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Biochemical Pharmacology 69 (2005) 1637–1645
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Muscarinic subtype affinity and functional activity profile of 1-methyl-2-(2-methyl-1,3-dioxolan-4-yl)pyrrolidine and 1-methyl-2-(2-methyl-1,3-oxathiolan-5-yl)pyrrolidine derivatives

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Received 13 January 2005; accepted 11 March 2005

Abstract

Starting from two previously studied muscarinic full agonists, characterized by a 1,3-dioxolane ((+)−**1**) and a 1,3-oxathiolane ((+)−**2**) cycle, two new series of muscarinic ligands were designed, obtained by the steric complication of the parent compounds produced by freezing the aminoalkyl chain into a pyrrolidine ring. Both tertiary amines and the corresponding iodomethyl derivatives were synthesised and studied, and several compounds of the series which behaved as muscarinic agonists have been selected, on the basis of preliminary binding experiments on rat cortex homogenates, for the present work.

Results are presented obtained from testing the affinity of the selected compounds against cloned human muscarinic receptors expressed in CHO cells, in order to evaluate subtype selectivity. Their functional activity on classical models of M_1–M_4 receptors, in guinea pig and rabbit tissues is also reported.

With respect to parent compounds, the new molecules present some selectivity toward hm2 receptors; fair M_2 selectivity is also evident in functional studies, where these compounds behave as partial agonists. Among the other compounds of the series (2*S*, 4′*R*, 2′*S*)-1,1-dimethyl-2-(2-methyl-1,3-dioxolan-4-yl)pyrrolidinium iodide (−)−**3** and (2*R*, 5′*S*, 2′*S*)-1-methyl-2-(2-methyl-1,3-oxathiolan-5-yl)pyrrolidine (+)−**5** present a promising pharmacological profile. Compound (−)−**3** shows modest hm2 selectivity in binding experiments but a clearcut M_2 selectivity in functional tests, where it behaves as a weak antagonist on M_1 and M_4 subtypes, as a weak full agonist on the M_3 subtype and as a potent partial agonist on M_2 subtype. Tertiary amine (+)−**5** presents a quite similar profile but appears more interesting since, lacking a permanent charge on the nitrogen atom, it may represent an interesting tool to study CNS muscarinic receptors.

Our results confirm that sterical complication of parent compounds (+)−**1** and (+)−**2** produces more selective muscarinic agonists.

Keywords: Muscarinic agonist; Subtype selectivity; Dioxolane derivative; Oxathiolane derivative; Binding studies; Functional studies

1. Introduction

Cholinergic compounds have been studied widely in the past decades to identify molecules useful for further characterization of muscarinic [1] and nicotinic [2] receptor subtypes. However, while characterization through antagonists has progressed satisfactorily, there are at present no agonists able to discriminate muscarinic subtypes [3]. Thus, new agonists, selective for one of the several muscarinic receptor subtypes, would be extremely useful not only to further characterize the receptors but also for their therapeutic potential in pathological states such as pain [4],

Abbreviations: CHO, Chinese hamster ovary; hm1–hm5, human muscarinic subtypes; M_1–M_5, muscarinic subtypes; K_i, inhibitor equilibrium dissociation constant; pK_i, -log K_i; pD_2, -log ED_{50}; K_b, antagonist affinity from functional tests; pK_b, -log K_b; McN-A-343, 4-(*N*-[3-chlorophenyl]-carbamoyloxy-2-butynyltrimethylammonium chloride; pCl-McN-A-343, (4-(*N*-[4-chlorophenyl]carbamoyloxy)-2-butynyltrimethylammonium chloride; NMS, *N*-methylscopolamine; K_D, affinity constant of the labeled ligand; B_{max}, total receptor density; N, non specific binding; PSS, physiological salt solution

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doi:10.1016/j.bcp.2005.03.009

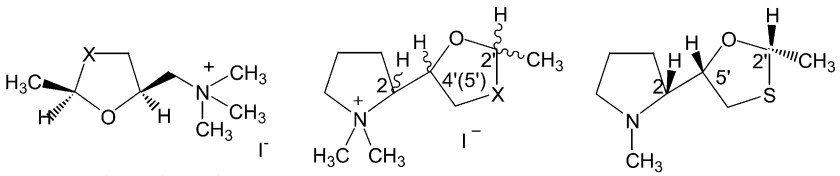


Plate 1.

schizophrenia [5] and Alzheimer's disease [6,7]. In both cases, the poor selectivity of the molecules studied has always represented the major problem.

For several years we have been working with cholinergic agonists, characterized by a pentatomic cycle, among them 1,3-dioxolanes and 1,3-oxathiolanes [8]. Iodomethylates of both series such as **(+)−1** and **(+)−2** (Plate 1), which have been extensively studied in our laboratory, show high agonistic activity on muscarinic as well as on nicotinic receptors [9–13]. As a matter of fact, compound **(+)−2** is one of the most potent cholinergics known so far. However, both compounds show modest selectivity between receptor subtypes. We reasoned that, by exalting the molecular complexity of the parent compounds, in particular through stereochemical complication in the proximity of the critical cationic head of the molecule, the chance of finding muscarinic and nicotinic agonists able to discriminate among cholinergic receptor subtypes would increase.

Therefore, we have designed and synthesized a series of compounds that are diastereomeric and enantiomeric isomers of 1-methyl-2-(2-methyl-1,3-dioxolan-4-yl)pyrrolidines, 1-methyl-2-(2-methyl-1,3-oxathiolan-5-yl)pyrrolidines and their iodomethylates. In preliminary assays, most of them showed fairly good affinity for the muscarinic receptors present in rat cortex and behaved as agonists, but surprisingly they did not show any nicotinic affinity up to a 10 μM dose, even if, as in nicotine, a pyrrolidine ring is present [14]. Here, we report the results of a study aimed at evaluating the potency and selectivity of the members of the series that showed appreciable muscarinic affinity on the cortex (shown in Plate 1). The pharmacological profile of the reference compounds **(+)−1** [15] and **(±)−2** [12], which are commercially available, was also assessed. Subtype affinity was evaluated in Chinese hamster ovary (CHO) cells expressing the five human muscarinic subtypes (hm1–hm5). Functional activity was evaluated on classical preparations (rabbit stimulated vas deferens, guinea pig stimulated left atria, guinea pig ileum and guinea pig lung strips) following reported previously methods [16,17].

2. Materials and methods

2.1. Drugs

The following drugs were used: McN-A-343 (RBI-Sigma); pCl-McN-A-343 (RBI-Sigma); carbachol (RBI-Sigma); yohimbine (RBI-Sigma); tripitramine (a gift from Prof. Melchiorre, Department of Pharmaceutical Sciences, University of Bologna, Italy); (+)-cis-dioxolane **((+)−1)** (RBI-Sigma); oxa-22 **((±)−2)** (RBI-Sigma); (−)-scopolamine methylbromide (Sigma); [^{3}H] *N*-methylscopolamine chloride (Perkin-Elmer Life and Analytical Science) specific activity range 2590–3200 GBq/mmol. Compounds **3–7** were synthesised as reported by Dei et al. [14]; compound **(+)-5** was used as oxalate salt. Other chemicals were of the highest quality commercially available. All the drugs were dissolved in the nutritive solutions immediately before use.

2.2. Binding studies

2.2.1. Cell culture and membrane preparation

CHO cells stably expressing cDNA encoding the five human muscarinic (hm1–hm5) receptors were generously provided by Prof. R. Maggio (Department of Neuroscience, University of Pisa, Italy). Growth medium consisted in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (Gibco, Grand Iland, NY), 100 units/ml each of penicillin G and streptomicyn, 4 mM glutamine (Sigma–Aldrich, Milano, Italy) and non essential aminoacids solution 100 × (Sigma–Aldrich, Milano, Italy) and 50 μg/ml of geneticin (Gibco, Grand Iland, NY) in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Confluent CHO cell lines were scraped, washed with buffer (25 mM sodium phosphate-containing 5 mM $MgCl_2$ at pH 7.4) and homogenized for 30 s using an Ultra-Turrax (setting 5). The pellet was sedimented 17,000 × *g* for 15 min at 4 °C and the membranes were resuspended in the same buffer, re-homogenized with Ultra-Turrax and stored at −80 °C [18]. An aliquot was taken for the assessment of protein content according to the method of Bradford [19] using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, München, Germany) and BSA was used as the standard.

2.2.2. Binding assay

The radioligand binding assay was run in polypropylene 96-well plates (Sarstedt, Verona, Italy) and performed for 120 min at room temperature in a final volume of 0.25 ml in 25 mM sodium phosphate buffer containing 5 mM $MgCl_2$ at pH 7.4. Final membrane protein concentrations

were 30 μg/ml (hm1), 70 μg/ml (hm2), 25 μg/ml (hm3), 50 μg/ml (hm4) and 25 μg/ml (hm5).

In homologous competition curves, [^{3}H] NMS was present at 0.2 nM in tubes containing increasing concentrations of unlabeled NMS (0.03–1000 nM) and at 0.075–0.2 nM in tubes without unlabeled ligand. In heterologous competition curves, fixed concentrations of the tracer (0.2 nM, ca. K_D) were displaced by increasing concentrations of several unlabeled ligands (0.01–1000 μM). All measurements were obtained in duplicate. At the end of the binding reaction, free radioligand was separated from bound ligand by rapid filtration through UniFilter GF/C plates (Perkin-Elmer Life and Analytical Science, Boston, MA) using a FilterMate Cell Harvester (Perkin-Elmer Life and Analytical Science, Boston, MA); after filtration, the filters were washed several times with ice cold buffer and allowed to dry overnight at room temperature under air flow, added of 25 μl of scintillation liquid (Microscint-20, Perkin-Elmer Life and Analytical Science, Boston, MA) and counted by TopCount NXT Microplate Scintillation Counter (Perkin-Elmer Life and Analytical Science, Boston, MA). The binding data were evaluated quantitatively with the weighted least-squares iterative curve fitting LIGAND program [20]; this analysis provides optimal estimates of binding parameters for the labeled ligand from the analysis of homologous curves: affinity constant (K_D), binding capacities (B_{max}) and nonspecific binding (N). Moreover, data from heterologous and homologous competition curves were simultaneously analyzed to obtain the K_i values for the unlabeled ligands. The computer program ALLFIT [21] was used for the analysis of sigmoidal dose response curves obtained in binding studies; the program uses the constrained four parameter logistic model to obtain estimates of half-maximal effective concentration (IC_{50}) values and the logit-log slope (''pseudo-Hill'' coefficient). All the quoted values are mean ± S.E.M.

2.3. Functional studies

2.3.1. General considerations

Male guinea pigs (200–300 g) and male New Zealand white rabbits (3.0–3.5 kg) were killed by cervical dislocation. The organs required were set up rapidly under 1 g of tension in 20 ml organ baths containing physiological salt solution (PSS) maintained at an appropriate temperature and aerated with 5% CO_2–95% O_2. Dose–response curves were constructed by cumulative addition of the reference agonist. The concentration of agonist in the organ bath was increased approximately 3-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Following 30 min of washing, a cumulative dose–response curve to the agonist under study was constructed. When the compound under study behaves as an antagonist, following 30 min of washing, tissues were incubated with the antagonist for 30 min, and a new dose–response curve to the agonist was obtained. Responses were expressed as a percentage of the maximal response obtained in the control curve. Contractions were recorded by means of a force displacement transducer connected to the MacLab system PowerLab/800. In addition, parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity.

In all cases, parallel experiments in which tissues received only the reference agonist were run in order to check any variation in sensitivity.

All animal testing was carried out according to the European Community Council Directive of 24 November 1986 (86/609/EEC).

2.3.2. Guinea-pig ileum

Two-centimeter-long portions of terminal ileum were taken at about 5 cm from the ileum–cecum junction and mounted in PSS, at 37 °C, of the following composition (mM): NaCl (118.0), $NaHCO_3$ (23.8), KCl (4.7), $MgSO_4{\cdot}7H_2O$ (1.18), KH_2PO_4 (1.18), $CaCl_2$ (2.52), glucose (11.7). Tension changes were recorded isotonically. Tissues were equilibrated for 30 min, and dose–response curves to carbachol were obtained at 30 min intervals, the first one being discarded and the second one being taken as the control.

2.3.3. Guinea-pig stimulated left atria

The heart was rapidly removed, and the right and left atria were separately excised. Left atria were mounted in PSS (the same used for ileum) at 30 °C and stimulated through platinum electrodes by square-wave pulses (1 ms, 1 Hz, 5–10 V) (tetra stimulus, N. Zagnoni). Inotropic activity was recorded isometrically. Tissues were equilibrated for 2 h and a cumulative dose–response curve to carbachol was constructed.

2.3.4. Guinea-pig lung strips

The lungs were rapidly removed and strips of peripheral lung tissue were cut either from the body of a lower lobe with the longitudinal axis of the strip parallel to the bronchus or from the peripheral margin of the lobe. The preparations were mounted, with a preload of 0.3 g, in PSS with the following composition (mM): NaCl (118.78), KCl (4.32), $CaCl_2{\cdot}2H_2O$ (2.52), $MgSO_4{\cdot}7H_2O$ (1.18), KH_2PO_4 (1.28), $NaHCO_3$ (25.0), glucose (5.55). Contractions were recorded isotonically at 37 °C after tissues were equilibrated for 1 h, then two cumulative dose–response curves to carbachol (0.01, 0.1, 1, 10, 100 μM) were obtained at 45 min intervals, the first one being discarded and the second one being taken as the control.

2.3.5. Rabbit stimulated vas deferens

This preparation was set up according to Eltze [17]. Vasa deferentia were carefully dissected free of surrounding tissue and were divided into four segments, two prostatic

portions of 1 cm and two epididymal portions of approximately 1.5 cm length. The four segments were mounted in PSS with the following composition (mM): NaCl (118.4), KCl (4.7), $CaCl_2$ (2.52), $MgCl_2$ (0.6), KH_2PO_4 (1.18), $NaHCO_3$ (25.0), glucose (11.1); 1 μM yohimbine and 0.01 μM tripitramine were included to block alpha$_2$-adrenoceptors and M_2 muscarinic receptors, respectively. The solution was maintained at 30 °C and tissues were stimulated through platinum electrodes by square-wave pulses (0.1 ms, 2 Hz, 10–15 V). Contractions were measured isometrically after tissues were equilibrated for 1 h, then a cumulative dose–response curve to pCl-McN-A-343 was constructed. On this preparation the pD_2 of the standard full agonist McN-A-343 is 6.33 ± 0.06.

3. Results

In Table 1 the saturation binding parameters for [^{3}H] NMS at cloned human muscarinic receptors in CHO cell membranes are reported. It may be seen that the affinity of the radioligand is similar for four subtypes (hm2–hm5), and our results are in agreement with literature data [22]. The [^{3}H] NMS affinity for hm1 receptor is about 2-fold higher than the value reported by the same author. The estimates of total receptor densities showed marked variation as previously described in the literature [18,23–25].

Only seven out of the 16 compounds reported in our previous paper [14] were taken into consideration in the present work, since the other compounds did not show affinity up to the dose of 100 μM for rat brain homogenates and were, therefore, considered inactive as muscarinic ligands. The results of binding on CHO cells expressing the five human muscarinic subtypes (hm1–hm5) and the functional activity on M_2, M_3, M_4 and putative M_1 muscarinic receptor models are reported in Tables 2 and 3, together with those of the parent compounds **(+)−1**, **(±)−2** and of the standard carbachol. In this respect, the presence of M_1 receptors in rabbit stimulated vas deferens has been questioned in favor of the M_4 subtype so that, currently, the subtype present in the tissue is still not defined adequately [23,26]. Binding data are reported as pK_i values (affinity). It is important to appreciate that the ''pseudo-Hill'' coefficients, when lower than unity, indicate binding heterogeneity. It is generally accepted that muscarinic agonists bind to high affinity (H) and low affinity (L) populations [27], and therefore, the pK_i reported values do not reflect the real binding behaviour of our compounds, and represent indeed ''apparent'' affinity values. Functional data are reported as pD_2 (−log ED_{50}) (agonistic potency), as pK_b (antagonist potency) and as α for intrinsic activity. With few exceptions, the new compounds are partial agonists ($\alpha < 1$) unlike the parent compounds that are full agonists ($\alpha = 1$).

3.1. 1,3-Dioxolanes (Table 2)

For this series previous research [14] indicated that only iodomethylates **(−)−3** and **(+)−4** showed muscarinic affinity and warranted further studies; the remaining methyl iodides and all four tertiary amines [14] did not show affinity up to the 100 μM dose. The lack of affinity of amines is in line with previous findings showing the tertiary amines of this family of cholinergic ligands to be, with few exceptions, some two orders of magnitude less affinitive and potent than the corresponding methyl iodides [11]. The binding affinity and functional activity of **(−)−3** and **(+)−4** are reported in Table 2. As regards the affinity for human muscarinic receptors expressed in CHO cells, the two compounds show a profile similar to that of parent compound **(+)−1** and carbachol, with a modest selectivity for hm2 receptors. In functional tests, the parent compound **(+)−1** remains the most potent in all the preparations, also if compared to the reference carbachol, but **(−)−3** shows a fair functional selectivity on the M_2 subtype, being a weak antagonist on M_1 and M_4 receptors and showing a pD_2 of 7.22 on M_2 and 5.32 on M_3 subtypes. Compound **(+)−4**, on the contrary, is less potent than **(+)−1** and carbachol on M_2 subtype and does not possess any remarkable subtype selectivity.

3.2. 1,3-Oxathiolanes (Table 3)

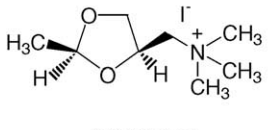
In this series, all four iodomethyl derivatives (**(−)−6, (+)−6, (−)−7, (+)−7**) were endowed with muscarinic affinity and were active on muscarinic functional models (Table 3). Moreover, unlike what happens in the 1,3-dioxolane series, where only quaternary compounds show some activity, the tertiary base **(+)−5** presents definite binding affinity and functional activity. Among these substances, compounds **(+)−6** and **(−)−7** show good affinity and moderate selectivity for hm2 subtypes, compared to the racemic parent compound **(±)−2**. As far as functional tests are concerned, compound **(+)−6** is a potent and selective partial agonist on M_2 receptors with a pD_2 of 7.25 against a pD_2 of 6.12 on M_3 subtypes and no functional activity on M_1 and M_4 subtypes, while compound **(−)−7** is equally selective but less potent.

Table 1
Saturation binding parameters for [^{3}H] NMS to cloned human muscarinic receptors in CHO cell membranes[a]

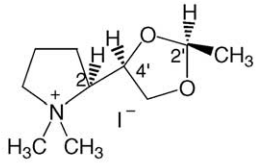
	hm1	hm2	hm3	hm4	hm5
B_{max} (fmol/mg prot)	1987 ± 258	538 ± 33	3444 ± 702	2565 ± 180	3163 ± 138
K_D (nM)	0.59 ± 0.12	0.13 ± 0.03	0.16 ± 0.04	0.14 ± 0.02	0.17 ± 0.05

[a] B_{max}: total receptor density, expressed as means ± S.E.M.; K_D: equilibrium dissociation constants determined by LIGAND program, data represent mean ± S.E.M.

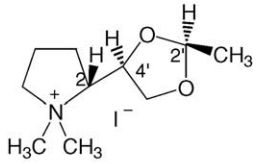
Table 2
Binding parameters and functional activities of dioxolane derivatives



(2S,4R)-(+)-1



(2S, 4'R, 2'S)-(-)-3



(2R, 4'R, 2'S)-(+)-4

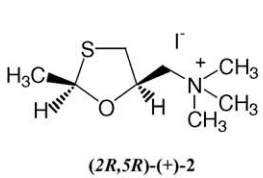
Compounds		Binding affinities[a]					Functional activities							
Compound	Stereochemistry	hm1	hm2	hm3	hm4	hm5	Rabbit vas deferens[b]		Guinea pig atrium (M_2[c])		Guinea pig ileum (M_3[c])		Guinea pig lung (M_4[c])	
		pK_i nH	pK_i nH	pK_i nH	pK_i nH	pK_i nH	α	pD_2 (pK_b)	α	pD_2	α	pD_2	α	pD_2
(+)-1	2S, 4R	4.96 ± 0.06 *1.00 ± 0.12*	5.92 ± 0.07 *1.09 ± 0.18*	4.87 ± 0.01 *0.91 ± 0.07*	5.05 ± 0.08 *0.78 ± 0.08*§	4.66 ± 0.09 *0.82 ± 0.26*	0.94 ± 0.01	6.91 ± 0.05	1	8.11 ± 0.15	1	8.15 ± 0.14	1	6.76 ± 0.07
(−)-3	2S, 4′R, 2′S	4.67 ± 0.09 *0.99 ± 0.37*	5.77 ± 0.13 *0.91 ± 0.09*	4.37 ± 0.10 *0.86 ± 0.14*	4.62 ± 0.08 *0.75 ± 0.05*§	4.64 ± 0.10 *0.78 ± 0.17*		(<5)	0.57 ± 0.02	7.22 ± 0.06	1	5.32 ± 0.23		(<5)
(+)-4	2R, 4′R, 2′S	4.28 ± 0.09 *0.73 ± 0.11**	5.30 ± 0.11 *0.89 ± 0.09*	4.33 ± 0.09 *0.72 ± 0.06**	4.76 ± 0.10 *0.78 ± 0.08**	4.28 ± 0.11 *0.85 ± 0.12*		(<5)	1	6.63 ± 0.06	0.94 ± 0.09	6.83 ± 0.18	0.93 ± 0.04	5.30 ± 0.23
Carbachol		4.42 ± 0.10 *0.63 ± 0.09**	5.92 ± 0.07 *0.98 ± 0.14*	4.36 ± 0.10 *0.57 ± 0.09**	5.20 ± 0.07 *0.61 ± 0.04*§	4.16 ± 0.09 *0.80 ± 0.23*			1	7.33 ± 0.08	1	6.68 ± 0.01	1	5.43 ± 0.03

[a] Binding parameters of muscarinic agonists at five human muscarinic receptor subtypes. The affinity estimates (as pK_i) were derived from both [^{3}H] NMS homologous and heterologous competition curves and represent the mean (± S.E.M.) of at least three experiments; the ''pseudo-Hill'' coefficients are given in italic and the values represent the mean (± S.E.M.) of at least three experiments, numbers labelled with (*) and (§) differ significantly from 1 ($^*p < 0.05$; $^§p < 0.001$).

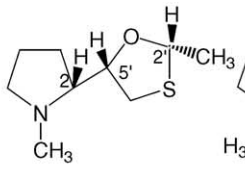
[b] α (±S.E.M.) and pD_2 (or pK_b in brackets) (±S.E.M.) values in functional tests on rabbit stimulated vas deferens. The presence of M_1 receptor in this tissue has been recently questioned (see text).

[c] α (±S.E.M.) and pD_2 (or pK_b in brackets) (±S.E.M.) values in functional tests on guinea pig atria, ileum and lung strips.

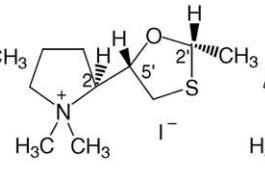
Table 3
Binding parameters and functional activities of oxathiolane derivatives



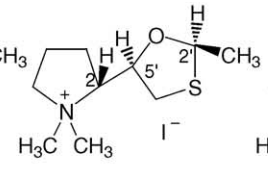
(2R,5R)-(+)-2



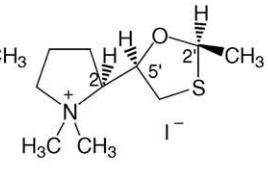
(2R, 5'S, 2'S)-(+)-5



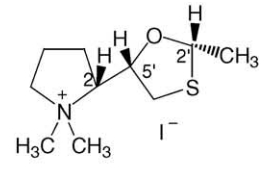
(2S, 5'S, 2'S)-(-)-6



(2R, 5'R, 2'R)-(+)-6



(2S, 5'R, 2'R)-(-)-7



(2R, 5'S, 2'S)-(+)-7

Compounds		Binding affinities[a]					Functional activities							
Compound	Stereochemistry	hm1	hm2	hm3	hm4	hm5	Rabbit vas deferens[b]		Guinea pig atrium (M_2[c])		Guinea pig ileum (M_3[c])		Guinea pig lung (M_4[c])	
		pK_I nH	pK_i nH	pK_I nH	pK_i nH	pK_i nH	α	pD_2 (pK_b)	α	pD_2	α	pD_2	α	pD_2 (pK_b)
(±)-2 [d]	2R*, 5R* [e]	4.96 ± 0.08 *0.94 ± 0.07*	5.68 ± 0.06 *1.04 ± 0.30*	5.07 ± 0.05 *0.75 ± 0.11*	5.19 ± 0.05 *0.82 ± 0.04*§	4.88 ± 0.08 *0.82 ± 0.26*	1	6.81 ± 0.09	1	7.13 ± 0.05	1	7.75 ± 0.02	0.92 ± 0.01	6.15 ± 0.04
(+)-5	2R, 5′S, 2′S	4.67 ± 0.10 *1.27 ± 0.31*	5.01 ± 0.09 *0.85 ± 0.14*	4.40 ± 0.11 *1.19 ± 0.12*	4.36 ± 0.08 *0.75 ± 0.08*	4.45 ± 0.11 *0.80 ± 0.07*		(<5)	0.69 ± 0.01	7.39 ± 0.08	0.48 ± 0.02	5.82 ± 0.03		(<5)
(+)-6	2R, 5′R, 2′R	5.08 ± 0.11 *0.74 ± 0.07*§	6.17 ± 0.09 *0.70 ± 0.05**	4.83 ± 0.12 *0.78 ± 0.16**	5.07 ± 0.06 *0.81 ± 0.11**	4.99 ± 0.10 *0.86 ± 0.10*		(<5)	0.75 ± 0.04	7.25 ± 0.09	1	6.12 ± 0.25	0.18 ± 0.01	[f]
(−)-6	2S, 5′S, 2′S	4.72 ± 0.08 *0.92 ± 0.07*	5.28 ± 0.08 *1.07 ± 0.10*	4.53 ± 0.09 *0.90 ± 0.10*	4.50 ± 0.06 *0.80 ± 0.06**	4.61 ± 0.10 *0.90 ± 0.16*		(4.66 ± 0.30)	0.56 ± 0.03	6.27 ± 0.22	0.78 ± 0.04	4.76 ± 0.10		(<5)
(+)-7	2R, 5′S, 2′S	4.85 ± 0.11 *0.79 ± 0.06**	5.45 ± 0.12 *0.83 ± 0.04*	4.65 ± 0.08 *1.30 ± 0.39*	4.74 ± 0.07 *0.74 ± 0.06**	4.63 ± 0.07 *0.84 ± 0.09*		(<5)	0.84 ± 0.01	7.72 ± 0.02	0.41 ± 0.05	6.60 ± 0.14	0.20 ± 0.03	[f]
(−)-7	2S, 5′R, 2′R	5.23 ± 0.08 *0.84 ± 0.04**	5.85 ± 0.09 *0.94 ± 0.06*	4.85 ± 0.06 *1.08 ± 0.22*	5.09 ± 0.09 *0.76 ± 0.05**	4.97 ± 0.10 *0.84 ± 0.09*		(5.10 ± 0.14)	0.53 ± 0.05	6.14 ± 0.08	0.59 ± 0.09	5.20 ± 0.11		(<5)
Carbachol		4.42 ± 0.10 *0.63 ± 0.09**	5.92 ± 0.07 *0.98 ± 0.14*	4.36 ± 0.10 *0.57 ± 0.09**	5.20 ± 0.07 *0.61 ± 0.04*§	4.16 ± 0.09 *0.80 ± 0.23*			1	7.33 ± 0.08	1	6.68 ± 0.01	1	5.43 ± 0.03

[a] Binding parameters of muscarinic agonists at five human muscarinic receptor subtypes. The affinity estimates (as pK_i) were derived from both [^{3}H] NMS homologous and heterologous competition curves and represent the mean (±S.E.M.) of at least three experiments; the ''pseudo-Hill'' coefficients are given in italic and the values represent the mean (±S.E.M.) of at least three experiments, numbers labelled with (*) and (§) differ significantly from 1 ($^*p < 0.05$; $^§p < 0.001$).

[b] pD_2 (or pK_b in brackets) values in functional tests on rabbit stimulated vas deferens. The presence of M_1 receptor in this tissue has been recently questioned (see text).

[c] α (±S.E.M.) and pD_2 (or pK_b in brackets) (±S.E.M.) values in functional tests on guinea pig atria, ileum and lung strips.

[d] The pD_2 values of the eutomer **(+)-2** on guinea pig atrium and ileum are reported in the cited references [10,13] and are 7.54 and 8.06, respectively.

[e] This compound is the racemic mixture of **(2*R*, 5*R*)-2** and **(2*S*, 5*S*)-2**. The notation **(2*R**, 5*R**)-2** indicates that the relative but not the absolute stereochemistry of the compound is defined.

[f] Intrinsic activity is too small to allow a sound evaluation of the relative pD_2 value.

The behaviour of compounds **(+)−5** and **(+)−7** is particularly interesting; while they do not show any impressive affinity and selectivity in binding studies, they do possess an interesting profile in functional tests. In fact **(+)−5** is the most M_2 selective, showing a pD_2 of 7.39 on M_2 against a pD_2 of 5.82 on M_3, while being a weak antagonist on M_1 and M_4 subtypes. On the other hand, compound **(+)−7** is the most potent M_2 agonist with a selectivity comparable to that of **(+)−6** and **(−)−7**.

4. Discussion

Since the compounds studied in the present and previous [14] works represent a small library of structurally identical diastereomers and enantiomers that are endowed with fairly different affinities and activities, it is apparent that stereochemistry plays a major role in their binding to muscarinic receptors. In particular, unlike the parent compounds, the active members of the series (**(−)−3**, **(+)−5**, **(−)−6**, **(+)−6**, **(−)−7**, **(+)−7**) appear to be more affinitive for one subtype (hm2), even if the differences are not impressive, thus fulfilling our expectations that the introduction of another stereogenic center, close to the amine function, would improve subtype selectivity. It can be observed, from a simple inspection of Tables 2 and 3, that binding selectivity derives from a reduction of affinity for hm1, hm3, hm4, hm5 subtypes rather than from its improvement on hm2 subtypes, suggesting that the introduction of the stereo center selectively impairs the binding to some subtypes. As concerns functional activity, the selectivity for M_2 muscarinic subtypes of these compounds is more impressive. Even in this case, M_2 selectivity seems due to a reduction of the potency on the other subtypes rather than to an increase of potency on M_2 receptor subtypes. Therefore, it is apparent that there are differences between the binding and functional assay results. As a matter of fact, cloned human muscarinic receptors expressed in CHO cells represent an artificial system loosely related to the rabbit and guinea pig tissues used for functional studies, not to mention genetic differences. For this reason the binding results were taken as a prescreening to select the compounds to evaluate in functional studies.

For the sake of clarity, the following discussion will examine separately the two series of compounds studied.

4.1. 1,3-Dioxolanes

The first thing to underline is that the absolute configuration of **(−)−3** and **(+)−4** is the same of the parent compound **(+)−1**, suggesting that the drugs bind to the same binding site. However, while the affinity of **(+)−4** is reduced for all subtypes with respect to the parent compound **(+)−1**, compound **(−)−3** maintains about the same affinity for the hm2 subtype, while for the other subtypes the affinity generally decreases. The fact that **(−)−3** is the 2-epimer of **(+)−4** demonstrates once more the importance of the stereochemical features of the molecule, in particular those of position 2. As far as functional activity is concerned, comparison of the pD_2 of **(−)−3** with that of parent compound **(+)−1,** confirms that selectivity for M_2 receptors arises from a selective reduction of potency on the other subtypes. Accordingly, compound **(−)−3**, with a pD_2 of 7.22, is some two orders of magnitude more potent on M_2 than on M_3 receptors and is a modest antagonist on M_4 and putative M_1 receptors.

4.2. 1,3-Oxathiolanes

Unlike what happens in the corresponding 1,3-dioxolane series, one of the four isomeric tertiary amines, **(+)−5**, shows affinity for human muscarinic receptors, in particular for the hm2 subtype (Table 3). As far as functional activity is concerned, **(+)−5** is a fairly potent M_2 agonist that is some two orders of magnitude more potent on M_2 than on M_1 and M_3 receptor subtypes; on M_4 subtype it behaves as a modest antagonist. This is quite an exciting result as the pharmacokinetic properties of **(+)−5** (that does not carry a permanent charge) could be most useful to study M_2 muscarinic subtypes in the CNS. This unusual affinity and potency of a tertiary amine like **(+)−5** could be related to the insertion of the tertiary nitrogen in a cycle, as happens with the closely related muscarinic agonist cevimeline (AF 102B) [28], recently approved for Sjogren's syndrome and in phase III trials for Alzheimer's disease. It is perhaps interesting that the absolute configuration at the oxathiolane positions (2′,5′) of **(+)−5** is the opposite of that of the parent compound (**(+)−2**) which carries a quaternary nitrogen.

In this class, all four isomeric methyliodides are endowed with good affinity for human muscarinic receptors and their stereoisomeric relationships offer a good opportunity to study the importance of the stereogenic centers on affinity and potency. Overall, the affinity trend parallels that found in the 1,3-dioxolanes series: the compounds appear to be slightly but firmly selective for the hm2 subtype, where they maintain the affinity of the parent compound, while affinity versus the remaining subtypes is generally depressed. Epimers **(+)−6** and **(−)−7**, that have the same absolute configuration of the parent compound **(+)−2**, as far as the stereogenic centres of the 1,3-oxathiolane nucleus are concerned, are the eutomers in the five receptors studied. However, **(+)−6** and **(−)−7** present opposite configurations at the pyrrolidine stereogenic center 2. This structural diversity does not affect the affinity for hm1, hm3, hm4 and hm5 subtypes, but introduces a different behaviour versus hm2 subtype, where **(+)−6** is more affinitive ($pK_i = 6.17$) than **(−)−7** ($pK_i = 5.85$) with a difference that is small yet significant. This result confirms that in these series of compounds, steric complication in alpha to the basic nitrogen can induce different pharmacological profiles in compounds that have the same stereochemistry in the rest of the molecule.

Functional studies confirm M_2 selectivity as a general feature of the series. Indeed, compounds **(+)−6**, **(−)−6**, **(+)−7** and **(−)−7** behave as modest antagonists or poor agonists on rabbit vas deferens and guinea pig lung. At the contrary, they act as partial or full agonists on guinea pig atrium and ileum, showing higher potency (1–2 orders of magnitude) on the former subtype. Interestingly, in the functional tests, the eutomer of the **(+)−7/(−)−7** couple is no longer **(−)−7**, as found in binding, but **(+)−7**. A complete analysis of the influence of the stereochemistry by simple inspection of the available data is not easy. A deeper understanding of its importance for muscarinic activity will likely be provided by the quantitative eudismic analysis [29,30] that is under way.

In conclusion, by steric complication of the potent muscarinic full agonists **(+)−1** and **(+)−2**, we have developed two new series of muscarinic partial agonists that are endowed with affinity and functional selectivity versus M_2 subtype. Among them, two compounds (**(−)−3**, **(+)−5**) are particularly interesting and show a promising pharmacological profile. Compound **(−)−3**, in binding experiments, presents a good selectivity for the hm2 subtypes that is confirmed and exalted in functional tests. Here, it behaves as a weak antagonist on M_1 and M_4 and as an agonist on M_2 and M_3 subtypes, showing a potency on M_2 subtype some two orders of magnitude higher than on M_3. The other somewhat more interesting compound is the tertiary amine **(+)−5** that, in functional studies, was fairly selective for M_2 subtypes and, lacking a permanent charge on the nitrogen, may be an interesting new tool to study CNS muscarinic receptors.

Acknowledgments

This research was supported by funds of the Italian Ministry of Instruction, University and Research (MIUR).

References

[1] Caulfield MP. Muscarinic receptors-characterization, coupling and function. Pharmacol Ther 1993;58:319–79.
[2] Chavez-Noriega LE, Crona JH, Washburn MS, Urrutia A, Elliott KJ, Johnson EC. Pharmacological characterization of recombinant human neuronal acetylcholine receptors h-alpha-2-beta-2, h-alpha-2-beta-4, h-alpha-3-beta-2, h-alpha-4-beta-2, h-alpha-4-beta-4 and h- alpha-7 expressed in Xenopus oocytes. J Pharmacol Exp Ther 1997;280:346–56.
[3] Korczyn AD. Muscarinic M1 agonists in the treatment of Alzheimer's disease. Exp Opin Inv Drug 2000;9:2259–67.
[4] Eisenach JC. Muscarinic-mediated analgesia. Life Sci 1999;64:549–54.
[5] Dean B, Bymaster FP, Scarr E. Muscarinic receptors in schizofrenia. Curr Mol Med 2003;3:419–26.
[6] Bartus RT. On neurodegenerative diseases, models, and treatment strategies: lessons learned and lessons forgotten a generation following the cholinergic hypothesis. Exp Neurol 2000;163:495–529.
[7] Gualtieri F. Cholinergic receptors and neurodegenerative diseases. In: Gulini U, Giannella M, Quaglia W, Marucci G, editors. Receptor chemistry towards the third millennium, vol. 85. Amsterdam: Elsevier Science B.V.; 2000. p. 89.
[8] Angeli P. Pentatomic cyclic agonists and muscarinic receptors: a 20 years review. Farmaco 1995;50:565–78.
[9] Melchiorre C, Angeli P, Giannella M, Gualtieri F, Pigini M, Cingolani ML, et al. Molecular requirements at the cholinergic receptors. Importance of ether oxygens in the dioxolane series. Eur J Med Chem 1978;13:357–61.
[10] Teodori E, Gualtieri F, Angeli P, Brasili L, Giannella M, Pigini M. Absolute configuration and cholinergic enantioselectivity of (+) and (−) cis-2-methyl-5-[(dimethylamino)methyl]-1,3-oxathiolane methiodide. J Med Chem 1986;29:1610–5.
[11] Triggle DJ. Ligands active at the cholinergic receptors. In: Triggle DJ, Triggle CR., editors. Chemical pharmacology of the synapse, vol. 291. New York: Academic Press, 1976. p. 361.
[12] Pigini M, Brasili L, Giannella M, Gualtieri F. Molecular requirements of the active sites of the cholinergic receptors. Eur J Med Chem 1981;16:415–9.
[13] Angeli P, Brasili L, Giannella M, Gualtieri F, Picchio MT, Teodori E. Chiral muscarinic agonists possessing a 1,3-oxathiolane nucleus: enantio- and tissue-selectivity on isolated preparations of guinea-pig ileum and atria and of rat urinary bladder. Naunyn Schmiedebergs Arch Pharmacol 1988;337:241–5.
[14] Dei S, Bellucci C, Buccioni M, Ferraroni M, Gualtieri F, Guandalini L, et al. Synthesis and cholinergic affinity of diastereomeric and enantiomeric isomers of 1-methyl-2-(2-methyl-1,3-dioxolan-4-yl)pyrrolidine, 1-methyl-2-(2-methyl-1,3-oxathiolan-5-yl)pyrrolidine and of their iodomethylates. Bioorg Med Chem 2003;11:3153–64.
[15] Belleau B, Puranen J. Stereochemistry of the interaction of enantiomeric 1,3-dioxolane analogs of muscarone with cholinergic receptors. J Med Chem 1963;6:325–8.
[16] Scapecchi S, Marucci G, Matucci R, Angeli P, Bellucci C, Buccioni M, et al. Structure-activity relationships in 2,2-diphenyl-2-ethylthioacetic acid esters: unexpected agonistic activity in a series of muscarinic antagonists. Bioorg Med Chem 2001;9:1165–74.
[17] Eltze M. Muscarinic M1 and M2 receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. Eur J Pharmacol 1988;151:205–21.
[18] Dörje F, Wess J, Lambrecht G, Tacke R, Mutschler E, Brann R. Antagonist binding profiles of five cloned human muscarinic receptor subtypes. J Pharmacol Exp Ther 1991;256:727–33.
[19] Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein, utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[20] Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for the characterization of ligand-binding systems. Anal Biochem 1980;107:220–39.
[21] De Lean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose–response curves. Am J Physiol 1978;235:E97–102.
[22] Christopoulos A, Sorman JL, Mitchelson F, El-Fakahany EE. Characterization of the subtype selectivity of the allosteric modulator heptane-1,7-bis-(dimethyl-3′-phthalimidopropyl)ammonium bromide (C_7/3-phph) at cloned muscarinic acetylcholine receptors. Biochem Pharmacol 1999;57:171–9.
[23] Budriesi R, Cacciaguerra S, Di Toro R, Bolognesi ML, Chiarini A, Minarini A, et al. Analysis of the muscarinic receptor subtype mediating inhibition of the neurogenic contractions in rabbit isolated vas deferens by a series of polymethylene tetra-amines. Br J Pharmacol 2001;132:1009–16.
[24] Buckley NJ, Bonner TI, Buckley CM, Brann MR. Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. Mol Pharmacol 1989;35:469–76.

[25] Maggio R, Barbier P, Bolognesi ML, Minarini A, Tedeschi D, Melchiorre C. Binding profile of the selective muscarinic receptor antagonist tripitramine. Eur J Pharmacol 1994;268:459–62.
[26] Caulfield MP, Birdsall NJ. International union of Pharmacology XVII classification of muscarinic acetylcholine receptors. Pharmacol Rev 1998;50:279–90.
[27] Kenakin T. Affinity. In: Kenakin T, editor. Pharmacologic analysis of drug-receptor interaction, vol. 242. Philadelphia: Lippincott-Raven Publishers; 1997. p. 88.
[28] Fisher A, Pittel Z, Haring R, Bar-Ner N, Kliger-Spatz M, Natan N, et al. M1 muscarinic agonists can modulate some of the hallmarks in Alzheimer's disease: implications in future therapy. J Mol Neurosci 2003;20:349–56.
[29] Gualtieri F. Definition of sets all important in correlations Pfeiffer's rule OK. Trends Pharmacol Sci 1990;11:315–6.
[30] Lehmann PA. Stereoisomerism and drug action. Trends Pharmacol Sci 1986;7:281–5.