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# Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology

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The synaptic enzyme acetylcholinesterase (AChE) terminates transmission at cholinergic synapses by rapidly hydrolysing acetylcholine. It is anchored within the synaptic cleft by a highly specialized anchoring device in which catalytic subunit tetramers assemble around a polyproline II helix. AChE is the target of nerve agents, insecticides and therapeutic drugs, in particular the first generation of anti-Alzheimer drugs. Both target-guided synthesis and structure-based drug design have been used effectively to obtain potent anticholinesterase agents. In addition, AChE is believed to play 'non-classical' roles in addition to its 'classical' role in terminating synaptic transmission (e.g. as an adhesion protein). It also accelerates assembly of Aβ into amyloid fibrils. Both of these actions involve the so-called 'peripheral' anionic site at the entrance to the active-site gorge. Novel anticholinesterases are targeted against this site, rather than against the active site at the bottom of the gorge.

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**Current Opinion in Pharmacology** 2005, **5**:293–302

This review comes from a themed issue on
Musculoskeletal
Edited by Daniel Bertrand and Ronald Hogg

Available online 20th April 2005

1471-4892/$ – see front matter


**DOI 10.1016/j.coph.2005.01.014**

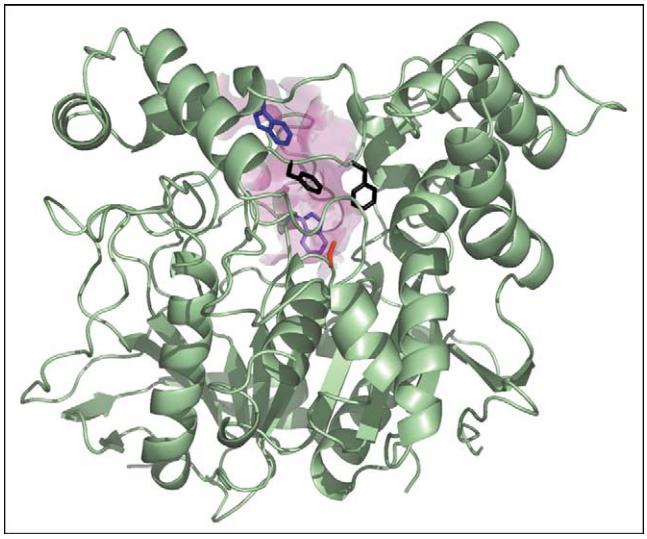
## Introduction

At the neuromuscular junction, the entire course of signal transmission — namely, the release of acetylcholine (ACh), its diffusion across the synaptic cleft, its reversible interaction with the nicotinic ACh receptor and, finally, hydrolysis by acetylcholinesterase (AChE) — occurs within a few milliseconds. The whole process must, therefore, be tightly integrated, both spatially and temporally [1,2]. In accordance with its physiological role, AChE has an unusually high turnover number, especially for a serine hydrolase, operating at a rate close to diffusion control, *viz* hydrolysing substrate so rapidly that the concentration around the enzyme molecule is depleted relative to its concentration in the bulk solution [3]. Furthermore, AChE occurs in an array of molecular forms, differing in both quaternary structure and the mode of anchoring within the synapse [4,5]; the expression pattern varies from tissue to tissue, even within the same animal [6], probably to satisfy the individual requirements of different types of synapse [7]. As a consequence of its key physiological role, AChE is the target of a repertoire of natural toxins and man-made poisons; these include alkaloids [e.g. galanthamine and (-)-huperzine A], the three-fingered polypeptide toxin fasciculin, synthetic organophosphate nerve agents, and organophosphate and carbamate insecticides [8]. AChE is also the target of drugs designed to combat neuromuscular disorders, such as myasthenia gravis and glaucoma [9], and most recently to alleviate the cholinergic deficiency associated with Alzheimer's disease [10]. Solution of the three-dimensional structure of *Torpedo californica* AChE (*Tc*AChE) [11] enhanced our understanding of the structural elements underlying its specificity and catalytic power. Subsequent elucidation of the human [12] and *Drosophila* [13] AChE structures permitted a structure-based approach to the design of anticholinesterase (antiChE) drugs and insecticides.

The three-dimensional structure of *Tc*AChE is displayed in Figure 1. Unexpectedly for such a rapid enzyme, its active site is located at the bottom of a deep and narrow cleft, named the active-site gorge, lined by 14 conserved amino acid residues [11]. The principal element of the so-called 'anionic' subsite of the active site is not a cluster of negatively charged residues, as had been assumed previously, but the indole ring of Trp84, with which the quaternary group of the choline moiety of ACh makes a cation-π interaction. Similarly, the principal element of the peripheral 'anionic' site at the top of the gorge, with which bisquaternary and peripheral site ligands interact, is the indole of Trp279. Thus, at this site, quaternary ligands interact with the enzyme via a cation-π interaction and potent bisquaternary ligands, such as decamethonium, align along the axis of the active-site gorge, making cation-π interactions with the two highly conserved tryptophan residues. At its other extremity, the acetyl moiety of ACh is confined by the so-called 'acyl pocket', of which Phe288 and Phe290 are prominent elements. Hydrolysis is accomplished by a Glu327–His440–Ser200 catalytic triad, similar to that found in other serine hydrolases, and a three-pronged oxyanion hole (not shown in Figure 1) serves to stabilize the transition state, as is, again, the case for other serine hydrolases.

For over 20 years, it has been suspected that AChE plays other roles, in addition to its 'classical' role in terminating

**Figure 1**



Three-dimensional structure of *Torpedo californica* acetylcholinesterase. The structure is presented as a ribbon diagram, with the N-terminus at the lower left, and the C-terminus at the upper right. The entrance to the active-site gorge is at the top, and the surface of the gorge is outlined as a pink surface. Trp 84, the key residue in the 'anionic' subsite of the active site, is represented as a blue stick model, as is Trp279, the key residue of the peripheral 'anionic' site at the gorge entrance. The active-site serine, Ser200, is shown as a red stick model, and Phe288 and Phe290, which line the acyl pocket, are shown as black stick models.

synaptic transmission [14–16]. It has been suggested that these 'non-classical' roles may be either catalytic (e.g. hydrolysis of ACh in a trophic context) or non-catalytic (e.g. 'moonlighting' as an adhesion protein or participating in haematopoiesis). In this review, we consider recent developments relating to both the classical and non-classical roles of AChE and their pharmacological consequences.

## Anchoring of synaptic acetylcholinesterase

As mentioned above, AChE occurs in an array of molecular forms that varies from tissue to tissue. These forms are generated by alternate splicing of the C-terminal exon followed by post-translational modification [5], and it appears that the polypeptide encoded by the T transcript, $AChE_T$, is that incorporated into synaptic AChE, at least in vertebrate fast muscles and in the central nervous system (CNS). At the neuromuscular endplate, one to three catalytic subunit ($AChE_T$) tetramers are attached to a triple-helical collagen tail, which anchors them to the basal lamina within the synaptic cleft, whereas in the CNS, a single tetramer is attached to a 20 kDa membrane-spanning polypeptide.

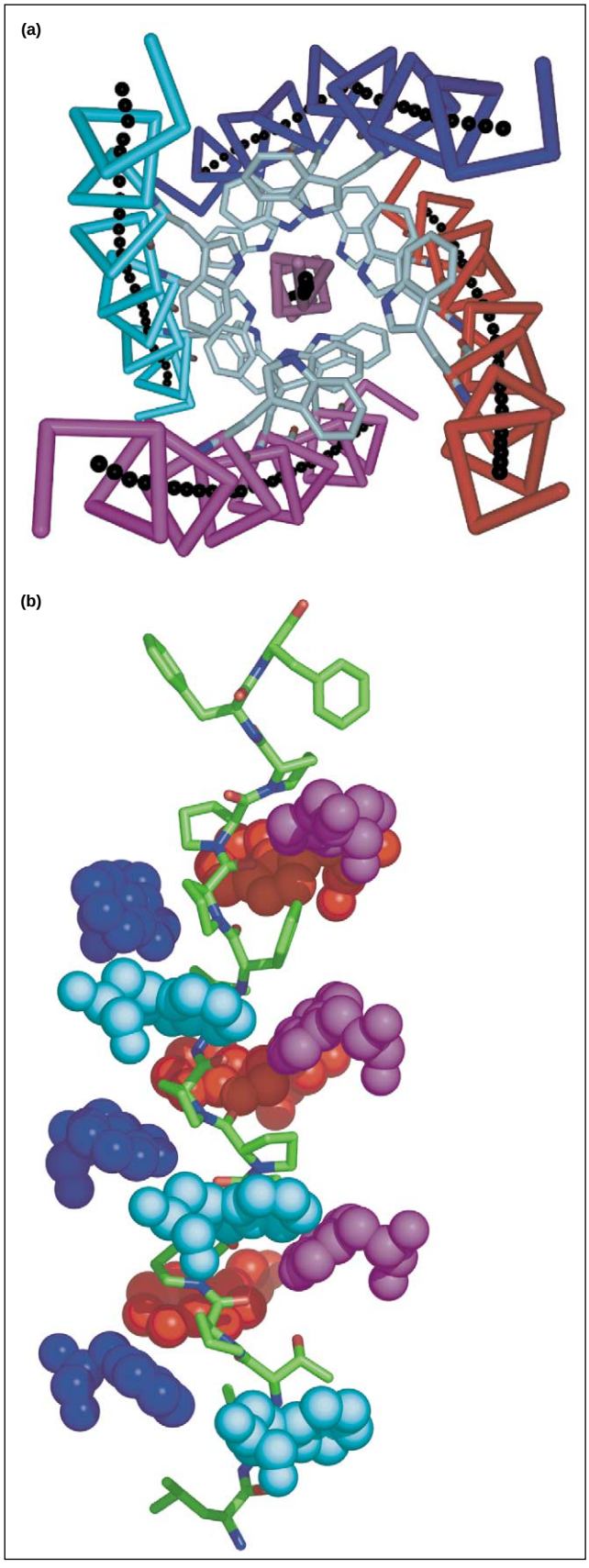
Cloning and expression of the collagen polypeptide ColQ [17] and, subsequently, of the 20 kDa polypeptide (PriMA) [18] permitted clarification of the mode of interaction of the $AChE_T$ subunits with the two anchors. Interaction involves a proline-rich attachment domain (PRAD) near the N-termini of both ColQ and PRiMA, with two cysteine residues adjacent to it that form disulfide bridges with a cysteine near the C-terminus of $AChE_T$. However, disulfide bond formation is not essential; indeed, synthetic polyproline is capable of tetramerizing $AChE_T$ subunits [19]. In the PRAD of human ColQ, eight out of 15 residues are prolines, and three of the remaining residues are phenylalanines; in the PRAD of human PRiMA, 14 out of 15 residues are prolines. The C-terminal sequence of $AChE_T$ responsible for interaction with the PRAD is a ~40 residue polypeptide named the tryptophan amphiphilic tetramerization (WAT) domain. This domain can replace the whole $AChE_T$ subunit in tetramer formation, and thus behaves as an autonomous interaction domain [20]. It contains three highly conserved tryptophan residues spaced seven residues apart.

The three-dimensional structure of the $[WAT]_4$PRAD complex was recently solved by X-ray crystallography [21••]. It reveals a novel supercoil structure in which four parallel WAT chains form a left-handed superhelix around an antiparallel left-handed PRAD helix resembling polyproline II (PPII; Figure 2). The three conserved tryptophans (W) in each of the WAT coiled coils serve as a WWW motif, making repetitive hydrophobic stacking and hydrogen-bond interactions with the PRAD. The hydrophobic contacts consist of a series of stacking interactions, mostly between indole rings of the tryptophans of WAT and the proline rings on PRAD. The polar interactions involve hydrogen bonds between tryptophan *N*1 atoms and the main-chain carbonyls of all the PRAD residues.

Interest in PPII helices has increased as some of the most common modular protein recognition domains in eukaryotic signal transduction pathways recognize proline-rich motifs [22]. Structures of complexes of SH3 domains with a repertoire of proline-rich motifs confirmed the prediction that aromatic residues of SH3 interlock with proline rings on one face of the PPII helix [23]. A set of nearly parallel aromatic residues forms a series of ridges and grooves on the domain surface, against which the PPII helix packs [24]. Not only do the indole rings fit well within the grooves of the PPII helix, but their *N*1 atoms also bond (via hydrogen bonds) to the main-chain carbonyl oxygens of PPII. The structure of the $[WAT]_4$PRAD complex, with four identical WWW motifs wrapped around the PRAD, displays these aromatic-PPII stacking and hydrogen bond interactions in their fullest form.

A variety of mutations in the *ColQ* gene result in the neuromuscular disease congenital endplate AChE deficiency, as a consequence of defective assembly or anchoring of the asymmetric forms of AChE [25]. The P59Q mutation in human ColQ [26] corresponds to P7 in the

**Figure 2**




Two views of the $[WAT]_4$PRAD crystal structure. **(a)** View of the $[WAT]_4$PRAD structure looking down the superhelical axis. Each of the four WAT chains (magenta, red, blue and cyan traces) assumes a coiled-coil structure, together forming a left-handed superhelical structure. The black dots trace their axes. The axis of the superhelix coincides with that of the PRAD helix (purple) which, itself, is not part of the superhelix. The WAT chains are related by an approximately four-fold symmetry, which also extends to the side chains, as illustrated by the conserved tryptophans. **(b)** Side view of the $[WAT]_4$PRAD structure, with the superhelical axis running vertically, showing stacking interactions between the WAT and PRAD helices. PRAD is depicted as a stick model. Only the tryptophans of the WATs are shown (in space-filling format), colour-coded as for the WAT chains in **(a)**. The tryptophan indole groups form a spiral staircase around the PRAD, fitting into grooves formed by its extended PPII helix. Reproduced with permission from [21••].

PRAD of human ColQ. Modeling of a glutamine residue replacing P59 suggests that the P59Q mutation not only affects attachment of each $AChE_T$ dimer to ColQ but also weakens the dimer–dimer interaction [21••]. This provides a plausible structural explanation for the failure to detect asymmetric forms of AChE in COS cells cotransfected with wild-type $AChE_T$ and the ColQ P59Q mutant [25].

## Target-guided synthesis of acetylcholinesterase inhibitors

In recent years, high-throughput screening of large libraries has been increasingly utilized for detection of novel lead compounds in drug discovery. Despite the technical advances in synthesis, purification and characterization of the huge number of compounds generated by such an approach, the fact that, typically, >99% of the compounds screened are inactive constitutes a serious drawback. Consequently, attempts are being made to produce only the active compounds of interest. Target-guided synthesis aims to achieve this objective by using the target protein as a template for assembling its own specific ligand from a collection of building blocks that adhere to a repertoire of subsites within the ligand-binding site and interact preferentially with each other [27].

As mentioned earlier, vertebrate AChE is the target of natural toxins and man-made poisons, as well as of drugs used for the treatment of neuromuscular disorders and the first generation of anti-Alzheimer drugs; invertebrate AChE is likewise the target of organophosphate and carbamate insecticides. There is thus an extensive literature dealing with the synthesis and characterization of lead antiChE compounds [28–31].

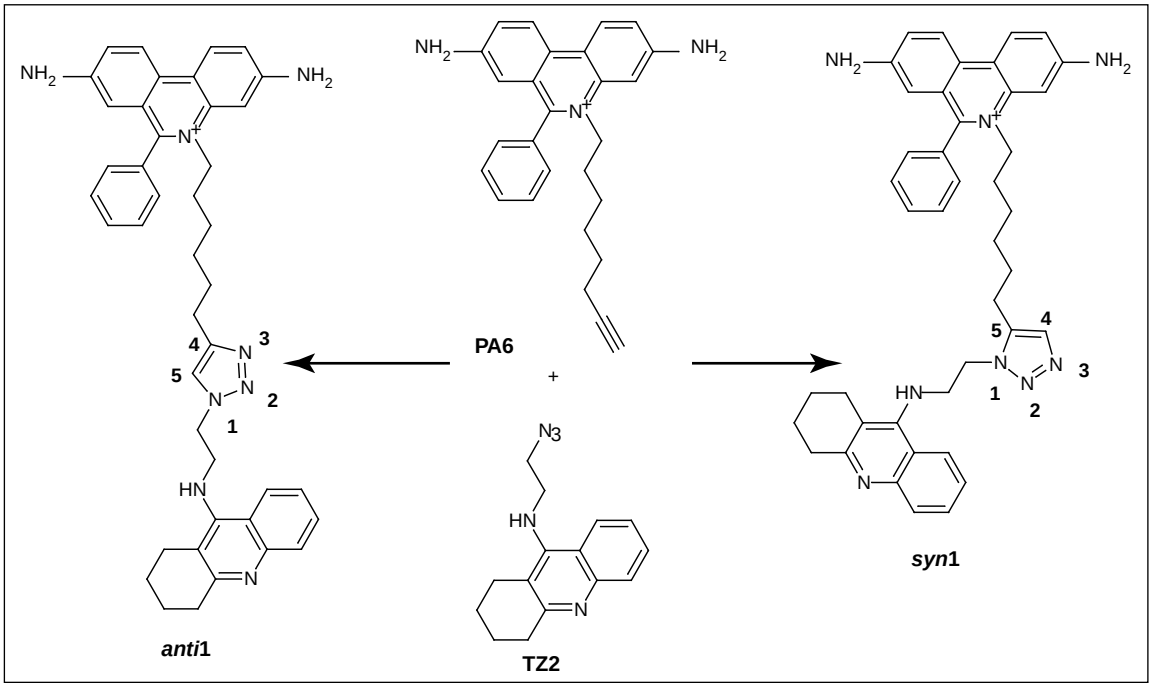
Although a combinatorial approach was earlier used to identify a potent bis-pyridinium inhibitor [32], a target-guided approach was only recently applied to AChE [33]. This involved utilization of a 'click reaction' — the Huisgen 1,3-dipolar addition of an azide and an acetylene — to yield a 1,2,3-triazole [34]. AChE was utilized as a 'reaction vessel', and the building blocks selected

contained as specificity elements two ligands, tacrine and phenylphenanthridinium, directed to the anionic subsite of the active site at the bottom of the active site gorge [35] and to the 'peripheral' anionic site near its entrance, respectively [36]. Both were linked via methylene spacers of varying lengths to the functional azide and acetylene groups, to yield a library of 49 pairs of compounds. These could potentially generate 98 bifunctional inhibitors of AChE that might assemble along its long and narrow active site gorge [11] owing to the proximity of the reactants. Each possible binary combination was incubated in the presence of *Electrophorus* AChE at room temperature, under which conditions a reaction in the absence of enzyme is negligible. Only for one combination — that of tacrine linked to azide via a two-carbon spacer (TZ2) with phenylphenanthridinium linked to acetylene via a six-carbon spacer (PA6), namely TZ2PA6 — was the corresponding triazole detected. The corresponding adduct could also be obtained by prolonged heating at 80 °C as two isomers, *syn*1-TZ2PA6 and *anti1*-TZ2PA6 (Figure 3), which could be separated and characterized.

The enzyme-catalyzed reaction generated predominantly the *syn1* isomer. The dissociation constants for a repertoire of AChEs from different species were 77–410 fM for the *syn1* isomer, making it the most powerful non-covalent AChE inhibitor described thus far, and 0.72–14 pM for the *anti1* isomer. Thus the more potent *syn1* isomer is that assembled by the enzyme, demonstrating the power of the experimental paradigm employed.

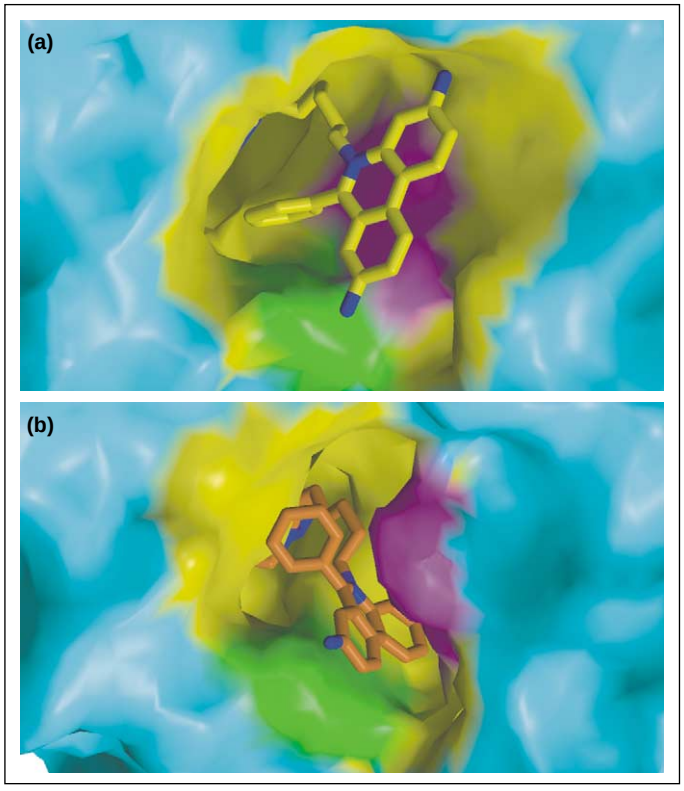
The structures of the complexes of both the *syn1*- and *anti1*-TZ2PA6 isomers with mouse AChE were solved by X-ray crystallography [37••]. The *syn1* isomer formed within the active site gorge freezes the AChE molecule in a unique and unanticipated conformation, whereas the complex of the *anti1* isomer adopts a conformation similar to that of the native enzyme. In both complexes, the tacrine moiety at the bottom of the gorge stacks against Trp86 in the anionic subsite of the active site, similarly to tacrine itself in the tacrine *Tc*AChE complex [35]. At the peripheral site in the *anti1*-TZ2PA6 complex, the phenanthridinium moiety stacks against the indole of Trp286 (Figure 4a), as observed for the decidium mouse AChE complex [38]. However, the triazole group in the *syn1* isomer aligns 2 Å deeper in the gorge; as the overall span of the linker is shorter in the *sy1n* than in the *anti1* isomer, the phenanthridinium moiety is pulled down the gorge into a region occupied by the indole of Trp286 in the peripheral site of the native enzyme. As a consequence, the side chain of Trp286 is dislodged from its native position, creating a much larger gorge opening, with the phenanthridinium being sandwiched between Trp286 and Tyr72 (Figure 4b). Clearly, the structure of the potent new inhibitor obtained by target-guided synthesis could not have been predicted by *in silico* modeling studies utilizing the native AChE structure as a template.

**Figure 3**




Structures of the *anti1* and *syn1* TZ2PA6 regioisomers formed by 1,3-dipolar cycloaddition. The phenylphenanthridinium, triazole, and tacrine moieties are shown from top to bottom. Reproduced with permission from [37••].

**Figure 4**




Views of the PAS region of mouse AChE bound to the phenylphenanthridinium moiety present in the *anti1* **(a)** and *syn1* **(b)** isomers. The mouse AChE molecular surfaces buried at the complex interfaces are shown in yellow, with the Tyr72 and Trp286 side chains highlighted in green and magenta, respectively. The area of the gorge-mouth openings in the *anti1* and *syn1* complexes are 14 $Å^2$ and 29 $Å^2$, respectively. Reproduced with permission from [37••].

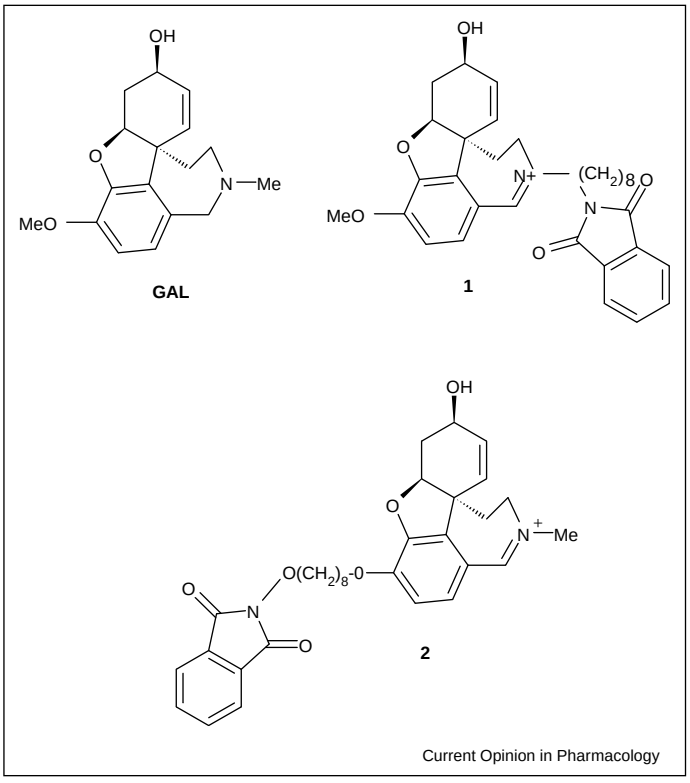
## Structure-based drug design

In recent years, structure-based drug design has had increasing success in providing lead compounds for drug development [39]. Use of X-ray or nuclear magnetic resonance structures of a complex of a ligand with its target protein permits the design of novel ligands so as to maximize interactions with the target, thereby increasing selectivity and potency. Use of an experimental structure provides a degree of certainty that cannot be achieved by *in silico* modeling and docking programmes, however sophisticated. Indeed, in several cases, docking procedures made assignments for ligand/AChE complexes that were invalidated by subsequent experimental data [40].

In many cases, binding of a ligand to AChE does not cause any substantial change in its structure [35,40,41], with the exception of the movement of the phenyl ring of Phe330 within the active site gorge in *Tc*AChE. However, significant, and in some cases, substantial changes do sometimes occur. One such case is the major conformational change produced upon binding of the *syn1*-TZ2PA6 isomer generated by target-guided synthesis described in the previous section [37••]. In the conjugates of *Torpedo* AChE both with the anti-Alzheimer drug rivastigmine [42] and with the nerve agent VX [43], a small but significant change occurs, namely disruption of the catalytic triad.

Upon binding at the bottom of the active site gorge of the bulky and rigid alkaloid galanthamine (GAL; licensed for treatment of Alzheimer's disease as Reminyl$^{TM}$), no substantial conformational change occurs [40,41]. In an attempt to enhance the affinity and selectivity of GAL for AChE, several bifunctional derivatives were synthesized, in which the GAL moiety was linked to a phthalimide moiety by a spacer [44]. One such bifunctional agent (compound 1 in Figure 5) displayed approximately 100-fold higher affinity for *Tc*AChE than did GAL itself. The crystal structure of the 1/*Tc*AChE complex reveals that the GAL moiety adopts the same orientation within the gorge as GAL itself [45••]. The eight-carbon spacer is aligned along the gorge, and the phthalimido group is stacked against Trp279 in the 'peripheral' site, similarly to the phenathridinium group of the *anti*-TZ2PA6 isomer complexed with mouse AChE described in the previous section [37••] or the aromatic ring system at the top of the active site gorge in the complex of *Tc*AChE with the widely prescribed anti-Alzheimer drug donepezil (Aricept$^{TM}$) [46]. Compound 2 has an affinity for *Tc*AChE

**Figure 5**




Structures of GAL and of two bifunctional derivatives (compounds 1 and 2).

similar to that of GAL. Consideration of its structure, in which the linker to the phthalimido group is attached on the opposite side of the GAL skeleton than in 1 (Figure 5), indicates that it would not fit into the active site of *Tc*AChE if its GAL moiety were to orient similarly to both GAL itself and compound 1. Indeed, when compound 2 was soaked into native *Tc*AChE crystals of the same trigonal form used to obtain the 1/*Tc*AChE complex, a marked degradation in diffraction quality was obtained, which precluded data collection. If, however, compound 2 was soaked into orthorhombic crystals, in which the packing of the protein molecules is very different, X-ray data could be collected; these data revealed that the GAL moiety is oriented much like in the GAL/*Tc*AChE and 1/*Tc*AChE complexes. Electron density corresponding to the initial methylene groups of the spacer is seen pointing in the direction of the acyl pocket, but the continuation of the spacer and the phthalimido group cannot be seen, nor can electron density corresponding to residues 284–288 in the acyl pocket. The interpretation offered is that the phthalimido group pierces the wall of the acyl pocket, protruding out into the solvent. As there is no symmetry-related protein molecule packed against the acyl pocket in the orthorhombic crystal form, in contrast to the trigonal form, this does not disrupt the crystal structure. Thus the prediction made on the basis of structure-based drug design is invalid. Although this is an extreme case, it shows the limitations of relying upon structure-based drug design that considers the protein as a rigid template. Furthermore, although some programmes use molecular dynamics or flexible docking, we are not aware of any programme that could cope with such drastic tearing of the fabric of the protein.

## Non-classical roles of acetylcholinesterase

Numerous reports suggest that AChE plays other roles in addition to its 'classical' function in terminating impulse transmission at cholinergic synapses. Such 'non-classical' functions might involve the hydrolysis of ACh in a non-synaptic context. Furthermore, the 26-residue C-terminal peptide ARP (acetylcholinesterase read-through peptide) of the read-through form of human AChE ($AChE_R$), which is produced when the *R* splice variant is induced by stress [47], has been reported to modulate haematopoietic differentiation [48]. We will, however, restrict ourselves to consideration of various phenomena that appear to involve the intact AChE molecule, but not its catalytic activity.

AChE displays sequence and putative structural homology to a group of neuronal adhesion proteins, including the mammalian neuroligins and the *Drosophila* proteins gliotactin and neurotactin [49••], which we have accordingly named CLAMs (cholinesterase-like adhesion mole-

cules) [50]. This suggests that one of the 'non-classical' roles of AChE may be as an adhesion protein involved in synaptic development and maintenance. Such a possibility is of particular interest as it has been reported that mutations in neuroligin genes are associated with autism, Asperger syndrome and/or mental retardation [51•,52•]. There is intriguing evidence that implicates AChE as a bone matrix protein [53], and it has recently been shown to interact with the basement membrane protein laminin [54•]. Moreover, Darboux *et al.* [55] have shown that chimeric proteins in which the extracellular domain of neurotactin had been replaced by either *Tc*AChE or *Drosophila* AChE display adhesive properties similar to those of wild-type neurotactin in cultured *Drosophila* S2 cells. An electrostatic motif at the mouth of the active site gorge of AChE, which is responsible for its unusually high dipole moment, is conserved in the CLAMs, and could be implicated in both their adhesive function and that of AChE itself [56].

Several groups have provided evidence for the involvement of AChE in neurite growth [57–59]. Although the pharmacological profiles of the effects of various antiChE agents on neurite growth are not easily interpreted in any of these studies, a pattern emerges that implicates the 'peripheral' site rather than the active site. Furthermore, in one case, neurite outgrowth induced by a catalytically inactive form of AChE has been reported [60].

Inestrosa and colleagues have shown that AChE accelerates the assembly of Aβ peptide into amyloid fibrils, probably through interaction at the 'peripheral' site [61–63]. Although the relevance of these observations to the pathology of Alzheimer's disease remains to be established, AChE is known to be associated with amyloid plaques [64]. It has also been shown that AChE/Aβ complexes display enhanced neurotoxicity compared with fibrils containing only Aβ [65]. Interestingly, the capacity of intact AChE to promote Aβ assembly can be mimicked by a hydrophobic peptide that contains residues belonging to the 'peripheral' site of *Tc*AChE [66] that had previously been shown to interact with liposomes [67].

In a pharmacological context, the studies briefly summarized above concerning the putative 'non-classical' activities of AChE appear to converge on the 'peripheral' anionic site. One could thus envisage that new categories of AChE inhibitors will be developed. Such leads might have dual specificity, being directed to both the active and 'peripheral; sites, as is the case for gorge-spanning inhibitors such as BW284C51 [68] and Aricept™ [46] (reviewed in [30]). Others might be directed, like the prototypic peripheral site inhibitor propidium [36] or the mamba venom toxin fasciculin [69,70], exclusively to the 'peripheral' site. As highlighted by Bourne *et al.* [37••], *syn1*-TZ2PA6, by disrupting the conformation of the peripheral site, might affect both its plaque-forming capacity and its adhesion functions. Piazzi *et al.* [71•] have also reported the design and synthesis of such dual-function lead compounds.

Although not associated with 'non-classical' functions of AChE, it is worth mentioning the recent description of another class of 'dual action' compounds for the treatment of Alzheimer's disease: the carbamate derivatives of N-propargylaminoindans and N-propargylphenethylamines [72]. The respective rasagiline- and selegiline-related series of compounds are designed to combine the inhibitory activities of both AChE and monoamine oxidase by virtue of their carbamoyl and propargylamine pharmacophores, and also to fulfil a neuroprotective role. In addition, it has been suggested that these novel AChE inhibitors regulate amyloid precursor protein metabolism, and thereby Aβ assembly, via activation of the mitogen-activated protein kinase pathway, resulting in enhancement of α-secretase activity [73]. Whether they also affect functions exerted by the peripheral anionic site (PAS) remains to be established.

## Conclusions

In this review, we first surveyed recent developments in our understanding of the structural elements underlying the unique anchoring devices utilized for the anchoring of AChE at cholinergic synapses. In particular, we showed how they make use of recognition elements involving stacking of aromatic rings against the proline rings of PPII helices, a device also utilized in signal transduction pathways. We then presented recent studies in which AChE served as a paradigm for development of selective high-affinity ligands by use of either target-guided inhibitor synthesis or structure-based drug design. Finally, we reviewed recent research on the 'non-classical' roles of AChE, which appear to be distinct from its 'classical' role in terminating transmission at cholinergic synapses. This research is resulting in the production of new classes of AChE inhibitors, targeted against its PAS, which might play dual roles in the context of Alzheimer's disease, by simultaneously inhibiting ACh hydrolysis and retarding assembly of the Aβ peptide to amyloid fibers.

## Acknowledgements

The authors acknowledge the support of the Kimmelman Center for Biomolecular Structure and Assembly (Rehovot, Israel), the Benoziyo Center for Neurosciences, the US Army Medical and Material Command under Contract No. DAMD17-02-1-0675. JLS is the Morton and Gladys Pickman Professor of Structural Biology.

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- •• of outstanding interest

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