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REVIEW ARTICLE

Synthetic polyamines: an overview of their multiple biological activities

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Abstract The binding of polyamines to a variety of receptors and other defined recognition sites has been widely reported. It is well-known that polyamines interact with aspartate, glutamate, and aromatic residues of a given receptor and/or enzyme mainly through the formation of ion bonds, since at physiological pH, protonation of amino groups is nearly complete. From this, the hypothesis arises that a polyamine may be a universal template able to recognize different receptor systems. This hypothesis suggests that both affinity and selectivity may be fine-tuned by inserting appropriate substituents onto the amine functions and by varying the methylene chain lengths between them on the polyamine backbone. In this paper, we detail several application of this design strategy aimed at discovering potent and selective polyamines able to bind neurotransmitter receptors and enzymes, such as muscarinic receptor subtypes, muscle-type nicotinic receptors and acethylcholinesterase.

Keywords Polyamines · Universal template · Methoctramine · MTDLs · Memoquin

Abbreviations

SAR	Structure–activity relationship
4-DAMP	4-diphenylacetoxy-*N*-methylpiperidine
nAChR	Nicotinic receptor
PhTX-433	Philanthotoxin-433
AD	Alzheimer's disease
Aβ	β-amyloid
AChE	Acetylcholinesterase
ACh	Acetylcholine
PAS	Peripheral anionic site
MTDL	Multi-target-directed ligand

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Introduction

Natural polyamines, such as putrescine, spermidine, and spermine are simple organic compounds bearing two or more amino groups charged at physiologically pH. They are widely distributed in living organism, where they are deeply involved in the regulation of cellular functions (Thomas and Thomas 2001). Due to their importance in modulating cell growth and death, polyamines could be either synthesized in the cells through a highly regulated metabolic pathway or imported from the extracellular space by specific transporters.

Their concentration is highly regulated: an excessive concentration of polyamines is associated with several pathological conditions. For example, an increase in polyamine levels has been noticed in several cancer cell lines. Further, polyamines are able to induce apoptotic cell death by triggering caspase cascade (Stefanelli et al. 1998) and, due to their positive charges at physiological pH, polyamines interact with DNA, RNA, phospholipids, and membrane proteins (Bachrach 2005).

In recent decades, medicinal chemists have carried out extensive studies on natural and synthetic polyamines due to their many physiological and/or pathological roles. Several studies suggest a strict relationship between polyamines and cellular proliferation. Thus, it is unsurprising that much effort has been directed towards developing

drugs able to act on polyamine-mediated biochemical pathways and to behave as anticancer agents (Casero and Marton 2007; Casero and Woster 2009; Gerner and Meyskens 2004; Wallace and Niiranen 2007). In addition to their possible use as antiproliferative agents, several synthetic polyamines have been designed and synthesized as receptor ligands (Andersen et al. 2006; Melchiorre et al. 2003; Stromgaard et al. 2005; Stromgaard and Mellor 2004).

In this brief overview, we discuss some of our research group's results in the polyamine field. These results have been obtained over the past two decades by applying the "Universal Template Approach". This approach allows us to design synthetic polyamines able to recognize different receptor systems and enzymes, and/or to discriminate between receptor subtypes (Melchiorre et al. 2003).

The "universal template approach"

The starting point in the development of the "universal template approach" was the observation that, in addition to its irreversible antagonism towards α-adrenoreceptors, benextramine (the prototype of polymethylene tetraamine disulfides) could competitively antagonize the cholinergic system, particularly the muscarinic receptor family (Benfey et al. 1979, 1980). Later, the finding that benextramine analogs lacking the disulfide bridge could discriminate between different muscarinic receptor subtypes (Melchiorre et al. 1987) led to the hypothesis that a polyamine backbone could be considered a universal template in the drug–receptor recognition process (Fig. 1). It was observed that, by inserting different substituents or by modifying the chain length between the nitrogen atoms of a polyamine scaffold, it was possible to modulate both affinity and selectivity for a given receptor system (Melchiorre 1988). The theory that a polyamine may act as a skeleton key able to recognize different receptor systems rests on the following.

Receptors are folded polypeptides which always contain the same amino acids albeit in different sequences and proportions. The polyamide backbone is a common feature of every protein and cannot be a tool for selectivity. Therefore, in most cases the lateral chains of the different amino acids play a role in the neurotransmitter–receptor and drug–receptor recognition process. Of these, aspartate, glutamate, and aromatic residues may have great importance for binding with cationic ligands by way of a cation–anion or a cation–π interaction. As proteins may bear several carboxylate and/or aromatic residues somewhere in their structure, it should be possible to design a lead compound with a polyamine backbone which is able to recognize multiple anionic sites of a given target protein. This ligand would be able to interact with all receptor proteins, provided that the distance separating the amine functions of the ligand fits the distance between the carboxylate or aromatic residues of the receptor. In other words, a polyamine could be considered a master key in the drug–receptor recognition process because its flexibility allows it to assume a suitable conformation for the interaction between protonated amine functions and receptor anionic sites. It has been reported that polyamines, such as spermine and homospermine are 85 and 97% tetracation at physiological pH, respectively, the remainder being the trication. Thus, a polyamine's cationic properties may help explain its ability to interact with biological counterions, that is, with a set of carboxylate anions fixed to the backbone of a receptor. Consequently, the distance between the cationic nitrogen atoms of a polyamine becomes critical in the drug–receptor recognition step. It is known that by increasing the number of interactions that take place between a receptor and a ligand increases the chances of distinguishing between different receptor systems. Thus, an appropriate modification of the chain length separating the nitrogen atoms of a polyamine might increase affinity, while the insertion of *N*-substituents might improve selectivity as well as affinity by increasing the overall number of contacts between a drug and a receptor. Many

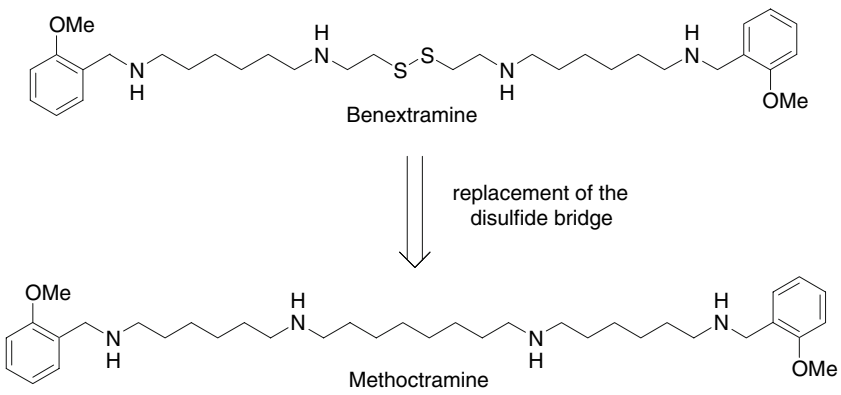

Fig. 1 Chemical structures of benextramine and methoctramine (Melchiorre et al. 1987)

naturally occurring or synthetic polyamines interact with a variety of ion channels, receptors, and enzymes, which may be activated or inhibited.

Looking for selective muscarinic receptors ligands

As mentioned above, our interest in developing polymethylene tetraamines as muscarinic receptors antagonists began when it was observed that benextramine, an α-adrenoreceptor irreversible antagonist, also displayed muscarinic receptor competitive antagonism with a significant selectivity towards cardiac muscarinic M_2 receptors (Benfey et al. 1979). It was found that the presence of a disulfide bridge is fundamental for α-adrenoreceptor irreversible inhibition through the formation of a covalent bond by way of a disulfide-thiol interchange reaction, whereas it is not important for the interaction with muscarinic receptors. In fact, the replacement of the disulfide bridge with two methylenes led to a benextramine analog that retained affinity for muscarinic receptors while losing affinity for α-adrenoreceptors (Melchiorre et al. 1987) (Fig. 1). Polymethylene tetraamines thus became the starting point for the design of a new class of antimuscarinic agents, sharing no structural homology with any other compound targeting the same receptor system.

Extensive structure–activity relationship (SAR) studies aimed at improving antimuscarinic activity and selectivity led to the discovery of methoctramine. It is classified as a selective muscarinic M_2 receptor antagonist and is widely used (Melchiorre et al. 1987) as a pharmacological tool for muscarinic receptor characterization and classification. Despite its high muscarinic M_2/M_3 receptor selectivity, methoctramine failed to discriminate between muscarinic M_1 and M_4 receptor subtypes. In fact, beside its high affinity for muscarinic M_2 receptors ($pK_i = 7.84 \pm 0.09$), it displayed slightly lower affinity for muscarinic M_1 ($pK_i = 7.43 \pm 0.11$) and M_4 ($pK_i = 7.58 \pm 0.13$) receptors and low affinity for muscarinic M_3 receptors ($pK_i = 5.96 \pm 0.18$), both in functional and binding assays (Melchiorre 1988, 1990; Melchiorre et al. 1989).

In particular, it was found that the antimuscarinic potency depends on three structural parameters: (i) the length of the carbon chain separating the nitrogen atoms; (ii) the substituents on the nitrogen atoms; (iii) the number of the nitrogen atoms (Melchiorre 1990). The biological activity of methoctramine is strictly dependent on the architecture of the polyamine backbone since the removal of a nitrogen atom as well as its replacement with an amide function led to a decrease in selectivity. Further, the number of methylene units separating either the inner nitrogen atoms or the inner from the outer nitrogen atoms is important for selectivity. Finally, the better substituent on the outer amine functions was the 2-methoxybenzyl group, which played an important role for both affinity and selectivity towards muscarinic M_2 receptors.

It was hypothesized that methoctramine, like any other polymethylene tetraamine, could behave as a bivalent ligand of muscarinic receptors. Structurally, such molecules incorporate two different pharmacophores linked through a spacer of variable length that may be responsible for selectivity by allowing bridging in one receptor subtype but not another. This is confirmed by the fact that, by changing the carbon chain length between the two terminal nitrogen atoms of methoctramine, the affinity for muscarinic M_2 receptors significantly changed. Moreover, the finding that four basic amine functions are required for optimum activity suggests that methoctramine interacts with four nucleophiles located on the receptor. The amino acidic sequence of cloned muscarinic receptors contains only five acidic residues: Asp 69, 97, 103 and 120 and Glu 382 (porcine cardiac sequence numbering) (Bonner 1989; Kostenis et al. 1998; Wess et al. 1995). Since Glu 382 is intracellularly located, the four Asp residues are responsible for the interaction with the four nitrogen atoms of methoctramine. In particular, Asp 69 is located in the second transmembrane segment while the other three residues are within the third transmembrane segment.

We hypothesized that two nitrogen atoms of methoctramine could interact with Glu 175 and Asp 173 (or Glu 172) on the external loop 4–5. Such interactions would allow methoctramine to penetrate into the third transmembrane domain where additional interactions with Asp 97 and 103 take place. We note that Asp 173 and Glu 172 and 175 are not conserved in the other muscarinic receptor subtypes which might explain for the selectivity of methoctramine (Melchiorre 1990) (Fig. 2).

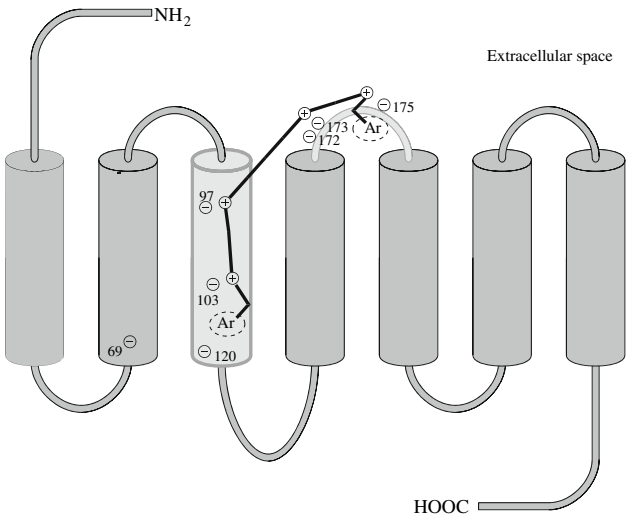

Fig. 2 Schematic representation of the interaction between methoctramine and muscarinic M_2 receptors

As previously reported, methoctramine, despite its high muscarinic M_2/M_3 receptors selectivity, failed to discriminate between muscarinic M_1 and M_4 receptor subtypes. Therefore, we tried to modify methoctramine's structure to obtain new polyamines endowed with an improved selectivity profile.

We focused on AQ-RA 741, a pirenzepine analog displaying selectivity for the muscarinic M_2 receptor subtype (Eberlein et al. 1989). An analysis of the tri-dimensional models of methoctramine and AQ-RA 741 revealed that in the extend conformation the distances between the two basic nitrogen atoms of AQ-RA 741 are very similar in comparison to the inner and the outer nitrogen atoms of methoctramine. Furthermore, the diamine derived from truncating methoctramine in two halves, which is closely related to AQ-RA 741, is neither affine nor selective towards muscarinic M_2 receptors. Therefore, we decided to replace the 2-methoxybenzyl groups of methoctramine with the tricyclic system of AQ-RA 741 (Melchiorre et al. 1993; Minarini et al. 1994) (Fig. 3).

Of the compounds synthesized, tripitramine, a non-symmetrically substituted tetraamine, was one of the most potent and selective muscarinic M_2 receptor antagonist available at that time, in both functional and binding assays, ($pA_2 = 9.75 \pm 0.02$; $pK_i = 9.54 \pm 0.08$; affinity profile, $M_2 >> M_4 = M_1 >> M_3$).

We applied a similar design strategy to obtain compounds selective for the M_1 receptor subtype. We focused on 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP), a relatively selective muscarinic M_1 and M_3 receptor antagonist (Barlow and Kitchen 1982). The 2-methoxybenzyl groups of methoctramine were replaced by the structural features of 4-DAMP and its conformationally constrained analog spiro-DAMP (Recanatini et al. 1995) (Fig. 4). From this study, spirotramine emerged as the most interesting compound displaying an inverse selectivity profile in comparison to methoctramine and tripitramine (Melchiorre et al. 1995). Spirotramine showed a higher affinity for muscarinic M_1 receptor and a significant lower affinity for all others muscarinic receptor subtypes (pK_i: $M_1 = 7.88 \pm 0.10$; $M_2 = 6.20 \pm 0.17$; $M_3 = 6.01 \pm 0.18$; $M_4 = 6.27 \pm 0.09$; affinity profile, $M_1 >> M_4 = M_2 = M_3$).

This study confirmed that an appropriate substitution on the terminal nitrogen atoms of a tetraamine backbone affords muscarinic ligands that display different selectivity profiles.

Cytotoxicity of methoctramine and related polyamines

Since many synthetic compounds with a polyamine backbone have a strong toxic effect, the cytotoxic effects of

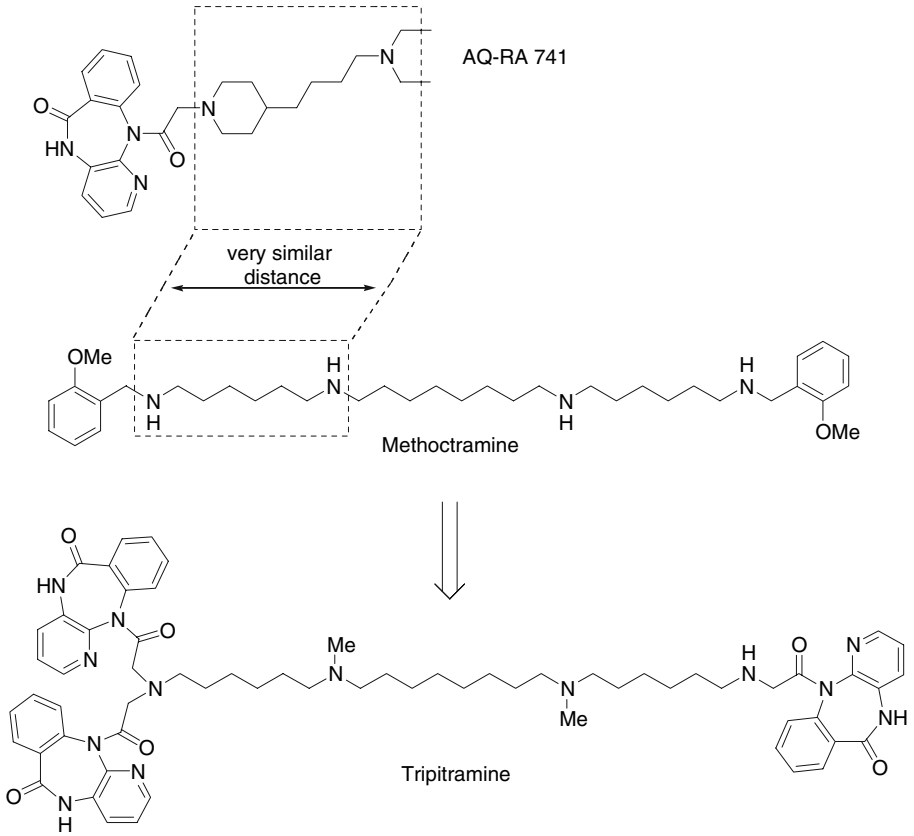

Fig. 3 Design strategy leading to tripitramine

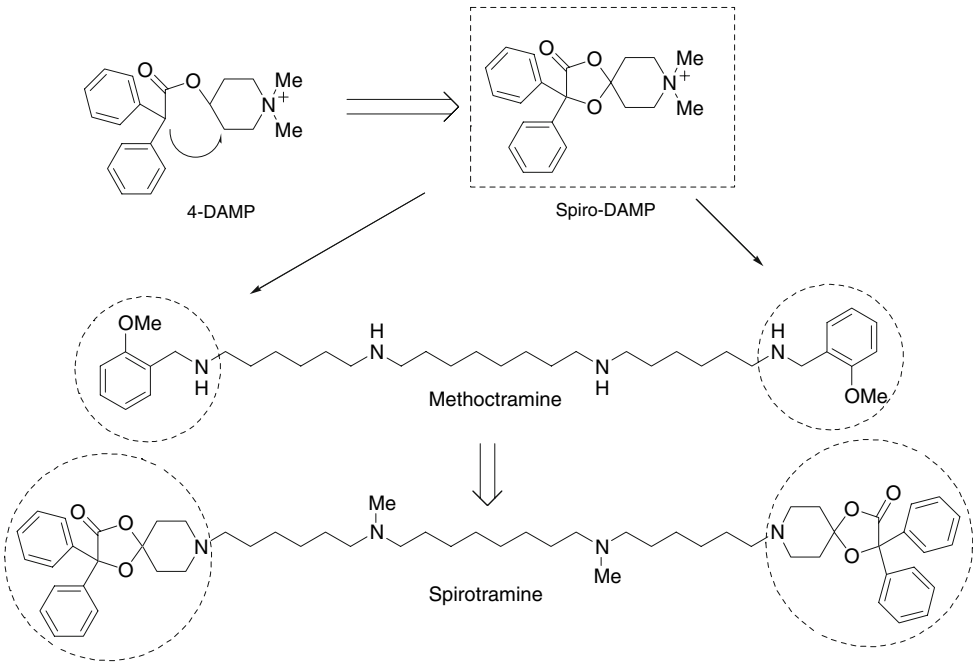

Fig. 4 Design strategy leading to spirotramine

methoctramine and related symmetrical analogs with a different carbon chain length between the inner nitrogen atoms were evaluated against a panel of cell lines (Zini et al. 2009). Methoctramine caused cell death at high micromolar concentrations only, while its pharmacological action is exerted at a nanomolar level. Increasing the chain length between the inner nitrogen atoms resulted in a significant increase in cytotoxicity. In particular, elevated cytotoxicity was associated with a 12-methylene chain spacer between the inner amine functions. H9c2 cardiomyoblasts were the most sensitive cells, followed by SH-SY5Y neuroblastoma, while HL60 leukemia cells were much more resistant. The highly toxic compound killed the cells in the absence of caspase activation, caused an increase in p53 expression and ERK1/2 phosphorylation, and led to oxidative stress.

Methoctramine and related compounds were also studied on the biosynthetic pathway of natural polyamines showing down-regulation of ornithine decarboxylase, the first enzyme of polyamine biosynthesis even at non-toxic concentration. They also caused a limited up-regulation of spermine/spermidine *N*-acetyltransferase, suggesting that interference in polyamine metabolism is not a primary mechanism of toxicity. Finally, methoctramine and its analogs bound to DNA with a higher affinity than spermine, but the correlation with their toxic effect was poor.

Polyamine as nicotinic receptor ligands

The muscle-type nicotinic receptor (nAChR) is a heteropentameric transmembrane ligand-gated ion channel, in which the five subunits are arranged around a central pore permeable for cations. The selectivity filter for cations is formed by several rings of negatively charged amino acid side chains protruding into the lumen of the pore. It was proposed that luminal non-competitive antagonists could enter the open channel and bind deep in it, close to the negatively charged selectivity filter. The highly positively charged polyamines would interact with the negatively charged amino acid residues of the selectivity filter (Hucho and Hilgenfeld 1989). Also in this case, for an optimal ligand receptor interaction, the distance separating the amine functions of the ligand should span the distance between the carboxylate residues of the receptor.

The observation that methoctramine antagonized the muscle-type nAChR at micromolar concentration (Melchiorre et al. 1987) prompted us to apply the universal template approach to designing polyamines as nAChR ligands (Bolognesi et al. 2002; Rosini et al. 1999, 2002). Furthermore, the wasp toxin philanthotoxin-433 (PhTX-433) and its synthetic analog PhTX-343, which both bear a polyamine scaffold, are well-known non-competitive antagonists of the muscle and neuronal nicotinic receptors. At submillimolar concentration, they competitively antagonize such receptors (Eldefrawi et al. 1988; Nakanishi et al. 1997; Rozental et al. 1989).

On this basis, methoctramine's structure was chemically modified to improve its affinity for nAChR. We studied the influence of the number of amine functions and the carbon chain length separating the amine functions. We also tried to constrain the highly flexible polyamine backbone in a macrocyclic structure. In addition, we synthesized hybrids of methoctramine and PhTX-343 to study the importance

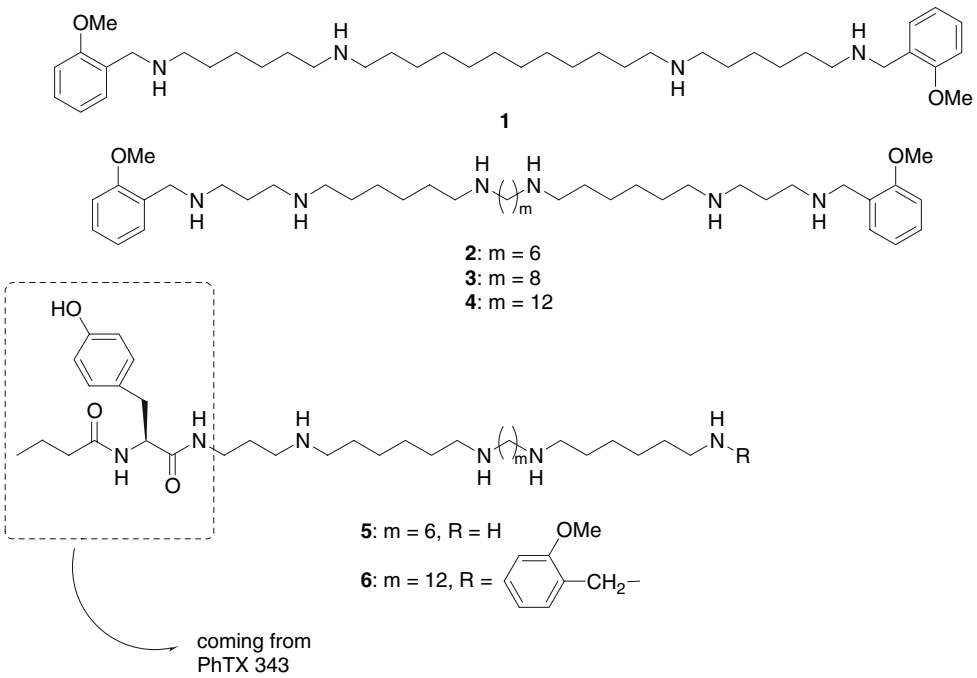

Fig. 5 Selected examples of methoctramine-related nicotinic receptor antagonists

of the 2-methoxybenzyl group (Fig. 5). In these studies, **1** emerged as the most active compound ($pIC_{50} = 7.02$), providing various information about the interaction between polyamines and the muscle-type nAChR (Rosini et al. 1999, 2002).

To further elucidate the mode of interaction of polyamines with the muscle-type nAChR we designed the photoaffinity label MR44, an asymmetric polyamino-amide that was 3- and 59-fold more potent than methoctramine and PhTX-343 at this receptor, respectively (Fig. 6).

Studies on the muscle-type nAChR of TE671 cells clearly showed that MR44 interacts with the closed channel conformation of this receptor. It was found that the aromatic moiety that carries the photolabile azido group binds to the upper part of the ion channel. This site lies within the hydrophobic sequence HWVY (residues α186–α189) containing three aromatic residues and is located close to the agonist-binding site (Bixel et al. 2001). The polycharged tail of MR44 may interact with the negative amino acid residues located deep in the lumen of the channel. In addition, it was demonstrated that two molecules of ^{125}I-MR44 labeled a receptor monomer with a 2:1 stoichiometry, whereas $^{125}I_2$-**7**, which is a symmetrical polyamino-amide, interacted with the receptor with a 1:1 stoichiometry. These results indicated that, at least with Torpedo nAChRs, symmetrically substituted tetraamines interact differently from monosubstituted tetraamines that bear a terminal primary amine function. Since two molecules of MR44 bind within the nAChR channel, each aromatic head interacts with one of the two α-subunits of the receptor monomer. Therefore, **7**, which binds the receptor with a 1:1 stoichiometry, should be in contact with the two α-subunits of the nAChR. **7**, probably assumes a folded U-shaped conformation where the positive tail reaches down the channel and each of the aromatic moieties interacts in the upper part with the α-subunits (Fig. 7).

Polyamines as multi-target-directed ligands in Alzheimer's disease

Alzheimer's disease (AD) is a devastating form of dementia. In recent decades, studies have shown that AD is a multifactorial pathology caused by genetic, environmental, and endogenous factors. These include excessive protein misfolding and aggregation, oxidative stress and free radical formation, impaired bioenergetics and mitochondrial abnormalities, and neuroinflammatory processes. β-amyloid (Aβ) peptide aggregates (Aβ plaques), and neurofibrillary tangles, composed of hyperphosphorylated tau protein, represent the two main hallmarks of AD correlated with the severity of the disease. It is well-known that AD is characterized by degradation of the cholinergic system together with alteration of other receptor systems, such as the glutamatergic and serotoninergic ones (Salloway et al. 2008). Until 2003, the only drugs available to offer symptomatic relief of AD were acetylcholinesterase (AChE) inhibitors such as donepezil, rivastigmine, galantamine, and tacrine (more recently retired from the market) (Fisher 2008). In addition, a non-competitive NMDA receptor antagonist, memantine, recently reached the market (Stys and Lipton 2007).

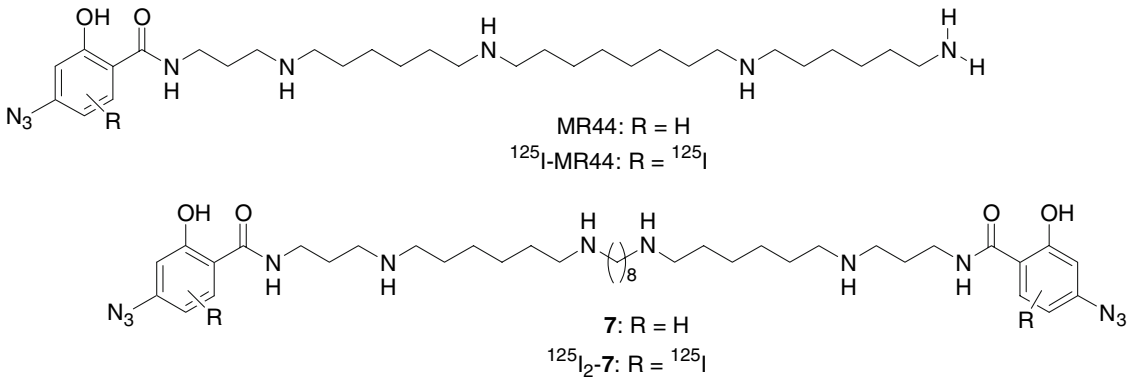

Fig. 6 Chemical structure of photoaffinity label compounds

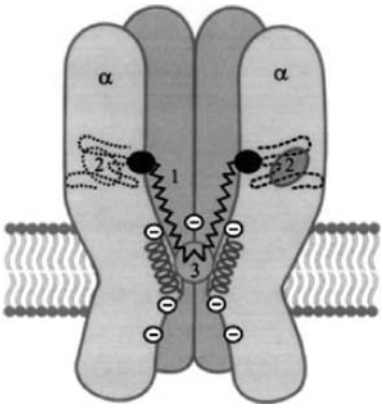

Fig. 7 Binding mode of $^{125}I_2$-**7** in the Torpedo nAChR. *1* $^{125}I_2$-**7**, *2* agonist-binding site, *3* high affinity non-competitive binding site (reprinted with permission from Rosini et al. 2002)

AChE inhibition remains the most important strategy for designing new potential anti-AD agents. This is because, in addition to its role in degradation of acetylcholine (ACh), AChE can accelerate Aβ aggregation through direct interaction with its peripheral anionic site (PAS) (Inestrosa et al. 1996). Therefore, molecules able to interact with both catalytic site and PAS, called "dual binding inhibitors", may lead to: (i) increase of the concentration of ACh in the synaptic cleft, (ii) inhibition of the Aβ aggregation induced by AChE (Bartolini et al. 2003; Inestrosa et al. 1996).

The currently marketed drugs for AD treatment are based on the so-called "one-molecule-one-target" paradigm. However, due to AD's multifactorial nature, drugs that hit more than one biological target may offer a better pharmacological approach to AD (the "one-molecule-multiple-targets" paradigm). Such molecules are known as multi-target-directed ligands (MTDLs) (Cavalli et al. 2008).

In addition to its inhibitory activity on adrenergic and muscarinic receptors, benextramine was observed to behave as an AChE reversible inhibitor ($pIC_{50} = 5.14$) and was able to antagonize presynaptic muscarinic M_2 autoreceptors (Fisher 2008). We thus began a research program aimed at developing polyamines endowed with multiple pharmacological activities for AD treatment. Several SAR studies performed on benextramine revealed that the 2-methoxybenzyl groups are important for AChE affinity (Melchiorre et al. 1998) while the disulfide bridge did not seem essential for the activity. Interestingly, AChE inhibition was increased by the transformation of the inner amine functions of methoctramine into amide groups as well as by the methylation of the nitrogen atoms. This transformation did not affect affinity for the presynaptic muscarinic receptors. The new lead compound obtained through these studies was called caproctamine. It was 42-fold more potent than benextramine in AChE inhibition. It displayed a well-balanced biological profile towards both AChE ($pIC_{50} = 6.77$) and muscarinic M_2 receptors ($pA_2 = 6.39$) (Melchiorre et al. 1998).

Caproctamine was further modified in order to verify the role of the substituents on the two aromatic rings and on the nitrogen atoms. Several caproctamine derivatives were synthesized and it emerged that the 2-methoxybenzyl groups were able to increase the basicity of the two benzylic nitrogen atoms, facilitating their protonation at physiological pH and their interaction with AChE sites (Tumiatti et al. 2003).

As a step forward in developing a potential anti-AD drug, we investigated the role of the octamethylene spacer separating the two amide functions. Due to its flexible inner polymethylene chain, caproctamine can assume several different conformations in solution. Therefore, we incorporated its inner chain into more constrained moieties such as dipiperidine and dianiline cycles (Fig. 8). The most potent AChE inhibitor of this series was the dipiperidino derivative **8** ($pIC_{50} = 8.48$). It had high AChE selectivity (AChE/BChE = 2570) and maintained good muscarinic M_2 receptor antagonism ($pK_b = 6.18$). Furthermore, **8** inhibited self-promoted and AChE-induced Aβ aggregation (Tumiatti et al. 2004).

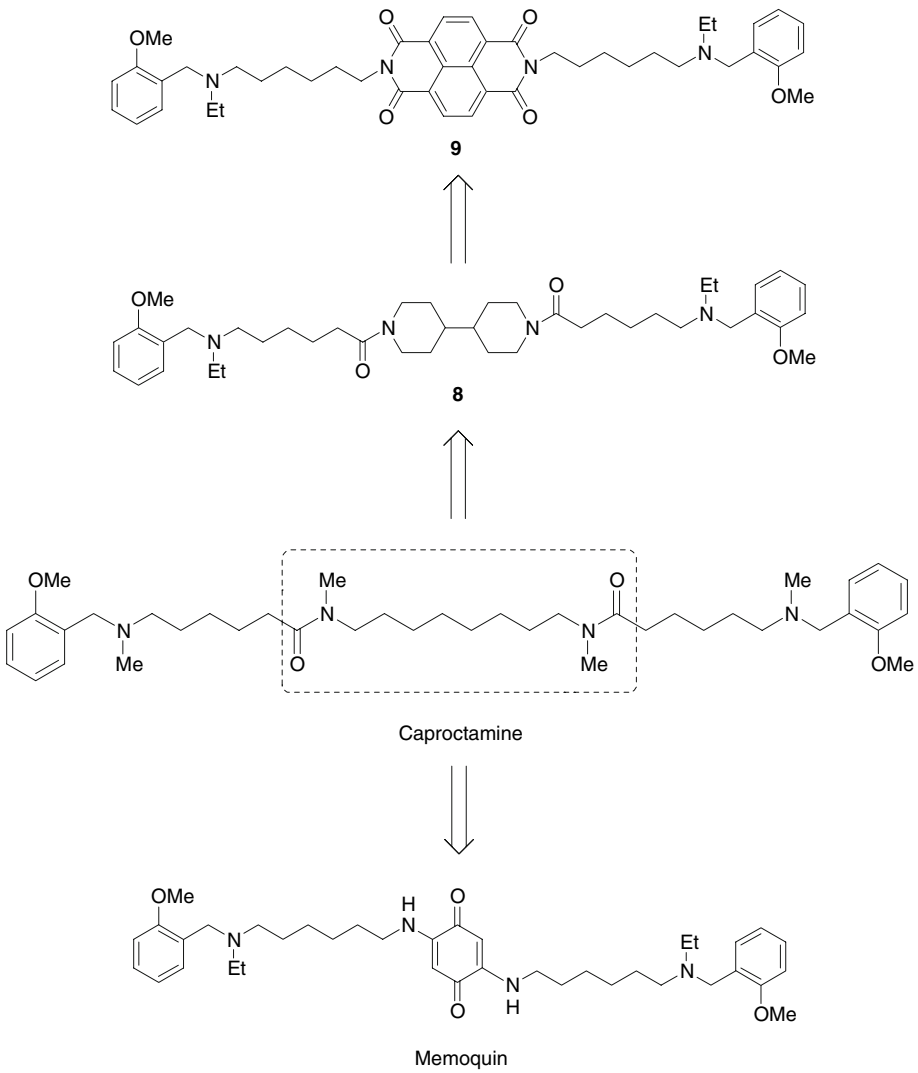

Fig. 8 Design strategy leading to MTDLs **8**, **9**, and memoquin as tools to investigate AD

Our study concluded that incorporating the inner carbon chain of caproctamine in a rigid skeleton improved and enlarged the biological profile of the prototype. This prompted us to replace the dipiperidino moiety of **8** with less flexible cyclic systems, leading to **9**, which is structurally characterized by a naphthalenetetracarboxylic diimide moiety (Fig. 8). **9** inhibited AChE in the subnanomolar range ($IC_{50} = 0.37$ nM) with an AChE/BChE selectivity ratio greater than 5,000, and with AChE-induced and self-promoted Aβ aggregation in the micromolar range (Tumiatti et al. 2008).

Following the same strategy, and since the biophoric space around the octamethylene spacer separating the two amide functions of caproctamine was quite "tolerant", we inserted a benzoquinone moiety in place of the octamethylene chain of caproctamine. This allowed us to obtain a new dual binding inhibitor endowed also with antioxidant properties. The resulting compound, called memoquin, showed high AChE inhibitory activity ($IC_{50} = 1.15$ nM) and was also able to block in vitro both self-induced and AChE-mediated Aβ aggregation, together with oxidative processes. Moreover, it caused an effective recovery from the cholinergic deficit, tau hyperphosphorylation, Aβ deposition, and behavioral abnormalities in AD11 anti-NGF mice, a comprehensive transgenic model of AD (Bolognesi et al. 2007).

Thanks to their exciting biological profiles, **9** and memoquin emerged as MTDLs able to hit several targets of the AD pathogenesis cascade.

Conclusions

The universal template approach has been our research group's driving force in developing synthetic polyamines able to hit different biological targets. We have demonstrated that the affinity and selectivity of a polyamine backbone can be modulated by both a suitable chain length

between the nitrogen atoms and by appropriate pharmacophores on these nitrogens. Herein, we have detailed only selected examples of the design strategy aimed at discovering potent and selective polyamines able to bind neurotransmitter receptors and enzyme proteins. We have also noted that the insertion of appropriate moieties on the terminal nitrogen atoms of a tetraamine scaffold leads to non-competitive antagonists of transient receptor potential vanilloid-type 1 responses to capsaicin (Mellor et al. 2004). It was shown that the interaction takes place with the acidic residues positioned at the pore entrance and at the end of the selectivity filter.

The most exciting result emerging from this paper is the discovery of new polyamine structure based MTDLs able to hit several targets of the AD pathogenesis cascade. Taken together, these data demonstrate that appropriate manipulations of polyamine ligands open encouraging perspectives in the discovery of new MTDLs for the treatment of other multifactorial diseases. It could also supply new tools to elucidate the complexity of their cellular biological pathways.

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