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Diphenidol-related diamines as novel muscarinic M_4 receptor antagonists

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Abstract—A series of hydrochloride derivatives **2a**–**9a** and quaternary ammonium derivatives **3b**–**9b** of diphenidol have been synthesized and characterized in receptor binding and cellular functional assays versus human muscarinic M_1–M_5 receptors expressed in CHO cells. Compound **8b**, a methiodide derivative with a bipiperidinyl moiety and a second diphenidol framework, showed a potent and selective M_4 activity as competitive antagonist. Moreover **8b**, acting as an allosteric modulator, was able to retard the dissociation rate of [^{3}H]-*N*-methylscopolamine from CHO-M_4 cell membranes exposed to atropine. Taken together, these data suggest that **8b** might open new avenues to the discovery of novel multivalent antagonists for the muscarinic receptors.
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Five distinct gene sequences coding for muscarinic M_1–M_5 receptors, members of the superfamily of G-protein coupled receptors, have been cloned. Muscarinic receptors are expressed in a complex, overlapping fashion in most tissues and modulate the activity of a large number of functions. These receptors display a high degree of sequence identity, and subtype-selective binding of agonists and antagonists is likely based on conformational dissimilarities.[1] Studies using knockout mice have attempted to clarify the main role of each muscarinic receptor subtype in different physiological functions. However, the loss of one of these subtypes can be compensated as a result of functional redundancy.[2] These complications have contributed to slow the discovery of selective and reversible M_4 antagonists, since so far only PD 102807[3] and few other benzoxazine analogues[4] have been described. The need of M_4-selective ligands is crucial to better explore the role played by this receptor subtype in the central nervous system[5] and in the peripheral tissues[6] and to investigate them as potential, novel drugs that may overcome the limitations of available, non-selective compounds.

In our recent work, we have synthesized novel muscarinic antagonists derived from diphenidol (1,1-diphenyl-4-piperidin-1-yl-butan-1-ol, **1**), an antiemetic agent used in the treatment of vomiting and vertigo and binding to M_1–M_4 receptors with superimposable affinity. Appropriate structural modifications of **1** led us to obtain M_2, M_3 as well as M_4 antagonists.[7] On the basis of the findings described in our previous works, we synthesized a novel series of diphenidol derivatives and characterized them in receptor binding and cellular functional assays versus human muscarinic M_1–M_5 receptors expressed in CHO cells. Thereon, we turned our attention to the basic centre and replaced the piperidine ring with some diamines, in order to evaluate the influence on affinity and/or selectivity of a second basic centre. At first, we considered the piperazine moiety, which may be used as a suitable basic portion for muscarinic antagonists,[8] and introduced some substituents on the second nitrogen atom, including a second diphenidol framework. Thereafter, we modified the flexibility and the length of the spacer linking the nitrogen atoms of the two diphenidol frameworks (Fig. 1).

The title compounds **2**–**9** were synthesized by direct mono- or di-alkylation with 4-chloro-1,1-diphenyl-butan-1-ol[9] (**10**) of the proper diamine (piperazine for **2** and **5**, 1-methylpiperazine for **3**, 1-phenylpiperazine for **4**, *N*,*N*′-dimethyl-ethane-1,2-diamine for **6**, *N*,*N*′-

Keywords: M_4-selective muscarinic antagonists; Diphenidol diamine derivatives; Muscarinic receptor binding assays; Functional studies; Allosteric modulation.
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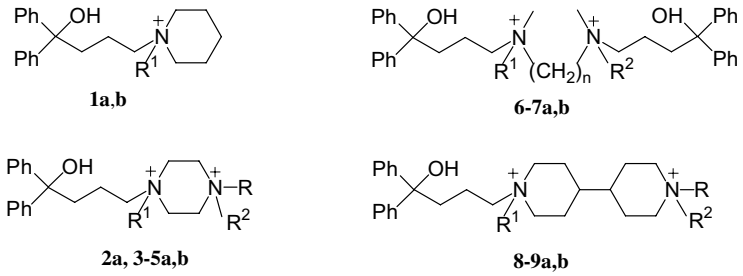


	R	R^1	R^2		R	n	R^1	R^2
1a	-	H	-	**6a**	-	2	H	H
1b	-	CH_3	-	**6b**	-	2	CH_3	CH_3
2a	H	H	H	**7a**	-	3	H	H
3a	CH_3	H	H	**7b**	-	3	CH_3	CH_3
3b	CH_3	-	CH_3	**8a**	$(CH_2)_3C(OH)Ph_2$	-	H	H
4a	Ph	H	-	**8b**	$(CH_2)_3C(OH)Ph_2$	-	CH_3	CH_3
4b	Ph	CH_3	-	**9a**	CH_3	-	H	H
5a	$(CH_2)_3C(OH)Ph_2$	H	H	**9b**	CH_3	-	CH_3	CH_3
5b	$(CH_2)_3C(OH)Ph_2$	CH_3	-					

Figure 1. Structure of diphenidol and target compounds.

dimethyl-propane-1,3-diamine for **7**, 4,4′-bipiperidine for **8** and 1-methyl-4,4′-bipiperidine[10] for **9**). The obtained tertiary amines[11] were converted to hydrochlorides **2a**–**9a** with hydrochloric acid[12] and to the corresponding methiodides **3b**–**9b**, bearing permanent positive charges, with methyl iodide[13] (Scheme 1). It was not possible to obtain compound **2b** in a pure form suitable for pharmacological assays.

All the synthesized compounds were tested for affinity versus human M_1–M_5 stable transfectants of CHO-K1 cells, using [^{3}H]-*N*-methylscopolamine chloride ([^{3}H]-NMS) as labeled ligand. Binding profile of the reference diphenidol hydrochloride (**1a**) and methiodide (**1b**)[7b] was also investigated in the present study. Binding data are reported as pK_i values (affinity) in Table 1. All the competition curves obtained with assayed compounds were compatible with the competitive inhibition of tracer binding.

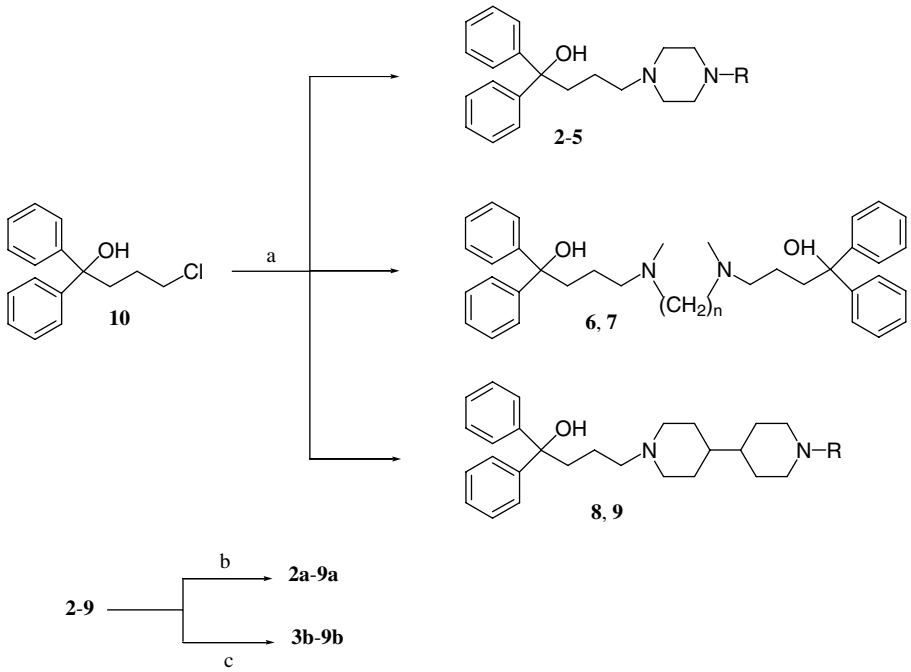
Reference compound **1a** showed a moderate affinity to human M_1–M_5 muscarinic receptors; however, it was not able to discriminate among the different muscarinic receptor subtypes. An appreciable increase of binding affinity to all muscarinic receptor subtypes was evidenced for the corresponding quaternary ammonium salt **1b**.

Replacement of the piperidine moiety with the piperazine ring (**2a**) did not cause any significant change in binding affinity at human M_1–M_5 subtypes, and the introduction of a substituent on 4-nitrogen atom of the piperazine (compounds **3a**–**5a**) was detrimental and led to drastic decrease of the binding affinity at all the five muscarinic subtypes.

Opening the piperazine ring, while maintaining the second diphenidol framework (**6a**), resulted in slight differences in affinity at human M_1–M_5 receptors, compared with **5a**. Interestingly, one-carbon homologation of the chain linking the two nitrogen atoms (**7a**) increased affinity of about or more than 2 orders of magnitude at human M_1–M_5 receptor subtypes. With respect to methiodide derivatives **3b**–**7b**, when compared to the corresponding tertiary amines, compounds **3b**–**6b** showed higher binding affinity at human M_1–M_5 receptors, while **7b** was less active. The better profile of the activity of quaternary ammonium derivatives is a common feature for most of the muscarinic antagonists.[14]

Compound **8a**, bearing a bipiperidinyl moiety and a second diphenidol framework, displayed a relevant affinity at the human M_4 receptor subtype (pK_i = 7.13), with a 12-fold enhanced affinity with respect to **1a** at this receptor subtype. Compound **8a** maintained a moderate affinity at the other receptor subtypes, comparable to those of **1a**. The corresponding quaternary derivative **8b** was the most interesting compound of this series, since it exhibited a noteworthy affinity at human M_4 receptor (pK_i = 7.90), similar to that found for the reference M_4 antagonist PD 102807 (ethyl 9-methoxy-2-methyl-3,11,12,14-tetrahydro-6a*H*-indolo[4′,5′:5,6][1,3]oxazino[2,3-*a*]isoquinoline-1-carboxylate),[3,4] maintaining a moderate affinity at the other muscarinic receptor subtypes. The following selectivity ratios were found: M_4/M_1 = 48, M_4/M_2 = 29, M_4/M_3 = 30 and M_4/M_5 = 47. A decrease in binding affinity was observed in derivative **9a**, where 4,4′-bipiperidine was preserved as diamine and a methyl group replaced the second diphenidol framework. The corresponding quaternary derivative **9b** showed affinity values similar to **9a**.

The affinity profile of derivatives **8a** and **8b** towards the human M_4 muscarinic receptor subtype was confirmed in functional assays. First, we investigated the effects of these compounds on GTP binding in membranes ob-



Scheme 1. Reagents and conditions: (a) diamine, 100–200 °C; (b) HCl, $CHCl_3/Et_2O$; (c) CH_3I, $CHCl_3$/acetone or $CHCl_3/Et_2O$.

Table 1. Binding affinities (pK_i) of the antagonists **1a**, **1b**, **2–9a**, **3–9b** and PD 102807 to human muscarinic receptors M_1–M_5

Compound	$pK_i \pm SEM$[a]				
	hM_1	hM_2	hM_3	hM_4	hM_5
1a	6.37 ± 0.09	5.55 ± 0.26	5.95 ± 0.13	6.04 ± 0.08	5.89 ± 0.25
1b	6.70 ± 0.05	6.56 ± 0.21	6.56 ± 0.21	6.54 ± 0.09	6.15 ± 0.20
2a	6.22 ± 0.07	5.14 ± 0.24	6.18 ± 0.12	5.93 ± 0.28	5.85 ± 0.30
3a	5.11 ± 0.24	4.45 ± 0.11	5.13 ± 0.34	5.01 ± 0.15	4.87 ± 0.21
3b	5.53 ± 0.27	4.63 ± 0.19	5.39 ± 0.26	5.32 ± 0.19	5.02 ± 0.22
4a	3.44 ± 0.34	3.34 ± 0.53	4.15 ± 0.23	4.00 ± 0.25	3.91 ± 0.31
4b	4.76 ± 0.10	4.59 ± 0.19	5.12 ± 0.47	4.64 ± 0.14	5.16 ± 0.26
5a	3.07 ± 0.26	3.75 ± 0.43	4.06 ± 0.19	4.65 ± 0.10	4.63 ± 0.21
5b	5.15 ± 0.26	5.64 ± 0.39	5.26 ± 0.35	5.91 ± 0.30	5.81 ± 0.18
6a	3.61 ± 0.16	3.67 ± 0.23	3.42 ± 0.53	3.79 ± 0.20	3.64 ± 0.26
6b	4.58 ± 0.17	4.32 ± 0.09	4.36 ± 0.23	4.95 ± 0.14	4.74 ± 0.45
7a	5.87 ± 0.14	5.26 ± 0.14	5.76 ± 0.19	6.21 ± 0.15	6.08 ± 0.13
7b	5.14 ± 0.14	4.92 ± 0.26	4.91 ± 0.29	4.95 ± 0.14	5.12 ± 0.16
8a	6.10 ± 0.16	6.06 ± 0.11	6.14 ± 0.32	7.13 ± 0.09	6.11 ± 0.33
8b	6.22 ± 0.06	6.44 ± 0.22	6.43 ± 0.09	7.90 ± 0.07	6.23 ± 0.25
9a	5.24 ± 0.17	5.40 ± 0.20	5.28 ± 0.15	5.93 ± 0.11	5.54 ± 0.25
9b	5.08 ± 0.15	5.36 ± 0.23	5.44 ± 0.11	5.57 ± 0.11	4.59 ± 0.32
PD 102807	5.64 ± 0.16	5.96 ± 0.27	6.14 ± 0.09	8.19 ± 0.15	5.74 ± 0.22

[a] Obtained in CHO-K1 cells stably expressing the human muscarinic M_1–M_5 receptors (mean of three experiments performed in triplicate). Further details are reported in Supplementary data.

tained from CHO cells stably expressing human M_1–M_5 receptors. We used a concentration-ratio analysis to estimate functionally the affinity constants (pK_b) of **8a** and **8b** and of the M_4 antagonist PD 102807,[3,4] at human M_1–M_5 receptors. The functional response was the stimulation by fixed concentrations of carbachol (10^{-12}–10^{-4} M) in the presence of europium-labeled GTP analogue (Eu-GTP) binding to cell membranes. The Eu-GTP assay measures the level of G-protein activation following agonist occupation of a G-protein coupled receptor, by determining the binding of the non-hydrolyzable analogue Eu-GTP to Galpha subunits. Thus, the assay measures a functional consequence of receptor occupancy at one of the earliest receptor-mediated events. The assay allows for traditional pharmacological parameters of potency, efficacy and antagonist affinity.[15] As reported in Table 2, **8b** displayed a noteworthy potency to prevent carbachol-stimulated binding of Eu-GTP in CHO/human M_4 cells. Compound **8b** showed a potency superimposable to that observed for PD 102807 whereas **8a** was less effective. As expected, in agreement with receptor binding data, **8a**, **8b** and PD 102807 were less effective in antagonizing carbachol-induced Eu-GTP binding to human M_1, M_2, M_3 and M_5

Table 2. Affinity constants (pK_b values) of **8a**, **8b** and PD 102807 in antagonizing carbachol-induced stimulation of Eu-GTP binding to CHO-M_1–M_5 cell membranes

Compound	$pK_b \pm SEM$[a]				
	hM_1	hM_2	hM_3	hM_4	hM_5
8a	5.92 ± 0.21	5.82 ± 0.27	6.14 ± 0.32	7.21 ± 0.14	6.40 ± 0.13
8b	6.04 ± 0.11	6.10 ± 0.22	6.25 ± 0.13	7.83 ± 0.18	6.02 ± 0.16
PD 102807	5.74 ± 0.18	5.55 ± 0.35	5.63 ± 0.16	7.90 ± 0.12	5.84 ± 0.21

[a] Obtained in CHO-K1 cells expressing the human muscarinic M_1–M_5 receptors (mean of three experiments performed in triplicate). Further details are reported in Supplementary data.

receptors. Compounds **8a**, **8b** and PD 102807 failed to affect basal Eu-GTP binding to CHO cell membranes expressing human M_1–M_5 (data not shown). Thereafter, to confirm the antagonist profile of **8a** and **8b** towards the human M_4 receptor, we evaluated the regulation of cAMP formation in CHO cells expressing the human M_4 receptor. Forskolin itself (10 μM) increased the rate of cAMP more than 12-fold in these cells. The muscarinic agonist carbachol (10^{-12}–10^{-4} M) inhibited, in a concentration-related manner, the forskolin-stimulated synthesis of cAMP (EC_{50} = 1870 nM). As shown in Table 3, **8b** and PD 102807 were very potent to reverse the inhibitory effect elicited by 5 μM carbachol whereas **8a** was less potent. All the other compounds were less effective (IC_{50} > 1 μM; data not shown). Compounds **8a** and **8b** or PD 102807 (from 0.1 nM to 1 μM), added to CHO cells, expressing human M_4 receptor, in the absence of carbachol did not modify forskolin-stimulated cAMP accumulation; thus, they do not exhibit any activity as muscarinic agonists (data not shown). Taken together, functional assay data confirmed the M_4-selective profile of compounds **8a** and **8b** and showed that they behave like competitive antagonists at M_4 receptor subtype.

The M_4 receptor binding affinity of compounds **8a** and **8b**, with a bipiperidinyl moiety and a second diphenidol framework, suggests that supplementary pharmacophores, such as bulky hydrophobic groups and a hydroxyl group, enhance the affinity by interaction with additional binding sites at M_4 muscarinic receptor subtype. In fact, compounds **9a** and **9b**, lacking the second 4-hydroxy-4,4-diphenyl-butyl moiety, showed a 16- and 214-fold decrease in affinity, respectively. Moreover, a proper spacer linking the two nitrogen atoms is crucial. Compounds **6a** and **6b**, which present a two-carbon chain as link between the nitrogen atoms of the two diphenidol units, exhibited very low affinity values. A one-carbon chain elongation (**7a** and **7b**) resulted in higher affinity values, even if modest. Considering M_1, M_2, M_3 and M_5 subtypes, the presence of the second diphenidol skeleton does not influence receptor interaction with respect to diphenidol, both as tertiary base and quaternary salt.

Interestingly, compound **8b** shows structural similarity to several alkane-bis-ammonium compounds, as W84 (hexamethylenebis[dimethyl(3-phthalimidopropyl) ammonium] dibromide), dimethyl-W84, the related DuoW and the eptane analogue C7/3′-phth, which have been characterized as allosteric modulators of muscarinic receptors,[17] including M_4 muscarinic receptor.[18] Indeed, muscarinic receptors can be modulated by ligands capable to interact with the acetylcholine binding pocket (the orthosteric site) and/or with allosteric sites.[19] The above-mentioned alkane-bis-ammonium compounds share a common pharmacophore consisting of two positively charged nitrogens, bridged by six or seven methylene groups, and two lateral aromatic moieties as crucial elements to obtain a high allosteric potency. Based upon these considerations, we evaluated the binding of **8b** to any allosteric site of the M_4 receptor. We measured the dissociation rate of [^{3}H]-NMS at the human M_4 muscarinic receptor, expressed in CHO cells, in experiments in which cell membranes were incubated with the radioligand and **8b** before radioligand dissociation was revealed by atropine (5 μM). In fact, an altered dissociation rate of the radioligand is unequivocally indicative of an allosteric action.[20] As expected, **8b** (10 nM), added to membranes incubated for 60 min in the presence of [^{3}H]-NMS, acted as a competitive antagonist and at this concentration almost halved the binding of [^{3}H]-NMS at the M_4 muscarinic receptor; interestingly, it was also able to retard the observed dissociation rate of the radioligand produced by atropine (Fig. 2). Under control conditions, the apparent dissociation rate constant of [^{3}H]-NMS ($k_{-1} = \ln 2/t_{1/2}$) was $0.03 \pm 0.004\ min^{-1}$ (mean ± standard error; $n = 4$) and it was decelerated in the presence of **8b** ($k_{-1} = 0.01 \pm 0.003\ min^{-1}$; mean ± standard error; $n = 4$). These results are consistent with an allosteric interaction between **8b** and the M_4 muscarinic receptor.

In summary, we have synthesized a series of diphenidol derivatives, which were evaluated both as hydrochlorides and as methiodides for their in vitro activity as muscarinic M_1–M_5 antagonists. Compounds **8a** and **8b**, bearing a bipiperidinyl moiety and a second diphenidol framework, showed an interesting selective M_4 antagonist activity. The quaternary ammonium derivative **8b** was the most potent M_4 antagonist and displayed, in receptor binding and in functional assays, a potency superimposable to that elicited by the reference M_4 antagonist PD 102807. Considering this also the corresponding hydrochloric salt **8a** exhibited a similar receptor profile, and the results obtained in our study provide the basis for further drug development. In addition, this idea is strengthened by the observation that **8b**, binding to the orthosteric and to a distinct allosteric site of the M_4 muscarinic receptor, might open new avenues to the discovery of novel multivalent antagonists[21] for the muscarinic receptors.

Table 3. EC_{50} values and their 95% confidence limits (CL_{95}) of **8a**, **8b** and PD 102807 in antagonizing carbachol-induced inhibition of forskolin-stimulated cAMP accumulation in CHO cells stably expressing hM_4 receptors

Compound	EC_{50} (nM) and CL_{95}[a]
8a	138 (82–163)
8b	67 (42–82)
PD 102807	54 (22–78)

The EC_{50} values and their 95% confidence limits (CL_{95}) were generated from dose–response curves using a computer program based on the method of Litchfield and Wilcoxon[16] (see also Supplementary data).
[a] Data are means (±SEM) of three experiments with incubations performed in triplicates.

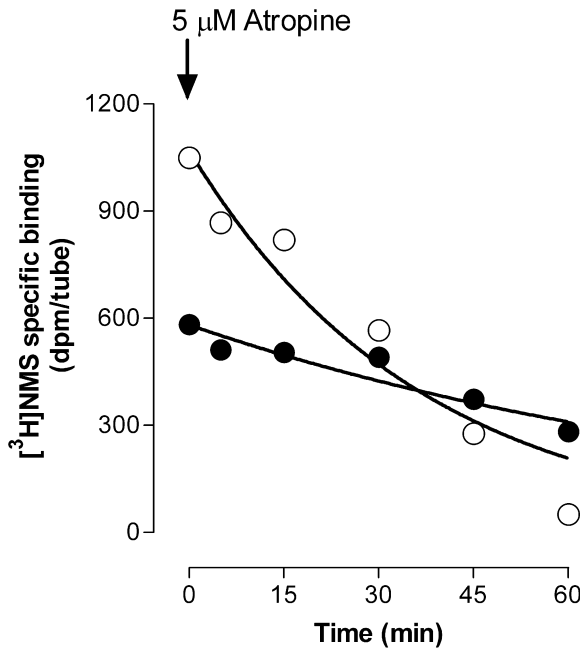


Figure 2. Effect of **8b** (10 nM) on the rate of [^{3}H]-NMS dissociation from CHO cell membranes expressing the human M_4 muscarinic receptor. Membranes were preincubated with 0.5 nM [^{3}H]-NMS for 120 min at 25 °C; then, radioligand dissociation was measured after the addition of 5 μM atropine (○). **8b** was added to the tubes 60 min before atropine (●). Data points represent means ± SEM of four experiments conducted in duplicate. [^{3}H]-NMS specific binding was calculated by subtracting the non-specific binding from the total binding as reported in Supplementary data. For the sake of clarity, error bars are not shown where they do not exceed 10% of the respective mean value.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.03.061.

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