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MINIREVIEW

Importance and Prospects for Design of Selective Muscarinic Agonists

J. JAKUBÍK, P. MICHAL, E. MACHOVÁ, V. DOLEŽAL

Department of Neurochemistry, Institute of Physiology, Czech Academy of Sciences, v.v.i , Prague, Czech Republic

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Abstract

There are five subtypes of muscarinic receptors that serve various important physiological functions in the central nervous system and the periphery. Mental functions like attention, learning, and memory are attributed to the muscarinic M_1 subtype. These functions decline during natural aging and an early deficit is typical for Alzheimer´s disease. In addition, stimulation of the M_1 receptor increases non-amyloidogenic processing of the amyloid precursor protein and thus prevents accumulation of noxious β-amyloid fragments. The selectivity of classical muscarinic agonists among receptor subtypes is very low due to the highly conserved nature of the orthosteric binding site among receptor subtypes. Herein we summarize some recent studies with the functionally-selective M_1 agonist xanomeline that indicate complex pharmacological profile of this drug that includes interactions with and activation of receptor from both orthosteric and ectopic binding sites, and the time-dependent changes of ligand binding and receptor activation. These findings point to potential profitability of exploitation of ectopic ligands in the search for truly selective muscarinic receptor agonists.

Key words

Cholinergic transmission • Xanomeline • Muscarinic receptor subtypes • G-proteins activation • Ectopic agonists

Corresponding author

V. Doležal, Department of Neurochemistry, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220 Prague 4, Czech Republic. Fax: +(420) 29644 2488. E-mail: dolezal@biomed.cas.cz

Introduction

Cholinergic neurons serve important functions both in the periphery and the central nervous system. Signals from presynaptic cholinergic terminal mediated by released acetylcholine (ACh) are transmitted to postsynaptic cells through two types of acetylcholine receptors, nicotinic and muscarinic. Nicotinic ACh receptors are non-selective cation channels whose activation leads to depolarization of postsynaptic cells. They are heteropentamers assembled from α, β, γ, δ, and ε subunits or in the case of certain central nicotinic receptors homopentamers composed of specific α-subunits only (Gotti *et al*. 2006). Muscarinic receptors belong to a family of GTP binding protein (G-protein)-coupled receptors that together represent the largest family of plasma membrane receptors (Lander *et al*. 2001, Fredriksson *et al*. 2003). Their stimulation leads to activation of specific G-proteins that transduce extracellular mediator messages to specific intracellular signaling pathways. A common feature of all G-protein-coupled receptors is that they are single proteins that comprise seven transmembrane domains. To date five subtypes of muscarinic receptors denoted as M_1-M_5 and encoded by five different genes have been discovered (Bonner *et al*. 1987, 1988, Peralta *et al*. 1987, Bonner 1989a,b).

Muscarinic and nicotinic receptors are widely expressed in both the central nervous system and in the periphery with distinct cellular as well as tissue localization of individual subtypes. Within the central nervous system, cholinergic neurotransmission is of utmost importance for mental functions such as learning, memory, and attention. The efficiency of mental functions becomes impaired during normal aging and this impairment has generally been ascribed to decreases in nerve cell number. Recent studies on primates and other mammals indicate, however, that atrophy of cholinergic

neurons in the basal forebrain may be more important for the impairment of mental functions than the comparatively small diminution of the total neuronal population in the brain (Smith *et al*. 1999, Conner *et al*. 2001, review Morrison and Hof 1997). Impaired neurotransmission *via* cholinergic synapses involves diminished release of acetylcholine, attenuation of the response of postsynaptic cells, and decreased density of the cholinergic innervation of the brain cortex and hippocampus. Recent studies indicate that the atrophy of basal forebrain cholinergic neurons is reversible (Smith *et al*. 1999, Conner *et al*. 2001). Loss of different types of neurons accompanied by deterioration of neural functions is known to occur in a number of neurodegenerative conditions such as Parkinson´s disease, Alzheimer´s disease, Huntington´s disease, Wilson´s disease and others. The reasons why certain groups of neurons degenerate, as well as the pathogenetic mechanisms responsible for the degeneration are not clear (reviewed in Sarter and Bruno 1998, 2004, Mehler and Gokhan 2000, 2001).

Roles of brain muscarinic receptors

As mentioned above, cholinergic transmission plays an important role in mental functions like attention, learning, and memory. These functions decline in the course of natural aging and accelerated deficit of cognitive functions is a typical symptom of Alzheimer's disease. At present, Alzheimer's disease is the most common neurodegenerative disease of the central nervous system whose incidence increases steadily. Original neurochemical findings of disturbances of acetylcholine metabolism (Bowen *et al*. 1976, Davies and Maloney 1976, Perry *et al*. 1977a,b, Sims *et al*. 1981, Francis *et al*. 1985) became basis for the „cholinergic hypothesis“ of Alzheimer´s disease (Bartus *et al.* 1982, Francis *et al*. 1999, Doležal and Kašparová 2003, Mesulam 2004). Since then a large body of evidence both supporting and questioning this hypothesis has accumulated (Bartus 2000).

One of the most important issues is whether disturbances of cholinergic mechanisms are present early in the pathogenesis of Alzheimer's disease or are simply a reflection of a general neurodegeneration that afflicts many neurotransmitter systems in the late or terminal stage of the disease. This issue is crucial for designing potential therapeutic approaches. In addition to the involvement of brain muscarinic transmission in mental functions it was demonstrated that stimulation of M_1 and M_3 subtypes of muscarinic receptors leads to non-amyloidogenic cleavage of the amyloid precursor protein (Buxbaum *et al*. 1992, Nitsch *et al*. 1992), leading to decreased production of noxious beta-amyloid fragments responsible for disease genesis and progression. The attenuation of muscarinic transmission early in pathogenesis of the disease may thus result in accelerated progression of the disease or could even participate as the primary insult in some of nonhereditary cases of the disease (Meier-Ruge 1994, 1996, Messier and Gagnon 1996, Hoyer 2000). Involvement of muscarinic receptors in regulation of the production of β-amyloid in human is further supported by the observation of its increased accumulation in Parkinson´s disease patients treated with antimuscarinic drugs (Perry *et al.* 2003). Additional support for muscarinic receptor signaling involvement comes from recent findings of early impairment of muscarinic receptor-G-protein coupling in a transgenic mice model of Alzheimer´s disease (Machová *et al*. 2008) correlating with the increase of amyloid-$\beta_{1\text{-}42}$ production (Savonenko *et al*. 2005) and the attenuation of M_1 receptor activation in Alzheimer´s brain cortex obtained at autopsy in human (Tsang *et al*. 2005).

Possibilities of muscarinic receptor subtype-specific interventions

Given the broad range of functions that muscarinic receptor subserve, it is of fundamental importance to find subtype-selective ligands for therapeutic use in specific disorders. Selectivity of physiological stimulation of muscarinic receptors is provided by the tissue localization of nerve endings and the firing pattern of cholinergic neurons. In contrast, exogenous ligands access all muscarinic receptors equally. Muscarinic receptors have a classical (orthosteric) binding site for natural or exogenous ligands located deep in a pocket created by the transmembrane segments of the receptor protein. This site is highly conserved among individual muscarinic receptor subtypes (Hulme *et al*. 2003). Some muscarinic antagonists display good subtype selectivity as their relatively bulky structure allows them to interact with less conserved amino acids adjacent to the orthosteric binding domain. In contrast, orthosteric agonists generally show poor selectivity as they are usually small and interact solely with few key amino acids in the conserved orthosteric binding site.

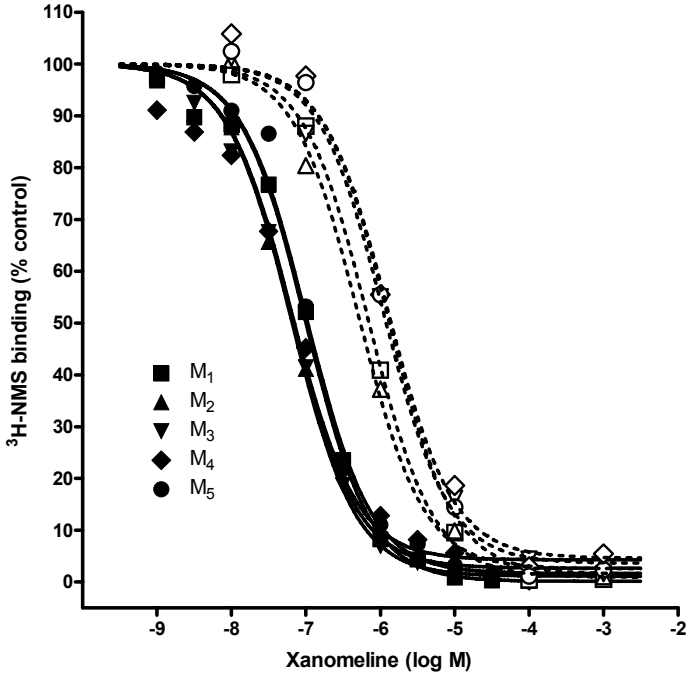

Fig. 1. Interactions of xanomeline with muscarinic receptor subtypes. **Upper panel:** Displacement of 1 nM [^{3}H]-N-methylscopolamine binding by the indicated concentrations of xanomeline (abscissa, log M) when present together with xanomeline (closed symbols) or after preincubation with the indicated concentrations of xanomeline followed by washing in the absence of free drug (open symbols). Data are derived from experiments performed on receptors expressed in membranes of CHO cells (Jakubík *et al*. 2006, Machová *et al*. 2007). Ordinate, specific [^{3}H]-N-methylscopolamine binding is expressed as percent of control binding in the absence of xanomeline treatment. **Lower panel:** IC_{50} of reversible and wash-resistant xanomeline binding.

	IC_{50} (log M)				
receptor subtype	**M_1**	**M_2**	**M_3**	**M_4**	**M_5**
reversible binding	**-7.00+/-0.04**	**-7.19+/-0.03**	**-7.19+/-0.02**	**-7.19+/-0.06**	**-6.99+/-0.02**
wash-resistant binding	**-6.16+/-0.02**	**-6.29+/-0.05**	**-5.95+/-0.06**	**-5.89+/-0.09**	**-5.89+/-0.05**

There are several possibilities for subtype-specific modulation of muscarinic transmission. A high degree of muscarinic receptor subtype selectivity can be achieved using allosteric modulators that increase or decrease affinity of classical agonists and antagonists including the natural ligand ACh. A large number of such allosteric ligands has been described (Tuček *et al*. 1990, Proška and Tuček 1994, Jakubík *et al*. 1995, 1997, Lazareno and Birdsall 1995, Doležal and Tuček 1998, Tuček *et al*. 1990, 1998, Lazareno *et al*. 2004, Jager *et al*. 2007). It has also been shown that allosteric modulators can directly induce activation of G-proteins in the absence of agonists (Jakubík *et al*. 1996, 1998). However, this type of activation is much less efficacious than that induced by full orthosteric agonists and is not prevented by the muscarinic antagonist atropine.

Still another type of muscarinic agonists exhibiting "functional selectivity" has recently been described. These particular ligands are represented by xanomeline (3-[3-hexyloxy-1,2,5-thiadiazo-4-yl]-1,2,5,6-tetrahydro-1-methylpyridine; Shannon *et al*. 1994, Bymaster *et al.* 1994, 1998) and AC-42 (4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine; Spalding *et al*. 2002, Langmead *et al*. 2006). They bind with comparable affinity to the orthosteric binding site of all subtypes of muscarinic receptors but in functional assays activate mainly the M_1 subtype with efficacy comparable to that of classical full agonists. Their functional outcome of receptor activation is prevented by atropine. It has been proposed that these atypical agonists attach to additional less conserved "ectopic" sites that are important for receptor activation.

Mechanisms of xanomeline action

Ectopic agonists thus represent a new class of muscarinic agonists that display functional selectivity. However, the molecular mechanisms determining this unique pharmacological profile are not known. We have recently been studying binding characteristics of xanomeline. Experiments revealed that it associates with M_1 receptors in an unusual way. Xanomeline displays two substantially different modes of binding; reversible binding with the orthosteric binding site and wash-resistant binding with a half-life more than 30 hours that takes place somewhere else (Jakubík *et al*. 2002). Reversibly as well as persistently bound xanomeline stimulates M_1 receptors and increases accumulation of

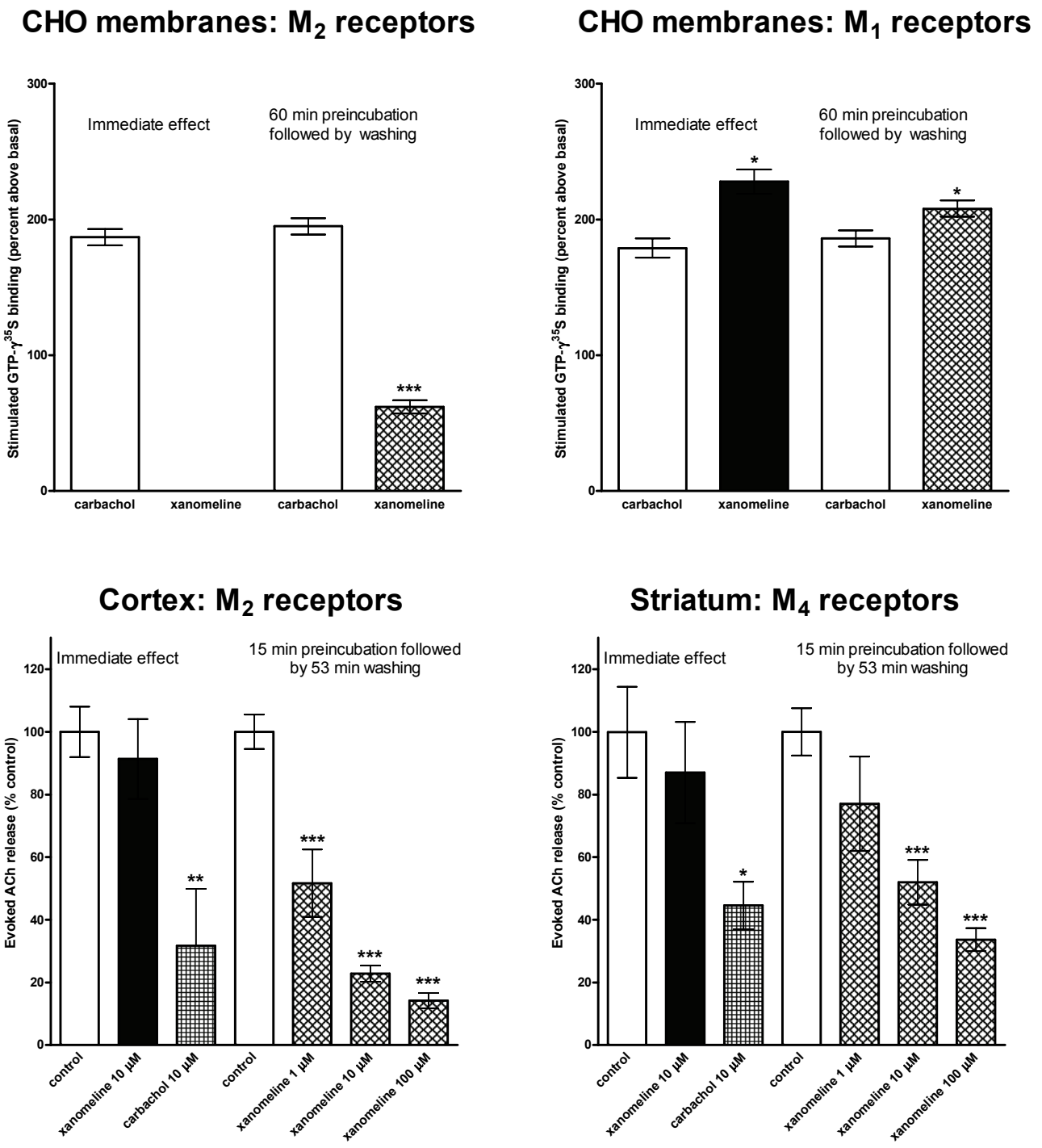

Fig. 2. Effects of free and wash-resistantly bound xanomeline in functional tests**. Upper row:** At M_2 receptors (left) expressed in CHO membranes immediate application of xanomeline does not stimulate GTP-γS binding. Significant stimulation amounting about one third of that induced by carbachol occurs after 60 min preincubation and removal of free xanomeline. Both free and wash-resistantly bound xanomeline at M_1 receptors stimulate GTP-γS binding to a similar extent that is significantly bigger than that induced by carbachol. Ordinate: values of induced increase of GTP-γS binding expressed as percent increase above basal values are derived from concentration-response measurements (Jakubík *et al.* 2006). EC_{50} values (log M) for carbachol are 6.12 and 6.06 at M_2 receptors and 6.08, and 6.07 at M_1 receptors without or with preincubation in the presence of carbachol, respectively. EC_{50} values for xanomeline are no effect and 5.81 at M_2 receptors and 8.04 and 5.98 at M_1 receptors without or with preincubation in the presence of xanomeline, respectively. Asterisks indicate significant difference from corresponding values in the presence of carbachol by t-test. **Lower row:** Unlike carbachol (10 μM) immediate application of xanomeline (10 μM) does not stimulate presynaptic inhibitory autoreceptors either in cortex (M_2 receptors; left graph) or in striatum (M_4 receptors; right graph). Significant concentration-dependent inhibition of stimulated ACh release becomes apparent after 15 min preincubation followed by 53 minutes washing, i.e. in the absence of free xanomeline (Machová *et al.* 2007). Ordinate: evoked ACh release is expressed as percent of control release in the absence of drugs. Asterisks indicate significant difference from corresponding controls determined by ANOVA and Dunnet´s test.

inositolphosphates in a classical antagonist-sensitive manner. However, xanomeline persistent binding can form even in the presence of orthosteric antagonists. The wash-resistant binding depends on the length of the O-alkyl chain of xanomeline which has to be at least O-butyl or longer (Jakubík *et al*. 2004). Only the reversible binding of xanomeline was detected on purified soluble receptors but both binding modes occurred on purified receptors reconstituted into liposomes. Wash-resistant binding appeared only when receptors were exposed to xanomeline after, but not before, reconstitution. These results indicate that the wash-resistant binding of xanomeline involves interhelical penetration of O-alkyl chain and its interaction with membrane lipids surrounding the receptor. However, this wash-resistant binding to the M_1 receptor as such cannot underlie functional selectivity of xanomeline because we demonstrated that it occurs with comparable affinity at all subtypes of muscarinic receptors (Jakubík *et al*. 2006, Machová *et al*. 2007; Fig. 1).

Kinetic and functional experiments on M_1 and M_2 receptors expressed in membranes of CHO cells revealed conspicuous differences in kinetics of wash-resistant xanomeline binding and receptor activation (Jakubík *et al*. 2006). The rate of formation of wash-resistant xanomeline binding is faster at M_1 than M_2 receptors at concentrations up to 3 μM but in either case does not correspond to a simple bimolecular reaction. Most remarkable xanomeline displays instant wash-resistant binding at M_1, but not M_2 receptors. In line with functional M_1 selectivity, xanomeline application immediately increases GTP-γS binding (indicator of receptor activation) with higher potency and efficacy than the classical full agonist carbachol whereas it has no effect at M_2 receptors (Fig. 2). Both free and wash-resistantly bound xanomeline activate M_1 receptor to a similar extent but with about 100 times lesser potency in the case of wash-resistant xanomeline (in the absence of free ligand). At M_2 receptors, unlike immediate application, wash-resistant xanomeline stimulates GTP-γS binding both in the absence and presence of free ligand to a similar extent that is, however, only about one third of the activation induced by carbachol (Fig. 2). Similar to the M_1 subtype, the potency is about 100 times higher in the presence of free ligand. Individual muscarinic receptor subtypes prefer coupling with specific G-proteins but agonist stimulation can also induce (although with lower potency and efficacy) activation of other G-proteins (Michal *et al*. 2001). The M_2 receptor subtype directly couples with all three major G-protein subclasses, i.e. $G_{i/o}$ (preferential), G_s, and $G_{q/11}$ (Michal *et al.* 2007). Wash resistantly bound xanomeline at M_2 receptors and both free and wash-resistant xanomeline at M_1 receptors activate coupling of preferential ($G_{i/o}$ and $G_{q/11}$) but also of non-preferential G-proteins. Discrimination by xanomeline among preferential and non-preferential G-proteins is better at M_1 than M_2 subtype.

These observations further support the concept of agonist-induced formation of multiple receptor conformations and emphasize the importance of kinetics of ligand binding and receptor activation in subtype selectivity. However, the above observations were derived from studies on receptors heterologously expressed in Chinese hamster ovary (CHO) cells. Applicability of these results to naturally expressed muscarinic receptors were demonstrated in *ex vivo* experiments with native tissue. Evoked ACh release from rat cortical and striatal slices is autoinhibited by presynaptic M_2 and M_4 receptors (Doležal and Tuček 1998, Zhang *et al*. 2002) that mediate this effect *via* the βγ dimer of the pertussis toxin-sensitive G-proteins $G_{i/o}$ (Doležal *et al*. 1989, Herlitze 1996). In line with reported M_1 selectivity, immediate application of xanomeline has no effect on the evoked ACh release in rat cortex or striatum. In contrast, short preincubation with xanomeline followed by extensive washing in both tissues results in a concentration-dependent permanent inhibition of evoked ACh release that amounts to the maximal inhibition achievable by the full agonist carbachol (Machová *et al.* 2007; Fig. 2). Inhibitory effects of wash-resistant xanomeline could not be abolished either by extensive washing in the presence of a classical antagonist, by the presence of antagonist during xanomeline treatment, or by irreversible blockade of the orthosteric binding site before xanomeline treatment. Delayed inhibitory effects of xanomeline treatment on evoked ACh release at concentrations up to 10 μM is fully antagonized by an orthosteric antagonist present during stimulation. However, inhibition of evoked ACh release after treatment with 100 μM is only partially sensitive to antagonist indicating receptor activation from an ectopic site.

Conclusions

Xanomeline demonstrates a complex pharmacological profile that involves reversible and

wash-resistant binding that results in full agonist activity at the M_1 muscarinic receptor or delayed wash-resistant partial agonist activity at M_2 receptors, or delayed wash-resistant full agonist activity at M_2 and M_4 receptors in native brain tissue. This complex profile that includes interactions with and activation of the receptor from both orthosteric and ectopic binding sites, and the time-dependent changes of ligand binding and receptor activation point to potential profitability of exploiting ectopic ligands in search for muscarinic receptor subtype-selective drugs.

Conflict of Interest

There is no conflict of interest.

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