Available online at www.sciencedirect.com
SCIENCE DIRECT®
Il Farmaco 58 (2003) 739–748
IL FARMACO
www.elsevier.com/locate/farmac

Synthesis and in vitro pharmacology of novel heterocyclic muscarinic ligands

Marco De Amici [a], Paola Conti [a], Ezio Fasoli [a], Elisabetta Barocelli [b], Vigilio Ballabeni [b], Simona Bertoni [b], Mariannina Impicciatore [b], Bryan L. Roth [c], Paul Ernsberger [d], Carlo De Micheli [a,*]

[a] *Istituto di Chimica Farmaceutica e Tossicologica, Università di Milano, Viale Abruzzi 42, Milan 20131, Italy*
[b] *Istituto di Farmacologia e Farmacognosia, Università di Parma, Parco Area delle Scienze 27/A, Parma 43100, Italy*
[c] *Department of Biochemistry and NIMH Psychoactive Drug Screening Program, SOM W438, Case Western Reserve University, 2109 Adelbert Rd., Cleveland, OH 44106, USA*
[d] *Department of Nutrition and NIMH Psychoactive Drug Screening Program, Case Western Reserve University, 2109 Adelbert Rd., Cleveland, OH 44106, USA*

Received 16 November 2002; accepted 15 January 2003

Abstract

A set of novel heterocyclic ligands (**7a**–**9a**, **7b**–**9b**, and **9c**) structurally related to oxotremorine **2** was designed, synthesized, and tested at muscarinic receptor subtypes. In the binding experiments at cloned hm1–5, the presence of the 2-methylimidazole/2-methyl-3-alkylimidazolium moiety in place of the pyrrolidine ring revealed, in derivatives **8a**, **8b**, and **9c**, a moderate selectivity for some receptor subtypes. The functional in vitro assays yielded results that correlated closely to binding data. In general, on passing from agonists bearing the pyrrolidine moiety to their analogues carrying the 2-methylimidazole function, the overall pharmacological efficacy profile is shifted from agonism toward partial agonism. The insertion of the 2-methyl-3-alkylimidazolium moiety advances the effect such that the compounds are pure antagonists. Quite similarly, chiral 3-oxo-Δ^2-isoxazoline (±)-**10** behaved as a weak antagonist unable to discriminate the different muscarinic receptor subtypes.

Keywords: Muscarinic ligands; Oxotremorine analogues; Muscarinic receptor subtypes; Functional assays; Cloned receptors

1. Introduction

The muscarinic acetylcholine receptors (mAChRs), which belong to the large superfamily of G protein-linked receptors, are expressed within the parasympathetic and central nervous systems, where they are involved in the excitatory or inhibitory modulation of a number of central and peripheral physiologic functions [1]. Molecular biological techniques have resulted in the cloning of five molecularly distinct mAChR (M_1–M_5) [2,3]. The M_1 receptors are found at high density in parasympathetic ganglia and neuronal tissues, whereas the M_2 and M_3 subtypes are mainly localized in peripheral effector organs such as heart (M_2) and smooth muscles, e.g. urinary bladder (M_3) and mucosal glands (M_3). The M_4 receptors have been identified peripherally in the lung [4] and centrally in the striatum using immunocytochemical and in situ hybridization techniques and are believed to be involved in the control of motor function [5] as well as in the mediation of antinociceptive effects [6]. Even though a better in vitro characterization of the M_5 receptors was recently achieved [7], at present their physiological role is far from being unequivocally established, although they are present mainly in the central nervous system [1,8].

The knowledge of the muscarinic receptor heterogeneity so far acquired through established conventional pharmacological approaches has been recently enriched by the advent of the genomic technology, which significantly contributes at providing the rationale for the identification of novel therapeutic targets for sub-

* Corresponding author.
E-mail address: carlo.demicheli@unimi.it (C. De Micheli).

0014-827X/03/$ - see front matter
doi:10.1016/S0014-827X(03)00113-7

type-selective muscarinic agents [1,9]. As a consequence, renewed efforts have been devoted to the search for new ligands able to selectively interact with the different muscarinic receptor subtypes. While a number of muscarinic antagonists have been developed in the past decades, there is still a demand for truly subtype-selective agonists, which could be clinically useful in treating, for example, Alzheimer's disease, pain, schizophrenia, and intestinal hypomotility [1,10–12].

In the past, our research group investigated the activity–selectivity profile of a set of chiral muscarinic ligands structurally related to natural muscarine [13–15]. Later on, we inserted the 3-oxo-isoxazolidinyl moiety of the highly potent muscarinic agonist azamuscarone **1** [16] (Fig. 1) into the molecular skeleton of oxotremorine **2** (Fig. 1), an efficacious muscarinic agonist moderately selective for the central nervous system [17]. In a series of related analogues, the pharmacological profile of representative derivatives, i.e. **3**–**5**, was studied in depth by taking into account both receptor occupancy [18] and in vivo tests [19]. It turned out that the oxotremorine-like derivative **3** exhibited a quite interesting analgesic activity.

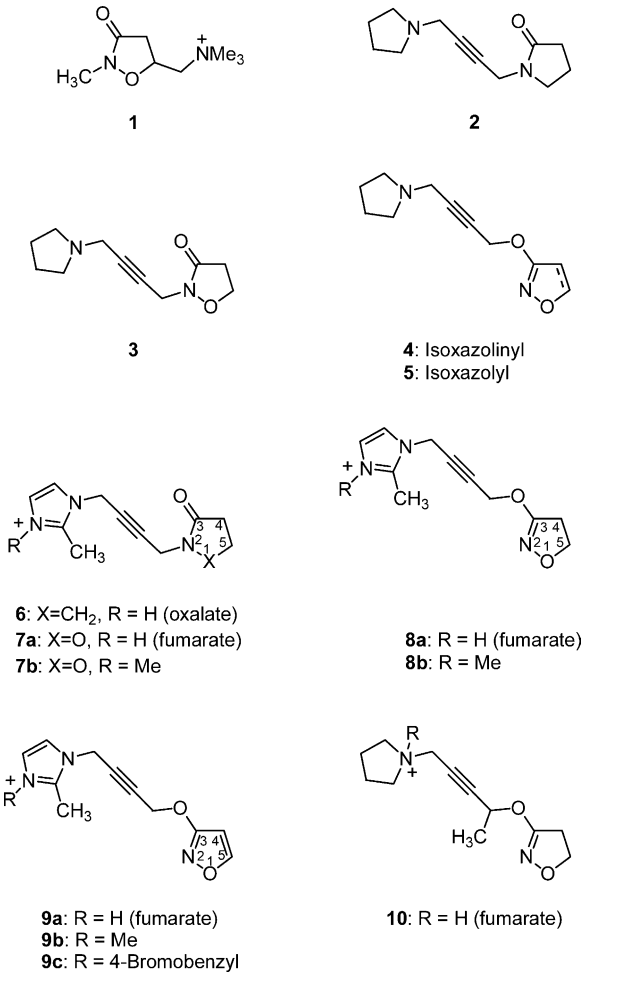


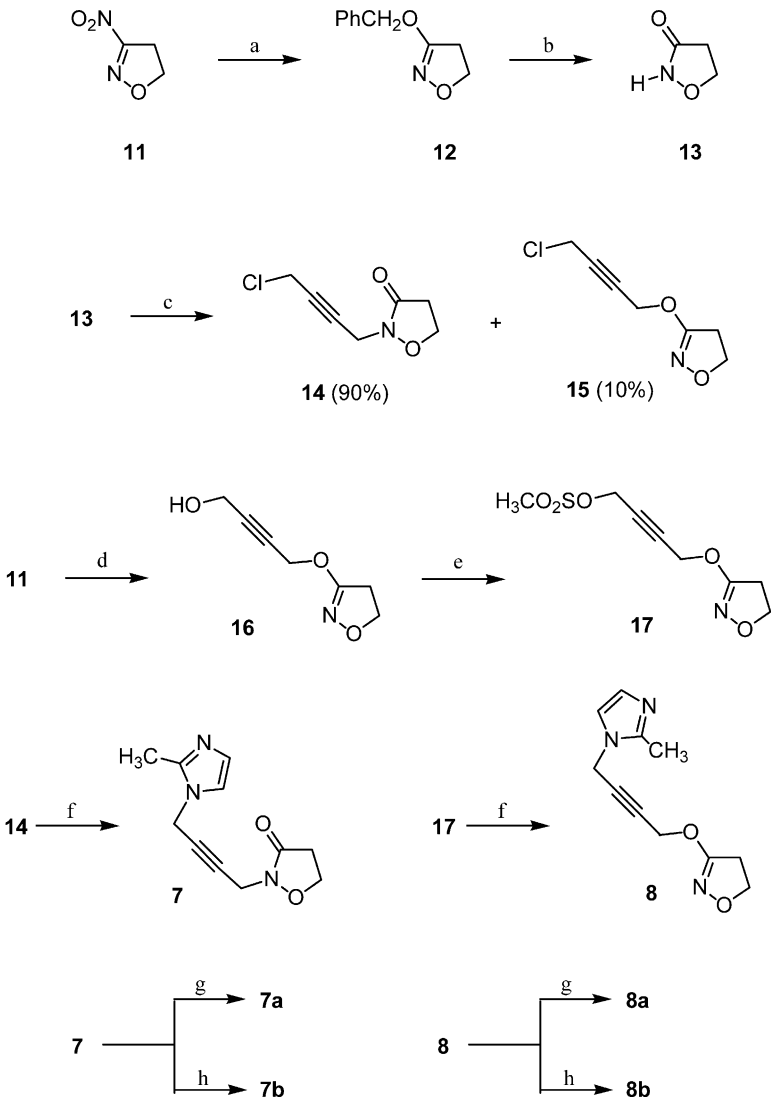
Fig. 1. Structure of model and target compounds.

As an extension of our previous studies, we now report the synthesis and the results of the pharmacological investigation of novel heterocyclic muscarinic ligands endowed with a butynyl side-chain. In particular, in our search for a bioisosteric replacement of the pyrrolidine function of derivatives **3**–**5**, we designed a set of derivatives where such a moiety was replaced by the 2-methylimidazole ring (Fig. 1, **7a**–**9a**). We also prepared the corresponding quaternary ammonium salts **7b**–**9b** and **9c**. Previous investigations showed that the exchange of the pyrrolidine nucleus of oxotremorine with the 2-methylimidazole ring, to generate **6** (Fig. 1), shifted the pharmacological profile from a full to a partial agonist [20]. Even more interesting, the insertion of the 2-methylimadazole nucleus in the skeleton of diphenylbutyramide derivatives, provided with an anti-muscarinic activity, enhanced the selectivity towards the M_3 receptor subtype [21]. Therefore, we designed and assayed novel ligands incorporating both the 2-methylimidazole and the 2-methylimidazolium nuclei, i.e. **7a**–**9a**, **7b**–**9b**, and **9c**, with the objective to uncover compounds with selective effects at specific receptor subtypes.

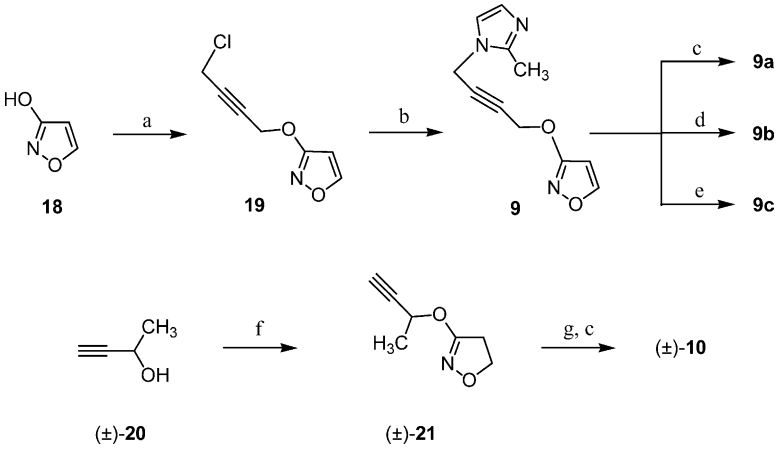
Moreover, in analogy to a modification previously performed on oxotremorine and its analogues [20,22], we synthesized the racemate of pyrrolidinyl derivative **10** (Fig. 1) to evaluate if the presence of the stereogenic center was able to alter the profile of compound **4**, the related non-chiral parent derivative. The new compounds were assayed at cloned human muscarinic receptor subtypes (hm1–5), expressed in Chinese Hamster Ovary (CHO) cells, and in functional tests at M_{1-3} tissue preparations.

2. Chemistry

The synthesis of target compounds was accomplished along the reaction sequences depicted in Schemes 1 and 2. Isoxazolidin-3-one (**13**) was conveniently prepared by transformation of known 3-nitro-Δ^2-isoxazoline (**11**) [23] into the corresponding 3-benzyloxy derivative **12**, which was then submitted to a catalytic hydrogenation (Scheme 1). Treatment of **13** with 1,4-dichloro-2-butyne, carried out in refluxing acetone in the presence of potassium carbonate, produced a 9:1 mixture of *N*- and *O*-alkynyl derivatives **14** and **15**, respectively. The two regioisomers were easily separated by column chromatography, and their structure was assigned by taking into account the difference in the chemical shifts of the α-CH_2 protons of the side-chain (δ 4.34 for **14** and δ 4.82 for **15**). The desired final salts **7a** and **7b** were obtained by reacting **14** with 2-methylimidazole followed by treatment of the intermediate free base **7** either with excess fumaric acid or methyl iodide (Scheme 1). In agreement with previously reported results [20], we



Scheme 1. (a) $C_6H_5CH_2OLi$, DMSO; (b)H_2-Pd/C, 5%; (c) 1,4-dichloro-2-butyne/K_2CO_3; (d) 2-butyne-1,4-diol/NaH/THF; (e) CH_3SO_2Cl/NEt_3; (f) 2-methylimidazole/CH_3CN; (g) $C_4H_4O_4$/2-propanol–MeOH; (h) CH_3I/2–propanol–ether.



Scheme 2. 1,4-Dichloro-2-butyne/K_2CO_3; (b) 2-methylimidazole/CH_3CN; (c) $C_4H_4O_4$/EtOH–ether; (d) CH_3I/2-propanol–ether; (e) *p*-$BrC_6H_4CH_2Br$/2-propanol–ether; (f) NaH-THF/**11**; (g) pyrrolidine/$(HCHO)_n$/CuCl/HOAc/dioxane.

assume that the alkylation step takes place at N-3. As a consequence, the reaction of **7** with methyl iodide gives rise to the 2,3-dimethylimidazolium iodide, i.e. **7b**.

Since the alkylation of intermediate **13** produced **15** in a very low yield, the same strategy could not be applied to the synthesis of the *O*-alkylated derivatives. For such a reason, we devised a new reaction sequence, which yielded exclusively the desired compounds. The reaction of 2-butyne-1,4-diol with 3-nitro-Δ^2-isoxazoline (**11**), in the presence of sodium hydride, produced alcohol **16** in 51% yield as a single product. Such an intermediate was then converted into the corresponding mesylate **17**, thus allowing the preparation of the *O*-alkynyl derivatives **8a** and **8b** (Scheme 1).

Following the procedure previously applied to **13**, the reaction of 3-hydroxyisoxazole (**18**) [24] with 1,4-dichloro-2-butyne gave isoxazolyl ether (**19**), precursor of the functionalized derivatives **9a**–**9c** (Scheme 2).

Finally, alkynyl-Δ^2-isoxazoline (±)-**21**, prepared from (±)-3-butyne-2-ol **20** and **11**, was submitted to a CuCl-catalyzed Mannich reaction in the presence of paraformaldehyde and pyrrolidine [25] (Scheme 2). The tertiary base (±)-**10**, so far obtained, was then transformed into the corresponding fumarate.

3. Results and discussion

The new compounds were tested for binding affinity at human muscarinic receptor subtypes (hm1–5) in transfected CHO cells labeled with [^{3}H]quinuclidinyl benzylate and the results are compared with those of well-recognized selective ligands (Table 1).

The potency and efficacy of the compounds under study were also evaluated towards some muscarinic receptor subtypes by performing the following four functional in vitro models: rabbit electrically stimulated vas deferens (M_1) [26], guinea pig electrically driven left atrium (M_2), guinea pig ileum (M_3), and urinary bladder (M_3; Table 2). Bethanechol and McN-A-343 were used as the reference agonists at M_{2-3} and M_1 subtypes, respectively.

Inspection of the data reported in Table 1 puts in evidence that among the 2-methylimidazole derivatives **7a**–**9a**, solely **8a** displays a certain degree of selectivity. Its affinity for hm2, hm3, and hm5 (pK_i values: 8.44, 8.60, and 8.21, respectively) is at least 10-fold higher than that associated to hm1 and hm4 (pK_i values: 7.04 and 7.40, respectively). The transformation of **8a** into the corresponding methylimidazolium methiodide **8b** gives rise to the appearance of a 10-fold selectivity for the hm3 receptor. As a matter of fact, derivative **8b** possesses a hm3/hm2 selectivity ratio of 14.6 which is higher than that reported for 4-DAMP (M_3/M_2 equal to 3.5 or 4.2) [27,28]. A similar profile holds true for **9c** since the derivative is a slightly selective hm3 ligand.

The results collected in the binding experiments were largely confirmed in the functional in vitro assays (Table 2). Comparison of the binding and function data by linear regression showed strong correlations for M_1 and M_2 receptors and a moderate correlation for M_3 subtype (Fig. 2). For M_1 and M_2 receptors, the six compounds tested in both assays fell very close to the line of identity, where binding affinity and functional activity show molar equivalency. Part of the deviation from perfect correlations may reflect species differences in ligand selectivity, as binding assays were carried out with the human form of the receptors and functional assays were performed in rabbits and guinea pigs. Compounds which were agonists (**8a** and **9a** for M_1 and compounds **7a**, **8a**, and **9a** for M_2) fell above the line of identity, indicating slightly greater functional activity relative to binding affinity. In contrast, antagonist compounds fell near or below the line of identity. The same trend was observed at M_3 receptors, but all compounds showed greater activity in binding than in functional assays, resulting in a downward shift of all points.

By taking into account the data collected in the functional tests (Table 2), the insertion of the 2-methylimidazole moiety shifts the pharmacological profile of the derivatives from a full ($\alpha = 1$) to a partial

Table 1
Binding affinity (pK_i, molar) of compounds **7a**–**7b**, **8a**–**8b**, **9a**, and **9c** at cloned human muscarinic receptor subtypes (hm1–5) expressed in CHO cells

Comp.	hm1	hm2	hm3	hm4	hm5
Pirenzepine	7.67±0.037				7.45±0.042
Methoctramine		7.11±0.036			
4-DAMP			9.17±0.042		
Tropicamide				8.46±0.021	
7a	6.92±0.036	7.14±0.030	6.99±0.101	7.06±0.034	9.29±0.037
7b	6.29±0.044	6.56±0.05	7.12±0.02	6.59±0.035	6.88±0.055
8a	7.04±0.007	8.44±0.080	8.60±0.022	7.40±0.046	8.21±0.086
8b	6.64±0.036	7.03±0.042	8.20±0.090	7.03±0.037	7.29±0.04
9a	5.96±0.018	6.55±0.050	6.96±0.022	6.33±0.022	6.68±0.056
9c	6.06±0.039	6.95±0.090	7.77±0.103	6.68±0.042	7.22±0.02

Table 2
In vitro functional activity of compounds **7a**–**7b**, **8a**–**8b**, **9a**–**9c**, and **10** at muscarinic receptor subtypes in rabbit vas deferens (M_1), guinea pig left atrium (M_2), guinea pig ileum (M_3) and urinary bladder (M_3)

Comp.	M_1			M_2			M_3					
	Rabbit vas deferens [a]			Guinea pig left atrium			Guinea pig ileum			Guinea pig urinary bladder		
	pEC_{50} [b]	ia [c]	pK_B [d]	pEC_{50} [b]	ia [c]	pK_B [d]	pEC_{50} [b]	ia [c]	pK_B [d]	pEC_{50} [b]	ia [c]	pK_B [d]
Bethanechol				5.83±0.04	1		6.51±0.07	1	–	4.96±0.03	1	4.38±0.33
McN-A-343	6.42±0.04	1	–	–			–			–		
7a		0	7.04±0.10	7.47±0.06	1	–	7.53±0.20	0.9	–	6.61±0.08	0.3	
7b		0	6.15±0.07		0	6.21±0.07		0	6.08±0.23		0	6.04±0.10
8a	7.43±0.04	1	–	8.09±0.15	1	–	7.94±0.17	0.7	–	6.51±0.24	0.3	
8b		0	6.72±0.12		0	6.79±0.23		0	6.91±0.02		0	7.0±0.16
9a	6.51±0.19	0.8	–	7.30±0.11	1	–	6.90±0.15	0.7	–	5.44±0.15	0.3	
9b		0	5.73±0.32		0	6.32±0.11		0	6.47±0.06		0	6.38±0.09
9c		0	6.36±0.17		0	7.07±0.24		0	6.44±0.12		0	5.93±0.09
10		0	6.93±0.06		0	6.54±0.14		0	6.35±0.29		0	6.55±0.05

[a] See Ref. [26].
[b] pEC_{50}±SE values are the negative logarithm of the agonist concentration that caused 50% of the maximum response attainable in that tissue.
[c] Intrinsic activity (α) measured by the ratio between the maximum response of the compound and the maximum response of the reference agonist.
[d] Apparent pK_B values±SE were calculated according to Furchgott [35].

agonist ($\alpha < 1$), as previously observed in the oxotremorine series [20]. In this frame, the profile of **7a** is worth noting since it shows a degree of functional selectivity. It behaves as a full agonist at guinea pig cardiac M_2 ($pEC_{50} = 7.47$), as a partial agonist in the two M_3 assays ($pEC_{50} = 7.53$, $\alpha = 0.9$ and $pEC_{50} = 6.61$, $\alpha = 0.3$, respectively) and as a pure antagonist at M_1 rabbit vas deferens receptors ($pK_B = 7.04$, $\alpha = 0$). As expected, based on the urinary bladder low coupling efficiency [29], compounds **7a**–**9a** display a 10–30-fold lower potency than that detected on ileum. The agonist effect of the studied compounds was competitively blocked by the classical antimuscarinic drug atropine and was unaffected by the presence of hexamethonium.

The transformation of the tertiary bases into quaternary ammonium salts further shifted the pharmacological profile towards pure antagonists ($\alpha = 0$). Once again, none of the ammonium derivatives showed selectivity for any of the muscarinic receptor subtypes. The 2-methylimidazolium methiodides, **7b**–**9b**, possess pK_B values in the range 5.73–7.00 at M_1–M_3 receptors. Quaternization of **9** with 4-bromobenzyl bromide to yield **9c** did not substantially alter the profile of the corresponding methiodide (**9b**). Such a modification, previously applied to a series of 2,2-diphenylbutyramides provided with potent antimuscarinic properties [30], significantly improved the M_3 versus M_2 selectivity.

Finally, we evaluated the influence of a substituent in the side-chain on both activity and selectivity. For such a purpose, the full muscarinic agonist **4** [17,18] (Fig. 1) was methylated at position 1 of the butynyl side-chain to produce derivative (±)-**10**, which became a weak non-

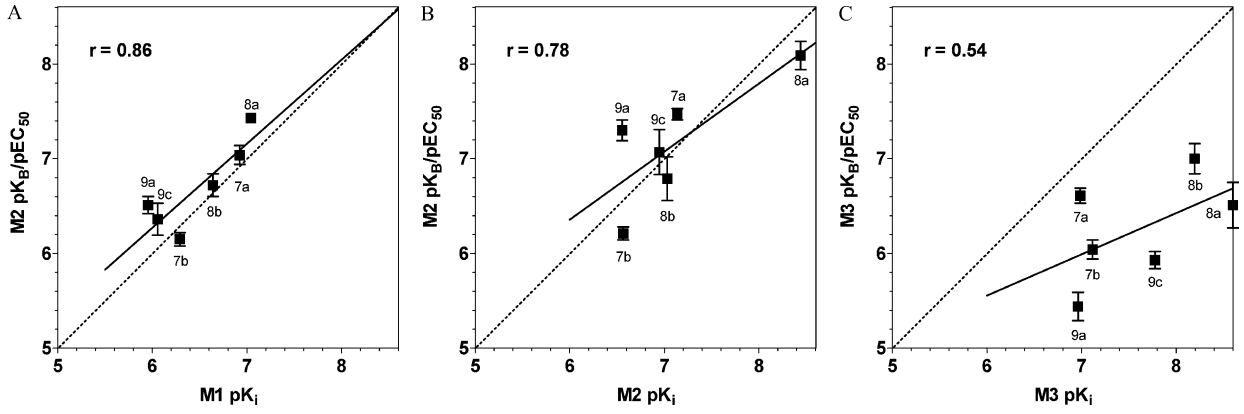


Fig. 2. Correlation of binding affinity (pK_i) to functional activity (pEC_{50} for agonists and pK_B for antagonists) for M_1 (A), M_2 (B), and M_3 (C) mAChRs. Dashed line represents the line of identity connecting identical values for the two axes. Solid line represents linear regression. Pearson correlation coefficients were as stated ($N = 6$). Error bars indicate standard error. For the binding data plotted on the horizontal axis, error bars are not visible since the errors were smaller than the size of the plotted symbol.

selective muscarinic antagonist (Table 2). This result parallels that previously observed on an analogue of oxotremorine [20,22].

In summary, the data collected on the novel oxotremorine-like compounds indicate that the presence of the 2-methylimidazole/2-methylimadazolium moieties strongly affects the intrinsic activity (ia) of the compounds. A selectivity between subtypes of 10-fold or more was noticed in some derivatives in the binding data, but selectivity was threefold or less in the in vitro functional assays.

4. Experimental

4.1. Chemistry

3-Substituted-Δ^2-isoxazolines, **11** [23] and **16** [17], isoxazolidin-3-one (**13**) [17], and 3-hydroxyisoxazole (**18**) [24] were prepared according to procedures described in the literature. Melting points were determined on a model B 540 Büchi apparatus and are uncorrected. Liquid compounds were characterized by the oven temperature for Kugelrohr distillations. ^{1}H NMR spectra were recorded with a Bruker AC-E 200 (200 MHz) spectrometer in $CDCl_3$ (unless otherwise specified) solutions. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in hertz. TLC analyses were performed on commercial silica gel 60 F_{254} aluminum sheets: spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution. Microanalyses (C, H, N) of new compounds agreed with the theoretical value within $\pm$0.4%.

4.1.1. 2-[4-(2-Methyl-1H-imidazol-1-yl)-2-butynyl]isoxazolidin-3-ones (7a and 7b)

A) To a solution of **13** [17] (1.80 g, 20.67 mmol) in acetone (30 ml) was added potassium carbonate (5.72 g, 41.38 mmol). The suspension was heated at reflux for 1 h and then cooled at room temperature. 1,4-Dichloro-2-butyne (12.13 ml, 0.124 mol) was then added and, after heating at reflux for 24 h, the crude reaction mixture was poured into water (100 ml) and extracted with dichloromethane (3 × 50 ml). The pooled organic extracts were dried over anhydrous sodium sulfate and concentrated at reduced pressure. The dark brown residue was chromatographed on silica gel (eluant: 40% ethyl acetate/petroleum ether) to give regioisomers **14** (1.42 g, 39.5% yield) and **15** (0.16 g, 4.5% yield).

2-(4-Chloro-2-butynyl)isoxazolidin-3-one (**14**): m.p., 73.5–74.5 °C (pale yellow prisms from 10% ethyl acetate/petroleum ether), R_f, 0.19 (eluant: 30% ethyl acetate/cyclohexane); ^{1}H NMR: 2.78 (t, 2H, H-4, J = 8.1 Hz), 4.14 (t, 2H, CH_2Cl, J = 1.8 Hz), 4.34 (t, 2H, CH_2N, J = 1.8 Hz), 4.38 (t, 2H, H-5, J = 8.1 Hz). Anal. $C_7H_8ClNO_2$ (C, H, N).

3-(4-Chloro-2-butynyl)oxy-Δ^2-isoxazoline (**15**): colorless liquid, b.p., 115–120 °C/0.5 mmHg; R_f, 0.61 (eluant: 30% ethyl acetate/cyclohexane); ^{1}H NMR: 3.0 (t, 2H, H-4, J = 9.6 Hz), 4.18 (t, 2H, CH_2Cl, J = 1.8 Hz), 4.43 (t, 2H, H-5, J = 9.6 Hz), 4.82 (t, 2H, O–CH_2C≡, J = 1.8 Hz). Anal. $C_7H_8ClNO_2$ (C, H, N).

B) To a solution of **14** (1.30 g, 7.49 mmol) in 10 ml acetonitrile was added 2-methylimidazole (1.23 g, 14.98 mmol). After stirring at reflux for 3 h, the solvent was removed at reduced pressure and the crude reaction mixture, acidified by addition of 10-ml 2-N HCl, was treated with ether (3 × 10 ml). The residual aqueous phase was made alkaline (pH 10) by portion-wise addition of solid K_2CO_3 and extracted with ethyl acetate (3 × 10 ml). The pooled organic extracts were dried over anhydrous sodium sulfate, the solvent was evaporated under vacuum, and the oily residue was purified by silica gel column chromatography (eluant: 2% methanol/dichloromethane) to afford 0.690 g (42% yield) of the desired tertiary amine.

2-[4-(2-Methyl-1*H*-imidazol-1-yl)-2-butynyl]isoxazolidin-3-one (**7**): pale yellow viscous oil, R_f, 0.30 (eluant: 5% methanol/dichloromethane); ^{1}H NMR: 2.40 (s, 3H, CH_3), 2.75 (t, 2H, H-4, J = 8.1 Hz), 4.28 (t, 2H, N–CH_2–C≡, J = 1.8 Hz), 4.34 (t, 2H, H-5, J = 8.1 Hz), 4.61 (t, 2H, CH_2–Imid., J = 1.8 Hz), 6.88 (s, 1H, H-4′), 6.91 (s, 1H, H-5′).

C) To a solution of fumaric acid (0.620 g, 5.34 mmol) in 15 ml of methanol–2-propanol (3:7) was added **7** (0.390 g, 1.78 mmol). The corresponding salt precipitated slowly on standing and was purified by crystallization.

2-[4-(2-Methyl-1*H*-imidazol-1-yl)-2-butynyl]isoxazolidin-3-one sesquifumarate (**7a**): m.p., 112.5–115 °C dec. (colorless prisms from absolute ethanol); ^{1}H NMR (CD_3OD): 2.63 (s, 3H, CH_3), 2.81 (t, 2H, H-4, J = 8.3 Hz), 4.40 (t, 2H, H-5, J = 8.3 Hz), 4.42 (t, 2H, N–CH_2–C≡, J = 1.7 Hz), 5.12 (t, 2H, CH_2–Imid., J = 1.7 Hz), 6.77 (s, 2H, HC=CH), 7.37 (d, 1H, H-4′, J = 2.1 Hz), 7.52 (d, 1H, H-5′, J = 2.1 Hz). Anal. $C_{17}H_{19}N_3O_8$ (C, H, N).

D) A solution of **7** (0.250 g, 1.14 mmol) in 2-propanol (5 ml) was treated with a fivefold excess of methyl iodide at room temperature. The corresponding salt precipitated upon addition of anhydrous ether.

2-[4-(2,3-Dimethyl-1*H*-imidazolium-1-yl)-2-butynyl]isoxazolidin-3-one iodide (**7b**): m.p., 140.5–142 °C (colorless prisms from 2-propanol); ^{1}H NMR (D_2O): 2.64 (s, 3H, CH_3), 2.92 (t, 2H, H-4, J = 8.5 Hz), 3.79 (s, 3H, N–Me), 4.43 (t, 2H, N–CH_2–C≡, J = 1.9 Hz), 4.47 (t, 2H, H-5, J = 8.5 Hz), 5.04 (t, 2H, CH_2–Imid., J = 1.9 Hz), 7.35 (d,

1H, H-4′, $J = 2.3$ Hz), 7.48 (d, 1H, H-5′, $J = 2.3$ Hz). Anal. $C_{12}H_{16}IN_3O_2$ (C, H, N).

4.1.2. 3-[4-(2-Methyl-1H-imidazol-1-yl)-2-butynyl]oxy-Δ^2-isoxazolines (**8a** and **8b**)

A) To a solution of **16** [17] (2.0 g, 12.89 mmol) and triethylamine (3.6 ml, 25.78 mmol) in dichloromethane (50 ml), a solution of methanesulfonyl chloride (2.50 ml, 25.78 mmol) in dichloromethane (15 ml) was added dropwise at 0 °C. After stirring for 1 h at room temperature, 1 N HCl (50 ml) was added, the phases were separated, and the aqueous layer was extracted with dichloromethane (3 × 30 ml). After the usual work up, the residue was column chromatographed (eluant: 50% petroleum ether/ethyl acetate) to give 2.62 g (87%) of the desired ester.

3-(4-Hydroxy-2-butynyl)oxy-Δ^2-isoxazoline mesylate (**17**): m.p., 49–50 °C (colorless prisms from 30% ethyl acetate/petroleum ether); R_f, 0.43 (eluant: 40% cyclohexane/ethyl acetate); ^{1}H NMR: 3.01 (t, 2H, H-4, $J = 9.4$ Hz), 3.17 (s, 3H, CH_3), 4.43 (t, 2H, H-5, $J = 9.4$ Hz), 4.83 (bs, 2H, O–CH_2C≡), 4.94 (bs, 2H, CH_2OSO_2). Anal. $C_8H_{11}NO_5S$ (C, H, N).

B) To a solution of **17** (1.80 g, 7.72 mmol) in 10 ml acetonitrile was added 2-methylimidazole (1.27 g, 15.44 mmol). After stirring at reflux for 1 h, the solvent was removed at reduced pressure and the residue was submitted to the same procedure above described for analogue **7**. Purification of the crude basic extract by silica gel column chromatography (eluant: 2% methanol/dichloromethane) afforded 1.23 g (73% yield) of the desired imidazole derivative.

3-[4-(2-Methyl-1*H*-imidazol-1-yl)-2-butynyl]oxy-Δ^2-isoxazoline (**8**): yellow viscous oil, R_f, 0.33 (eluant: 5% methanol/dichloromethane); ^{1}H NMR: 2.39 (s, 3H, CH_3), 2.99 (t, 2H, H-4, $J = 9.6$ Hz), 4.40 (t, 2H, H-5, $J = 9.6$ Hz), 4.65 (t, 2H, CH_2–Imid., $J = 1.9$ Hz), 4.78 (t, 2H, O–CH_2–C≡, $J = 1.9$ Hz), 6.91 (s, 1H, H-4′), 6.94 (s, 1H, H-5′).

C) To a solution of fumaric acid (0.763 g, 6.57 mmol) in 25 ml of methanol–2-propanol (3:7) was added **8** (0.480 g, 2.19 mmol). The corresponding salt precipitated slowly on standing and was purified by crystallization.

3-[4-(2-Methyl-1*H*-imidazol-1-yl)-2-butynyl]oxy-Δ^2-isoxazoline sesquifumarate (**8a**): m.p., 121.5–123 °C dec. (colorless prisms from absolute ethanol); ^{1}H NMR (CD_3OD): 2.63 (s, 3H, CH_3), 3.05 (t, 2H, H-4, $J = 9.7$ Hz), 4.40 (t, 2H, H-5, $J = 9.7$ Hz), 4.89 (bs, 2H, O–CH_2–C≡), 5.11 (bs, 2H, CH_2–Imid.), 6.75 (s, 2H, HC=CH), 7.32 (d, 1H, H-4′, $J = 2.0$ Hz), 7.51 (d, 1H, H-5′, $J = 2.0$ Hz). Anal. $C_{17}H_{19}N_3O_8$ (C, H, N).

D) A solution of **8** (0.410 g, 1.87 mmol) in 2-propanol (10 ml) was treated with a fivefold excess of methyl iodide at room temperature. The corresponding salt precipitated upon addition of anhydrous ether.

3-[4-(2,3-Dimethyl-1*H*-imidazolium-1-yl)-2-butynyl]oxy-Δ^2-isoxazoline iodide (**8b**): m.p., 132.5–133.5 °C dec. (colorless prisms from 2-propanol); ^{1}H NMR (D_2O): 2.65 (s, 3H, CH_3), 3.12 (t, 2H, H-4, $J = 9.7$ Hz), 3.79 (s, 3H, N–Me), 4.44 (t, 2H, H-5, $J = 9.7$ Hz), 4.86 (bs, 2H, O–CH_2–C≡), 5.08 (bs, 2H, CH_2–Imid.), 7.37 (d, 1H, H-4′, $J = 2.0$ Hz), 7.49 (d, 1H, H-5′, $J = 2.0$ Hz). Anal. $C_{12}H_{16}IN_3O_2$ (C, H, N).

4.1.3. 3-[4-(2-Methyl-1H-imidazol-1-yl)-2-butynyl]oxyisoxazoles (**9a**, **9b**, and **9c**)

A) The 3-substituted isoxazole (**19**) was prepared in 36% yield from 3-hydroxyisoxazole (**18**) [24] according to the procedure above described for the preparation of **14** and **15**, by increasing the overall refluxing time to 48 h.

3-(4-Chloro-2-butynyl)oxyisoxazole (**19**): colorless liquid, b.p., 125–130 °C/0.5 mmHg; R_f, 0.32 (eluant: 10% ethyl acetate/cyclohexane); ^{1}H NMR: 4.20 (s, 2H, CH_2Cl), 4.94 (s, 2H, O–CH_2C≡), 6.01 (d, 1H, H-4, $J = 1.8$ Hz), 8.16 (d, 1H, H-5, $J = 1.8$ Hz). Anal. $C_7H_6ClNO_2$ (C, H, N).

B) Intermediate **19** (2.20 g, 12.82 mmol) was reacted with 2-methylimidazole (2.11 g, 25.7 mmol) following the protocol previously applied to **14**. The corresponding tertiary base (1.475 g, 53% yield) was obtained in a pure form after silica gel column chromatography (eluant: 2% methanol/dichloromethane).

3-[4-(2-Methyl-1*H*-imidazol-1-yl)-2-butynyl]oxyisoxazole (**9**): light yellow viscous oil, R_f, 0.38 (eluant: 5% methanol/dichloromethane); ^{1}H NMR: 2.39 (s, 3H, CH_3), 4.67 (bs, 2H, CH_2–Imid.), 4.91 (bs, 2H, O–CH_2–C≡), 6.0 (d, 1H, H.4, $J = 2.0$ Hz), 6.93 (s, 1H, H-4′), 6.95 (s, 1H, H-5′), 8.13 (d, 1H, H-5, $J = 2.0$ Hz).

C) Derivative **9** (0.450 g, 2.07 mmol) was reacted with excess fumaric acid or excess methyl iodide according to the procedures applied to **7** and **8**.

3-[4-(2-Methyl-1*H*-imidazol-1-yl)-2-butynyl]oxyisoxazole sesquifumarate (**9a**): m.p., 120.5–122 °C (colorless prisms from absolute ethanol); ^{1}H NMR (CD_3OD): 2.58 (s, 3H, CH_3), 4.98 (bs, 2H, O–CH_2–C≡), 5.08 (bs, 2H, CH_2–Imid.), 6.18 (d, 1H, H-4, $J = 2.0$ Hz), 6.75 (s, 2H, HC=CH), 7.24 (d, 1H, H-4′, $J = 2.0$ Hz), 7.44 (d, 1H, H-5′, $J = 2.0$ Hz), 8.45 (d, 1H, H-5, $J = 2.0$ Hz). Anal. $C_{17}H_{17}N_3O_8$ (C, H, N).

3-[4-(2,3-Dimethyl-1*H*-imidazolium-1-yl)-2-butynyl]oxyisoxazole iodide (**9b**): m.p., 137–138 °C dec.

(colorless prisms from 2-propanol); ^{1}H NMR (CD_3OD): 2.70 (s, 3H, CH_3), 3.89 (s, 3H, N–CH_3), 5.03 (bs, 2H, O–CH_2–C≡), 5.20 (bs, 2H, CH_2–Imid.), 6.19 (d, 1H, H-4, $J = 2.0$ Hz), 7.56 (d, 1H, H-4′, $J = 2.0$ Hz), 7.65 (d, 1H, H-5′, $J = 2.0$ Hz), 8.45 (d, 1H, H-5, $J = 2.0$ Hz). Anal. $C_{12}H_{14}IN_3O_2$ (C, H, N).

D) A solution of **9** (0.300 g, 1.38 mmol) in 10 ml of ether/2-propanol (4:1) was treated with 4-bromobenzyl bromide (0.552 g, 2.21 mmol) at room temperature. The corresponding ammonium salt precipitated on standing.

3-[4-(2-Methyl-3-(4-bromobenzyl)-1*H*-imidazolium-1-yl)-2-butynyl]oxyisoxazole bromide (**9c**): m.p., 139–141 °C dec. (colorless prisms from acetone–ether); ^{1}H NMR (CD_3OD): 2.71 (s, 3H, CH_3), 5.05 (t, 2H, O–CH_2–C≡, $J = 1.5$ Hz), 5.22 (t, 2H, CH_2-Imid., $J = 1.5$ Hz), 5.45 (s, 2H, CH_2Ar), 6.19 (d, 1H, H-4, $J = 1.9$ Hz), 7.31 (d, 2H, Ar, $J = 8.6$ Hz), 7.63 (d, 1H, H-4′, $J = 2.1$ Hz), 7.67 (d, 2H, Ar, $J = 8.6$ Hz), 7.73 (d, 1H, H-5′, $J = 2.1$ Hz), 8.44 (d, 1H, H-5, $J = 1.9$ Hz). Anal. $C_{18}H_{17}Br_2N_3O_2$ (C, H, N).

4.1.4. (±)-3-[4-(1-Pyrrolidinyl)-1-methyl-2-butynyl]oxy-Δ^2-isoxazoline (*10*)

A) To a solution of (±)-3-butyn-2-ol (**20**) (1.80 g, 25.68 mmol) in 25 ml anhydrous THF was added sodium hydride (0.72 g, 30 mmol) under an inert atmosphere. After stirring at room temperature for 30 min, a solution of **11** (1.0 g, 8.61 mmol), in anhydrous THF (10 ml) was added dropwise. The reaction mixture was heated at reflux for 18 h, then cooled at 0 °C. After cautious addition of water (20 ml) and concentration at reduced pressure, the residual aqueous phase was extracted with ethyl acetate (4 × 30 ml). After the usual work up, the residue was purified on a silica gel column (eluant: 30% ethyl acetate/petroleum ether) to give 0.540 g (45%) of the intermediate alkyne.

(±)-3-(1-Methyl-2-propynyl)oxy-Δ^2-isoxazoline (**21**): colorless oil, b.p., 85–90 °C/0.3 mmHg; R_f, 0.23 (eluant: 20% ethyl acetate/cyclohexane); ^{1}H NMR: 1.61 (d, 3H, CHCH_3, $J = 7.2$ Hz), 2.56 (d, 1H, H–C≡C, $J = 1.9$ Hz), 2.98 (t, 2H, H-4, $J = 9.8$ Hz), 4.40 (t, 2H, H-5, $J = 9.8$ Hz), 5.25 (dq, 1H, CHCH$_3$, $J = 7.2$ and 1.9 Hz). Anal. $C_7H_9NO_2$ (C, H, N).

B) A solution of alkyne (±)-**21** (0.478 g, 3.44 mmol) in 4 ml of dioxane was added to a stirred suspension of pyrrolidine (334 μl, 4.0 mmol), cuprous chloride (26 mg, 0.26 mmol), glacial acetic acid (0.1 ml), and paraformaldehyde (0.106 g, 3.53 mmol) in dioxane (5 ml). The reaction mixture was stirred at 50 °C for 18 h, then the solvent was removed at reduced pressure. After addition of 10-ml 2-N HCl and extraction with ether (3 × 10 ml), the residual aqueous phase was made alkaline (pH 10) by portion-wise addition of solid K_2CO_3, and extracted with ethyl acetate (3 × 10 ml). After the usual work up, the oily residue was purified by silica gel column chromatography (eluant: 5% methanol/ethyl acetate) to give the desired pyrrolidine derivative (0.329 g, 43% yield).

(±)-3-[4-(1-Pyrrolidinyl)-1-methyl-2-butynyl]-oxy-Δ^2-isoxazoline: yellow oil, R_f, 0.40 (eluant: 5% methanol/dichloromethane); ^{1}H NMR: 1.56 (d, 3H, CHCH_3, $J = 7.2$ Hz), 1.77 (m, 4H, β pyrrolidine), 2.59 (m, 4H, α-pyrrolidine), 2.94 (t, 2H, H-4, $J = 9.8$ Hz), 3.43 (s, 2H, CH_2N), 4.37 (t, 2H, H-5, $J = 9.8$ Hz), 5.24 (q, 1H, CHCH$_3$, $J = 7.2$ Hz).

C) The corresponding fumarate was prepared by reacting the tertiary base (0.300 g, 1.35 mmol) with a threefold excess of fumaric acid.

(±)-3-[4-(1-Pyrrolidinyl)-1-vmethyl-2-butynyl]-oxy-Δ^2-isoxazoline fumarate (**10**): m.p., 154–156 °C dec. (colorless prisms from absolute ethanol); ^{1}H NMR (CD_3OD): 1.60 (d, 3H, CHCH_3, $J = 7.1$ Hz), 2.11 (m, 4H, β-pyrrolidine), 3.03 (t, 2H, H-4, $J = 9.7$ Hz), 3.39 (m, 4H, α-pyrrolidine), 4.13 (s, 2H, CH_2N), 4.39 (t, 2H, H-5, $J = 9.7$ Hz), 5.24 (q, 1H, CHCH$_3$, $J = 7.1$ Hz), 6.73 (s, 2H, HC=CH). Anal. $C_{16}H_{22}N_2O_6$ (C, H, N).

4.2. Pharmacology

Stable cell lines expressing the hm1–hm5 muscarinic receptors were obtained via the resources of NIMH PDSP and were tested for receptor expression by radioligand binding to L-quinuclidinyl[phenyl-4-^{3}H]benzylate ([^{3}H]QNB). Saturation experiments were performed using 5–2500 pM [^{3}H]QNB in 10 mM potassium phosphate buffer, pH 7.4, to determine the K_d of [^{3}H]QNB for each receptor subtype. Competition binding experiments were performed using 180 pM [^{3}H]QNB and 10–50 μg of membrane protein. All binding experiments were performed in the presence of 1% DMSO. Non-specific binding was defined by 0.5 μM atropine. After equilibrium was reached (120 min incubation at 25 °C), bound and free radioactivity were separated by filtration using Whatman GF-C filters. K_i values were derived from IC_{50} values using Cheng–Prusoff equation [31].

Male guinea pigs (250–350 g) and New Zealand white rabbits (3.0–3.5 kg; Morini, S. Polo, Italy) were used. The tissues for in vitro experiments were removed from animals fasted 24 h before the experiments and killed by CO_2 inhalation. Isolated preparations were set up following the techniques previously described [17].

4.2.1. Electrically stimulated rabbit vas deferens

According to Eltze [32], the prostatic portion of each vas deferens was mounted in a 10-ml organ bath, containing a modified Krebs solution (mM composition: NaCl, 134; KCl, 3.4; $CaCl_2$, 2.8; KH_2PO_4, 1.3; $NaHCO_3$, 16; $MgSO_4$, 0.6; glucose, 7.7) kept at 31 °C and bubbled with 95% O_2–5% CO_2. Yohimbine (1.0 μM) was present throughout the experiment to prevent prejunctional α_2-adrenoceptors stimulation. For isometric recordings, the tissues were left to equilibrate for 4 min under a passive load of 0.75 g before electric field stimulation through platinum electrodes was applied by square-wave pulses (0.5 ms, 0.05 Hz, supramaximal intensity 450 mA; LACE Elettronica Mod. ES3, Ospedaletto PI, Italy).

4.2.2. Electrically stimulated guinea pig left atrium

The left atria were mounted in 20 ml organ baths under 0.5 g tension at 33 °C, immersed in a modified Krebs-Henseleit solution (mM composition: NaCl, 118.9; KCl, 4.6; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25; $MgSO_4 \cdot 7H_2O$, 1.2; glucose, 11.1), gassed with a 95% O_2–5% CO_2 mixture. After a period of stabilization of 45 min, tissues were electrically stimulated through platinum electrodes by square-wave submaximal pulses (2 Hz, 5 ms, 5 V), and inotropic activity was recorded isometrically.

4.2.3. Guinea pig ileum

Ileal segments 2–3 cm long were set up under 1.0 g tension at 37 °C in 10 ml organ baths filled with Krebs-Henseleit solution (see above), bubbled with carbogen. Tissues were allowed to equilibrate for 45 min and afterwards contractile responses were isometrically recorded.

4.2.4. Guinea pig urinary bladder

Four strips (5×10 mm) were prepared from each urinary bladder according to Ringdahl [33] and were longitudinally suspended under 2.0 g tension at 35 °C in Krebs-Henseleit solution (see above) bubbled with 95% O_2–5% CO_2 and, after 45 min of equilibration, contractile responses were isometrically recorded.

4.2.5. Protocols

Agonist concentration–response curves were constructed in each tissue by cumulative application of concentrations of the test compounds [34]. The agonist potency was expressed as $pEC_{50}(-\log EC_{50})$ calculated by linear regression analysis using the least-square method. ia was calculated as a fraction of the maximal response to the reference full agonist, Bethanechol or McN-A-343. Concentration–response curves of the agonists were reconstructed in the presence of atropine 0.1 μM and hexamethonium 100 μM. When the compounds were tested as antagonists, a dose–response curve to the full agonists, Bethanechol or McN-A-343 was repeated after 30 min incubation with the test compounds (10 nM–10 μM). The potency of the compounds acting as antagonists was expressed as pK_B value, the calculated molar concentration of the test compounds that causes a twofold increase in the EC_{50} values of the muscarinic agonists used in the functional tests, calculated according to Furchgott's method [35]. All data are expressed as means of 6–8 experiments.

Acknowledgements

This research has been supported by a grant (COFIN 1999) from Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR, Rome). The authors wish to thank Dr. Giuseppe Domenichini (University of Parma) for his skilful technical assistance. Additional support from the NIMH PDSP and KO2MH01366 to BLR is acknowledged.

References

[1] R.M. Eglen, A. Choppin, N. Watson, Therapeutic opportunities from muscarinic receptor research, TiPS 22 (2001) 409–414.
[2] M.P. Caulfield, Muscarinic receptors: characterization, coupling and function, Pharmacol. Ther. 58 (1993) 319–379.
[3] J. Wess, Molecular biology of muscarinic acethylcholine receptors, Crit. Rev. Neurobiol. 10 (1996) 69–99.
[4] J.L. Chelala, A. Kilani, M.J. Miller, R.J. Martin, P. Ernsberger, Muscarinic receptor binding sites of the M_4 subtype in porcine lung parenchyma, Pharmacol. Toxicol. 83 (1998) 200–207.
[5] C. Bolden, B. Cusak, E. Richelson, Antagonism by antimuscarinic and neuroleptic compounds at the five cloned human muscarinic cholinergic receptors expressed in Chinese hamster ovary cells, J. Pharmacol. Exp. Ther. 260 (1992) 576–580.
[6] M.J. Sheardown, H.E. Shannon, M.D. Swedberg, P.D. Suzdak, F.P. Bymaster, P.H. Olesen, C.H. Mitch, J.S. Ward, P. Sauerberg, M_1 receptor agonist activity is not a requirement for muscarinic antinociception, J. Pharmacol. Exp. Ther. 281 (1997) 868–875.
[7] N. Watson, D.V. Daniels, A.P. Ford, R.M. Eglen, S.S. Hegde, Comparative pharmacology of recombinant human M_3 and M_5 muscarinic receptors expressed in CHO-K1 cells, Br. J. Pharmacol. 127 (1999) 590–596.
[8] R.M. Eglen, S.R. Nahorski, The muscarinic M_5 receptor: a silent or emerging subtype?, Br. J. Pharmacol. 130 (2000) 13–21.
[9] N.J.M. Birdsall, N.M. Nathanson, R.D. Schwarz, Muscarinic receptors: its a knockout, TiPS 22 (2001) 215–219.
[10] M.P. Caulfield, N.J.M. Birdsall, International Union of Pharmacology. XVII. Classification of muscarinic acethylcholine receptors, Pharmacol. Rev. 50 (1998) 279–280.
[11] J.L. Ellis, D. Harman, J. Gonzales, M.L. Spera, R. Liu, T.Y. Shen, D.M. Wipij, F. Zuo, Development of muscarinic analgesics derived from epibatidine: role of the M_4 receptor subtype, J. Pharmacol. Exp. Ther. 288 (1999) 1143–1150.
[12] P. Sauerberg, L. Jeppesen, T.H. Olesen, T. Rasmussen, M.D. Swedber, M.J. Sheardown, A. Fink-Jensen, A. Thomsen, H. Thogersen, K. Rimvall, J.S. Ward, D.O. Calligaro, N.W. Delapp, F.P. Bymaster, H.E. Shannon, Muscarinic agonists with antipsychotic-like activity: structure–activity relationships of 1,2,5-

thiadiazole analogues with functional dopamine antagonist activity, J. Med. Chem. 41 (1967) 4378–4384.
[13] M. De Amici, C. Dallanoce, C. De Micheli, E. Grana, A. Barbieri, H. Ladinsky, G.B. Schiavi, F. Zonta, Synthesis and pharmacological investigation of the enantiomers of muscarone and *allo*-muscarone, J. Med. Chem. 35 (1992) 1915–1920.
[14] M. De Amici, C. De Micheli, T. Gianferrara, E. Grana, G. Dondi, H. Ladinsky, G.B. Schiavi, Synthesis and muscarinic activity of the chiral forms of methylenemuscarones, Farmaco 48 (1993) 1349–1357.
[15] P. Angeli, F. Cantalamessa, R. Cavagna, P. Conti, M. De Amici, C. De Micheli, A. Gamba, G. Marucci, Synthesis and pharmacological characterization of enantiomerically pure muscarinic agonists: difluoromuscarines, J. Med. Chem. 40 (1997) 1099–1103.
[16] M. De Amici, C. De Micheli, E. Grana, R. Rodi, F. Zonta, M.G. Santagostino-Barbone, Synthesis and pharmacological investigation of cholinergic ligands related to muscarone, Eur. J. Med. Chem. 24 (1989) 171–177.
[17] C. Dallanoce, P. Conti, M. De Amici, C. De Micheli, E. Barocelli, M. Chiavarini, V. Ballabeni, S. Bertoni, M. Impicciatore, Synthesis and functional characterization of novel derivatives related to oxotremorine and oxotremorine-M, Bioorg. Med. Chem. 7 (1999) 1539–1547.
[18] E. Barocelli, V. Ballabeni, S. Bertoni, C. Dallanoce, M. De Amici, C. De Micheli, M. Impicciatore, New analogues of oxotremorine and oxotremorine-M: estimation of their in vitro affinity and efficacy at muscarinic receptor subtypes, Life Sci. 67 (2000) 717–723.
[19] E. Barocelli, V. Ballabeni, S. Bertoni, M. De Amici, M. Impicciatore, Evidence for specific analgesic activity of a muscarinic agonist selected among a new series of acetylenic derivatives, Life Sci. 68 (2001) 1775–1785.
[20] M.W. Moon, C.G. Chidester, R.F. Heier, J.K. Morris, R.J. Collins, R.R. Russell, J.W. Francis, G.P. Sage, V.H. Sethy, Cholinergic activity of acetylenic imidazoles and related compounds, J. Med. Chem. 34 (1991) 2314–2327.
[21] H. Miyachi, H. Kiyota, H. Uchiki, M. Segawa, Synthesis and antimuscarinic activity of a series of 4-(1-imidazolyl)-2,2-diphenylbutiramides: discovery of potent and subtype-selective antimuscarinic agents, Bioorg. Med. Chem. 7 (1999) 1151–1161.
[22] B. Ringdahl, D.J. Jenden, Affinity, efficacy and stereoselectivity of oxotremorine analogues for muscarinic receptors in the isolated guinea-pig ileum, J. Mol. Pharmacol. 23 (1983) 17–25.
[23] P.A. Wade, Synthesis of 3-substituted 2-isoxazolines and 5,6-dihydro-1,2,4*H*-oxazines, J. Org. Chem. 43 (1978) 2020–2023.
[24] I. Iwai, N. Nakamura, Studies on acetylenic compounds. XLIV. Synthesis of 3-aminoisoxazoles and 3-hydroxyisoxazoles (3-isoxazolones), Chem. Pharm. Bull. 14 (1966) 1277–1286.
[25] B.M. Nilsson, H.M. Vargas, U. Hacksell, Urea and 2-imidazolidone derivatives of the muscarinic agents oxotremorine and *N*-methyl-*N*-(1-methyl-4-pyrrolidino-2-butynyl)acetamide, J. Med. Chem. 35 (1992) 3270–3279.
[26] R. Budriesi, S. Cacciaguerra, R. Di Toro, M.L. Bolognesi, A. Chiarini, A. Minarini, M. Rosini, S. Spampinato, V. Tumiatti, C. Melchiorre, Analysis of the muscarinic receptor subtype mediating inhibition of the neurogenic contractions in rabbit isolated vas deferens by a series of polymethylene tetramines, Br. J. Pharmacol. 132 (2001) 1009–1016
This functional test is commonly used to characterize ligands for M_1 receptors. However, recent results by Melchiorre et al. suggest that inhibition of the neurogenic contractions in this tissue preparation could be mediated by M_4 receptors:
[27] C. Melchiorre, A. Minarini, S. Spampinato, V. Tumiatti, Design, synthesis and biological activity of some tetramines related to methoctramine and 4-DAMP, Bioorg. Med. Chem. Lett. 5 (1995) 785–790.
[28] P. Angeli, Pentatomic cyclic antagonists and muscarinic receptors: a 30-year review, Farmaco 53 (1998) 1–21.
[29] B. Ringdahl, M. Roch, D.J. Jenden, Regional differences in receptor reserve for analogs of oxotremorine in vivo: implications for development of selective muscarinic agonists, J. Pharmacol. Exp. Ther. 242 (1987) 464–471.
[30] H. Miyachi, H. Kiyota, M. Segawa, Design, synthesis and antimuscarinic activity of some imidazolium derivatives, Bioorg. Med. Chem. Lett. 9 (1999) 3003–3008.
[31] Y.-C. Cheng, W.H. Prusoff, Relationship between the inhibition constant Ki and the concentration of inhibition which causes fifty percent inhibition (IC_{50}) of an enzymatic reaction, Biochem. Pharmacol. 22 (1973) 3099–3108.
[32] M. Eltze, Muscarinic M_1- and M_2-receptors mediating opposite effects on neuromuscolar transmission in rabbit vas deferens, Eur. J. Pharmacol. 151 (1988) 205–221.
[33] B. Ringdahl, Selectivity of partial agonists related to oxotremorine based on differences in muscarinic receptor reserve between the guinea ileum and urinary bladder, Mol. Pharmacol. 31 (1987) 351–356.
[34] J.M. Van Rossum, Cumulative does–response curves. II. Technique for the making of dose–response curves in isolated organs and the evaluation of drug parameters, Arch. Int. Pharmacodyn. Ther. 143 (1963) 299–330.
[35] R.F. Furchgott, P. Bursztyn, Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors, Ann. NY Acad. Sci. 144 (1967) 882–898.