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New advances in pharmacological approaches to the cholinergic system: an overview on muscarinic receptor ligands and cholinesterase inhibitors

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Abstract

The cholinergic system is expressed in neuronal and in non-neuronal tissues. Acetylcholine (ACh), synthesized in and out of the nervous system can locally contribute to modulation of various cell functions (e.g. survival, proliferation). Considering that the cholinergic system and its functions are impaired in a number of disorders, the identification of new pharmacological approaches to regulate cholinergic system components appears of great relevance. The present review focuses on recent pharmacological drugs able to modulate the activity of cholinergic receptors and thereby, cholinergic function, with an emphasis on the muscarinic receptor subtype, and additionally covers the cholinesterases, the main enzymes involved in ACh hydrolysis.

The presence and function of muscarinic receptor subtypes both in neuronal and non-neuronal cells has been demonstrated using extensive pharmacological data emerging from studies on transgenic mice. The possible involvement of ACh in different pathologies has been proposed in recent years and is becoming an important area of study. Although the lack of selective muscarinic receptor ligands has for a long time limited the definition of therapeutic treatment based on muscarinic receptors as targets, some muscarinic ligands such as cevimeline (patents US4855290; US5571918) or xanomeline (patent, US5980933) have been developed and used in pre-clinical or in clinical studies for the treatment of nervous system diseases (Alzheimer' and Sjogren's diseases). The present review focuses on the potential implications of muscarinic receptors in different pathologies, including tumors. Moreover, the future use of muscarinic ligands in therapeutic protocols in cancer therapy will be discussed, considering that some muscarinic antagonists currently used in the treatment of genitourinary disease (e.g. darifenacin, patent, US5096890; US6106864) have also been demonstrated to arrest tumor progression in nude mice.

The involvement of muscarinic receptors in nociception also is over-viewed. In fact, muscarinic agonists such as vedaclidine, CMI-936 and CMI-1145 have been demonstrated to have analgesic

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effects in animal models comparable or more pronounced to those produced by morphine or opiates.

Likewise, the crucial role of cholinesterases (acetylcholinesterase and butyrylcholinesterase) in neural transmission is discussed, as large number of drugs inhibiting cholinesterase activity have become of increasing relevance particularly for the treatment of neurodegenerative disorders. Herein we summarize the current knowledge of the cholinesterase inhibitors with particular attention to recent patents for Alzheimer's disease drugs.

Keywords

muscarinic receptors; acetylcholinesterase; butyrylcholinesterase; neurological diseases; tumors; nociception

Introduction

Acetylcholine as a neurotransmitter and signaling molecule

Acetylcholine (ACh) is one of the best characterized neurotransmitters. Its central roles in cholinergic areas and synapses both within the central (CNS) and peripheral nervous system (PNS) are well known.

ACh was the first molecule identified as neurotransmitter; it also seems to be phylogenetically one of the older signalling molecules. In fact ACh has been detected in bacteria, protozoa, fungi, algae and primitive plants, indicating that the cholinergic system was widely distributed in living organisms before its appearance in the nervous system [1].

During embryogenesis (e.g. sea urchin, chick) ACh has been described to act as a morphogen that controls cell migration [2, 3]. In adult mammals, its presence in different tissues such as endothelium, keratinocytes [4], lymphocytes [5] and lung bronchial epithelia [6] has been described, as well as its ability to regulate basic cell functions including growth, survival, differentiation and apoptosis. In several cases, the ACh present in several tissues is not necessarily derived from cholinergic innervation; in fact ACh, locally synthesised by various cell types (e.g. lymphocytes, lung epithelium) and binding to muscarinic or nicotinic receptors, may often activate an autocrine loop [7, 8]. In early embryo stage development, ACh modulates neurogenesis by controlling neural stem and progenitor cell proliferation, as well as neuron and glial cell survival and differentiation, well prior to it acquiring its more classic neurotransmitter function [9–14].

The involvement of ACh in the modulation of inflammatory states has also been reported. Recent data have demonstrated that ACh, derived from parasympathetic innervations inhibits the release of tumor necrosis factor (TNF- α) and interleukin 1 (IL-1) from macrophages via the activation of nicotinic receptors, which supports the existence of a "cholinergic anti-inflammatory pathway" [15]. Moreover, lymphocytes are capable of both synthesizing ACh and responding to cholinergic stimulation, which suggests that the inflammatory state may be modulated by ACh released both from parasympathetic and immune systems [16, 17]. In addition, the microglial cells in the CNS, are also able to respond to ACh stimulation,

inhibiting the release of TNF- α . These data also suggest the existence of a “brain cholinergic anti-inflammatory pathway”, mediated by $\alpha 7$ nicotinic receptors, that may become of interest in the treatment of several neuropathologies in which local inflammation plays a crucial role [18].

The components of the cholinergic system including the enzymes and proteins involved in ACh synthesis, release and neurotransmitter hydrolysis have been largely studied and the gene coding for multiple nicotinic receptor subunits and muscarinic receptor subtypes have been thoroughly identified. Their binding sites and effector domains have been described in detail; the signal transduction pathways downstream of receptor activation have been characterized and they include ion channels, G-proteins and several enzymes.

On the basis of these considerations, it appears evident that the broader role of ACh should be reconsidered. The present knowledge of the physiological roles of ACh and of its possible involvement in pathological states opens new perspectives for the development of new therapeutic strategies in a number of common disorders.

Muscarinic receptor subtypes: structure and function

ACh binds two types of receptors: the ionotropic family of nicotinic receptors and the metabotropic family of muscarinic receptors.

In the central nervous system, nicotinic and muscarinic receptor subtypes are present both on neurons [19] and glial cells [14, 20, 21], where they mediate various physiological effects (e.g. motor control, temperature regulation and memory, synapse function and plasticity) either as auto- or heteroreceptors. Within the peripheral nervous system, muscarinic receptors appear largely involved in several functions, such as smooth muscle contraction, glandular secretion and the regulation of cardiac rate [22].

Muscarinic receptors belong to the family of transmembrane receptors coupled to G-proteins. Five muscarinic receptor subtypes have been cloned and characterized in different mammalian and non-mammalian species; they show a high degree of homology that is also maintained across different species [23, 24]. Variability between subtypes is only limited to the carboxy- and amino terminals of the proteins and to the third cytoplasmic loop [25].

M1, M3 and M5 receptors, via the activation of G-proteins, stimulate IP3 hydrolysis and intracellular calcium mobilization (Fig. 1) [26, 27]. They also modulate phospholipase A2 and phospholipase D in different cell lines and primary cultures [28, 29]. M2 and M4 receptors inhibit adenylate cyclase activity thereby decreasing cAMP intracellular levels (Fig. 1). They are also able to modulate the activity of K⁺ and Ca⁺⁺ channels [27]. Muscarinic receptor subtypes can additionally activate small G proteins, such as Rho GTPase and recruit new effectors including IP3K and MAPK/ERK kinases [9, 30]. These latter signalling pathways appear to play important roles in the control of cell growth and proliferation, as indicated by studies on glial cell primary cultures [13, 14, 31].

Pharmacology

The identification of selective ligands for muscarinic receptors has encountered relative difficulties due to the high degree of homology between subtypes and the frequent co-expression of more than one receptor in tissues. In turn, the lack of selective agonists and antagonists has for a long time hampered the design of therapeutic treatments with muscarinic receptors as targets.

Recently the development of muscarinic receptor knockout mice or the induced expression of a single receptor, using transfection of specific constructs in cell lines, has led to a better understanding of the functions of each receptor subtype and has allowed the generation and testing of new ligands, capable of discriminating and selectively activating the receptor subtypes [32].

The characterization of antagonists for the five muscarinic receptors has been made practical by radioligand binding techniques, using [³H]N-methylscopolamine as a ligand, on membrane preparations from cells transfected with constructs for a single muscarinic receptor subtype. The affinity constants (K_d) values obtained, have allowed the characterization of the selectivity or non-selectivity of a wide number of ligands [26].

An important property of muscarinic receptors is the presence of specific binding sites which, if occupied by ligands, modify binding to the ACh recognition site. The identification of these allosteric compounds has produced other relevant information on muscarinic receptor heterogeneity [33, 34]. Several allosteric ligands for muscarinic receptors have been described, most of which are able to exhibit negative cooperativity with non-selective agonists and antagonists [22]. For example, gallamine and methoctramine are allosteric mediators that show negative cooperativity with the antagonist *N*-methylscopolamine (NMS), and act as selective allosteric antagonists of the M2 receptor subtype [35].

Recently it has been proposed that polyamines may represent a universal template in the design of new selective muscarinic ligands. Indeed, it has been confirmed that appropriate substituents on the terminal nitrogens of a tetramine template can tune both affinity and selectivity for muscarinic receptors. An example is C-tripitramine that is able to significantly discriminate M1 and M2 receptors *versus* the other subtypes [36].

Among the antagonists, there are toxins isolated from the snake venom of the green (*Dendroaspis angusticeps*) and black mamba (*Dendroaspis polylepis*). These toxins (MT1, MT2, MT7) show a high selectivity for specific muscarinic subtypes (e.g. MT7 for the M1 subtype) [37, 38].

The chemical structures of major muscarinic antagonists and their selectivity are reported in Table 1.

The identification of muscarinic agonists has proven to be even more complex. Extensive studies in systems expressing both native or transfected receptor subtypes, indicated that the efficacy of an agonist is not only dependent on the receptor subtype selective binding affinity, but can also be modified by the specific cellular or tissue context (e.g. the reserve of

the specific receptor subtype, resistance of the receptor subtype to extended exposure to the agonist) [22].

Selective binding of a single receptor subtype followed by its functional activation has been applied to the design of muscarinic agonists with potential for use in the treatment of specific pathologies. An example is the agonist Vedaclidine (LY297802) (see Table 4) which shows a functional selectivity for the M4 subtype and good antinociceptive properties [39].

Generally, when we consider molecules with agonist function, we think to ligands able to interact with the orthosteric site of the receptor. When considering the high homology of the ACh binding site between all muscarinic receptor subtypes, more recently the possibility has emerged to modulate the seven transmembrane spanning receptors (e.g. muscarinic receptors) using allosteric ligands. Allosterism is a complex biochemical mechanism based on the ability of some molecules to bind at a topographically allosteric site, to induce modification in term of inhibition or activation the binding sites of an orthosteric ligand. New advances in molecular pharmacology have supported the development of new allosteric modulators for different enzymes, ion channels and G-protein- coupled receptors (GPCRS). Allosteric modulators can act with three different modalities: as positive allosteric modulators (PAMs) that potentiate the receptor response, as negative allosteric modulators (NAMs), which decrease activity of the receptors and finally as silent allosteric modulators (SAMs) that bind at allosteric sites, and can block the PAMs or NAMs binding and interfering with orthosteric ligands [40]. Allosteric agonists offer several advantages; the first may be dependent on high variability of allosteric binding sites that may allow the achievement of higher binding selectivity. Moreover, allosteric ligands are saturable, and hence when the allosteric binding sites are all occupied, no further additional effects are observable [40]. Allosteric agonists for muscarinic receptors have been recently generated; many of these have been tested for the treatment of neurological/psychiatric disorders, such as 1H-pyrrolo[3,2-C]quinolin-4-(5H)-one analogues (WO2012154731A1) that have been characterized as allosteric modulators of M4 muscarinic receptor subtypes [41].

Therapeutic applications of muscarinic receptor ligands

Extensive preclinical pharmacological data together with clinical studies are now available to evaluate the therapeutic potential of select muscarinic receptor antagonists in defined pathologies, such as for genitourinary and respiratory disorders. The M3 antagonists solifenacin (US6017927; US7230030B2), and darifenacin (US5096890; US6106864) have been approved and adopted for the treatment of urinary bladder smooth muscle disorders (see Table 2) [42–44]. Similar results have been reported for the non-selective muscarinic antagonist tolterodine (US5382600). Studies *in vivo* have demonstrated that in both mice as well as humans, following oral administration, tolterodine is extensively metabolized in the liver and generates an active metabolite, 5-hydroxymethyl metabolite (5-HMT) [45]. Its ability to bind muscarinic receptors in a more selective way in bladder rather than in submaxillary gland has provided it a relevant advantage for the treatment of patients with an overactive bladder [45].

More recently, it has been approved as new antimuscarinic drug in the treatment of overactive bladder and in urinary incontinence. Fesoterodine fumarate (Toviaz, Patent

number 7,384,980, by Schwarz Pharma Productions, distributed by Pfizer, Inc.) (Fig. 2) is a compound that like tolterodine, is hydrolyzed by non-specific esterases to 5-hydroxymethyl tolterodine (5-HMT), that represents its active metabolite [46, 47].

Although well tolerated, high doses of fesoterodine can induce side effects such as dry mouth, dyspepsia, dry eyes and urinary retention.

All cell types present within the respiratory tract express muscarinic and nicotinic receptors. For this reason, the use of muscarinic receptor antagonists has been proposed for the treatment of chronic obstructive pulmonary disease and in bronchodilator therapies [48–50]. The primary muscarinic receptor antagonists used as bronchodilators are ipratropium and oxitropium, (antagonists for M1/M3 receptors) and, more recently, tiotropium (non-selective) (Table 2). In particular, tiotropium, has provided major advantages considering that its single daily administration shows higher efficacy and less side effects than the use of iprotropium [51]. The advantage of these products, when compared to atropine or other muscarinic antagonists, result from their pharmacokinetics; in particular, the presence of a quaternary ammonium in their chemical formula allows for a local effect reducing their absorption by bronchi [51]. More recently several new muscarinic antagonists have been determined able to counteract both genitourinary and respiratory tract disorders (e.g. asthma, pulmonary fibrosis, allergic rhinitis) (see Table 3).

Of equal note, several pharmacologically interesting muscarinic agonists have likewise been developed. The muscarinic agonist Cevimeline ((+/-)-cis-2-methylspiro[1,3-oxathiolane-5,3'-quinuclidine] monohydrochloride hemihydrate) (US4855290; US5571918) has been approved and appears highly effective for the treatment of the autoimmune disorder, Sjogren's disease [52], a syndrome primarily affecting salivary and lachrymal glands, that results in a dry mouth and dry eyes (Table 4). The relevance and involvement of muscarinic receptors in this pathology is supported by evidence that Sjogren's patients produce autoantibodies to M3 receptors, and that the levels of these autoantibodies may represent a clinical marker for the identification of the syndrome [52]. Also notable is that polypeptides capable of enhancing odorant muscarinic receptor activation for the treatment of olfactory dysfunctions such as anosmia, hyposmia, dysomia and olfactory agnosia have been also generated (US 20130004983A1) [53].

The reported broad clinical application of muscarinic receptor ligands suggest that they could be extended to treatment of other pathologies [54]; indeed, new therapeutic treatments may be developed where the cholinergic system and, in particular, muscarinic receptors appear to play a relevant role as epitomized in Alzheimer's disease (AD) where muscarinic agonists have long been assessed; albeit, thus far without clinically translatable efficacy.

Muscarinic receptor drugs in the treatment of neurological/psychiatric disorders

The well known roles of muscarinic receptors within the nervous system and their possible relevance in central diseases has allowed the identification of novel therapeutic candidates based on the use of muscarinic agonists and antagonists in the treatment of an array of neurological diseases [42]. As mentioned above, the low selectivity and efficacy of muscarinic agonists have been a serious problem for their use in clinical studies. However

ligands such as the M1 agonists alvaneline and xanomeline (US5980933)(Table 4) have been evaluated in clinical trials for neurological pathologies such as AD [78, 79].

M1 receptors are largely expressed in the hippocampus and cortex, indicating relevant roles in learning and memory [19]. In addition to the involvement of the cholinergic system in cognitive function, M1 receptors have been demonstrated to control amyloid precursor protein (APP) processing and the generation of the neurotoxic APP fragment, amyloid β -peptide ($A\beta$). These findings support the hypothesis that M1 receptors may represent an interesting target for AD treatment [78]. Xanomeline, the first M1 agonist used in clinical trials (Table 3), significantly improved cognitive function in AD [80], albeit this was accompanied by significant gastrointestinal side-effects together with other cholinergically mediated actions (e.g. hyper-salivation). As described above, the discovery of allosteric modulators of muscarinic receptors is supporting the development of selective ligands for different receptor subtypes. Currently, allosteric modulators for M1 receptor subtypes (VU0357017 and VU0364572) have been produced, that more than likely will be assessed in AD [81].

It is known that Parkinson's disease is associated with the loss of dopaminergic neurons; this induce an imbalance between dopaminergic and cholinergic effects with an excess of cholinergic effects. Muscarinic receptor antagonists used as antiparkinsonians include trihexyphenidyl also called benhexol (US 6929801) [82] (Fig. 3A). This compound is frequently used for the prevention and the treatment of dyskinesia. An alternative for the treatment of Parkinson's disease is Benztropine mesilate (known as benztropine or as Cogentin) (US 2008/0226559) [83] (Fig. 3B). This is an anticholinergic drug also used for the treatment of dystonia, a rare disorder that causes abnormal muscle contraction, and results in twisting postures of the limbs, trunk, or face. Additionally, in patients with schizophrenia benztropine reduces the adverse side effects of antipsychotic treatments [84].

Muscarinic receptors and tumors

Cancer is caused by the accumulation of mutations of oncogenes and tumor suppressor genes, which eventually result in unlimited cell proliferation and altered cell adhesion, thus favouring metastasis formation. Some properties of tumor cells suggest that embryonic genes may be reactivated during malignant growth. For example, muscarinic receptors are expressed by neural crests during their migration from the neural tube [2]. Although their expression has not been found in mature melanocytes, they are present in human melanoma and melanoma cell lines indicating that the expression of muscarinic receptors, can be reactivated during melanocyte transformation [85].

Muscarinic receptors are expressed in several primary and metastatic tumors, such as on colon, ovary, prostate and lung carcinomas [85–89], in breast cancer, in astrocytomas and glioblastomas [90–99]. In some of these tumors (e.g. small cell lung carcinoma, SCLC) ACh is probably synthesised by the tumor cells considering the co-localization of the ACh biosynthetic enzyme, choline acetyltransferase (ChAT) and muscarinic receptors. This implies that the ACh ability to modulate tumor cell proliferation and/or migration might be dependent on an autocrine mechanism mediated by muscarinic and/or nicotinic receptors [97].

Although some of these tissues could express different ACh receptors, the M3 receptor subtype appears to be the primary one involved in the progression of several tumors (Table 5). Indeed its selective activation increases tumor cell proliferation through the modulation of several signal transduction pathways, including calcium mobilization, PI3K and MAPK/ERK activation [89–93].

In melanoma cell lines and in mammary adenocarcinoma, muscarinic receptors can also modulate chemotaxis and angiogenesis, suggesting in addition, their involvement in metastasis formation [85, 91, 92].

Muscarinic receptors appear, likely, to be involved in brain tumors. Patients with brain tumors often show altered levels of ACh in their cerebrospinal fluid; moreover the presence of muscarinic receptors in astrocytoma and in glioma cell lines has been widely described [90, 94–96, 99]. In astrocytoma cell lines ACh has been shown to stimulate proliferation and activate both ERK1/2 and PI3K pathways, mediated through M3 muscarinic receptor activation. Glioma cell lines have, likewise, been shown to respond to cholinergic stimulation decreasing their migratory ability [100]. Moreover, as assessed in *in vitro* studies, it has been demonstrated that the M2 muscarinic agonist arecaidine is capable to retard in glioblastoma cell lines (U87MG and U251MG), cell cycle progression with a significant decrease of cells in S phase. Moreover, M2 receptor activation reduces cell survival, inducing a dramatic apoptosis in both cell lines [94–96]. Together, these data suggest that M2 receptor activation in glioblastoma cell lines severely impairs both cell proliferation and survival and thus suggests that it may provide a new and promising tool for glioblastoma therapy. In light of these data, an agonist for M2 receptor may have a strategic relevance for the glioblastoma therapy. Although complete elucidation of its function (agonist or antagonist) remains to be undertaken, at least one compound has been determined able to bind M2 receptors, (WO04057344A1) [101], and hence may provide a lead for further research. However, considering the difficulty of finding ligands that combine high selectivity and reasonable pharmacokinetics for *in vivo* use as a drug treatment., together with the possible array of associated side effects, compounds able to inhibit (US8193161) [102] or active G α i-protein, the main effector downstream of M2 receptor activation, have been also considered and may represent an alternative strategy to effectively modulate the activity of M2 receptor.

Finally, in some tumors, such as ovarian carcinoma, the expression of muscarinic receptors negatively correlates with patient survival; this promotes a correlation between muscarinic receptor activity and tumor malignancy [86].

The role of the mAChRs in tumor progression is confirmed by the exposure of tumor cells to muscarinic antagonists, which impair cell proliferation both *in vitro* and *in vivo* [89].

As an example, darifenacin (Table 2) used in the treatment of overactive bladder for its high degree of M3 selectivity and for its *in vivo* stability [103], has been tested in *nude* mice to evaluate its ability to block tumor growth of SCLC xenografts. The data reported indicate that darifenacin inhibits tumor growth and decreases MAPK phosphorylation in a dose-dependent manner, suggesting that, the use of this drug could also be considered for the

treatment of other tumor types in which M3 receptors have been demonstrated to stimulate cell proliferation [104] (Table 4).

In vitro studies have demonstrated that the antibiotic migrastatin (Fig. 4) [105], is able to inhibit cell migration and anchorage-independent growth of SCLC and human esophageal cancer cells (EC17), by acting as a mAChR antagonist. Although currently remains unknown which muscarinic subtypes were inhibited by migrastatin, its ability to affect mAChRs activation has been clearly demonstrated in human neuroblastoma SK-N-SH cells, in rat bladder smooth muscle cells and in CHO cells expressing M1-M5 receptors [105].

In conclusion, several studies *in vitro* and *in vivo* have clearly demonstrated that muscarinic antagonists and some selective agonists, such as arecaidine, are able to counteract the growth and/or the invasiveness of several tumors. This suggests that muscarinic antagonists, already used in the treatment of other disorders, might be considered as new potential therapeutic tools for cancer therapy [104].

Muscarinic receptors and nociception

As previously reported, muscarinic receptors are widely distributed both in the central and peripheral nervous system [19, 106, 107]. It is known that centrally administered muscarinic agonists cause analgesic effects via spinal and supraspinal mechanisms [108]. The analgesia induced by cholinergic agonists is comparable to that obtained with morphine administration [109]. Therefore, the identification on one hand of receptor subtypes involved in this response and on the other of muscarinic ligands capable of selectively activating these receptors, is of considerable interest for possible therapeutic application.

Knockout mice for muscarinic receptor subtypes used in common analgesia tests (e.g. hot-plate test or tail-flick) have allowed the demonstration of the role of M2 and M4 receptor subtypes in mediating the analgesic effects of muscarinic agonists both at the spinal cord and brain level [110]. Although the pre-synaptic M3 receptor also appears to be involved in the nociception-induced by formalin [111], the M2 receptor appears predominant as the mediator of the analgesic response due to its high expression levels in the spinal cord.

M2 and M4 receptors are also able to suppress the transmission of noxious stimuli at the peripheral level. Data obtained on peripheral nociceptors, isolated from the skin saphenous nerve preparation, have demonstrated that selective activation of M2 receptors reduces the responsiveness of peripheral nociceptors to various noxious stimuli and inhibits CGRP release [112, 113]. These data have also been confirmed in animal models in which the orofacial formalin test has been performed [114].

The relevant role of M2/M4 receptors in reducing pain sensitivity, implies that selective ligands for these subtypes should receive more attention as potential targets in anti-nociception therapy.

Muscarinic agonists centrally administered, cause a decrease in body temperature whereas, when peripherally administered induce excessive saliva secretion. These effects are dependent on M1 and M2 receptor activation [49]. Moreover M2 agonist administration also causes cardiovascular side effects.

The variety of effects caused by M2 activation suggests that attention should be focused on M4 agonists. Vedaclidine (LY297802) is one of the first M4 agonists proposed for pain therapy. At low doses it displays a good anti-nociceptive action, without parasympathetic effects [74] (Table 5). In 1999, Ellis and collaborators synthesized two epibatidine analogs (CMI-936 and CMI-1145) that in mice showed a strong anti-nociceptive action mediated by muscarinic receptors; unlike that of epibatidine that interacted with peripheral and central nicotinic receptors. The intrathecal injection of muscarinic M4 toxin (MT-3) blocks the anti-nociception effects produced by CMI-936 and CMI-1145, indicating that these compounds specifically bind M4 receptors [115].

Altogether these data imply that the use of selective agonists for M4 receptors may reduce the undesirable muscarinic side effects deriving from the stimulation of other muscarinic subtypes, and represent an important alternative to the use of opiates in pain therapy.

However, a better knowledge of the downstream mechanisms associated with M2/M4 activation in the modulation of nociception could greatly favour the discovery of other potential and more effective targets for nociceptive therapy.

Moreover, the identification of first allosteric potentiators of M4 muscarinic receptors may represent a new therapeutic strategy also for the treatment of pain (US8071776; WO2012154731A1) [116, 41].

Cholinesterases

Acetylcholinesterase (AChE) is a single enzyme encoded by a single gene although several isoforms exist due to variable splicing of exons. AChE can have a variety of quaternary structures. The catalytic monomer can associate with other monomers to form complexes that are different in their hydrodynamic parameters and ionic or hydrophobic interactions. Molecular forms of the enzyme are of two types: Globular (G) and Asymmetric (A), and according to the number of catalytic monomers in them they are named Gi, G2, G4 and A4, Ag and Aji. The globular forms can have different amphiphilicity depending on their eventual attack to the plasma membrane or not (G2a type I form is attached to the membrane through a GPI anchor, and the G4a form is anchored through its 20 kDa hydrophobic P subunit). The other forms are fully soluble (G4na) or are weakly associated to the membrane, being easily solubilized without detergents (G4a type II and *Gidi* type II). The asymmetric forms are formed by three tetramers of catalytic subunits attached to the basal lamina through a collagen-like tail (ColQ). The catalytic monomer H in humans is unique to cells of the haematopoietic lineage. In the central nervous system, the G4a *Vfpe* II, G2a type II, G4 and G4na forms are usually found, while the asymmetric forms are found in the postsynaptic space of the neuromuscular junction. Most isoforms are found in the central and peripheral nervous systems [117]. Isoforms have also been characterized in hematopoietic cells, as well as in tumor cells [118, 119]. AChE expression is not restricted to cholinergic neurons. AChE has been shown to be expressed in hematopoietic cells, osteoblasts, vascular endothelial cells, leukocytes, and to be induced in various cell lines undergoing apoptosis.

Although the primary role of AChE is hydrolysis of ACh, alternative functions, that are independent of catalytic activity, for AChE, such as involvement in cell adhesion, proliferation, and neurite outgrowth in diverse types of neurons, including non-cholinergic neurons [120–124], the inhibition of proliferation of multipotent stem cells and regulation of apoptosis [125] have been also demonstrated. Other functions attributed to AChE include promotion of amyloid fiber assembly and amidase activities [126].

In 1932 the enzymatic activity that catalysed the hydrolysis of esters of choline, including butyrylcholine, succinylcholine and acetylcholine, was named butyrylcholinesterase (BuChE, EC 3.1.1.8), also known as pseudo-cholinesterase or nonspecific cholinesterase. BuChE is a serine hydrolase that catalyses the hydrolysis of esters of choline [127]. BuChE is expressed in distinct populations of neurons [128], is a co-regulator of cholinergic neurotransmission [129], and seems to be involved in some aspects of the development of the nervous system [130].

BuChE is structurally and functionally related to AChE, and like AChE, BuChE has a serine amino acid residue that is essential for its catalytic activity. BuChE and AChE belong to the α/β -fold family of proteins because they contain a central β -sheet that is surrounded by α -helices 3 [131]. Each catalytic subunit of human BuChE has 574 amino acid residues [132].

There are different molecular forms of BuChE, including monomers and oligomers that consist of identical catalytic subunits [133]. The various molecular forms of BuChE are identified according to their monomeric and oligomeric nature. The symmetric monomeric form is called the globular form (G1). The dimeric form, designated as the G2 form, consists of two monomers that are joined by a disulfide bridge formed between the cysteine 571 residues of each monomer 47. Two G2 forms can be held together by hydrophobic interactions to form a tetramer, the G4 globular form. These three molecular forms (G1, G2 and G4) are all symmetrical, hydrophilic, and are found primarily in the soluble form.

Cholinesterase Inhibitors

An alternate mechanism to directly stimulating muscarinic and/or nicotinic receptor post-synaptic elements can be achieved through the use of cholinesterase inhibitors. Such drugs primarily act by inhibiting the enzyme AChE, which is chiefly responsible for metabolizing and ending the pharmacologic action of the neurotransmitter ACh in human and mammalian brain. This has the effect of augmenting the cholinergic function of the brain, and has provided the principle treatment approach in the current symptomatic therapy for AD. In this regard, the cholinesterase inhibitors (donepezil, galantamine and rivastigmine) (Table 6a) are administered either orally (once or twice daily), or via transdermal patch, as available [134–137]. A key difference between administration of cholinesterase inhibitors *versus* direct agonists of either the muscarinic or nicotinic system is that whereas receptor subtype selective actions cannot be achieved via cholinesterase inhibition, the temporal pattern of cholinergic brain activation is both maintained and augmented.

Acetyl- and Butyrylcholinesterase

Two classes of cholinesterase enzyme coexist in the organism and possess an array of both cholinergic and non-cholinergic functions, depending on their time of expression, location

and specific subtype form [138, 139]. Within the brain, however, AChE predominates and in humans accounts for some 90% of cholinesterase activity, with the sister enzyme butyrylcholinesterase (BuChE, EC 3.1.1.8) accounting for the residue [140, 141]. With regard to cholinergic function, in general AChE is principally allied to neurons, whereas BuChE is largely localized in and secreted from glial cells [141–143]. Studies by Darvesh and colleagues [141, 143–145], however, demonstrate that particular neurons possess BuChE rather than AChE, and their role in cholinergic function is supported by their colocalization and/or very close proximity to choline acetyltransferase (ChAT), the rate-limiting enzyme that catalyzes the synthesis of ACh [146]. Indeed, BuChE associated neurons appear throughout the brain and spinal cord [143]. For example, some 10% – 15% of cholinergic neurons within human hippocampus and amygdala appear to express BuChE rather than AChE [143–145]. AChE and BuChE share approximately 65% amino-acid sequence homology albeit encoded by different genes on human chromosomes 7 (7q22) and 3 (3q26), respectively [138–139]. Nevertheless, both highly efficiently bind and cleave ACh, although with slightly different kinetics, to render the identical metabolic products choline and acetic acid [138, 139, 141, 147–149]. Despite this, the relative involvement of BuChE in the regulation of ACh levels has, until recently, been largely underestimated. AChE, originally, and more recently BuChE have been fully sequenced and, in particular for BuChE, are heavily glycosylated. Specifically, human AChE bears three carbohydrate asparagine-linked residues and BuChE has nine, which can impact their aqueous solubility, stability, functional conformation and hence their biological activity [138, 139, 141, 150, 151]. Furthermore, both enzymes can form disulfide bridges with defined cysteines along their sequence to support their dimerization to yield multiple structural enzyme subunit forms at different body sites within the brain, for example, the three globular forms (G forms) comprising one (G1), two (G2) or four (G4) catalytic subunits predominantly exist. Albeit different functions have been assigned to different regions of both AChE and BuChE, and the majority have yet to be elucidated, the sites associated with ACh hydrolysis are internalized in both enzymes. Explicitly, AChE and BuChE both possess a primarily hydrophobic active narrow gorge, characterized by X-ray crystallography to be in the order of 20Å deep for AChE [151, 152], into which ACh diffuses and then becomes cleaved. On entering this active gorge, ACh orientates to optimally bind within two defined locations: (i) within a catalytic region at the base of the gorge (sometimes called the acylation site) and (ii) within a choline-binding site midway along it, which is often termed the cationic- π site.

The Basis of Acetyl- and Butyrylcholinesterase Function and Inhibition

The acylation/catalytic site affords the fundamental chemistry that largely underpins the actions of both AChE and BuChE and comprises a catalytic triad of Ser₂₀₃, His₄₄₇ and Glu₃₃₄ for human AChE [138, 139, 141, 150], and Ser₁₉₈, His₄₃₈ and Glu₃₂₅ for human BuChE [147, 150]. The substrates ACh as well as inhibitors such as physostigmine, can bind covalently to the active site subsequent to the attack at the carbonyl group of the substrate by the Ser hydroxyl anion that leads to a tetrahedral alkoxo anion enzyme-substrate intermediate. The His and Glu act as proton acceptors/donors during the hydrolysis process and, thereby, provide a charge relay system [153]. Such acid/base interactions between these three catalytic triad amino acids promote nucleophilic attack at the carbonyl carbon of the acyl-group by the resulting alkoxide group of the Ser. This generates a transient tetrahedral

enzyme-substrate intermediate that is an oxyanion. This tetrahedral intermediate then collapses, the choline moiety leaves and the serine becomes acylated. Thereafter, the attack of a water molecule at the Ser acyl group again generates a stabilized tetrahedral intermediate that then collapses to restore a free catalytic site. The midpoint choline/cationic- π site within the gorge of both AChE and BuChE contains amino acids (Trp₈₆ and Tyr₃₃₇ in AChE, Trp₈₂ and Ala₃₂₈ in BuChE) that interact with quaternary amino group within the choline residue of ACh to optimally orientate the molecule within the lower acylation/catalytic site [138, 139, 141, 150–153]. The described catalytic mechanism of AChE and BuChE hydrolysis efficiently supports the cleavage of some 1.6×10^4 ACh molecules per second [141, 150–155]; an amount that, under normal conditions, is limited solely by the rate of ACh diffusion into the enzyme gorge. Blockade of the gorge and, thereby, substrate approach to the catalytic triad within the acylation/catalytic site will clearly reduce the ability of AChE and/or BuChE to metabolize ACh and prolong its pharmacologic action. Cholinesterase inhibitors have been generated, either as natural products (e.g., (–)-physostigmine and galantamine) or synthetic ones (e.g., donepezil and rivastigmine) on a number of diverse chemical scaffolds (Table 6a). Consequent to their three-dimensional structures and placement of specific reactive chemical moieties within them, they have the ability to both fit within and bind to amino acids that both line and form the structure of the internalized gorges of AChE and BuChE [141, 151, 153]. A high degree of amino acid homology (up to 52%) exists in the gorges within AChE and BuChE, which underpins their similar metabolism of ACh; however, key structural difference have allowed the development of selective inhibitors for each and, additionally, underpin the broader substrate acceptance (promiscuity) of BuChE. As an example, the inhibitors (–)-physostigmine and rivastigmine (cLog P value 2.1), which both co-inhibit AChE and BuChE and thus are unselective cholinesterase inhibitors, possess carbamate groups and consequently primarily act by binding (carbamylating) the catalytic Ser within the acylation/catalytic site. Rather than forming a highly transient acyl-enzyme complex, as per ACh, both inhibitors generate far more stable (but reversible) carbamylated-enzyme complexes that time-dependently impair further catalytic activity. As with ACh, defined interaction with both inhibitors occur within the choline/cationic- π region to optimally orientate the inhibitors to support approach and interaction with the catalytic Ser. By contrast, the natural product galantamine (Clog P value 1.02) is considered to chiefly bind within the choline/cationic- π site and with preference for AChE (Table 6a) [156]. The benzylpiperidine-based agent donepezil (cLog P value 4.6) binds within a different region still [157], the peripheral anionic site (PAS) that resides at the entrance of the gorge, and the compound extends more deeply into the mid cationic- π site. The PAS is particularly important within AChE, as it provides a regulatory site via which chiefly high ACh levels but other ligands too, can induce noncompetitive inhibition. Interaction with key amino acids (human AChE: Trp₂₈₆ Tyr₇₂, Tyr₁₂₄, Tyr₃₄₁ and Asp₇₄) [152–158] within this binding domain can induce conformational changes deeper within the gorge that impact the acylation site via a relay whose mechanism remains to be fully characterized. In large part, the three-dimensional structures of both BuChE and AChE are comparable to two hemispheres that sandwich the different binding domains involved in the catalysis of ACh and other substrates between two loops (the acyl- and omega-loops) that form the sidewalls of the active site gorges of each enzyme [138, 139, 141, 147, 150–153]. The gorge for BuChE, however, is sizably bigger

than that of relatively narrow AChE volume: BuChE 500 Å³ *versus* 300 Å³ for AChE [141, 147, 151, 153]. In this regard, of the amino acid residues that demarcate the active site gorge of AChE, fourteen are aromatic and of these, six key ones are replaced by smaller aliphatic or polar residues within BuChE that together, with other changes, provides BuChE a larger and less restrictive gorge with regard to the differential size and three-dimensional shape of the substrates and inhibitors that these enzymes have the capacity to bind [141, 147, 151–154]. Within the PAS, in particular, key aromatic residues critical to donepezil interaction in AChE are absent in BuChE, and hence donepezil has a high selectivity for AChE (Table 5) [157]. Additionally and contrary to AChE, the presence of excess substrate induces the activation of BuChE, rather than its inhibition [141, 147, 153]. In synopsis, the similarities in the binding domains of AChE and BuChE, consequent to their high degree of amino acid homology, underpin their serine hydroxylase activities and largely alike metabolism of ACh. However, specific amino acid changes define the differential three-dimensional structures of their internalized gorges and, consequently, their selectivity or lack of selectivity for the currently clinically available anticholinesterases.

With increasing evidence that selective BuChE inhibition may have relevance as a palliative treatment for AD, and potentially have disease-modifying actions [141, 147–149, 153, 158], attention has developed on BuChE-selectivity to define its actions in humans. In this regard, specific BuChE inhibitors have been generated around a number of chemical scaffolds [153, 154, 159–162] (Table 6a, 6b). An ideal example is that of (–)-physostigmine that unselectively co-inhibits both AChE and BuChE with high and essentially similar potency. On this hexahydropyrroloindole carbamate backbone, successful generation of close structural analogues that selectively inhibit BuChE or AChE or yet other targets has proved possible. (–)-Physostigmine, similar to ACh, enters the gorge of both AChE and BuChE and interacts with the very same binding domains - the acylation and cationic- π sites – and reversibly carbamylates either enzyme consequent to attack of the carbonyl function of the methylcarbamate of (–)-physostigmine on the catalytic triad [159, 161]. Decarbamylation, resulting in restoration of enzymic activity, occurs within approximately an hour to thereby generate a pharmacodynamic inhibition half-life ($T_{1/2}$) of some 30 min [154, 163]. Increasing the complexity of the carbamate moiety to a phenyl group slows the decarbamylation rate allowing a lengthy reversible enzyme inhibition, as found for phenserine [154, 163, 164]. The increased lipophilicity associated with replacing the methylcarbamate of (–)-physostigmine (cLog P value 0.42) with the phenylcarbamate of (–)-phenserine (cLog P value 2.2) augments lipid solubility to support transport across cellular membranes, such as the gastrointestinal and blood-brain barrier [161, 163–167]. As important, moreover, this aryl group provides needed structural rigidity (that an alkyl group of equal carbons cannot generate). The unsubstituted phenylcarbamate present in (–)-phenserine (Table 6b) provides it a 70-fold selectivity for AChE *vs* BuChE consequent to hydrophobic π - π stacking electron interactions of its phenylcarbamate between flanking phenyl groups of two phenylalanines (human AChE: Phe₂₉₅ and Phe₂₉₇) that form the base of the AChE catalytic gorge [161]. These two aromatic amino acids are replaced by smaller aliphatic ones in human BuChE (Leu₂₈₆ and Val₂₈₈), thereby creating a deeper gorge within BuChE to accommodate longer inhibitors. In this regard, (–)-N¹, N⁸-bisnorcymserine (cLog P value 1.80) that bears a *para*-isopropyl substitution on the phenylcarbamate group provides

a high selectivity for BuChE (110-fold) with an unusually high potency. Initial studies on human BuChE provided an IC_{50} of 1 nM [159]; however, more in depth enzyme kinetic studies have revealed a value of 0.228 nM and a K_i of 0.131 nM [168]. Such levels are predicted as readily achievable in humans and clinical trials of the agent have been initiated.

Pharmacological effects of BuChE inhibition

When administered systemically to rodents, AChE as well as unselective cholinesterase inhibitors that enter the brain consequent to their high lipid solubility (cLog P values), as would be expected, elevate endogenous extracellular levels of brain ACh in a concentration-dependent manner [163, 169–175], with particularly high concentrations inducing an inverted U-shaped curve likely consequent to presynaptic autoreceptor stimulation [176]. The development of knockout (–/–) mice for AChE and BuChE, separately, has substantially added to our broader understanding of the function of these enzymes in health and disease [177–178]. Interestingly, selective BuChE inhibitors similarly augment brain endogenous ACh concentrations [169] and, as confirmed in AChE –/– mice, achieve this via action on BuChE [170]. In a recent study, Pepeu and colleagues [172] matched the time-dependent rise in cortical extracellular ACh levels (a 2- to 3-fold elevation) achieved by systemic administration of donepezil (AChE selective: 1 mg/kg, i.p.), rivastigmine (cholinesterase unselective: 0.6 mg/kg, i.p.) and (–)-N¹-phenethylcymserine (BuChE selective: 5 mg/kg, i.p.), as determined in rat by *in vivo* microdialysis coupled to HPLC. This similar ACh rise, however, was achieved by a donepezil-induced 27% inhibition of brain AChE without BuChE effect, a rivastigmine induced 40% AChE and 25% BuChE inhibition, and by a (–)-N¹-phenethylcymserine-induced 39% BuChE inhibition without impact of AChE [172]. This is notable in light of the knowledge that rat, similar to healthy human brain, primarily comprises 90% AChE and 10% BuChE. Thus, a 39% inhibition of BuChE – comprising 10% of brain cholinesterase activity, elevated brain ACh levels to the same amount as a 27% inhibition of AChE comprising 90% of brain cholinesterase activity [172], and underlies that both AChE and BuChE co-regulate brain cholinergic function [169–170, 177–179].

Selective AChE, BuChE as well as unselective cholinesterase inhibition augment long-term potentiation (LTP) (assessed in rodent brain slices) [169, 180, 181], a marker of synaptic strength and plasticity that underlies the molecular basis of memory formation [182]. Likewise, AChE, BuChE as well as unselective cholinesterase inhibition improve a number of measures of cognitive performance in a variety of animal models [183–186]. FDA-approved AD treatment with donepezil, rivastigmine, and galantamine is based on several large clinical studies in patients diagnosed with mild to moderate disease [134–137]. However consequent to patent expirations that it was for rivastigmine in 2007 (US Patent No. 5,602,176 (priority date 1987) [187], galantamine in 2008 (US Patent No. 4,663,318) [188], donepezil (US Patent No. 5,985,864) [189] and memantine in 2010 (US Patent No. 7,810,643 B2) [190], each can be administered as generics – unless the transdermal route is preferred (e.g., Novartis rivastigmine transdermal Patent No. 6,316,023, issued 2001) [191]. These cholinesterase inhibitors are generally agreed to provide symptomatic relief in AD, albeit that additional cholinergic/non-cholinergic mediated actions have been described in cellular and animal models that have potential to impact AD relevant targets [137, 185, 192]; their translational impact to the clinic remains unknown. As examples among many,

galantamine has been reported to provide nAChR-mediated protection of neuronal cell cultures from A β -induced neurotoxicity [193] and donepezil to lower t-phosphorylation by actions on GSK-3 β and provide neuroprotection [194]. However, a number of drawbacks are associated with these AChE and unselective cholinesterase inhibitors, including peripheral and centrally mediated adverse effects (i.e., nausea, vomiting, diarrhea, tremors and sleep disturbances) as well as their effectiveness in only a limited number of patients for a limited time duration.

A loss of cholinergic neuron presynaptic elements in AD progression is well characterized [195, 196] and matched by a decline in AChE levels within the hippocampus and cerebral cortex [140, 197]. Indeed, the primary drug target of AChE inhibitors – AChE, is routinely lost by some 45% in key brain regions during disease progression; potentially underpinning the limited window of efficacy of AChE inhibitors. In contrast, BuChE activity concurrently rises by up to 2-fold [140, 147, 148, 197], likely consequent to the elevated number of glial cells found in AD brain. A disparity, thereby, develops between reduced ACh synthesis/availability and one of its main hydrolysing enzymes increased BuChE further supporting the potential use of BuChE selective inhibitors in AD. In preclinical toxicological studies, BuChE-selective (–)-cymserine analogues and in particular (–)-N¹, N⁸-bisnorcymserine (Patent No. 6,683,105, issued 2004) lack the traditional dose-limiting cholinergically-mediated adverse profile associated with AChE and unselective cholinesterase inhibitors (e.g., centrally mediated tremor, lacrimation and salivation) [169]. A Phase 1 clinical trial involving dose escalation studies of (–)-N¹, N⁸-bisnorcymserine is ongoing < <http://www.clinicaltrials.gov/ct2/show/NCT01747213>>, and since this is the first BuChE-selective inhibitor to enter the clinic it should prove interesting to see whether preclinical studies of safety and efficacy translate to humans.

Multifunctional anti-cholinesterases

It has been recognized that neuropathological events that likely underpin the development and progression of AD that were originally considered separate, are complexly linked. For example, biochemical cascades that cause the increased generation of A β from amyloid precursor protein (APP) may induce tau phosphorylation or alter ACh synthesis or cholinergic receptor expression [198–200]. In an equal way, changes in cholinergic function feed back on APP processing and consequent A β generation. With the understanding that current clinically approved cholinesterase inhibitors have limited efficacy, despite their numerous promising pharmacological actions in cellular and animal models, investigators have turned to the development of multifunctional anticholinesterases with the hopes of targeting more than one mechanism involved in AD. There are a plethora of targets (e.g. neurotrophic support, loss of essential growth factors within brain, inflammation, tau, APP, apoptosis), and the concept is to combine within a single molecule the required properties to impact two or more different targets. Examples among many include the elegant work of Youdim and colleagues [201–202], Han and colleagues [203, 204]. In these studies, different moieties associated with anticholinesterase action the carbamate group [201, 202] and the backbone of tacrine [65, 66] have been combined with other pharmacologically interesting scaffolds to generate single compounds possessing multiple action. In this regards, ladostigil [(N-propargyl-(3R) aminoindan-5yl)-ethyl methyl carbamate] is a AChE/BuChE inhibitor

with brain selective inhibitory actions on monoamine oxidase (MAO)-A and -B, and the tacrine dimer bis(propyl)-cognitin and analogues combine anticholinesterase actions with NMDA, GABA_A receptor and nitric oxide synthase inhibition [201–204].

An interesting but contrasting example is where anticholinesterase action can be dialed out of a compound as in the clinical AD experimental drug Posiphen ((+)-phenserine), which is the chirally pure (+)-enantiomer of chirally pure (–)-phenserine (Table 6b). (–)-Phenserine and Posiphen have three-dimensionally different chemical structures, and demonstrate how the chirality of a single methyl group (in the 3a*S* or 3a*R* positions, respectively) with no capability of chiral switching between the two can abolish anticholinesterase action at both AChE and BuChE (Tables 6). However, Posiphen, similar to (–)-phenserine, dramatically lowers the rate of synthesis of APP and its generation of the AD toxin, Aβ [205–208]. This action can hence be clearly defined as non-cholinergically mediated and, indeed, has been characterized to occur via translational regulation of APP synthesis at the level of the 5′-untranslated region of APP mRNA [209]. This involves an iron response element that is additionally found in the Parkinson's disease protein α-synuclein, that both compounds can also downregulated [210]. Posiphen [208], due to its lack of cholinesterase inhibitory action, can be administered in doses dramatically greater than (–)-phenserine [164–167], both preclinically and clinically, and in addition to its tolerability has a different half-life and profile.

Conclusions and future development

Considering the heterogeneous expression of the cholinergic system components in different tissues both the neuronal and non-neuronal type, their dis-regulated expression and function appear more frequently associated to different disorder types. For this reason, chemical and molecular research combined with animal models, the use of a number of toxins, together with selective muscarinic agonists/antagonists and AChE/BuChE inhibitors have defined how the cholinergic system functions in health and disease, and how it may be advantageously regulated for use in medical therapy.

With regard to the five muscarinic receptor subtypes, the lack of selective ligands capable of binding to specific subtypes has, for a long time, limited the development of therapies based on cholinergic receptor activation; since the use of the muscarinic agonists often has been encumbered by adverse effects, such as a decrease of body temperature, excessive saliva secretion and alterations in cardiac rate.

In the latter decade the use of knockout mice [215] and development of molecular biochemistry has allowed a more precise definition of the role of each muscarinic subtype and the generation of new selective muscarinic ligands with minimal side effects.

Recently new therapeutic applications of muscarinic agonists and antagonists are emerging. Muscarinic receptors have been found to be involved in several nervous system disorders such as AD (M1 receptor), Parkinson's diseases (M4 receptor) and schizophrenia (M5) and the use of muscarinic ligands in the treatment of these pathologies is now being reconsidered [42, 215]. In fact, some muscarinic ligands, such as cevimeline or xanomeline, have or being evaluated for neurological disorders (e.g. schizophrenia, AD).

A further important aspect is that some M3 muscarinic antagonists, such as solifenacin, darifenacin and the non selective antagonist tolterodine, have been approved and adopted for the clinical treatment of urinary bladder smooth muscle disorders or for chronic obstructive pulmonary disease.

Considering the ability of muscarinic receptor activation to induce tumour cell proliferation [44, 104], the use of muscarinic antagonists might also be considered for the design of new protocols for cancer treatment. As previously described, in different tumor types, the M3 receptor appears to be the primary subtype involved in tumor progression since its activation can enhance tumor cell proliferation, migration or angiogenesis. The concept to use M3 muscarinic antagonists to counteract tumor progression is supported by the evidence that, in *nude mice*, M3 antagonist darifenacin is able to inhibit growth of SCLC xenograft, decreasing MAPK phosphorylation. However, more recently the demonstration that the selective activation of M2 receptors proved able to counteract *in vitro* glioblastoma cell proliferation and survival [95, 96], suggest that the identification of selective agonists for these receptors may have relevance for brain tumor therapy, and is worthy of further investigation.

The ability of some muscarinic receptor subtypes (M2/M4) to produce analgesic effects also suggests a relevant role for ACh and muscarinic receptors in the modulation of *neurogenic inflammation* and encourages the search for new muscarinic agonists for pain therapy. Although still limited, new therapeutic perspectives for muscarinic ligands in the treatment of several diseases both in the nervous system and in other tissues are emerging. The search for novel drugs with higher selectivity and reduced side effects is currently active, as indicated by patents obtained for fesoterodine fumarate (US 5935958) [51] and other compounds targeted to muscarinic receptors (US007151115B2; US20070054911A1) [216, 217] (see also table 3). More recently, in spite of a high homology between muscarinic receptors, the identification of allosteric modulators of cholinergic receptors underpins a real possibility of soon obtaining new pharmacological drugs able to selectively modulate cholinergic receptor activity by binding to allosteric sites.

Several neurological disorders are associated with altered production of ACh. The use of anticholinesterases (differentially selective for AChE or BuChE) appears of great relevance considering that the inhibition of the enzymes involved in ACh-hydrolysis produces small but, nevertheless, clinically significant and consistent improvements in cognitive measures in subjects with AD. Furthermore, they are reported to mitigate AD related behavioral problems and declines in activities of daily living. Regrettably, not all AD patients respond and the described benefits are generally of limited duration [134–137]. Moreover their use was restricted in fuction of side effects reported in some patients. Certainly, anticholinesterases will continue to have a role in AD therapy, as standalone as well as in combination treatment. However, there remains much room for improvement in this drug class potentially gained by the development of BuChE inhibitors as well as agents with additional actions that impact disease progression. Moreover, new strategies implicating molecular modifications of drugs already used in AD therapy appear necessary to improve the activity of these molecules and reduce the side effects [189, 218].

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Fig. 1.
Main signal transduction pathways coupled to muscarinic receptor subtypes

Fig. 2.
Chemical structure of fesoterodine fumarate (Toviaz)

A **B**

Fig. 3.
Chemical structures of trihexyphenidyl (benzhexol) (A) and benztropine (B)

Fig. 4.
Chemical structure of migrastatin

Fig. 5.
Chemical structures of the epibatidine analogs with anti-nociceptive properties. A) (–)-epibatidine; B) CMI-936; C) CMI-1145.

Table 1

Muscarinic antagonists

Antagonists	Receptor subtypes	Chemical structure
Pirenzepine	M1>M4
Methoctramine	M2>M4
Gallamine	M2
4-DAMP	M3>M1>M2
Tropicamide	M4

Table 2
Muscarinic receptor antagonists approved for therapeutic protocols or used in clinical trials

Antagonists	Receptor selectivity	Chemical structures	Status	Patents and company	Therapeutic application
Solifenacin	M3		Phase III	US2006017927 US2004186061 <i>Schwarz Pharma AG</i> [55, 56]	Overactive bladder
Darifenacin	M3		Phase III	US5096890 US2006106864 <i>Pfizer Inc</i> [57, 58]	Overactive bladder
Ipratropium	M1/M3		Approved	US3505337 <i>Boehringer Ingelheim</i> [59]	Chronic obstructive pulmonary disease
Oxitropium	M1/M3		Approved	US3472861 US007271197B2 US2005256201 <i>Glaxo Group Limited</i> [60, 61, 62]	Chronic obstructive pulmonary disease

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Antagonists	Receptor selectivity	Chemical structures	Status	Patents and company	Therapeutic application
Tiotropium	Non selective		Approved	US5610163 <i>Boehringer Ingelheim</i> [63]	Chronic obstructive pulmonary disease
Tolterodine	Non selective		Approved	US5382600 <i>Pharmacia</i> [64]	Overactive bladder

Table 3

New patents for muscarinic receptor antagonist drugs

Chemical name	Patent	Biological activity	Company
bi-aryl8-azoniabicyclo[3.2.1] octane	US8183257 [65]	Muscarinic receptor antagonists	Glaxo Group Limited
bi-aryl8-azoniabicyclo[3.2.1] octane	EP 1740177B1 [66]	Muscarinic receptor antagonists	Glaxo Group Limited
bi-aryl8-azoniabicyclo[3.2.1] octane	US 20120157491A 1 [67]	Muscarinic receptor antagonists	Glaxo Group Limited
3,3 diphenylpropylamines (derivatives)	US 7,855,230B2 [68]	Muscarinic receptor antagonists	UCB Pharma GmbH
—	US 8309572 [69]	Muscarinic receptor antagonists	Glaxo Group Limited
7-azoniabicyclo [2.2.1.] heptane	US 20120029044A1 [70]	Muscarinic receptor antagonists	none

Table 4

Muscarinic receptor agonists approved for therapeutic protocols or used in clinical trials or in animal models.

Agonists	Receptor selectivity	Molecular structures	Status	Patents and company	Therapeutic application
Alvamefine	M1		Phase III discontinued	US5998404 US006486163B1 US20030119880A1 [71, 72]	Alzheimer's disease
Xanomeline	M1>M4		Phase III	US5980933 <i>Eli Lilly and Company</i> [73]	Alzheimer's disease
Cevimeline	M1		Approved	US4855290 US5571918 [74, 75]	Sjogren's disease
Vadacilidine (LY297802)	M4		Phase II	US006323194B1 [76]	Antinociceptive effects [77]

Table 5

Muscarinic receptor subtypes involved in different tumor types

Tumour types	Muscarinic subtypes involved in tumor progression	Biological activity	Authours
Melanoma	M3>M5	Cell migration	[85]
Colon cancer cell lines	M3	proliferation	[88]
Small cell lung carcinoma (SCLC)	M3	proliferation	[89]
Prostate Cancer Cells	M3	proliferation	[87]
Cells derived from benign prostate hypertrophy (BPH)	M3	proliferation	[87]
Breast cancer	M3	proliferation	[93, 98]
Mammary Adenocarcinoma	M3	proliferation, angiogenesis	[92]
Astrocytoma	M3	proliferation, inhibition of cell migration	[90, 99]
Glioblastoma	M2	Inhibit cell proliferation and survival	[95, 96]

Table 6

a Chemical structures of clinical and common anticholinesterases, with their IC ₅₀ values (concentration required to inhibit 50% enzyme activity).	
(-)-Physostigmine, eserine (<i>Antilirium</i>) IC ₅₀ AChE = 28 ± 2 nM IC ₅₀ BuChE = 16 ± 3 nM Selectivity: 2-fold BuChE
Donepezil (<i>Aricept</i>) IC ₅₀ AChE = 22 ± 8 nM IC ₅₀ BuChE = 4150 ± 1700 nM Selectivity: 188-fold AChE Patent [189]
Galantamine (<i>Reminyl</i> , <i>Razadyne</i> , <i>Nivalin</i>) IC ₅₀ AChE = 800 ± 60 nM IC ₅₀ BuChE = 7300 ± 830 nM Selectivity: 9-fold AChE Patent [188]
Rivastigmine (<i>Exelon</i>) IC ₅₀ AChE = 4150* ± 160 nM IC ₅₀ BuChE = 37 ± 5 nM *erythrocyte AChE assay – however, considered to be far more potent against brain AChE, and therefore ChE unselective Patent [191]
Tacrine, tetrahydroaminoacridine (<i>Cognex</i>) IC ₅₀ AChE = 190 ± 40 nM IC ₅₀ BuChE = 47 ± 10 nM Selectivity: 4-fold BuChE
(-)-Phenserine IC ₅₀ AChE = 22 ± 14 nM IC ₅₀ BuChE = 1560 ± 45 nM Selectivity: 70-fold AChE [21, 28, 32] Patent [211]
(-)-N ¹ , N ⁸ -Bisnorcymserine IC ₅₀ AChE = 110 ± 2 nM IC ₅₀ BuChE = 1 ± 0.1 nM Selectivity: 110-fold BuChE [26, 35] Patent [212]

b Chemical structures of clinical and common anti-cholinesterases	
Posiphen (also known as (+)-phenserine) IC_{50} AChE = >10,000 nM IC_{50} BuChE = >10,000 nM Selectivity: no cholinergic activity Patent [213]
Phenothiazine aryl and alkylaryl analogues: IC_{50} BuChE as low as 3.5 nM without detectable AChE activity [27, 29] Patent [214]
BW 284c51 (irreversible) IC_{50} AChE = 980 ± 550 nM IC_{50} BuChE = 340,000 nM Selectivity: 347-fold AChE
Iso-OMPA (irreversible) IC_{50} AChE = 18 ± 8 nM IC_{50} BuChE = 48,000 nM Selectivity: 2666-fold BuChE