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Perspective

Therapeutic Opportunities for Muscarinic Receptors in the Central Nervous System

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Introduction

The neurotransmitter acetylcholine binds to two types of cholinergic receptors: the ionotropic family of nicotinic receptors and the metabotropic family of muscarinic receptors (Figure 1). Nicotinic receptors are ligand-gated ion channels that modulate cell membrane potentials. Muscarinic receptors belong to the large superfamily of plasma membrane-bound G protein-coupled receptors (GPCRs). To date, five subtypes of muscarinic receptors have been cloned and sequenced from a variety of species including mammalian (human, cow, pig, rat, mouse) and nonmammalian (chicken, *Drosophila melanogaster*, frog) and show a remarkably high degree of sequence similarity across species as well as across receptor subtype. The M_1-M_5 muscarinic receptors are expressed predominantly within the parasympathetic nervous system which exerts excitatory and inhibitory control over central and peripheral tissues and participate in a number of physiologic functions including heart rate, arousal, cognition, sensory processing, and motor control. Availability of molecular probes and receptor subtype-selective antibodies has provided detailed knowledge of receptor distribution and shown significant overlap in a wide variety of brain regions and peripheral tissues. Muscarinic agonists such as muscarine and pilocarpine and antagonists such as atropine have been known for over a century, but little progress has been made in the discovery of receptor subtype-selective compounds making it difficult to assign specific functions to the individual receptors. Recently, muscarinic receptor knockout mice have been developed and some improvement has been made in the selectivity of ligands that will hopefully generate a new understanding of the role of each receptor in physiological processes and especially brain function.

This review will focus on the pharmacology of the muscarinic receptor family and therapeutic opportunities for muscarinic intervention in disorders of the central nervous system. Discussion of the recent advances in the pharmacology and molecular biochemistry of muscarinic receptors will be followed by summaries of the involvement of this family of receptors in psychosis, Alzheimer's disease, and Parkinson's disease. Due to their broad central and peripheral distribution, muscarinic receptors have shown promise as targets for the treatment of such diverse diseases such as glaucoma, bradycardia, gastric acid secretion, and asthma and as antispasmotics for GI disturbances. Additional relevant reviews have discussed these topics including specific discussion of muscarinic receptor characterization,[1−3] smooth muscle function,[4] molecular analysis,[5] ion channel function,[6] subtype-specific toxins,[7] signal transduction,[8] and pharmacology.[9]

Muscarinic Receptor Structure and Function

M_1-M_5 Receptor Genes. The first pharmacological evidence for the presence of multiple subtypes of muscarinic receptors appeared in the early 1950s[10] and became more evident as differences in tissue responses to various muscarinic ligands were observed. Before cloning revealed at least five receptor subtypes, pharmacological evidence was able to resolve at least three

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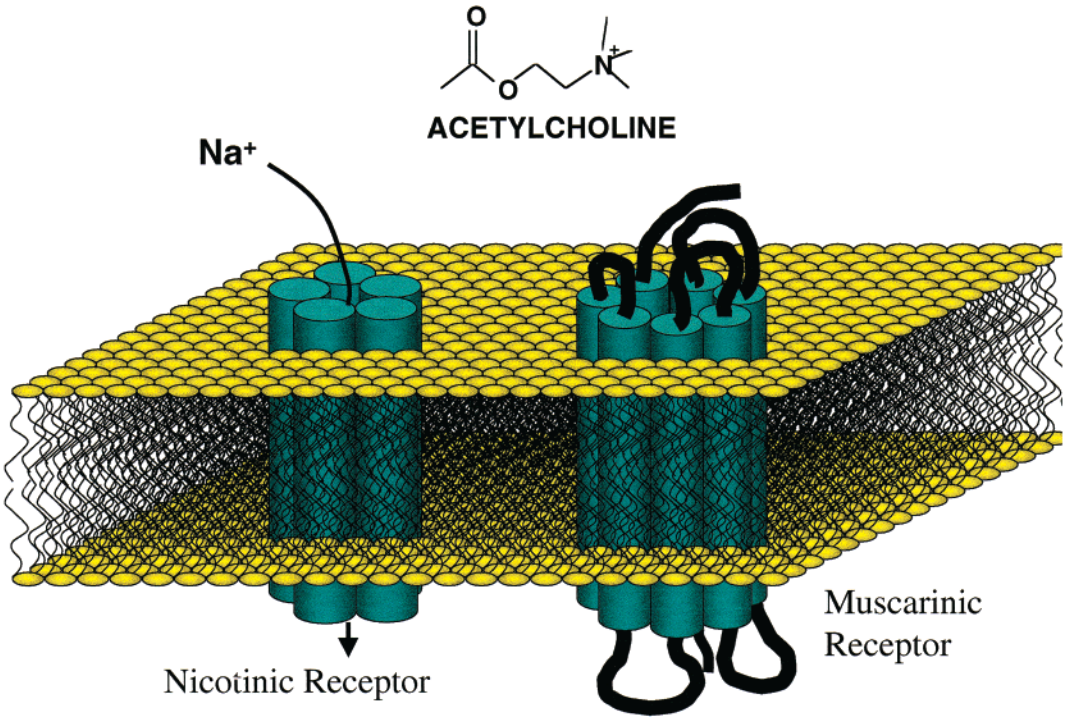


Figure 1. Cartoon of the two types of acetylcholine receptors. Acetylcholine receptors are integral membrane proteins that occur as either nicotinic-sensitive ionotropic or muscarinic-sensitive metabotropic receptors. Nicotinic receptors are ion channels created by the assembly of five protein subunits. Muscarinic receptors belong to the superfamily of GPCRs which are characterized as a single peptide which weaves in and out of the plasma membrane creating seven hydrophobic membrane spanning domains and three extracellular and cytoplasmic loops.

pharmacologically defined subtypes classified by capital M_1-M_3. Genes encoding the M_1 and M_2 receptors were cloned first by Numa and colleagues[11] followed by genes encoding the M_3-M_5 receptors by Bonner et al.[12,13] No convincing pharmacological or molecular evidence suggesting that additional members of this family exist has been provided to date. A recent meeting of the IUPHAR panel on GPCR nomenclature has suggested a common designation for the five muscarinic receptor subtypes denoted by capital M_1-M_5 to reflect their order of discovery.[3] The five known muscarinic receptor subtypes share common characteristics with the superfamily of GPCRs and especially those that bind small molecules or neurotransmitters.[14] The muscarinic receptors are single integral plasma membrane glycoproteins coded by genes that lack introns. Their topology upon folding has recently been shown to be similar to the well-characterized photoreceptor rhodopsin which is believed to have seven hydrophobic α-helical domains spanning the plasma membrane seven times connected by hydrophilic intracellular and extracellular loops.[15,16]

Progress in developing therapeutically useful muscarinic agents has been slow based on the current inventory of relatively nonselective ligands for the five receptor subtypes. This difficulty may be due to the significant amino acid sequence identity between the five receptors possibly creating similarity across all five binding domains. One must keep in mind, however, that examples exist of single amino acid differences in GPCR subtypes that yield significant differences in ligand binding affinity.[17] X-ray crystallography of the prototypic GPCR, bacteriorhodopsin, has been used to predict determinants of ligand−receptor interactions through binding site modeling. However, a high-resolution crystal structure does not currently exist for the muscarinic or other related GPCRs. Therefore, receptors have been modeled based on the low-resolution structure of rhodopsin[18] and computer modeling of the sequences of known GPCRs.[19−22] These models predict that the seven transmembrane spanning domains form a pocket that defines the ligand-binding domain. A two-dimensional electron crystallographic map of bovine rhodopsin confirmed the expectation that the seven hydrophobic helical bundles form a cylindrical type core with four of the bundles almost perpendicular to the membrane and three take on a more tilted orientation.[20] A recent 2.8 Å three-dimensional crystal structure of rhodopsin was completed that will no doubt help refine our predictions about muscarinic receptor structure and function.[16]

The critical amino acid residues involved in acetylcholine binding have been identified by covalent affinity-labeling and mutagenesis experiments. Acetylcholine is predicted to interact with amino acids present in the outer regions of the binding pocket about 10−15 Å from the surface.[1] In the M_1 receptor, a critical asparagine residue (Asp105) is involved in binding of the positively charged amino headgroup of acetylcholine and most equivalent muscarinic ligands based on binding of radiolabeled acetylcholine mustard[23] and propylbenzylcholine mustard.[19,24] Mutagenesis of this site resulted in a reduction in ligand affinities.[25,26] Asp105 is conserved in most GPCRs that bind biogenic amine type neurotransmitters. Six additional residues critical for acetylcholine binding (Thr231, Thr234, Tyr148, Tyr506, Tyr529, Tyr533) are conserved across the five muscarinic receptors but not other GPCRs. Two tryptophans in the M_3 receptor (Trp503, Trp143) which are conserved across many GPCRs were found to be critically involved in high-affinity ligand binding and are predicted to face the ligand-binding cavity.[27]

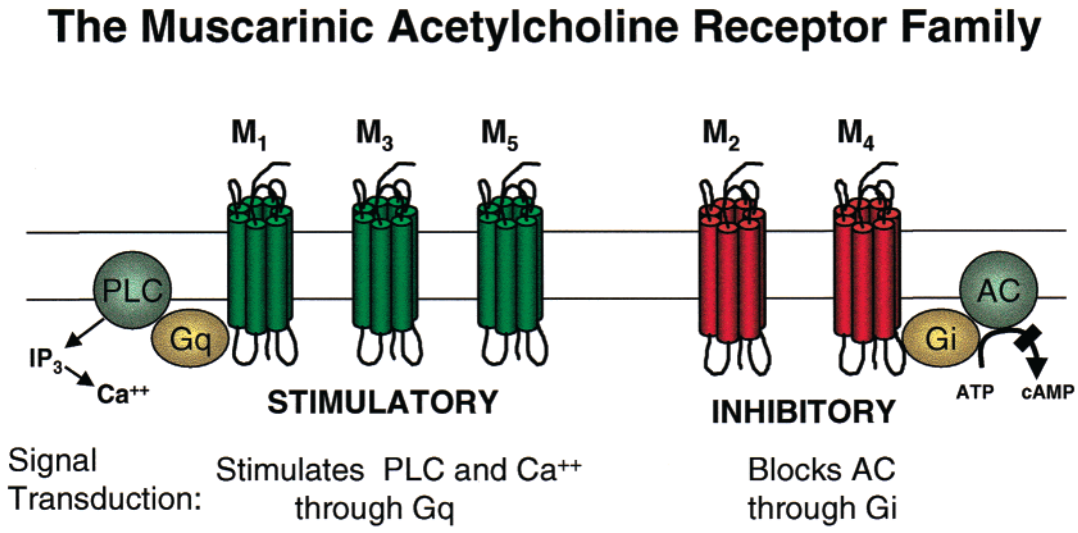


Figure 2. Family of five muscarinic acetylcholine receptors. The muscarinic receptor family has five known members designated M_1−M_5 based on the order in which they were cloned. The M_1, M_3, and M_5 stimulatory receptors couple primarily to the mobilization of intracellular calcium. The increase in cytoplasmic calcium occurs following stimulation of a signaling cascade which begins with coupling to the heterotrimeric G protein, Gq, followed by stimulation of the effector enzyme, phospholipase C, which releases inositol-1,4,5-trisphosphate (IP_3). The M_2 and M_4 inhibitory receptors negatively modulate adenylate cyclase to reduce cytoplasmic levels of cAMP.

Competitive antagonists are thought to bind to the same critical residues facing the binding pocket but appear to also interact with additional determinants which influence their affinity for each receptor. For example, mutation of Ser120 to Ala resulted in a loss of antagonist binding affinity with little effect on agonist binding.[28] Moreover, replacement of Asn507 with Asp, Ser, or Ala resulted in a differential loss of antagonist affinity based on the structural class of compound.[29]

Signal Transduction. The physiological response to muscarinic receptor stimulation is mediated through a cascade of protein interactions including G protein activation followed by stimulation or inhibition of an effector channel or enzyme.[8] Tissue localization, pre- or postsynaptic location, complement of signal transduction machinery contained in the cell, and receptor subtype expression pattern influence the biological consequence of muscarinic receptor stimulation. The functional activity of M_1, M_3, and M_5 receptors is most commonly associated with their ability to mobilize intracellular calcium through stimulation of phospholipase Cβ (Figure 2). Phospholipase Cβ activation releases IP_3 which stimulates the IP_3 ion channel receptor to release Ca^{2+} from intracellular compartments resulting in a transient cytosolic calcium flux. The M_2 and M_4 receptors inhibit adenylate cyclase activity reducing the local concentrations of cAMP (Figure 2). They also block the activity of voltage-operated calcium channels involved in neurotransmitter release. More recently, the signal transduction pathways associated with muscarinic receptors have expanded to include more effectors and to include pathways normally thought to interact exclusively with growth factors.[8]

In addition to their ability to stimulate phospholipase C through Gq, the M_1, M_3, and M_5 receptors have also been shown to stimulate phospholipase A_2[30−33] and phospholipase D[34−38] in various cell lines and primary cultured cells. Phospholipase A_2 or D activation has a common requirement for extracellular calcium. A cation channel mediating muscarinic receptor-dependent calcium influx has been initially characterized and may provide the regulatory step essential for phospholipase activation.[8,36] These studies showed that the muscarinic receptor-operated calcium channel is voltage-independent, has a low conductance, and in the cell lines studied may be under the regulation of a small-molecular-weight G protein. Expression studies with a mammalian homologue of the *Drosophila* Trp/Trpl cation channels indicated that the Trp6 channel was under the regulation of the M_5 receptor.[39] Receptor-operated calcium channels may be a key signaling pathway for other calcium-mobilizing receptors. Additional signal transduction activation has been associated with the M_1, M_3, and M_5 receptors including the inefficient stimulation of adenylate cyclase through both Gs and G protein-independent pathways.[40−43]

The M_2 and M_4 receptors couple primarily to the inhibition of adenylate cyclase but also interact with Gs to stimulate adenylate cyclase.[40−42,44] Pertussis toxin treatment, which blocks Gi/Go function, increases the likelihood of Gs and muscarinic receptor interaction suggesting that G protein stoichiometry within the cell plays a key role in directing the signaling potential of each receptor. M_2 and M_4 receptors stimulate phospholipase C in some cell lines which is thought to be mediated in part by $\beta\gamma$-subunits released following heterotrimeric G protein activation.[45] A novel signaling pathway involving the activation of phospholipase A_2 by Gi-coupled receptors, including the M_2 and M_4 receptors, is stimulated when phospholipase A_2 is preactivated either directly or with calcium-mobilizing agonists.[46] In this study, M_2 and M_4 receptors augmented purinergic receptor-stimulated phospholipase A_2 activation. This may be physiologically relevant since the purinergic receptor agonist, ATP, is co-released with many neurotransmitters, including acetylcholine, and may serve to prime arachidonic acid mobilizing or other signal transduction pathways.

The regulation of muscarinic receptor function and trafficking by kinase enzymes has been extensively

studied and has been recently reviewed.[47,48] Muscarinic receptors regulate kinase and phosphatase activity either through heterotrimeric G protein activation and subsequent second-messenger production or through the direct activation of small-molecular-weight G proteins.[49−53] Hence, pathways normally thought to be mediated by growth factor receptors have been linked to GPCRs in ways that are just beginning to be appreciated. Many of the recent discoveries of muscarinic receptor signaling have been initially observed in ectopic expression models using mammalian cell lines. For the most part, however, signal transduction observed in cell lines has also been observed when receptors are studied in primary culture or tissue slice preparations. Due to the large overlap in expression patterns between the five muscarinic receptors in brain, it has been difficult to ascribe a specific signaling pathway to a single receptor subtype without selective pharmacological agents.

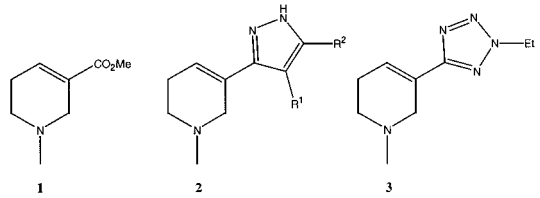
Pharmacology

Sources of Muscarinic Receptor Subtypes. Before the cloning of the muscarinic receptors, isolated tissues served as sources of putative pure muscarinic receptor subtype responses for many decades. Superior cervical ganglion has been used as a predominant M_1 receptor functional model[54,55] as well as vas deferens.[56] Guinea pig bladder has served for M_3 receptor responses,[57] and cardiac tissue, especially isolated atria, as a source of M_2 receptor.[58] The M_5 receptor is localized primarily in the substantia nigra and VTA.[59] However, localization of the M_5 receptor in other brain regions and periphery has been controversial.[60] This is due to its low expression and lack of tools for its positive identification other than by PCR, which provides identification of mRNA but no quantitative measure of protein levels. Only one source of discrete M_5 receptor expression has been described to date in a human melanoma cell line, A2058.[61] In this cell, M_5 receptors uncharacteristically coupled to the inhibition of adenylate cyclase. Pharmacological approaches have also been applied to M_5 receptor characterization and localization and suggest that the distribution may be wider than previously thought.[60] Cloning and ectopic expression of human and rat M_1−M_5 muscarinic receptor cDNA in cell lines has provided a source of pure receptor subtype for binding and functional studies. Considerable information has accumulated using these cells about the molecular structure, pharmacology, and signal transduction of each subtype. The recent challenge has been to correlate data derived from the molecular studies to tissue and in vivo pharmacology.

The recent creation of mice bearing a genetic deletion for the M_1, M_2, and M_4 receptors has provided an opportunity to investigate physiological responses to ligand challenge in animals and tissues devoid of one or more receptor subtypes. For example, M_1 receptor knockout mice lost their seizure response to pilocarpine suggesting that M_1 receptors play a role in the etiology of epilepsy.[62] Superior cervical ganglion cells isolated from M_1 knockout mice no longer displayed M current inhibition following application of a muscarinic agonist verifying its predominant functional M_1 receptor content. Spontaneously beating isolated atria normally show a reduction in the number of beats per unit time following addition of a muscarinic agonist to the bath media. M_2 knockout mice did not respond to muscarinic agonist application indicating that regulation of heart rate is predominantly regulated through the M_2 receptor.[63] However, a more complex regulation is likely to occur in vivo and includes central and ganglionic involvement. M_2 receptors were also shown to play a major role in the regulation hypothermia, tremor, and analgesia in mice. Remarkably, M_4 knockout mice respond similarly to wild-type mice following muscarinic agonist-induced analgesia, tremor, salivation, or hypothermia. M_4 knockout mice were hyperactive and hyperresponsive to D_1 dopamine agonists, amphetamine, and apomorphine suggesting a role in regulating dopamine release and dopamine-mediated locomotor activity.[64] Using M_1, M_2, and M_4 knockout mice, Hille and colleagues were able to show that N and L type voltage-gated calcium channels were differentially regulated by the muscarinic subtypes in mouse superior cervical ganglion neurons.[65] They found that the fast regulatory pathway was M_2 receptor-mediated, the slow second-messenger-dependent pathway was M_1-mediated, and neither was regulated by M_4 receptors. Additional studies with M_3 and M_5 single knockout and combinations of double-muscarinic receptor knockout mice will no doubt enhance our understanding of the physiological role of each member of this receptor family.

Subtype-Selective Muscarinic Agonists. The development of muscarinic receptor subtype-selective compounds has been challenging, and on detailed examination the relative selectivity has been limited. This review will concentrate on compounds that have appeared more recently in the literature and for which some claim for muscarinic subtype selectivity has been mentioned.[66,67]

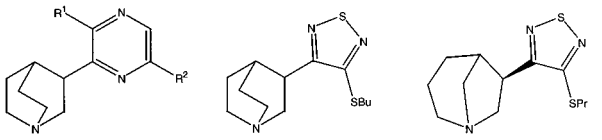
A popular strategy for improving both metabolic stability and muscarinic subtype selectivity has been to replace the ester group of the muscarinic agonist arecoline (**1**) or conformationally more rigid azabicyclic analogues with bioisosteric heterocycles. For instance, evaluation of pyrazole analogues **2** detected compounds that show M_3 agonist selectivity compared to M_1 agonist activity in tissues.[68] By contrast, Lu 25-109 (**3**) in which a tetrazole replaces the ester group of **1** shows partial M_1 agonist activity in tissues and in an M_1 cell line but M_2 and M_3 antagonist activity in tissues.[69]



1 **2** **3**

Among a series of azabicyclic pyrazines **4** is a partial M_1-selective agonist that has 5-fold greater M_1 efficacy than the selective M_1/M_3 agonist L 689-660[70,71] (**5**), less M_3 agonist activity than **5**, and no M_2 agonist activity in the guinea pig atrium.[72] Among a series of 1,2,5-thiadiazolyl azabicyclics that have muscarinic activity, LY297802 (**6**) is found to produce potent muscarinically mediated antinociceptive activity.[73,74] While **6** is a M_1 agonist in the rabbit vas deferens, M_2 and M_3 antago-

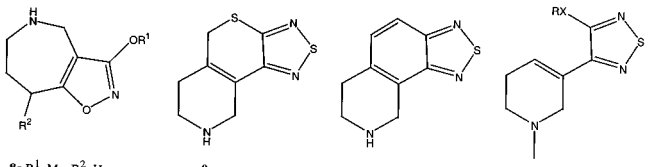
nism or weak partial agonism was demonstrated in other tissues. Subsequently, studies using other muscarinic agonists lacking M_1 agonist activity in tissues and M_1 cell lines showed that M_1 agonist activity is not required for antinociception.[75] However, structurally related PTAC (**7**) shows muscarinic antagonist activity in M_1, M_3, and M_5 cell lines but partial agonist effects in M_2 and M_4 cell lines.[76,77] Functional dopamine antagonist activity and antipsychotic-like activity could be demonstrated with **7** in several different tests, but **7** has no affinity for dopamine receptors suggesting a potentially new therapeutic use for muscarinic agonists that may be mediated through M_2 or M_4 receptor subtypes.



4 R^1=Ohexyl, R^2=H
5 R^1=H, R^2=Cl

6 **7**

Restricting the rotation of the heterocyclic ester bioisostere within this structural class has not dramatically improved muscarinic subtype selectivity. Rigid arecoline analogues **8a**−**c** show some M_2/M_4 selectivity when compared across muscarinic receptor subtype cell lines using R-SAT technology,[78] but the selectivity appears to be an artifact of the test system since standard muscarinic agonists show similar selectivity.[79] In tissues, the 8-methyl derivative **8d** shows some functionally selective partial M_1 agonist activity.[80] The fused heterocyclic analogues of the muscarinic analgesics such as **9** and **10** do not show improved muscarinic subtype selectivity compared to **11a**, nor is functional selectivity observed in receptor subtype clones.[81]

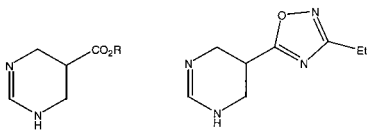


8a R^1=Me, R^2=H
8b R^1=Et, R^2=H
8c R^1=propargyl, R^2=H
8d R^1=propargyl, R^2=Me

9 **10**

11a RX = SMe
11b RX = Ohexyl

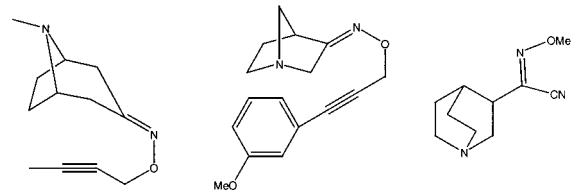
Replacement of the tetrahydropyridine ring of arecoline analogues with a tetrahydropyrimidine as in **12a,b** and **13** provides selective M_1 efficacy in cell lines compared to efficacy in M_2 and M_3 cell lines.[82]



12a R=Et
12b R=propargyl

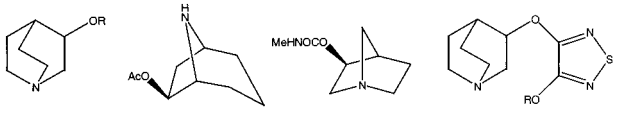
13

Because oximes have electrostatic properties similar to esters, an alternative strategy of replacing an ester group with an oxime has been frequently used in muscarinic agonist research. On the basis of binding studies in tissues, tropinone oxime **14** appears to have some selectivity for M_1 receptors over M_2 and M_3 receptors, but the compound did show functional agonism in an M_3 preparation but not in an M_2 assay.[83] CI-1017 (**15**) has 8-fold greater affinity for M_1 receptors compared to M_2 receptors as well as functional selectivity at M_1 and M_4 receptors compared to M_2, M_3, and M_5 receptors using both second-messenger assays and R-SAT technology.[84] Additional promising oximes from this series have also been described.[85] Sabcomeline (SB 202026, **16**) does not show selective affinity for muscarinic subtypes, but it is a potent and efficacious M_1 agonist in the rat cervical ganglion while in other tissues and in vivo assays it is a much less potent and efficacious M_2 and M_3 agonist.[86,87]



14 **15** **16**

Rigid analogues of acetylcholine have also been pursued as a strategy for obtaining muscarinic subtype selectivity. A relatively older example that has seen continued development until very recently is talsaclidine (**17**), an analogue of aceclidine (**18**).[88] Talsaclidine shows only minor differences in affinity among the M_1−M_5 receptor subtypes. However, in functional assays, **17** is most potent and efficacious in cells expressing M_1 receptors, slightly less in M_3 cells, and inactive in M_2 cells. Tissue assays also suggest reasonable M_1 selectivity. Nortropane (**19**), has 12-fold higher affinity for M_2 receptors compared to M_4 receptors but much higher selectivity for M_2 receptors compared to M_1 and M_3 receptors.[89] Functional assays demonstrate that **19** is an agonist at M_1−M_4 receptors but only a partial agonist at M_3 receptors. WAY-131256 (**20**), a rigid carbamate analogue of acetylcholine, does not show selective affinity among the M_1−M_5 receptor subtypes, and **20** is comparably potent agonist at both M_1 and M_2 receptors.[90] By contrast, in vivo studies suggested that **20** is selective for M_1 receptors compared to M_2 and M_3 receptors. Thiadiazole ester bioisosteres of aceclidine (**21a,b**) are as comparably efficacious and potent as the functionally selective M_1 agonist xanomeline (**11b**) in an M_1 cell line and have very little M_3 activity in vivo.[91]

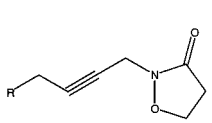


17 R = propargyl
18R = acetyl

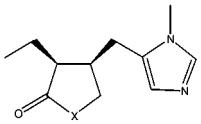
19 **20**

21a R = O-pentyl
21b R = O-hexyl

Oxotremorine analogues **22a,b** have selective affinity for M_1 receptors in rat brain compared to M_2 receptors in rat heart, and binding index studies suggest that **22a** is a partial muscarinic agonist while **22b** is a full agonist.[92] Newer, subtype-selective analogues of pilocarpine (**23**) have not been revealed but thiopilocarpine (SDZ-ENS 163, **24**) continued into clinical trials.[93] SDZ-ENS 163 acts in vitro and in vivo as a partial agonist at M_1 and M_3 receptors but as an antagonist at M_2 muscarinic receptors. However, **24** did not distinguish between m_1 and m_3 receptors in radioligand binding studies.[94]

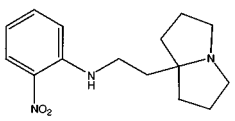


22a R = NMe_2
22b R = NMe_3^+



23 X = O
24 X = S

Some compounds seemingly unrelated to classical muscarinic agonists have shown some hint of muscarinic subtype selectivity. For instance, certain small-molecular-weight proteins in snake venoms appear to have high selective affinity for M_1 receptors compared to M_2 receptors, and they also stimulate M_1 receptors containing tissues without producing effects on M_2 and M_3 receptor-mediated tissues.[95,96] The antipsychotic clozapine, which has high muscarinic affinity but only modest M_1 subtype selectivity, was found to be a potent agonist in M_4 cell lines but an antagonist in the other muscarinic receptor subtype cell lines.[97] Subsequently, a few, but not all, antipsychotic agents have also been found to have M_4 agonist activity in cells expressing the receptor, but they did not modulate cAMP levels in tissue.[98] This result is consistent with a weak partial agonism that is most evident in cell lines expressing high levels of receptor. This is supported by more recent evidence showing that clozapine behaved as a weak partial agonist at M_1-M_4 receptors expressed in cell lines.[99] Although perhaps inspired by the M_1 agonist R46559,[100] SK-946 (**25**) is a structurally unique diamine that has selective affinity for M_1 receptors compared to M_2 receptors in rat brain and which stimulates M_1 receptors in rat fetal hippocampal neuronal cells.[101,102] Two seemingly unique classes of amidinoquanidines **26**[103] and amidines **27**[104] have also been recently disclosed that may have some functional muscarinic subtype selectivity.



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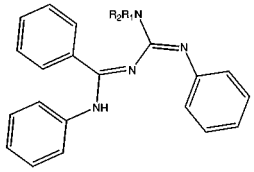
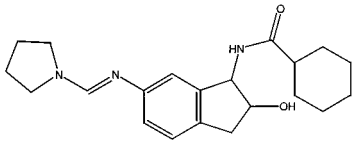


26



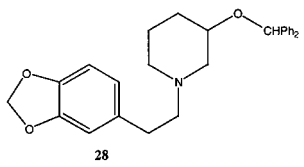
27

The cholinergic hypothesis of dementia and Alzheimer's disease and the marginal clinical success of acetylcholinesterase inhibitors continue to support the development of selective M_1 agonists for the treatment of these diseases. However, none of the functionally selective M_1 agonists reported to be in clinical trials have emerged into clinical practice suggesting that considerably more effort is needed to adequately test the M_1 cholinergic hypothesis. More recently, the potential use of selective agonists in the management of pain and in the treatment of schizophrenia has also gained attention. Although the muscarinic subtypes associated with these treatments in humans is not yet well-resolved, the M_2 and M_4 subtypes seem to be highly implicated. The therapeutic uses of M_3-selective muscarinic agonists remain unrealized although the predominance of M_3 receptors on smooth muscle and exocrine glands suggests treatments for dysfunction in these tissues. The potential therapeutic uses of M_5-selective agonists also remains an unknown especially because of the low abundance of this receptor in the brain and the absence of a pharmacological defined test for this subtype. In all cases, the development of potent and efficacious, molecularly defined, subtype-selective agonists would be of great benefit in clarifying and realizing the therapeutic potential of muscarinic agonists.

Subtype-Selective Muscarinic Antagonists. The methods used to determine the muscarinic subtype selectivity of antagonists have been no more consistent than for muscarinic agonists. Most of the caveats used in assessing muscarinic agonist subtype selectivity should be equally applied to claims for muscarinic antagonist subtype selectivity. Additional factors to consider with muscarinic antagonists are their potentials to not only act as antagonists but to also act as allosteric modulators of muscarinic receptors.[105] Among the therapeutic uses suggested for subtype-selective antagonists, the treatment of peripheral smooth muscle disorders such as bladder, airway, and bowel disorders with M_3 antagonists has been particularly of interest, while antagonists of M_2 and M_4 receptors have been suggested as treatments for movement disorders, dementia, cardiac disorders, and pain.

Certain arylalkylamines have shown selectivity for M_3 receptors in tissues, and three of these (**28**−**30**) have been evaluated clinically as potential treatments for irritable bowel syndrome, obstructive airway diseases, and urinary incontinence, respectively. Radioligand binding studies in tissues suggests that **1** has higher affinity for M_3 receptors than for M_1, M_2, or M_4 receptors.[106] Functional tissue assays confirm that **28** is selective for M_3 receptors compared to M_1 and M_2 receptors, but the magnitude of the selectivity is dependent on the particular M_3 tissue preparation used. While **29** shows 4-fold functional selectivity for inhibition of muscarinic receptors in the urinary bladder compared to muscarinic receptors in the parotid glands in vivo, this selectivity cannot be attributed to activity at a single muscarinic receptor subtype.[107−109] In cloned human muscarinic (M_1-M_5) receptors, **29** does not discriminate among the subtypes in binding experiments nor are there large differences in affinity for muscarinic receptors in specific tissues (M_1-M_3). A similar profile is presented by the major metabolite of **29**.[109] Comparisons of muscarinic antagonists with different in vivo and in vitro selectivities led to the conclusion that a balance between M_2 and M_3 affinity was required for bladder selectivity. Darifenacin (**30**) not only is a more potent antagonist in tissues controlled by M_3 receptor subtypes compared to tissues controlled by M_1 and M_2 receptor subtypes but also appears to be selective for M_3 receptors in smooth muscle preparations compared to M_3 receptors in glandular tissue.[107] More recently, the M_3 selectivity of **30** was confirmed in binding experiments with cloned human muscarinic receptor subtypes where **30** shows a 47-fold separation between M_2 and M_3 affinity.[106,110]

Arylalkylamine NPC-14695 (**31**) also shows higher affinity for tissue M_3 receptors than for the M_2 or M_1 receptor.[111] The observed 11- and 37-fold separation



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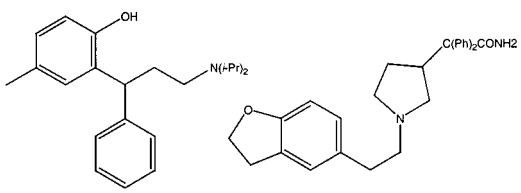
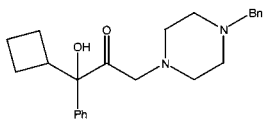


29 **30**

between bladder function and mydriasis and salivation, respectively, also suggests selectivity for smooth muscle M_3 receptors in vivo. High selectivity for bronchial smooth muscle muscarinic receptors compared to muscarinic receptors in the heart and salivary gland is also seen with **31**. Vamicamide (**32**) shows similar potency as an M_1 and M_3 antagonist in tissue preparations but is less potent in M_2 tissue.[112] The ability of **32** to inhibit bladder contractions yet have little effect on heart rate and poorly penetrate the blood−brain barrier suggests that **32** might be useful for the treatment of bladder hyperactivity. KRP-197 (**33a**) has subnanomolar affinity for M_3 receptors in the ileum, comparable affinity for M_1 receptors, but 13-fold lower affinity for M_2 receptors in the atrium and is also suggested to be a potential treatment for urinary incontinence.[113,114] Structurally related imidazole **33b** has somewhat lower M_3 affinity than **33a** but has higher selectivity for M_3 receptors than **33a**. In contrast to other compounds within this structural class, the *S*-isomer of commercially available antihistamine dimethindine (**34**) exhibits higher affinity for M_2 receptors than for M_1, M_3, or M_4 receptors in tissues in both functional and binding assays.[115]



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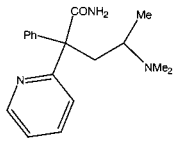

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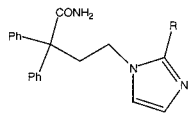
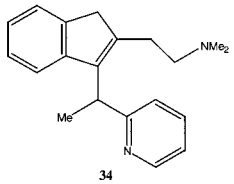
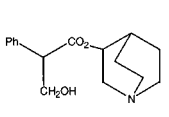


33a R=Me
33b R=*i*Pr



34

Complex esters of amino alcohols have been shown to have extremely varied muscarinic subtype-selectivity profiles. For instance, like many of the arylalkylamines, quinuclidinyl tropate **35** shows selectivity for M_3 receptors in functional assays in tissues with 4-fold selectivity over M_4 receptors but greater separations between M_3 and M_1 or M_2 activity.[116] The M_3 subtype selectivity of **35** is also supported by binding experiments in cloned receptors (M_1−M_4) where **35** has 10-fold higher affinity for M_3 receptors than for M_4 receptors. Tissue binding assays suggest that LG50643 (**36**) has greatest affinity for M_3 receptors, 2.7-fold greater than for M_1 receptors, and 15-fold greater than for M_2 receptors.[117] In trachea preparations, functional M_3 versus M_2 selectivity was demonstrated by showing a 7-fold separation between postjunctional and prejunctional potency. In contrast, *S*-ET-126 (**37**) is functionally an M_1 antagonist in tissues with 8-fold selectivity over M_2 activity and even less M_3 antagonist activity.[118] Extensive molecular modification of 2-ethylthio diphenylacetate **38** did not lead to improved M_2 selectivity in functional tissue assays.[119] Highest M_2 selectivity was usually found among quaternary derivatives. Binding studies in cloned muscarinic receptor subtypes have not confirm the M_2 subtype selectivity of **38**.[120] Tropane ester PG-9 (**39**) produces antinociception in several standard tests and is highly potent as an antagonist in the putative M_4-selective guinea pig uterus assay.[121] In tissues selectively controlled by M_1−M_3 receptors, **39** is at least a 10-fold less potent antagonist. Microdialysis experiments show that **39** increases release of acetylcholine in the rat cerebral cortex within the antinociceptive dose range suggesting that nociception produced by **39** may be mediated through inhibition of presynaptic M_4 receptors.



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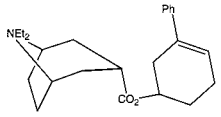

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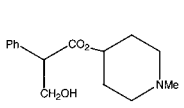

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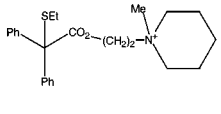

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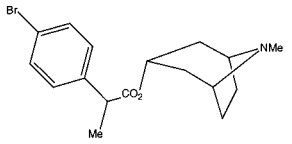
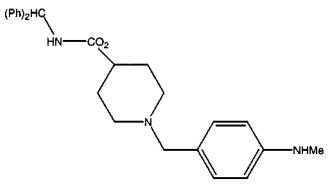


39

Carbamates of many of these structurally related amino alcohols also show M_3 selectivity. YM-58790 (**40**) has high affinity for both M_1 and M_3 receptors in tissue binding assays but 11-fold lower affinity for M_2 receptors.[122] In vivo, **40** is distinguished from other members of a series by the 6.7-fold separation between antagonism of M_3-mediated responses in the bladder and salivary glands as well as a lack of blockade of M_2-mediated bradycardia. YM-46303 (**41**) has higher muscarinic affinity than **40** but is somewhat less selective in vivo.[123] YM-53705 (**42**) has M_3 subtype affinity comparable to that of **40** but greater separation (10-fold) between effects on the bladder and on salivary glands.[124]



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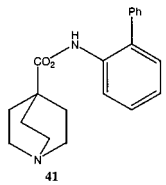

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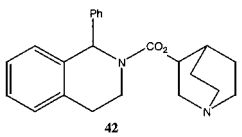
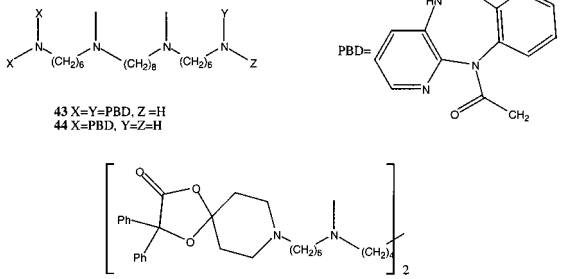


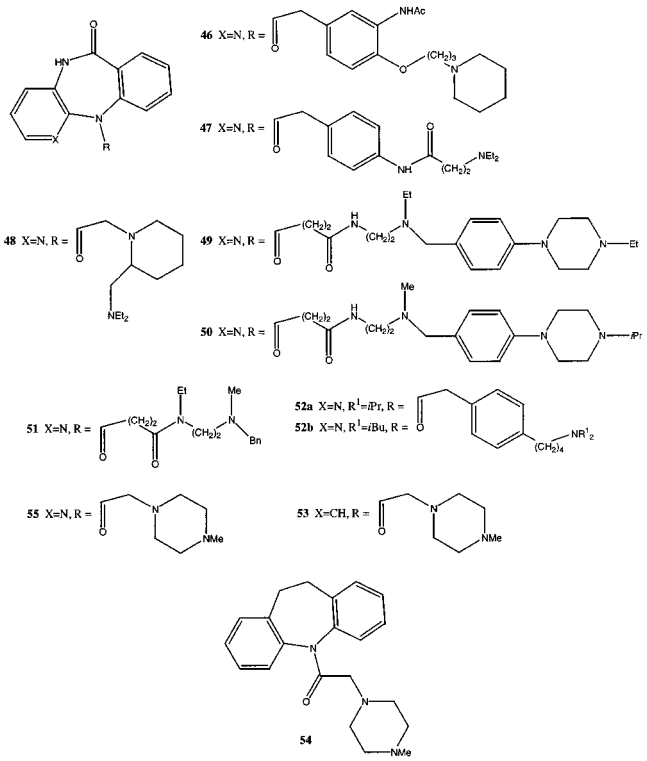
42

Tripitramine (**43**) incorporates structural elements of the two M_2-selective muscarinic antagonists methoc-

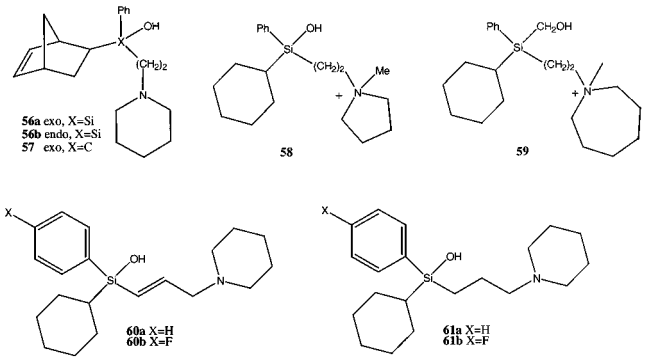
tramine[125] and AQ-RA 741.[126,127] In functional assays in tissues, **43** blocks M_2 receptors 1600-fold better than M_3 receptors and in tissue binding assays is equally selective for M_2 over M_3 receptors. The order of affinity for the muscarinic subtypes is $M_2 > M_1 > M_4 > M_3$ with only a 2-fold selectivity between M_1 and M_4 receptors. In cloned human muscarinic receptors, **43** is selective for M_2 receptors, but the magnitude of the selectivity between M_2 and M_1 receptors (6-fold) is significantly less than that seen in tissue binding experiments.[128] By contrast, dipitramine (**44**) has highest affinity for M_1 receptors in the M_1-M_4 tissue binding assays but there is only a 5-fold separation between M_1 and M_2 affinity.[127] Spirotramine (**45**) has lower affinity for M_1 receptors than **44,** but **45** does show higher M_1 selectivity than **44** in tissue binding assays.[129] Recently, additional structure−activity studies were reported in this structural class, but clearly superior subtype selectivity was not obtained.[130]



Dibenzodiazapenes continue to provoke interest as potential subtype-selective muscarinic antagonists, particularly as M_2 antagonists. Both YM59981 (**46**) and YM55758 (**47**) have much higher M_2 affinity than the prototypical M_2-selective antagonist AF-DX 116 (**48**) in rat heart binding assays, and they also have greater separation (>40-fold) between M_2 and M_3 affinity.[131] Modest separation between M_2 and M_1 affinity (4- and 6.3-fold, respectively) is also seen. Both **46** and **47** are distinguished within their chemical series by high oral activity and poor brain penetration, advantageous properties for peripherally acting cardiac agents. Similarly, succinamides YM43571 (**49**) and YM47244 (**50**) also have higher M_2 affinity and selectivity than **48** and better M_2/M_1 and M_2/M_3 selectivity (>10 and >79, respectively) than **46** and **47**.[132] A large separation between M_2- and M_3-mediated effects is also seen in vivo with **49** and **50**, but the M_2 effects are noncompetitive and there is some concern about the oral bioavailability of the compounds. An earlier succinamide **51** has somewhat lower M_2 affinity and M_2/M_3 selectivity than **49** and **50** and comparable M_2/M_1 selectivity (16-fold), but the in vivo M_2 antagonist activity is also noncompetitive.[133] Attempts to develop selective M_2 antagonists that cross the blood−brain barrier as potential dementia treatments led to phenylacetamides **52a**,**b**.[134] Both **52a**,**b** have higher affinity for M_2 receptors than **48** in tissue binding assays, and both show 7-fold higher affinity for M_2 receptors than for M_1 receptors. In contrast to earlier M_2-selective compounds, **52b** does penetrate the blood−brain barrier. Desaza analogues **53** and **54** of the M_1-selective antagonist pirenzepine (**55**) that have a greater potential for blood−brain barrier penetration have also been prepared.[135] While **53** has higher affinity for cloned M_1 receptors than **55**, the M_1 selectivity of **53** is no better than that for **55**. Both **54** and **55** had comparable M_1 affinity, but the M_1/M_4 selectivity of **54** (10- fold) was higher than for **55** (4-fold).

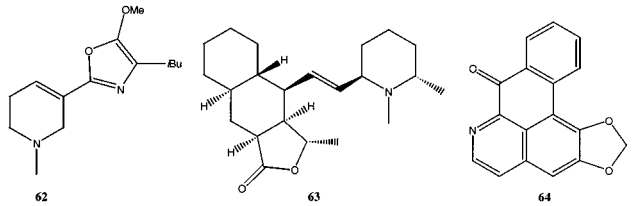


Both **56a**,**b**, silicon-containing analogues of the relatively M_1-selective antagonist piperiden (**57**), have essentially the same subtype affinity and order of subtype selectivity ($M_1 > M_4 > M_3 > M_2$) as **57**.[136] Among a series of stable, enantiomerically pure homologues of silatricyclamol iodide **58**, (*R*)-**59** has the highest M_1 affinity and selectivity in functional assays but the M_1 affinity and selectivity in binding assays is less.[137] Unsaturated analogues **60a**,**b** of the relatively M_3-selective antagonists hexahydrosiladifenidols **61a**,**b** have higher affinities but lower receptor subtype selectivity than the parent compounds.[138]

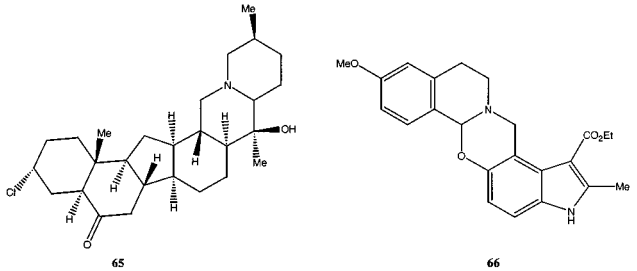


A variety of miscellaneous structures have also been found to have muscarinic antagonist subtype selectivity. The tetrahydropyridine **62** that is a heterocyclic ana-

logue of a number of muscarinic agonists has modest selectivity for M_1 receptors compared to M_2 receptors in tissue binding assays.[139] A number of similar heterocyclic analogues have been described as muscarinic antagonists, but there is insufficient data for determining if they have subtype selectivity.[140] Reduction of the furanone ring of himbacine (**63**) gives compounds with diminished M_2 subtype affinity but which retain some selectivity compared to M_1 subtype affinity.[141] A number of other modifications of himbacine that may lead to subtype selectivity have appeared in abstract form.[142] Liriodenine (**64**) is 10-fold more potent in inhibiting muscarinic-induced contractions in trachea and ileum than in atrial preparations suggesting some potential for M_3 subtype selectivity.[143] 3-α-Chloroimperialine (**65**) is a potent muscarinic antagonist that has 6-fold higher affinity for cloned M_2 receptors than M_4 receptors and even greater selectivity compared to M_1 and M_3 receptors.[144] Isoquinoline **66** does not have particularly high affinity for cloned muscarinic receptors but did show 10-fold selectivity for M_4 receptors compared to M_3 receptors and even greater selectivity compared to the other muscarinic subtypes.[145] Functional assays in muscarinic subtype cell lines confirmed that **66** shows highest potency in blocking muscarinic agonist-induced responses in an M_4 cell line, but **66** is only 10-fold less potent in blocking similar effects in an M_2 cell line. Certain snake toxins have also been demonstrated to selectively bind to and antagonize responses at the M_4 receptor subtype.[146−149]



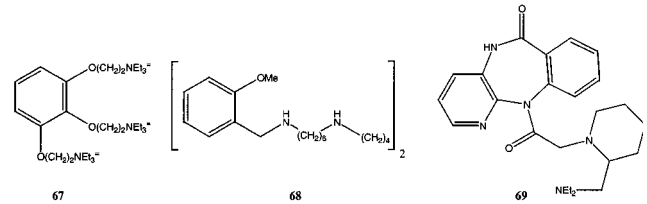
62 **63** **64**



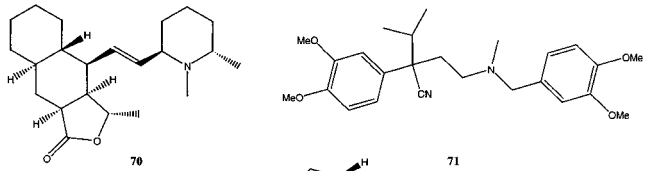
65 **66**

Subtype-Selective Allosteric Modulators. The development of muscarinic agonists and antagonists with high degrees of subtype selectivity has remained relatively elusive probably because of the close sequence homology within the ligand-binding pocket among the muscarinic receptor subtypes. The development of subtype-selective allosteric modulators may be a more promising prospect because of the much more varied sequences in the extracellular domains of the muscarinic subtypes where the allosteric modulators are presumed to bind to the receptor.[150] Another potential advantage of allosteric modulators is the potential for either enhancement or diminution of a particular subtype modulatory effect by acetylcholine or an exogenous muscarinic agonist or antagonist. The magnitude of any increase or decrease in subtype activity could also be controlled by the magnitude of cooperativity produced by a particular allosteric modulator. Regardless of these potential advantages, the number of reports of allosteric modulators conferring muscarinic subtype selectivity remain relatively sparse and the potencies and additional activities of most of these modulators suggest that they are only suitable as lead structures.

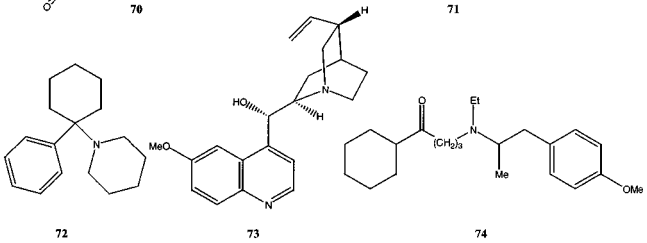
The rank order of allosteric-mediated negative cooperativity for *N*-methylscopolamine (NMS) binding in M_1-M_3 receptors with the cardiac-selective muscarinic antagonists gallamine (**67**), methoctramine (**68**), AF-DX116 (**69**), and himbacine (**70**) is $M_2 > M_1 > M_3$.[151] A similar rank order of negative cooperativity is seen with the ion channel blockers verapamil (**71**), PCP (**72**), quinidine (**73**), and secoverine (**74**).[151]



67 **68** **69**



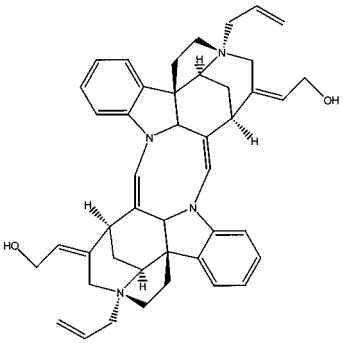
70 **71**



72 **73** **74**

A number of neuromuscular blocking agents have been found to produce differential modulation of muscarinic subtypes through allosteric interactions. Alcuronium (**75**) increases the binding of NMS to M_2 and M_4 receptors but inhibits the binding to M_1, M_3, and M_5 receptors.[152] Brucine (**76**) increases the affinity of acetylcholine for M_1 and M_3 receptors but produces different patterns of affinity augmentation at receptor subtypes with other muscarinic agonists.[153] Other workers also found that acetylcholine affinity for M_1 receptors is increased almost 2-fold by **76**, while M_3 receptor affinity is increased 3-fold by the brucine derivative **77** and 2.6-fold by **78**.[154] These compounds produce no increases or less of an increase in affinity for acetylcholine at the other muscarinic receptor subtypes. By contrast, all three compounds and strychnine (**79**)[155] increase NMS binding at M_2 receptors by at least 2-fold while smaller increases in affinity are seen at the other receptor subtypes. N-Substituted strychnine analogues show various patterns of positive and negative cooperativity with NMS binding, but none of these derivatives increase acetylcholine binding.[154]

Eburnamonine (**80**) augments the binding of acetylcholine to M_2 and M_4 receptors and produces positive cooperativity at M_2 receptors with other muscarinic agonists.[153] Vincamine (**81**) produces positive allosteric



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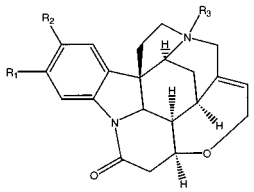
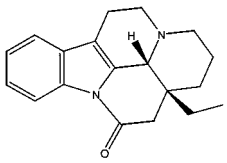


76 R_1R_2=OMe, R_3=H
77 R_1R_2=OMe, R_3=CH_2Cl
78 R_1R_2=OMe, R_3=O
79 R_1R_2=H, R_3=H

effects with some agonists at some subtypes.[153] Recently, a second distinct allosteric site sensitive to staurosporine and related indolcarbazoles has been described for M_1-M_4 receptors which is distinct from the site which binds strychnine-related compounds.[156] This opens up additional avenues for drug development based on allosteric modulation of muscarinic receptors.



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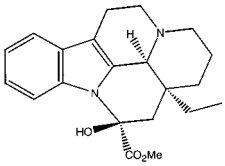

81

The therapeutic potential of allosteric modulators should span all of the potential uses of selective agonists and antagonists since the therapy would only depend on the subtype being addressed and the degree and direction of the cooperativity produced by the modulator. Clearly, the work on allosteric modulators of muscarinic receptor subtypes remains in relative infancy compared to work toward subtype-selective muscarinic agonists and antagonists. Progress in the development of subtype-selective modulators should benefit from the application of broad screening techniques that are now pervasive in the pharmaceutical industry.

Schizophrenia

Schizophrenia is a severe psychosis afflicting more than 1% of the population worldwide, and about 100 000 new cases are diagnosed each year. The predominant symptoms of schizophrenia are classified as positive (hallucinations, delusions, vocal outbursts, hearing of voices), negative (attention deficits, social withdrawal, poverty of thought and speech, anhedonia, anergia), and cognitive (deficits in short term and executive memory). Classical neuroleptics, such as haloperidol (Haldol) and chlorpromazine (Thorazine), are effective in treating the positive symptoms of the disease and were discovered based on their ability to block dopamine receptors.[157] However, these compounds induce side effects such as extrapyramidal symptoms (EPS) and hyperprolactinemia. Newer "atypical" antipsychotics, such as clozapine and olanzapine, are effective against both positive and negative symptoms, have fewer tendencies to cause EPS and hyperprolactinemia, and therefore provide a significant improvement over previous treatments. However, these agents require several weeks before their therapeutic effects are fully realized and are relatively ineffective against cognitive deficits. Moreover, clozapine can induce a potentially fatal blood disease, agranulocytosis, in a small percentage of patients and therefore requires constant blood monitoring during treatment. Alternative targets for therapeutic intervention have been proposed including serotonin, dopamine, glutamate, and muscarinic receptors in an attempt to provide drug therapies for treatment-resistant patients, increase the speed of therapeutic benefit, and improve the cognitive symptoms.

Muscarinic receptors are particularly compelling as therapeutic targets for the treatment of schizophrenia based on evidence that muscarinic antagonists produce a psychosis in humans with symptoms similar to the positive and negative behaviors and cognitive deficits associated with the disease.[158−162] Hallucinations are one of the most debilitating components of schizophrenia and degenerative neurological diseases. Certain drugs and substances of abuse can induce hallucinations in humans suggesting that receptors for these compounds may play a role in generating hallucinations and may be attractive therapeutic targets to treat these debilitating symptoms. For example, ketamine and phencyclidine (NMDA receptor ligands), muscimol and β-carbolines (GABA receptor ligands), LSD (serotonin receptor ligand), and cocaine (dopamine transporter ligand) induce hallucinations that resemble those seen in neurological diseases. Cholinergic antagonists induce a psychosis that includes delusions and hallucinations which are most likely mediated through muscarinic receptors since nicotinic receptor antagonists, such as mecamylamine, are not inherently hallucinogenic. In particular, the muscarinic antagonists, quinuclidinyl benzilate (QNB), scopolamine, and atropine induce a psychosis in humans that includes schizophrenic-like auditory and visual hallucinations.[158,159,161,162] At low doses, the antagonists induce visual hallucinations that involve clearly defined objects or people and auditory hallucinations that include music and voices that inspire dialogue. At higher doses of muscarinic antagonists, however, the hallucinations progress to severe delusions and loss of awareness. Patients with a previous history of psychosis, those suffering schizophrenia, and Parkinson's patients being treated with anticholinergics are particularly susceptible to muscarinic antagonist-induced hallucinations.[161,163,164]

The pathophysiology of schizophrenia is thought to be related to a relative increased dopaminergic neurotransmission.[165] This hypothesis is supported by pharmacological evidence that agents that stimulate the dopamine system induce the positive symptoms of schizophrenia and antidopaminergics relieve these symptoms.[157,166] Interest in cholinergic treatment of schizophrenia predates the dopamine hypothesis.[167] Early clinical studies with cholinergic agonists[168,169] and the acetylcholinesterase inhibitor physostigmine[170,171] suggested that cholinergic receptor stimulation improved the psychotic symptoms of schizophrenics. However, this approach had limited acceptance due to the poorly tolerated cholinergic side effects associated with these nonselective agents. An understanding of the relationship between the cholinergic and dopaminergic system was emerging which suggested that they were normally in balance and reciprocally regulated each other. It was speculated that in schizophrenia, an imbalance existed

between the dopamine and acetylcholine system that could possibly be corrected with muscarinic agonist therapy.[171,172] Since the dopamine and muscarinic systems reciprocally modulate each other, and since muscarinic antagonists induce the positive symptoms of schizophrenia, it follows that muscarinic agonists may be useful in reducing the positive symptoms associated with schizophrenia and other psychosis.

Additional evidence has accumulated supporting the hypothesis that muscarinic agents may be useful in the treatment of psychosis based on an interaction with the dopamine system. Pharmacological evidence showed that the dopamine and muscarinic systems reciprocally modulate each other.[173−175] Muscarinic agonists or cholinesterase inhibitors were shown to depolarize neurons expressing dopamine receptors[176] and increase dopamine release in the nucleus accumbens when injected near the VTA.[177] Dopamine release may be modulated through a muscarinic heteroreceptor on dopaminergic neurons with pharmacological properties of an M_1 receptor.[178−180] More recently, xanomeline, which has a mixed partial agonist and antagonist profile at the muscarinic receptor subtypes, reduced psychotic symptoms of patients with Alzheimer's disease including hallucinations and delusions.[181] A chemically related muscarinic compound, PTAC, with partial M_2/M_4 agonist and $M_1/M_3/M_5$ antagonist properties was suggested as a potential treatment for schizophrenia based on preclinical data with similarities to atypical neuroleptics such as clozapine and olanzapine.[76] PTAC inhibited conditioned avoidance responding in rats without inducing catalepsy seen with similar doses of haloperidol suggesting that its action is not due to nonspecific motor effects. PTAC also blocked apomorphine-induced climbing in mice suggesting that its mechanism of action involved modulation of the dopamine system. Moreover, in in vivo electrophysiological experiments, PTAC inhibited the number of spontaneous firing A10 dopamine cells in the VTA relative to the substantia nigra but had little if any effect on the firing of substantia nigra A9 dopamine cells suggesting low EPS liability. In contrast, haloperidol blocks the spontaneous firing of both A9 and A10 cells which may contribute to the EPS seen with this compound. Atypical neuroleptics with little motor side effects such as clozapine and olanzapine also do not appreciably affect A9 cell-firing rates. PTAC, however, inhibited A10 cell firing after both acute and chronic treatment suggesting that it may have a rapid onset of action. PTAC had no affinity for either dopamine or serotonin receptors suggesting it has a unique mode of action through muscarinic receptors.

Identifying the muscarinic receptor subtype(s) associated with dopamine regulation is a challenging problem since all five muscarinic receptors are found in brain areas rich in dopamine neurons. However, some differences in receptor distribution have been observed for both mRNA and protein. Of the five known muscarinic receptor subtypes, M_1 and M_4 receptors are most abundant in forebrain areas thought to be involved in schizophrenia (cerebral cortex, basal ganglia, hippocampus).[182] M_1 and M_4 receptors in particular are colocalized with dopamine receptors in caudate-putamen and cerebral cortex.[183,184] More recently, mice lacking the M_4 receptor were hyperactive and hyperresponsive to DA_1 agonists suggesting a role for the M_4 receptor in the tonic suppression of the dopamine system.[64]

The M_2 receptor follows an opposite distribution pattern with highest densities in brainstem and in lower abundance than M_1 and M_4 in the forebrain. M_2 receptors are located on cholinergic interneurons of the striatum and tegmental cholinergic neurons and are considered to be the predominant autoreceptor regulating acetylcholine release.[184,185] It has been suggested that the psychotogenic actions of antimuscarinic agents may be mediated predominantly through the M_2 autoreceptors located on Ch5 and Ch6 cholinergic tegmental neurons.[186] The Ch5 and Ch6 neurons project to dopamine-rich areas of the VTA and substantia nigra and are the only known cholinergic inputs to these areas.[187−190] In support of this proposal, postmortem brain tissue obtained from schizophrenics was found to have a higher density of Ch5 and Ch6 cholinergic fibers.[191] It should be noted that some investigators have not found changes in cholinergic neurons of schizophrenic brain tissue.[192] M_2 receptors are also widely distributed on noncholinergic cells,[183,193] and their function on these cells is unknown.

The M_5 receptors are predominantly localized to the substantia nigra pars compacta and VTA and are thought to be the receptor responsible for acetylcholine-mediated dopamine release. It has been suggested that it is the only muscarinic receptor subtype co-localized with D_2 receptor mRNA in the substantia nigra and VTA.[183] Recent studies using RT-PCR and a pharmacological labeling approach suggest that the M_5 receptor is more widely distributed than previously considered.[60,194] M_5 receptor distribution is distinct from the other four subtypes with significant labeling in outer layers of the cortex, hippocampus, caudate putamen, olfactory tubercle, and highest in substantia nigra. M_3 receptors are found in cerebral cortex, thalamus, and hippocampus but not in the basal ganglia[195] and may have similar functions as the M_1 receptor in these areas.

As described above, the molecular mechanism of antimuscarinic-induced psychosis has not been clearly defined and would benefit from receptor subtype-selective pharmacological agents. Treatment of schizophrenia with muscarinic agents may provide significant therapeutic utility when treating the positive and negative symptoms with the added benefit of improving cognitive function. A muscarinic ligand might also be useful in treating the psychosis and dementia associated with neurodegenerative diseases such as Alzheimer's disease, Lewybody disease, and Parkinson's disease. The complexity of the cholinergic nervous system and the overlapping distribution of the five muscarinic receptor subtypes within the brain present a significant challenge to the development of muscarinic-based therapeutics for psychosis and other indications.

Alzheimer's Disease

Alzheimer's disease (AD) is a disorder characterized by progressive age-related impairment of neurological function. The clinical manifestations include progressive loss of cognition (dementia) as well as psychosis, loss of reasoning ability, agitation, reduction in personal care, and other disturbances of affect.[196−198] Patients are usually impaired in two or more areas of cognition

including, memory, language, visiospatial function, and attention. Postmortem brain tissue samples from AD patients consistently show a neuropathology characterized by progressive accumulation of amyloid plaques and neurofibrillary tangles, shrinkage and death of neurons, and inflammatory changes marked by accumulation of activated microglia.[199−202] These changes are associated with degeneration of neurons expressing cholinergic, serotonergic, glutamanergic, and neuropeptide neurotransmitter systems.[203]

The loss of cholinergic neurons in particular may contribute to the etiology of AD due to their localization in memory-forming areas of the brain and participation in cognitive function. Anatomical evidence has shown that cholinergic innervation projects from the nucleus Basalis of Meynart to hippocampus, amygdala, and neocortex,[204−207] which are considered to be neural substrates for learning and memory[208−210] and for visual representation and judgment.[211] Cerebral cortex, hippocampus, and amygdala show extensive AD pathology,[212,213] reduction in acetylcholine levels,[205,214−216] and loss of cholinergic innervation.[209,210,217,218] The loss of the acetylcholine-synthesizing enzyme, choline acetyltransferase, was observed in cerebral cortex and hippocampus from postmortem AD tissue.[214,215,219]

Cholinesterase Inhibitors. The cholinergic hypothesis assumes that replacement of acetylcholine, which is progressively lost during the course of AD, would potentially delay and possibly restore losses in cognitive abilities. Two approaches have been used to test this hypothesis including using cholinesterase inhibitors to maintain synaptic levels of acetylcholine[220,221] or alternatively administration of synthetic agonists to stimulate acetylcholine receptors of either the muscarinic or nicotinic family.[222,223] Cholinesterase inhibitors were shown to reverse cognitive loss in rodents following lesions of the cholinergic system projecting to hippocampus and cortex.[224,225] Previous studies showed cognitive loss in rats after lesions were made in cholinergic memory circuits.[226−229] Using another approach, spatial memory impairment in rats resulting from lesions of the basal forebrain cholinergic projection system were partially reversed by brain implantation of acetylcholine-producing cells.[230] On the basis of a plethora of preclinical and clinical data, cholinesterase inhibitors are currently the only approved treatment for cognitive deficits associated with AD. Donepezil (Aricept), tacrine (Cognex), and rivastigmine (Exelon) are approved for use in the United States, and three compounds have received European approval, tacrine being the first and, more recently, donepezil and rivastigmine (ENA-713). Other compounds under investigation include galantamine, physostigmine, eptastigmine, and metrifonate. Currently it is believed that cholinesterase inhibitors act primarily by increasing available acetylcholine within the active synapse thereby restoring deficient cholinergic neurotransmission. Mechanistically, tacrine, donepezil, galantamine, and physostigmine are reversible inhibitors of acetylcholinesterase and butyrylcholinesterase, rivastigmine is a pseudoirreversible inhibitor of these enzymes, while metrifonate is considered to be an irreversible inhibitor. Early clinical trials with cholinesterase inhibitors, such as tacrine, showed modest but statistically insignificant effects on cognitive performance even when combined with the acetylcholine precursor lecithin.[231] Subsequent trials allowing increased dosing showed significant improvement in measures of cognitive performance but were still modest in outcome due in part to dose limiting side effects including GI disturbances and hepatotoxicity.[232,233] Donepezil was shown to be potentially more effective than tacrine due to a lower side effect profile and possibly due to its higher selectivity for acetylcholinesterases over butyrylcholinesterases. However, only modest improvements in cognitive function were observed in early stage AD patients with this compound.[234] Physostigmine has undergone clinical testing and consistent with this class of compounds has shown marginal effectiveness with both statistically significant[235,236] and insignificant outcomes.[237]

Cholinesterase inhibition has several obvious drawbacks. These compounds depend on an intact cholinergic system still capable of synthesizing acetylcholine; therefore, they may be most useful in early stages of AD and lose effectiveness over time. The presence of M_2 autoreceptors would potentially respond to acetylcholine and have opposing actions on acetylcholine release. Selective cholinesterase inhibitors, free of dose-limiting side effects, are not currently available, and current compounds may not allow sufficient modulation of acetylcholine levels to elicit the full therapeutic response. For this reason alternative approaches have been considered including direct stimulation of either muscarinic or nicotinic acetylcholine receptors.

Muscarinic Receptor Agonists. Pharmacological evidence suggests that muscarinic and possibly nicotinic receptors play an important role in learning and memory. For example, muscarinic antagonists disrupt short-term memory in rats[238−240] and mice.[241] The muscarinic receptor antagonist scopolamine disrupts memory in humans[242] which can be reversed with the cholinesterase inhibitor physostigmine.[235] Nicotinic receptors may also offer a viable therapeutic target for cognitive enhancement due to their location in memory-forming areas of the brain. The $\alpha4\beta2$ receptor subtype may be the most interesting target of the nicotinic receptor family due to its higher abundance in brain, though levels of this receptor are several orders of magnitude lower in density compared to muscarinic receptors. Nicotinic receptors are located on presynaptic cholinergic terminals, and nicotinic agonists increase acetylcholine release resulting in a feed-forward effect on cholinergic transmission. However, nicotinic antagonists do not induce cognitive impairment seen with muscarinic antagonists suggesting a less direct involvement in the memory process.

Determination of which of the five muscarinic receptor subtypes are involved in memory formation and storage has been hampered by the lack of receptor subtype-selective ligands. Due to its high abundance in hippocampus and cortex, the M_1 receptor may be largely involved in memory formation.[62,243] Moreover, pirenzepine, which is an M_1 receptor-preferring antagonist, disrupts memory in rats and mice[238,240,241] though involvement of other subtypes cannot be completely ruled out. A recent study found that muscarinic agonist potentiation of *N*-methyl-D-aspartate receptor currents in rat hippocampal neurons, believed to be a predomi-

nant mechanism for cholinergic modulation of hippocampal function, was mediated by M_1 receptors, supporting the role of this subtype in learning and memory.[244] These findings suggest that the M_1 receptor may be an attractive target for cognitive enhancement in AD. Radioligand binding studies on postmortem brain tissue from AD patients demonstrated a loss of M_2 receptors that are thought to be predominantly presynaptic and a preservation of postsynaptic M_1 receptors.[245] M_1 agonist replacement therapy has been considered by several groups as potentially more promising than development of cholinesterase inhibitors, since the latter approach depends on acetylcholine release from presynaptic neurons that are progressively lost during the course of the disease. The first attempts at muscarinic replacement therapy were unsuccessful due to the lack of selectivity of the muscarinic agonists and associated muscarinic side effects. Cholinergic side effects include salivation, lacrimation, and GI disturbances. This is supported by the lack of effectiveness of bethanecol and arecoline in the treatment of AD.[246,247] Development of muscarinic compounds that are truly receptor subtype selective has not been achieved leaving the hypothesis partly untested. The first clinical evidence that a direct muscarinic agonist with significant M_1 activity would improve cognitive function in patients with AD was shown with xanomeline.[181] The xanomeline trial resulted in significant improvements in cognition, despite dose-limiting adverse events. Its favorable effects on psychotic-like behaviors of AD suggest that muscarinic agonists may be useful for the treatment of schizophrenia or other psychosis.

Use of direct-acting agonists for the M_1 receptor in the treatment of AD may have limitations. A recent report demonstrated an apparent decrease in the levels of M_1 receptor protein in AD brain tissue as determined by immunoprecipitation with subtype-selective antibodies, although the same study showed no change in M_1 receptors as determined by radioligand binding in agreement with previous work.[248] Although not demonstrated directly, this finding suggests altered M_1 receptor structure in AD brain, a suggestion further supported by studies demonstrating impaired M_1 receptor functional activity in the disease.[249−251] More recently reduction in high-affinity binding of M_1 receptors in AD brain was noted suggesting reduced efficiency of receptor−G protein interactions resulting in loss of signal transduction efficiency.[252] It is not clear if sufficient M_1 receptor reserve exists in AD brain areas regulating cognitive function to compensate for these partial reductions in muscarinic receptor function. It may also be possible to delay or greatly reduce the loss of M_1 receptor function if M_1 agonist replacement is initiated early in the disease.

In addition to the involvement of the cholinergic system in cognitive function, muscarinic receptors have been implicated in the control of amyloid precursor protein (APP) processing. The etiology of AD is in part due to the abnormal processing of APP leading to the formation and deposition of a neurotoxic fragment, amyloid-β peptide (Aβ). The senile plaques of AD consist predominantly of a 39−43-residue of Aβ. Aβ is produced by proteolysis of the much larger APP. Nitsch and colleagues first demonstrated that muscarinic agonist stimulation of HEK293 cells transfected with M_1 and M_3 receptors (but not M_2 or M_4) resulted in increased release of a soluble N-terminal derivative of APP (APPs).[253] AAPs is derived from the secretory pathway whose activation is believed to reduce the formation of amyloidogenic Aβ.[254] Subsequently, it was shown that activation of M_1 receptors on cells containing either normal or Swedish mutant forms of APP resulted in significant reductions in Aβ release into the medium.[255] In addition to the muscarinic receptors, other GPCRs linked to the stimulation of phosphatidylinositol hydrolysis (i.e. $5HT_{2a}$, $5HT_{2c}$, $mGluR_{1\alpha}$, bradykinin) have also been shown to stimulate nonamyloidogenic APPs release from transfected cells.[256] In this study, release of APPs could be stimulated in superfused brain slices by electrical depolarization and a significant component of this effect could be blocked by atropine indicating involvement of muscarinic receptors. These workers also demonstrated that APPs release from brain slices could be stimulated by agonists with partial selectivity for M_1 receptors, but not by nonselective agonists. Important observations on APP processing in vivo have been made by Haroutunian and colleagues who demonstrated that excitotoxic lesions of the basal forebrain cholinergic projection system in rats resulted in upregulation of APP mRNA in the cortex accompanied by increased levels of a soluble APP derivative in the CSF.[257] Lesions of rat basal forebrain cholinergic neurons in vivo with 192IgG-saporin have been shown to increase the amount of membrane-associated APP and reduce the amount of soluble (presumably secreted) APP in the cortex, changes that were reversed by restoring cholinergic function via implantation of fibroblasts secreting nerve growth factor.[258] Signal transduction associated with M_1 receptor activation leads to modulation of APP release.[254] These studies indicate that M_1 receptors in vivo may affect APP processing in the same way as observed in cell systems in vitro. In addition, muscarinic agonists have been shown to reduce the phosphorylation state of tau protein in cultured cells as well as in vivo.[259,260] Hyperphosphorylated tau is the major component of neurofibrillary tangles in AD brain.[261] Therefore, muscarinic receptor-mediated regulation of APP processing and tau phosphorylation suggest the possibility that muscarinic agonist replacement may have the added benefit of affecting the progression of AD by inhibiting those processes which contribute to accumulation of plaques and tangles.

AD like other neurodegenerative diseases is the result of multiple abnormalities, all of which have not been fully recognized. The development of a therapeutic designed to help a patient repair or recover from a genetic defect may be beneficial but may only impact a fraction of the total patient population. A full understanding of this disease could easily require decades of challenging research that may do little to help a rapidly aging population of middle-aged adults. It has been shown that palliative treatments, though far from ideal, can significantly reduce the emotional and financial burdens of both patient and family. An approach focused on reducing or delaying cognitive decline and difficult behaviors through agonist replacement therapy has yet to be adequately evaluated.

Parkinson's Disease

Parkinson's disease is characterized by four predominant symptoms: bradykinesia (slow and difficult movement), muscular rigidity, resting tremor that abates during a voluntary movement, and problems maintaining balance which is seen as a disturbed gait and possible falling. The symptoms result from a loss of dopaminergic neurons that project to the striatum (caudate and putamen) from the substantia nigra pars compacta beyond what is normally expected during the aging process. Late stage disease progresses from akinesia to immobility. A parkinsonism-like state can be induced by dopamine-blocking drugs such as the neuroleptics, haloperidol and chlorpromazine, and the antiemetics, prochloperazine and metoclopromide. Other neurological diseases also can induce these symptoms such as less common neurodegenerative diseases and stroke. Current treatments include muscarinic antagonists such as benztropine and trihexyphenidyl and dopamine replacement regiments such as the dopamine precursor levodopa or the dopamine agonists bromocriptine and pergolide.

As in schizophrenia, the predominant neurochemical abnormality in parkinsonism results from disturbances in the balance between the cholinergic and dopaminergic systems. Loss of dopaminergic neurons results in a predominance of the cholinergic system resulting in the tremor and movement disorders. This hypothesis is supported by clinical observations showing that cholinergic agonists and cholinesterase inhibitors induce or exacerbate parkinsonian tremors.[262−266] Muscarinic antagonists, although effective at treating movement disorders associated with parkinsonism, have a tendency to induce undesirable side effects including psychosis as described above. Anticholinergics currently prescribed are relatively nonselective for the individual muscarinic receptor subtypes providing little information at the mechanistic level as to their sites of action. Animal models have helped investigators understand the neurochemical basis for Parkinson's disease in relationship to the dopamine system, but little information is available about which muscarinic receptor subtypes modulate the dopamine system in the basal ganglia.

Recent studies have focused on vacuous jaw tremors in rodents as a model of human resting parkinsonian tremor. Muscarinic agonists such as pilocarpine induce a parkinsonian jaw tremor in rodents that oscillates at a similar frequency to jaw and hand tremors observed in human Parkinson's patients.[267] Pharmacological characterization of the muscarinic subtypes involved in the jaw tremor is consistent with regulation by the M_4 receptor.[268] Dopamine depletion studies and direct injections of muscarinic agonists and antagonists into brain regions indicated that regulation of tremor is initiated predominantly in the ventrolateral striatal area and that the substantia nigra also plays an important role.[269] The distribution of muscarinic receptors in basal ganglia is described above and indicates that all five receptor subtypes, in particular the M_1, M_4, and M_5 receptors, are expressed in significant levels in the neostriatum and on dopamine cells. Selective muscarinic ligands and muscarinic receptor knockout mice should help support the hypothesis that the M_4 receptor plays a key role in tremor control. These data suggest that a selective M_4 antagonist might offer an improvement over current muscarinic antagonist therapies for parkinsonian tremor.

Conclusion and Future Directions

As described in this review, muscarinic ligands have had a long history in the treatment of central nervous system disorders. Previous compounds, including those more recently developed such as xanomeline and milameline for AD-related cognitive decline, offer limited therapeutic benefit due to their lack of selectivity resulting in dose-limiting side effects. Advances in molecular biochemistry and genetics have provided a new collection of research tools creating new therapeutic development opportunities for muscarinic and other receptor families. The discovery and development of more selective ligands will occur through screening of larger and more chemically diverse compound libraries with the help of sensitive in vitro assays run in parallel across receptor families. Knockout mice and other forms of genetically manipulated animal models will deepen our understanding of receptor subtype function and help unravel their complex interactions with other proteins. Hopefully the genetic revolution will help physicians improve their detection, and possibly prediction of neural disease, allowing earlier therapeutic intervention during and before the disease process. Even with improved diagnostics and knowledge of disease mechanism, the ultimate goal of halting or preventing disease progression may be years away. For this reason, palliative treatments for AD and other diseases still remain very attractive as primary and adjunctive therapies.

The cholinergic hypothesis predicts that loss of cholinergic function plays a significant role in the cognitive decline observed in AD.[270] Two or more decades of effort leave cholinesterase inhibitors as the only FDA-approved treatments currently available for AD-related cognitive decline. The marginal efficacy of cholinesterase inhibitors and difficulty of developing selective muscarinic agonist-based therapeutics has softened the enthusiasm for the muscarinic agonist approach to the treatment of cognitive decline, psychosis, and other CNS diseases. The new challenge is to provide solid evidence for the role of the M_1 receptor in modulating postsynaptic function and demonstrating that an M_1 agonist alone is sufficient to restore cognitive acuity. It will also be important to understand the relative contribution of M_2 receptor blockade and if this activity alone is sufficient to achieve robust therapeutic effects superior to current therapies. Most likely, the progressive basal forebrain cholinergic deafferentation that occurs in AD will quickly obviate this mechanistic approach. Combinations of M_1 agonism, M_2 antagonism, and cholinesterase inhibition will be investigated for efficacy and will be optimized based on patient response and genetic profile. Preclinical models of cognitive performance are now providing better information about efficacy at the various cognitive domains such as attention, recent and long-term memory, spatial learning, and possibly executive function. It will be important to determine if therapeutic balance of nicotinic receptor versus muscarinic receptor modulation has the potential to provide even greater benefit and which cognitive domains are most impacted.

Treatments for schizophrenia and psychosis have centered on modulation of the dopamine system, and remarkable improvements have been made in treating both positive and negative symptoms associated with the disease using atypical antipsychotic medications. However, atypical antipsychotics such as clozapine are based on complex mechanisms that are not fully understood and have limited usefulness in treating cognitive deficits associated with psychosis. Reciprocal modulation between the dopamine and acetylcholine systems suggests that muscarinic receptor-based medications would offer an alternative approach that could also restore cognitive deficits. The challenge is to understand the relative contributions of each muscarinic receptor subtype, and possibly nicotinic receptor subtypes, to the positive, negative, and cognitive symptoms of psychosis.

Pharmacological and behavioral data support a role for the M_2 and M_3 receptors in mediating many of the parasympathomimetic cholinergic symptoms associated with muscarinic ligands. Recent studies using muscarinic receptor knockout mice have largely supported previous pharmacological findings. Therefore, stimulation of M_2 and M_3 receptors appears to be responsible for many of the side effects observed with previous muscarinic agonists. If M_2 and M_3 receptor activity is avoided in future compounds, will they retain their usefulness as therapeutics? The answer to this key question will determine the success of muscarinic-based efforts since M_2 and M_3 receptors are located in areas of the brain involved in cognition, psychosis, and movement control. Peripheral cholinergic-regulated physiology is regulated in part through central mechanisms. The role of each muscarinic receptor subtype in central versus peripheral physiology has yet to be explored in detail. Successful design of novel selective ligands devoid of cholinergic side effects will require a deeper understanding of these interactions especially as they affect the cardiovascular and gastrointestinal systems. Although the use of receptor knockout animals has already been helpful in this regard, there are concerns about the limitations of this approach. The nervous systems of receptor knockout animals may undergo significant developmental changes in brain architecture to compensate for loss of the gene target. These potential rearrangements may confound interpretation of data derived from these animals. Alternative genetic approaches are possible which provide conditional and localized regulation of receptors in adult animals. Hopefully these models will be available soon. The slow decline in interest in cholinergic-based therapeutics that has occurred over the past decade is beginning to be revitalized through the influence of modern molecular biochemistry. In general, GPCRs have proven to be useful therapeutic targets for many historical and modern pharmaceuticals. On the basis of recent learning, the next decade should prove particularly exciting and productive for the muscarinic receptor family.

Biographies

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