterocyclic rings of both tacrine and (-)-huperzine A might occupy proximal regions in the active site, and subsequently the hybrid should exhibit increased potency relative to the parent compounds. The most potent compound, huprine X, has one of the highest affinities yet reported, as noted in its binding to human AChE with an inhibition constant of 26 pM [67].

Molecular modeling studies in conjunction with molecular dynamics and free energy calculations [68-70] allowed us to propose a putative binding mode that has been recently confirmed by solving the X-ray structure of the *Tc*AChE-huprine X complex [71]. The basic structural features are: i) the quinoline ring is stacked between the rings of Trp84 and Phe330; ii) the amino group occupies a position similar to that of the amino group in tacrine and the protonated amino group in (-)-huperzine A; the amino group is well hydrated and forms water-mediated contacts to residues such as Asp72 and Ser122; iii) the ring NH group is hydrogen-bonded to the carbonyl oxygen of His440; and iv) the bicyclo[3.3.1]nonadiene unit of the (-)-enantiomer fits into a hydrophobic pocket formed by residues Tyr121, Phe290, Phe330 and Phe331. Accordingly, huprines can be viewed as truly structural hybrids of tacrine and (-)-huperzine A, as far as the 4-aminoquinoline of huprines occupies the same position of the corresponding unit of tacrine, and the carbobicyclic unit of huprine is placed in a region close to the position occupied by the corresponding subunit of (-)-huperzine A.



(-)-huprine

Fig. (7). Schematic representation of the (-)-enantiomer of huprine.

The close structural resemblance between (-)-huprine, tacrine, and (-)-huperzine A suggests that attachment of substituents to equivalent positions of (-)-huprine and tacrine/(-)-huperzine A should lead to analogous changes in the potency. This theoretical prediction was confirmed by the available experimental data [68-70]. Thus, attachment of chlorine at position 3 leads to around 7-fold increase in the inhibitory potency (Table 4), as also found for tacrine (see Table 1). Likewise, replacement of methyl by ethyl at position 9 slightly increases the inhibitory potency (Table 4), but bulkier substituents decrease the binding affinity. A similar effect is also found upon replacement of methyl by phenyl in (-)-huperzine A [53]. The increased binding affinity obtained upon chlorination at position 3 is related to the fact that chlorine fills a hydrophobic cavity formed by Met436, Ile439 and Trp432. The slight increase in binding affinity observed upon replacement of methyl by ethyl at position 9 reflects the better desolvation of the inhibitor.

Table 4. Pharmacological Data of Selected Derivatives of (-)-Huprines

Compound [a]	IC_{50} (nM) [b]	IC_{50} (nM) [c]
R_3: H; R_9: CH_3	47.1	n.d.
R_3: H; R_9: CH_2CH_3	27.4	n.d.
R_3: F; R_9: CH_3	3.49	n.d.
R_3: Cl; R_9: CH_3	1.15	0.32
R_3: F; R_9: CH_2CH_3	6.73	2.11
R_3: CH_3; R_9: CH_2CH_3	4.5	n.d.
R_3: Cl; R_9: CH_2CH_3	1.30	0.32

a [68-70]. b Bovine erythrocytes. c Human erythrocytes.

Donepezil and N-Benzylpiperidines

Donepezil (Aricept®), also known as E2020, (Fig. **8**) was the second FDA-approved drug for the treatment of mild to moderate AD. Donepezil, which binds to AChE in the nanomolar range [72,73], adopts a unique orientation along the active site gorge extending from the anionic subsite of the active site to the peripheral site through aromatic interactions with conserved aromatic amino acid residues (Fig. **9**) [74]. The benzyl ring interacts through π-π stacking with the indole ring of Trp84. The charged nitrogen of the piperidine ring makes a cation-π interaction with the phenyl ring of Phe330, whose orientation is similar to that seen in the complex with galanthamine and decamethonium. The indanone ring stacks against the indole ring of Trp279, and there are water-mediated contacts that seem to be crucial for binding and specificity.

Owing to the superior pharmacological properties, including high efficacy, safety profile and selectivity, a large interest has been devoted to the design of novel N-benzylpiperidine derivatives, particular attention being paid to the replacement of the indanone moiety by a variety of chemical systems [75-78]. Likewise, a large number of theoretical studies, including both 3D QSAR [79-81] and docking [82-85] analyses have been performed on N-benzylpiperidine derivatives.

A convergent approach consists of the development of aminopyridazines derivatives as AChE inhibitors, exemplified in the case of minaprine (Fig **8**) which, besides its original antidepressive properties, presents a weak, reversible AChE inhibitory activity and a weak, but highly selective affinity for muscarinic M1 receptor [86]. To further

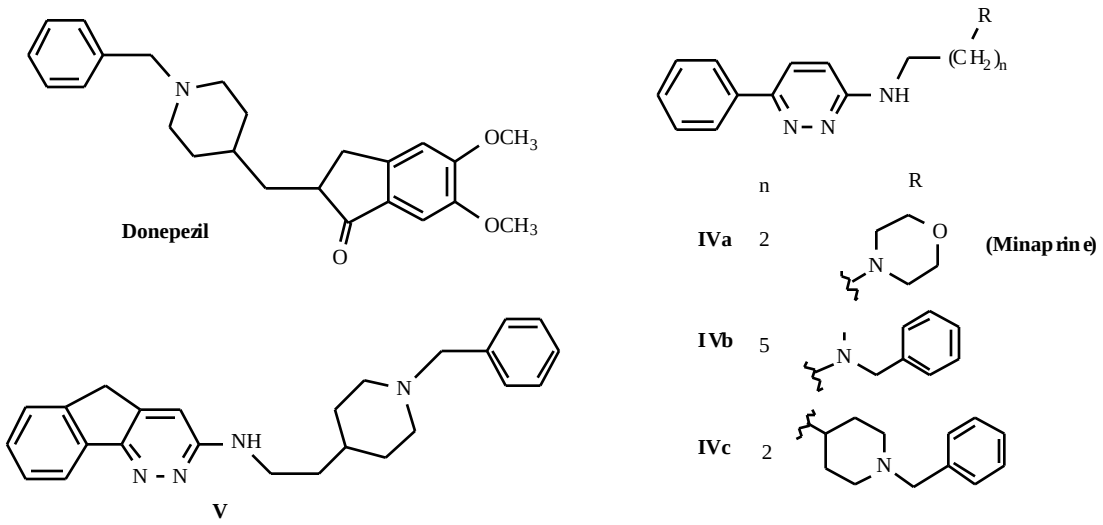


Fig. (8). Schematic representation of donepezil and N-benzylpiperidine analogues.

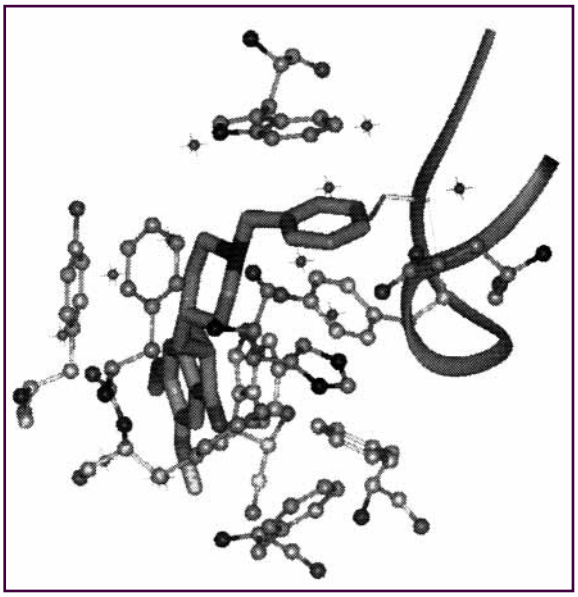


Fig. (9). Detail of the 3D structure of the AChE complex with donepezil.

investigate aminopyridazines as AChE inhibitors, a SAR study yielded compound IV (Fig. **8**), which is 5000-fold more potent compared to minaprine (Table 5) [87]. Interestingly, the highest potency, together with the best discrimination between AChE and BchE, is associated with the inclusion of the N-benzylpiperidine ethyl moiety, which is also present in donepezil. Further research effort has allowed the design of compound V (Fig. **8**), which has an IC_{50} of 10 nM on electric eel AChE [88], thus representing a 60000-fold increase in potency relative to minaprine. Docking studies [88] show that the benzylpiperidine moiety occupies a similar position and orientation than donepezil in its complex with AChE. Indeed, the arylpyridazine part interacts with the indole ring of Trp279 in a similar way as found for the indanone ring of donepezil.

Table 5. AChE Inhibitory Activity of Aminopyridazine Derivatives

Compound [a]	IC_{50} (μM) [b]	IC_{50} (μM) [c]
IVa (minaprine)	600	n.d.
IVb	0.74	0.93
IVc	0.12	0.14

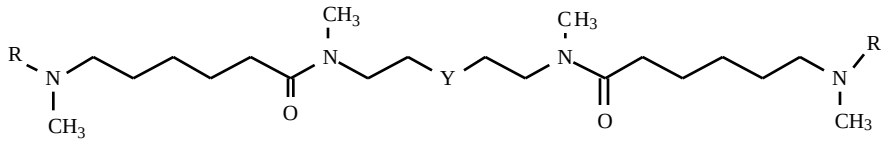
a [87]. b Electric eel. c Human erythrocyte.

Dual Binding Site Inhibitors

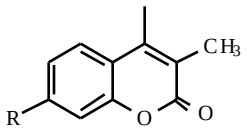
The existence of both anionic and peripheric binding sites (Trp84 and Trp279) inspired the design of ligands able to interact simultanenously with both sites [see ref. 89 for review]. The first of these compounds was based on the bis-tetrahydroaminoacridine motif [90]. The homodimeric bis-tacrine analogue is 149-fold more potent as AChE inhibitor than tacrine, and near 100-fold more selective for AChE than for BChE [91-93]. The optimum huperzine A-tacrine dimeric derivative is 13-fold more potent than (-)-huperzine A, and 25-fold more potent than tacrine [94]. Other studies have examined the inhibitory potencies of novel tacrine-related compounds [95], heterodimeric derivatives of huperzine A [96] and galanthamine [97], which were between 2 and 5-fold more potent than tacrine.

Other Noncovalent Inhibitors

Alternative strategies are based on the use of polyamine compounds having affinity for both AchE and muscarinic M2 receptors [98]. Melchiorre *et al.* have investigated the AChE inhibition of polyamine compound, such as caproctamine (Fig. **10**), which exhibited an inhibitory potency similar to that of tacrine (Table 6). Docking of caproctamine showed that this compound is able to bind simultaneously at both the active and peripheral sites of AchE. Particularly, the *o*-methoxybenzylamine moiety interacts with a set of residues surrounding Trp84, while, at the opposite end, the second *o*-methoxybenzylamine unit can reach Trp279. Moreover, the intermediate chain establishes hydrophobic interactions with several aromatic residues of the gorge.



Caproctamine (R= 2-MeOC $_6$H$_5$CH$_2$; Y=(CH $_2$)$_4$)



X (R= O(CH $_2$)C$_6$H$_4$-3'-Cl)

Fig. (10). Schematic representation of several noncovalent compounds with AChE inhibitory activity.

Table 6. K_I Values for the Inhibition of AchE for Caproctamine in Comparison with Tacrine

Compound	K_I (μM) [a]
tacrine	0.151
caproctamine	0.104

a Human erythrocytes; [98].

The observation that some compounds can behave as inhibitors of both MAO (mainly MAO-B) and AchE has raised the interest in exploring their therapeutic potential. This is the case of some coumarins derivatives (Fig. **10**), which were found to inhibit AChE in the micromolar range, the most active compound having a K_I value of 3.40 μM [99,100]. However, the SAR study showed no clear relationship between MAO and AChE inhibition. Based on these preliminar results, better assessment and optimization of AChE inhibitory activity without loss of MAO-B inhibition can be an interesting therapeutic strategy.

FUTURE PROSPECTS

The acceptance of the cholinergic hypothesis for the palliative treatment of AD has been limited by the modest efficacy displayed by AChE inhibitors and the occurrence of adverse side-effects. However, its interest has been stimulated by recent evidences that cholinergic pharmacotherapy might be related to aggregation and deposition of Aβ peptide [101-103]. Accordingly, novel compounds that might increase the level of ACh and simultaneously interfere with processing of Aβ peptide appears to be a challenging strategy. Future trends seem also to address the development of combined therapeutic approaches that exploit inhibition of AChE and control of other cognition-mediated components, such as muscarinic and nicotinic effectors or non-cholinergic agents. Combined with the structural information available for inhibitors of diverse chemical complexity, computational approaches can be valuable in the design of compounds with improved pharmacological profile.

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LIST OF ABBREVIATIONS

AD	=	Alzheimer's disease
Aβ	=	β-amyloid peptide
ACh	=	Acetylcholine
AChE	=	Acetylcholinesterase
TcAChE	=	Torpedo californica AChE
SAR	=	Structure-activity relationships

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