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Novel oxotremorine-related heterocyclic derivatives: Synthesis and in vitro pharmacology at the muscarinic receptor subtypes

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Abstract—A set of novel heterocyclic ligands (**6**–**27**) structurally related to Oxotremorine **2** was designed, synthesized and tested at muscarinic receptor subtypes (mAChRs). In the binding experiments at cloned human receptors (hm1–5), compounds **7** and **15** evidenced a remarkable affinity and selectivity for the hm2 subtype. The in vitro functional assays, performed on a selected group of derivatives at M_1, M_2, and M_3 tissue preparations, singled out the 3-butynyloxy-5-methylisoxazole trimethylammonium salt 7 as a potent unselective muscarinic agonist [pEC_{50}: 7.40 (M_1), 8.18 (M_2), and 8.14 (M_3)], whereas its 5-phenyl analogue 12 behaved as a muscarinic antagonist, slightly selective for the M_1 subtype [pK_B: 6.88 (M_1), 5.95 (M_2), 5.53 (M_3)]. Moreover, the functional data put in evidence that the presence of the piperidine ring may generate a functional selectivity, e.g., an M_1 antagonist/M_2 partial agonist/M_3 full agonist profile (compound **21**), at variance with the corresponding quaternary ammonium salt (compound **22**) which behaved as a muscarinic agonist at all M_{1-3} receptors, with an appreciable selectivity for the cardiac M_2 receptors.

1. Introduction

The muscarinic acetylcholine receptors (mAChRs) are prototypical members of the superfamily of G protein-linked receptors.[1–3] The muscarinic actions of acetylcholine (ACh) are mediated by five molecularly distinct mAChR subtypes (M_1–M_5)[1–3] whose activation produces either an excitatory or an inhibitory modulation of a number of central and peripheral physiological functions.[4] Different experimental approaches, including immunohistochemical and mRNA hybridization techniques, have shown that mAChRs are widely expressed and critically regulate a number of physiological processes.[5,6] The M_1 receptors are expressed at high density in parasympathetic ganglia and forebrain areas including the cerebral cortex, hippocampus, and striatum where they are implicated in learning and memory. The M_2 subtype is localized at presynaptic level in the brain and in peripheral effector organs such as heart (M_2) or smooth muscles where it participates in a synergistic way with the M_3 subtype in the regulation of the contractility of respiratory, gastrointestinal, and genitourinary tracts. The M_4 receptors, identified peripherally in the lung[7] and centrally in the striatum, are believed to be involved in the control of motor functions,[8] cognitive processes,[9] and antinociception.[10,11] The M_5 receptor mRNA has been identified in various peripheral and cerebral blood vessels and it has been demonstrated that dopaminergic neurons innervating the striatum almost exclusively express the M_5 receptor subtype;[12] however, its physiological role is far from being completely established.[13] Recently, emerging evidence indicates that acetylcholine acts also in a paracrine fashion through the activation of the different muscarinic receptor subtypes located in several non-innervated tissues such as keratinocytes, lymphocytes, placenta, and endothelial and ocular lens cells.

Keywords: Muscarinic ligands; Oxotremorine-related compounds; Muscarinic receptor subtypes; Cloned receptors; Binding assays; Functional tests.
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As autocrine mediator, acetylcholine seems to be involved in the regulation of cell growth or proliferation and in the release of smooth muscle relaxing agents.[4]

During the past two decades, exploration of the role of specific mAChR subtypes (M_1–M_5) in mediating the diverse physiological actions of ACh has been the subject of detailed investigations. Such a knowledge is essential for the development of novel therapeutic approaches aimed at enhancing or inhibiting specific muscarinic receptor-controlled responses. This is particularly true for central mAChR subtypes, which regulate an extraordinarily large number of cognitive, behavioral, sensory, motor, and autonomic functions, whose imbalance is implicated in the pathophysiology of several CNS diseases, i.e. Alzheimer's and Parkinson's diseases, pain, schizophrenia, depression, and epilepsy.[14,15] To this end, the discovery of new agonists[4] has been a goal pursued by numerous research groups in the effort to obtain selective mAChR ligands clinically useful in the palliative treatment of the above-cited CNS neurodegenerative diseases as well as in the therapy of peripheral dysfunctions, e.g., intestinal hypomotility. However, the task of assigning specific physiological functions to the five mAChR subtypes has proved very challenging, due to the lack of agonists provided with a high degree of selectivity for the individual subtypes[16] and to the coexpression of multiple mAChRs in a variety of organs, tissues, or cell types.[5,6] These difficulties have been partially circumvented by the generation of mutant mouse strains deficient in each of the five mAChR subtypes.[8]

In the past, our research group investigated the pharmacological profile of a group of muscarinic ligands structurally related to natural Muscarine **1**[17–19] and to Oxotremorine **2**[20–23] (Fig. 1). The pharmacological profile of representative derivatives, i.e. **3**–**5**, was studied in depth by taking into account both receptor occupancy and in vivo tests. It emerged that the nonselective oxotremorine-like derivative **3** exhibited quite an interesting analgesic activity.[24] 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. On one hand, we prepared the new 5-methyl/phenyl isoxazole derivatives **6**–**12** (Fig. 1) structurally related to **5**, on the other, we synthesized the group of derivatives **13**–**27** (Fig. 1), in which the terminal pyrrolidine function of compounds **3**–**5** was replaced by more steric demanding nonquaternized and quaternized moieties. To investigate the effect of these structural modifications on the activity/selectivity profiles at the five muscarinic receptor subtypes, the target derivatives **6**–**27** were assayed at cloned human muscarinic receptor subtypes (hm1–5), expressed in Chinese Hamster Ovary (CHO) cells. Some of them were also evaluated in functional tests at tissue preparations which contain M_{1-3} receptors.

2. Chemistry

The synthesis of target compounds was accomplished along the reaction sequences depicted in Scheme 1–4. Derivatives **6**–**12** (Fig. 1) were readily prepared by reacting known 3-hydroxy-5-methylisoxazole **28a**[25] or 3-hydroxy-5-phenylisoxazole **28b**[25] with 1,4-dichloro-2-butyne to yield intermediates **29a**, **29b**, which were sequentially treated with either dimethylamine or pyrrolidine to produce the corresponding free bases **30a**, **30b** and **31a**, **31b**, respectively (Scheme 1). Treatment of the intermediates **30a** and **31a** with fumaric acid or excess methyl iodide afforded fumarates **6** and **8** and iodomethylates **7** and **9**, respectively. The 5-phenyl-3-oxyisoxazolyl oxalate **10** and fumarate **11**, and methiodide **12** were likewise prepared from **30b** and **31b** (Scheme 1). Similarly, the reaction of 3-hydroxyisoxazole **32**[26] with 4-chlorobut-2-yn-1-ol gave intermediate **33**, which was reacted with *N*-hydroxyphthalimide under the Mitsunobu protocol[27–29] to yield derivative **34** (Scheme 2). Treatment of this intermediate with hydrazine produced in good yield the oxime ether **35**, which was condensed with 3-quinuclidone and then transformed into either fumarate **13** or iodomethylate **14**.

Isoxazolidin-3-one derivative **37**[20] was reacted with four different amines (Scheme 3) to generate amines **38**–**41**, which were subsequently transformed into either oxalates **15**, **18**, iodomethylates **16**, **17**, **19**, and hydrochoride **20**. In a parallel way, mesylate **42**[20] (Scheme 4) was converted into final salt derivatives **21**–**27**.

3. Results and discussion

The new derivatives **6**–**27** were assayed for binding affinity at human muscarinic receptor subtypes (hm1–5) in transfected CHO cells labeled with [^{3}H]quinuclidinyl benzylate, and the results were compared with those of well-recognized selective ligands and with the structurally related derivative **3** (Table 1). The compounds (10 μM) were preliminarily tested at hm2 and hm5, representative of the two subgroups (M_2, M_4 and M_1, M_3, M_5, respectively) of muscarinic receptors. The derivatives which displayed a percent inhibition of the radioligand binding higher than 50% were then assayed at all five receptor subtypes in order to evaluate their K_i values (nM).

The affinity, potency, and efficacy of compounds **6**–**9**, **11**–**15**, **21**, and **22** were then evaluated by performing the following three functional in vitro assays: rabbit electrically stimulated vas deferens (M_1),[30] guinea pig electrically driven left atrium (M_2), and guinea pig ileum (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 in the set of novel derivatives **6**–**27**, compounds **7** and **15** displayed a remarkable affinity and selectivity for the hm2 subtype; the K_i values obtained for **7** and **15** (50 and 40 nM, respectively) at hm2 receptors were lower than those of the reference isoxazolidinyl derivative **3** (K_i = 70 nM). A qualitative analysis of the structure–activity relationship evidences that replacement of the pyrrolidine moiety in the isoxazoli-

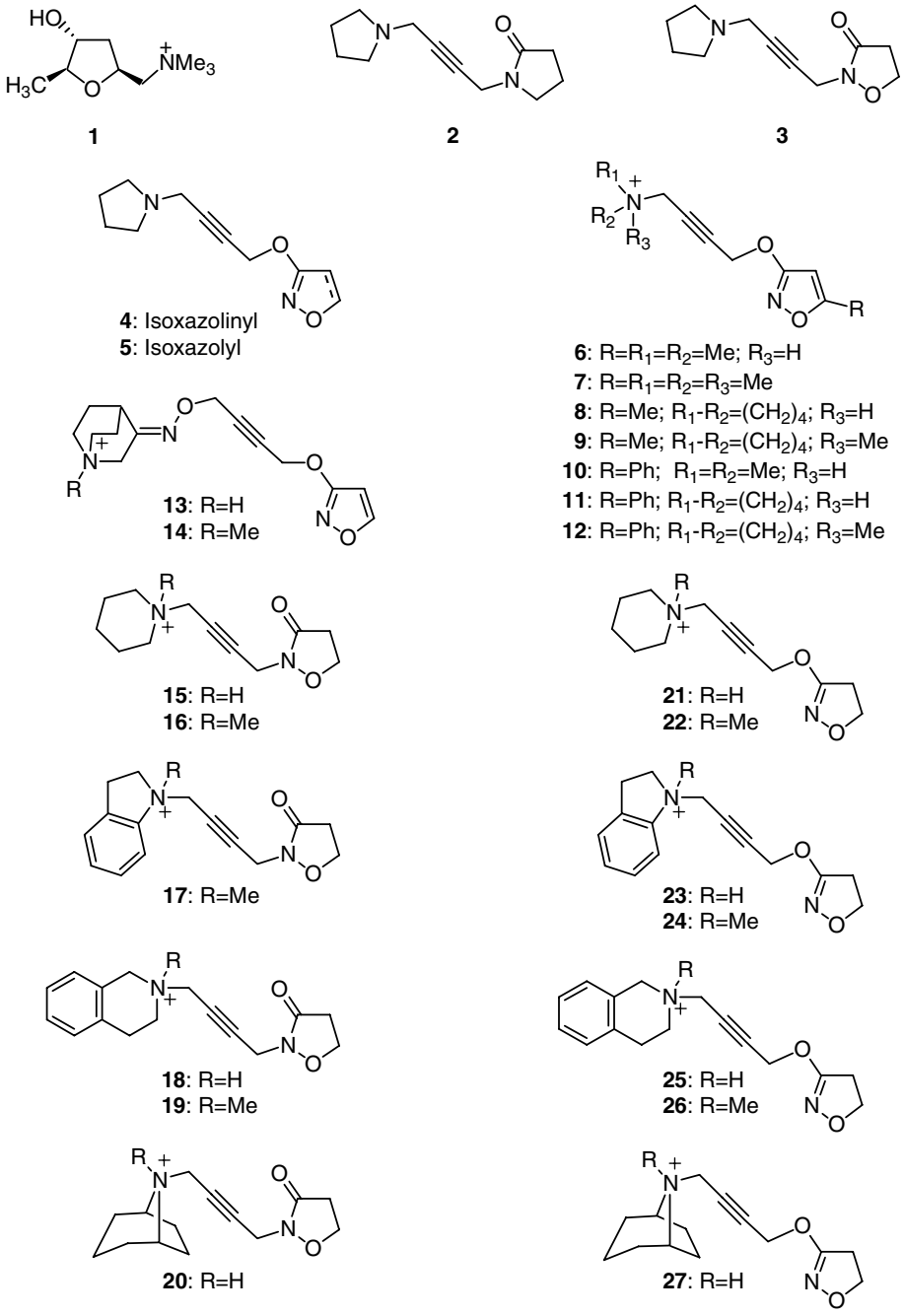
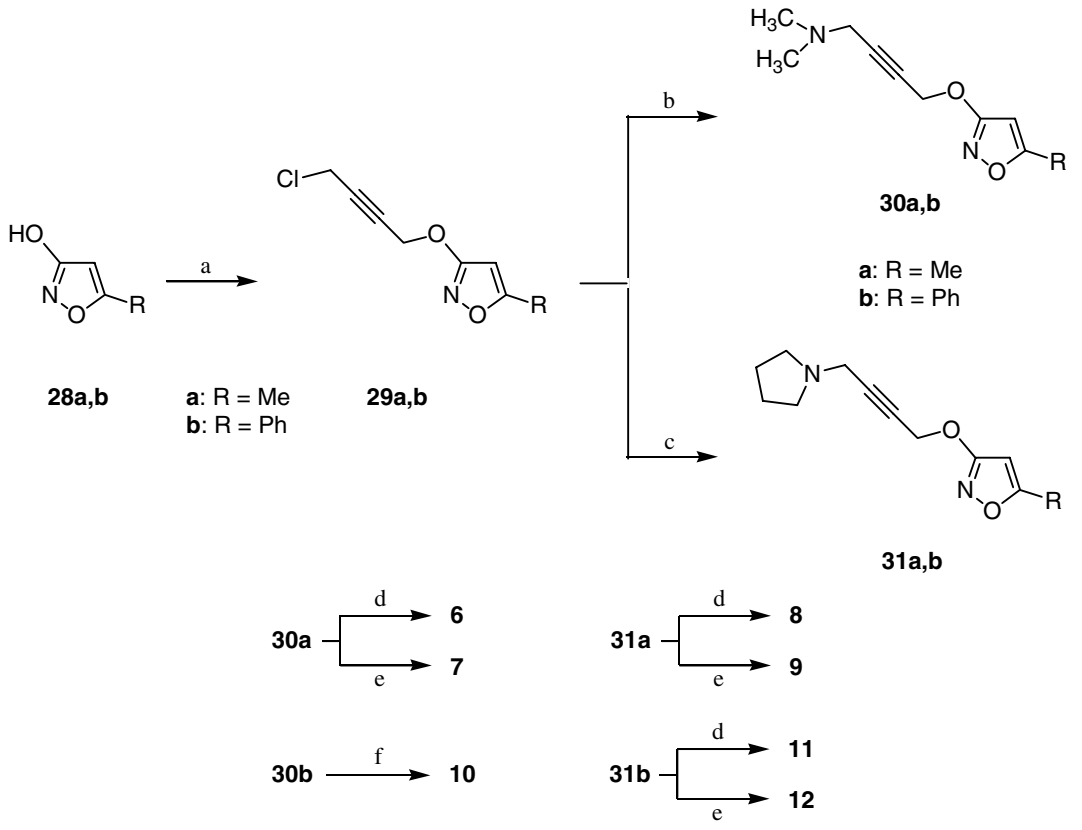


Figure 1. Structure of model and target compounds.

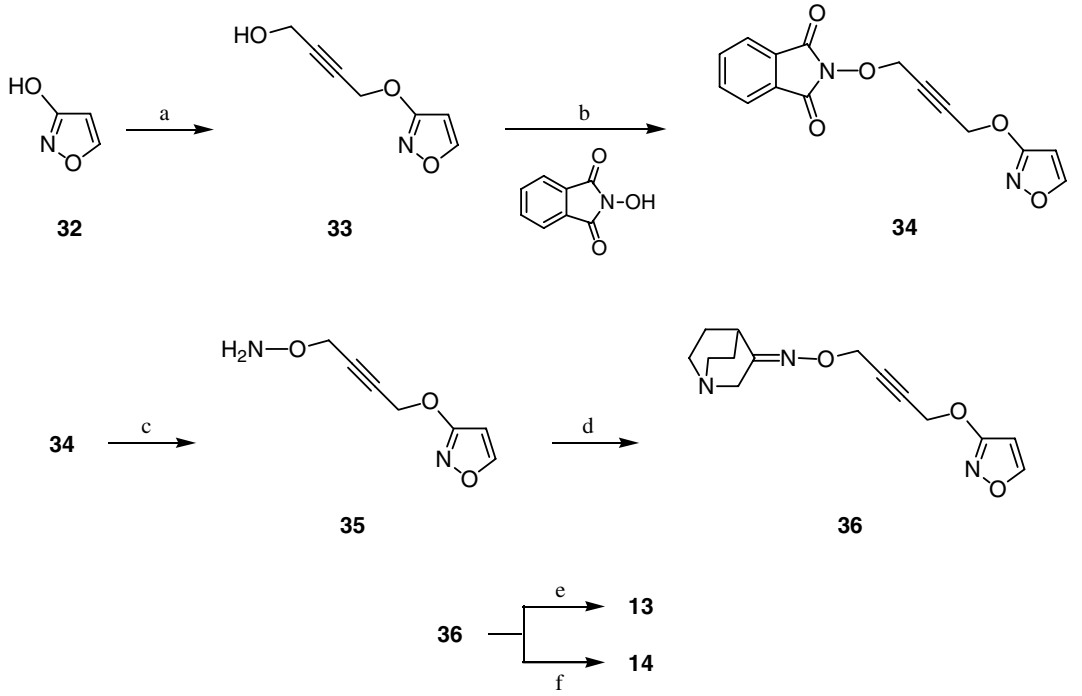
din-3-one derivative **3** with the piperidine ring (**15**) almost doubles the affinity at hm2 receptors. This results in increased selectivity of **15** for hm2, as indicated by affinity ratios, over hm1 (20), hm3 (13), hm4 (20), and hm5 (14) subtypes. The corresponding affinity ratios for the parent compound **3** were 13, 5.6, 3.2, and 7.3, respectively. Worth noting, the corresponding methiodide **16** is devoid of any affinity for the five mAChR subtypes.

Even higher values of hm2 selectivity characterize the affinity profile of 5-methyl-3-oxyisoxazolyl trimethylammonium salt **7**, e.g., hm1/hm2 > 200; hm3/hm2 80; hm4/hm2 14, hm5/hm2 126 at variance with the corresponding dimethylamino derivative **6** which is a feeble ligand for hm2 and hm4 (K_i values 3.8 and 1.1 μM, respectively) and completely inactive at hm1, hm3, and hm5.

A set of derivatives (**6**–**9**, **11**–**15**, **21**, and **22**) was selected for evaluation in functional assays to assess their pharmacological profile (Table 2). In this group of compounds, we chose to insert also derivatives **8** and **14**, characterized by a negligible binding affinity, and **13**, whose binding data were unavailable. The most interesting results refer to the comparison of the data of derivative **3** with those of its homologue **15** as well as the data of derivative **6** with those of its methiodide **7**. As shown in Table 2, the replacement of the pyrrolidine moiety

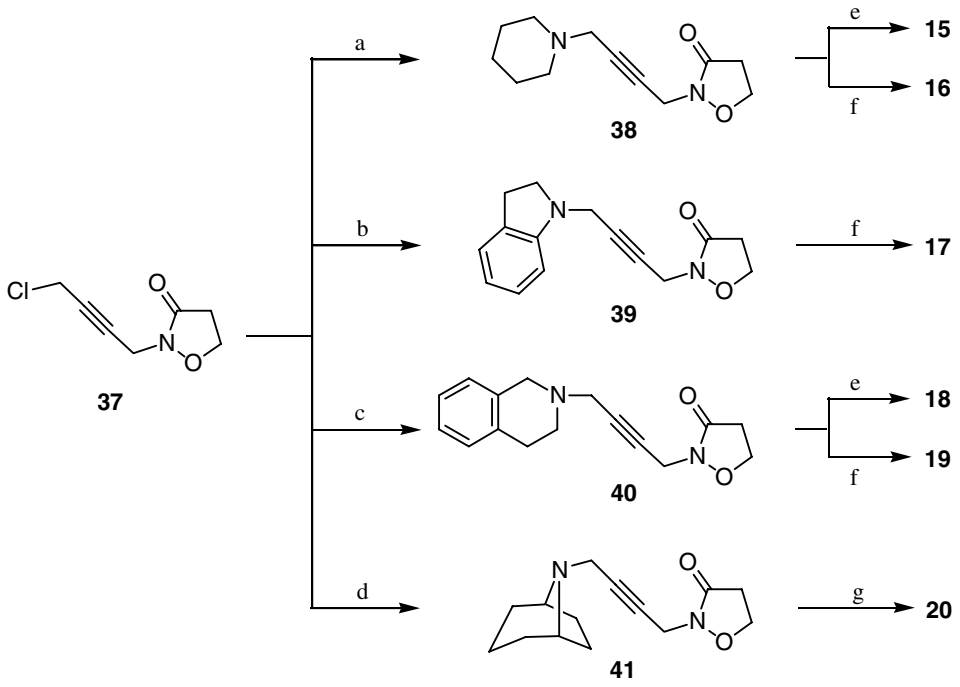


Scheme 1. Reagents: (a) 1,4-dichloro-2-butyne, K_2CO_3, acetone; (b) $NHMe_2$, DMF; (c) Pyrrolidine, DMSO; (d) $C_4H_4O_4$, 2-propanol-MeOH; (e) CH_3I, MeOH-ether; (f) $C_2H_2O_4$, ether.

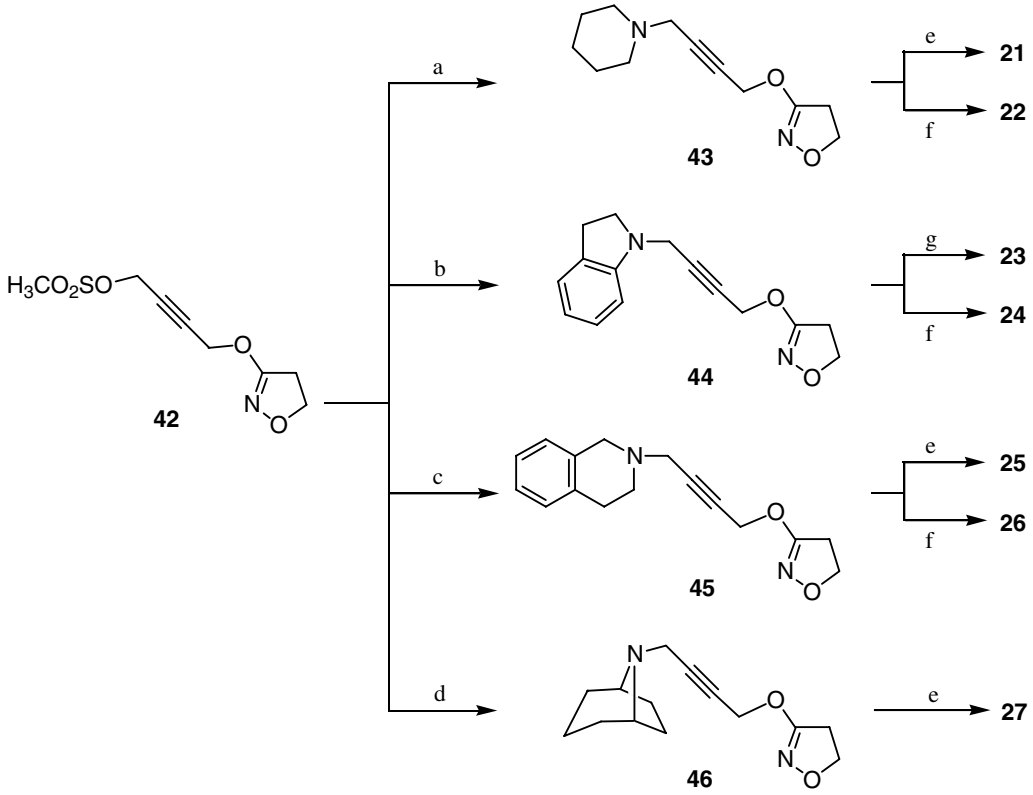


Scheme 2. Reagents: (a) K_2CO_3, acetone, 4-chlorobut-2yn-1-ol; (b) PPh_3, DEAD, THF; (c) $H_2NNH_2{\cdot}H_2O$, Et_2O; (d) 3-quinuclidone, MeOH; (e) $C_4H_4O_4$, 2-propanol-MeOH; (f) CH_3I, 2-propanol.

(derivative **3**) with its piperidine homologue (derivative **15**) brings about a sharp change in the pharmacological profile. Indeed, compound **3** is a relatively potent, nonselective muscarinic agonist (pEC_{50} = 7.31–7.98 at



Scheme 3. Reagents: (a) Piperidine, Cs_2CO_3, acetone; (b) Indoline, MeOH; (c) 1,2,3,4-Tetrahydroisoquinoline, MeOH; (d) Nortropane, Cs_2CO_3, MeOH; (e) $C_2H_2O_4$, 2-propanol-MeOH; (f) CH_3I, MeOH-Et_2O; (g) HCl-Et_2O.



Scheme 4. Reagents: (a) Piperidine, MeOH; (b) Indoline, MeOH; (c) 1,2,3,4-Tetrahydroisoquinoline, MeOH; (d) Nortropane, Cs_2CO_3, MeOH; (e) $C_2H_2O_4$, 2-propanol-MeOH; (f) CH_3I, MeOH-Et_2O; (g) HCl-Et_2O.

M_1–M_3 subtypes; $\alpha = 1$), whereas **15** turns out to be a functionally selective derivative characterized by a moderate antagonist activity at M_1 and M_3 receptors ($pK_B = 6.45$ and 5.55, respectively; $\alpha = 0$) and a partial agonism, with exiguous intrinsic activity, at the M_2 subtype ($pK_B = 5.94$; $\alpha = 0.2$) (Table 2).

Table 1. Binding affinity (K_i, nM) of compounds **6**–**27** at cloned human muscarinic receptor subtypes (hm1–5) expressed in CHO cells

Compound	hm1	hm2	hm3	hm4	hm5
Pirenzepine	21.38				35.48
Methoctramine		10.38			
4-DAMP			0.68		
3	905	70	392	222	510
6	>10^4	3796	>10^4	1095	>10^4
7	>10^4	50	3995	682	6310
8		>10^4			>10^4
9	7680	2041	7386	5932	7514
10		>10^4			>10^4
11	6141	>10^4	4013	5478	6885
12	3376	>10^4	1448	5986	1836
13		nt[a]			nt[a]
14		>10^4			>10^4
15	819	40	537	792	544
16		>10^4			>10^4
17		>10^4			>10^4
18		>10^4			>10^4
19		>10^4			>10^4
20		>10^4			>10^4
21	4550	876	3919	6041	5139
22	7367	219	4740	1681	4116
23		>10^4			>10^4
24	>10^4	4099	5539	>10^4	>10^4
25		>10^4			>10^4
26	6958	474	887	2265	5664
27		>10^4			>10^4

[a] nt, not tested.

As previously observed for structurally related analogues,[20] the transformation of the dimethylamine moiety into the corresponding trimethylammonium salt gives rise to a substantial change in the functional profile. As a matter of fact, whereas the dimethylamino derivative **6** behaves as a moderately potent agonist at M_2 and M_3 receptors and as a partial agonist at M_1 receptors, the corresponding methiodide **7** becomes a potent full agonist at all three receptor subtypes with a 100-fold increase in both potency and affinity, e.g., pEC_{50} 7.40 (M_1), 8.18 (M_2), and 8.14 (M_3) for **7** versus pEC_{50} 5.46 (M_1), 6.29 (M_2), and 6.10 (M_3) for **6**.

The replacement of the *N*,*N*-dimethylamino or trimethylammonium head with the pyrrolidine nucleus yields a remarkable drop in the ability to bind and to activate the muscarinic receptors as demonstrated by the pK_B or pEC_{50} values calculated for compounds **8** and **9** in comparison with those of derivatives **6** and **7**. The further substitution of the methyl group with the more lipophilic and larger phenyl group generates the 3-butynyloxy-5-phenylisoxazole derivative **11**, behaving as a full M_1 agonist ($pEC_{50} = 4.35$), a partial M_2 agonist ($pEC_{50} = 4.66$, $\alpha = 0.64$), and an M_3 antagonist ($pK_B = 4.94$, $\alpha = 0$), and the corresponding methiodide **12**, behaving as an antagonist at the muscarinic receptors with a weak M_1 selectivity ($pK_B = 6.88$).

According to the change of the pharmacological profile observed on passing from compound **3**–**15**, in the 3-butynyloxy-Δ^2-isoxazoline series replacement of the pyrrolidine moiety with the piperidine ring caused the conversion of a nonselective agonist [compound **4**, $pEC_{50} = 6.76$ (M_1), 7.0 (M_2), 7.41 (M_3)] into a functionally selective M_1 antagonist/M_2 partial agonist/M_3 full agonist (compound **21**, M_1: $pK_B = 5.91$; M_2: $pEC_{50} = 5.96$, $\alpha = 0.6$; M_3: $pEC_{50} = 5.75$, $\alpha = 1$), although characterized by a reduced potency. Conversely, its

Table 2. In vitro functional activity of compounds **6**–**9**, **11**–**15**, **21**, and **22** at muscarinic receptor subtypes in rabbit vas deferens (M_1), guinea pig left atrium (M_2) and guinea pig ileum (M_3)

Compound	M_1 Rabbit vas deferens[a]				M_2 Guinea-pig left atrium				M_3 Guinea-pig ileum			
	pEC_{50}[b]	ia[c]	pK_D[d]	pK_B[e]	pEC_{50}[b]	ia[c]	pK_D[d]	pK_B[e]	pEC_{50}[b]	ia[c]	pK_D[d]	pK_B[e]
Bethanechol					6.19 ± 0.07	1.0	5.37 ± 0.16		6.37 ± 0.05	1.0	4.90 ± 0.13	
McN-A-343	6.40 ± 0.04	1.0	7.20 ± 0.28									
3[f]	7.31 ± 0.11	1.0	6.52 ± 0.20		7.73 ± 0.10	1.0	6.73 ± 0.29		7.98 ± 0.12	1.0	6.91 ± 0.28	
4[f]	6.76 ± 0.16	1.0	6.44 ± 0.18		7.00 ± 0.07	1.0	6.40 ± 0.11		7.41 ± 0.03	1.0	6.38 ± 0.39	
6	5.46 ± 0.19	0.6	5.45 ± 0.24		6.29 ± 0.20	1.0	3.56 ± 0.22		6.10 ± 0.07	1.1	4.40 ± 0.11	
7	7.40 ± 0.09	0.9	6.97 ± 0.06		8.18 ± 0.14	1.0	7.03 ± 0.12		8.14 ± 0.12	1.0	6.17 ± 0.13	
8		0		5.66 ± 0.27		0		4.81 ± 0.07	4.82 ± 0.12	0.4	4.81 ± 0.37	
9		0		6.01 ± 0.01	6.60 ± 0.06	1.0	6.72 ± 0.32		6.26 ± 0.11	0.9	5.78 ± 0.47	
11	4.35 ± 0.03	1.0	nt[g]	nt[g]	4.66 ± 0.06	0.6	nt[g]	nt[g]		0		4.94 ± 0.15
12		0		6.88 ± 0.15		0		5.95 ± 0.25		0		5.53 ± 0.12
13		0		5.85 ± 0.49	5.51 ± 0.06	0.7	5.97 ± 0.10		5.99 ± 0.09	0.9	5.44 ± 0.08	
14	5.37 ± 0.27	0.9	3.82 ± 0.16		<4				5.45 ± 0.07	0.7	5.03 ± 0.48	
15		0		6.45 ± 0.07		0.2		5.94 ± 0.05		0		5.55 ± 0.04
21		0		5.91 ± 0.08	5.96 ± 0.29	0.6	6.22 ± 0.34		5.75 ± 0.01	1.0	4.66 ± 0.21	
22	6.10 ± 0.12	0.4	6.51 ± 0.19		7.34 ± 0.05	1.0	6.74 ± 0.18		6.57 ± 0.08	1.0	6.53 ± 0.19	

[a] See Ref. 30.
[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) was measured by the ratio between the maximum response of the compound and the maximum response of the reference agonist.
[d] Apparent pK_D ± SE values were calculated according to Furchgott[33] and McKay.[34]
[e] Apparent pK_B ± SE values were calculated according to Furchgott.[33]
[f] All the reported values were taken from the literature.[20,21]
[g] nt, not tested.

corresponding methiodide **22** becomes a full agonist of higher potency at M_2 and M_3 subtypes (pEC_{50} = 7.34 and 6.57, respectively), and a partial agonist at M_1 receptors (pEC_{50} = 6.10; α = 0.4). Methiodide **22** is the only compound under study which shows an agonist profile coupled with a degree of M_2 selectivity.

In summary, the data collected on the novel Oxotremorine-like compounds indicate the 3-butynyloxy-5-methyl-isoxazole moiety as a scaffold suitable to generate potent muscarinic ligands. These results lay the basis for further investigations aimed at finding out the structural determinants needed to discriminate the different muscarinic receptor subtypes.

4. Experimental

4.1. Chemistry

4.1.1. Materials and methods. 3-Hydroxy-5-methylisoxazole **28a**,[25] 3-hydroxy-5-phenylisoxazole **28b**,[25] 3-hydroxyisoxazole **32**,[26] isoxazolidin-3-one **37**,[20] and 3-substituted-Δ^2-isoxazoline **42**[20] 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 Varian Mercury 300 (300 MHz) spectrometer in $CDCl_3$ (unless otherwise specified) solutions at 20 °C. 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, Cl, and N) of new compounds agreed with the theoretical value within ± 0.4%.

4.1.2. Synthesis of compounds 6–12. To a solution of **28a**[25] (3 g, 30.28 mmol) in acetone (40 mL) was added potassium carbonate (8.3 g, 60.05 mmol). The suspension was heated at reflux for 1 h, then cooled at rt 1,4-Dichloro-2-butyne (17.59 mL, 0.180 mol) was then added dropwise and, after heating at reflux for 24 h, the crude reaction mixture was poured into water (120 mL) and extracted with dichloromethane (3× 60 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: 10% ethyl acetate/petroleum ether) to give derivative **29a** (1.95 g, 35% yield).

4.1.2.1. 3-[(4-Chlorobut-2-yn-1-yl)oxy]-5-methylisoxazole (29a). Light yellow oil, bp 130–135 °C/0.5 mm Hg. R_f 0.38 (10% ethyl acetate/petroleum ether). ^{1}H NMR: 2.32 (s, 3H), 4.20 (t, 2H, J = 1.8), 4.90 (t, 2H, J = 1.8), 5.65 (s, 1H). Anal. Calcd for $C_8H_8ClNO_2$: C, 51.77; H, 4.34; N, 7.55. Found: C, 52.02; H, 4.09; N, 7.31.

The procedure described above for the synthesis of **29a** was similarly applied to **28b**[25] to give derivative **29b** in 30% yield.

4.1.2.2. 3-[(4-Chlorobut-2-yn-1-yl)oxy]-5-phenylisoxazole (29b). Yellow oil, bp 165–170 °C/0.5 mm Hg. R_f 0.56 (15% ethyl acetate/petroleum ether). ^{1}H NMR: 4.20 (t, 2H, J = 1.8), 4.98 (t, 2H, J = 1.8), 6.19 (s, 1H), 7.43 (m, 3H), 7.71 (m, 2H). Anal. Calcd for $C_{13}H_{10}ClNO_2$: C, 63.04; H, 4.07; N, 5.66. Found: C, 63.40; H, 3.82; N, 5.38.

A solution of **29a** (0.49 g, 2.64 mmol) in *N*,*N*-dimethylformamide (13 mL) and a tenfold excess of dimethylamine was stirred at rt in a sealed metal container for 3 h. The crude reaction mixture was concentrated at reduced pressure (50 °C/0.5 mm Hg) and the residue, dissolved in 2 N HCl (5 mL), was treated with diethyl ether (3 × 5 mL). The residual aqueous phase was made alkaline by portionwise addition of solid K_2CO_3 and extracted with ethyl acetate (3 × 5 mL). After the usual work-up, the oily residue was purified by silica gel column chromatography (eluant: 5% methanol/dichloromethane) to afford the desired tertiary amine **30a** (0.31 g, 60%).

4.1.2.3. *N*,*N*-Dimethyl-4-[(5-methylisoxazol-3-yl)oxy]-but-2-yn-1-amine (30a). Colorless oil, bp 120–125 °C/0.5 mm Hg. R_f 0.30 (5% methanol/dichloromethane). ^{1}H NMR: 2.28 (s, 6H), 2.32 (s, 3H), 3.31 (t, 2H, J = 1.8), 4.88 (t, 2H, J = 1.8), 5.65 (s, 1H). Anal. Calcd for $C_{10}H_{14}N_2O_2$: C, 61.84; H, 7.27; N, 14.42. Found: C, 61.65; H, 7.48; N, 14.19.

The procedure described above for the synthesis of **30a** was applied on a comparable amount of **29b** to afford derivative **30b** in 52% yield.

4.1.2.4. *N*,*N*-Dimethyl-4-[(5-phenylisoxazol-3-yl)oxy]-but-2-yn-1-amine (30b). Yellow oil, bp 170–175 °C/0.5 mm Hg. R_f 0.43 (5% methanol/dichloromethane); ^{1}H NMR: 2.46 (s, 6H), 3.52 (t, 2H, J = 1.8), 4.86 (t, 2H, J = 1.8), 5.62 (s, 1H), 7.28 (m, 3H), 7.52 (m, 2H). Anal. Calcd for $C_{15}H_{16}N_2O_2$: C, 70.29; H, 6.29; N, 10.93. Found: C, 69.95; H, 6.55; N, 10.78.

A parallel protocol was applied to the reaction of **29a** (1 g, 5.39 mmol) with pyrrolidine (900 μL, 10.77 mmol) in dimethylsulfoxide (5 mL) for 3 h. After the above-described work-up, the oily residue was purified by silica gel column chromatography (eluant: 2% methanol/dichloromethane) to provide the desired tertiary amine **31a** (0.56 g, 47%).

4.1.2.5. 5-Methyl-3-[(4-pyrrolidin-1-ylbut-2-yn-1-yl)oxy]-isoxazole (31a). Pale yellow oil, bp 155–160 °C/2 mm Hg. R_f 0.50 (5% methanol/dichloromethane). ^{1}H NMR: 1.58 (m, 4H), 2.10 (s, 3H), 2.38 (m, 4H), 3.21 (t, 2H, J = 1.9), 4.62 (t, 2H, J = 1.9), 5.41 (s, 1H). Anal. Calcd for $C_{12}H_{16}N_2O_2$: C, 65.43; H, 7.32; N, 12.72. Found: C, 65.12; H, 7.09; N, 13.05.

Intermediate **29b** was similarly reacted with pyrrolidine to give derivative **31b** in 80% yield.

4.1.2.6. 5-Phenyl-3-[(4-pyrrolidin-1-ylbut-2-yn-1-yl)oxy]-isoxazole (31b). Mp 64–65 °C (yellow prisms from diisopropyl ether). R_f 0.33 (5% methanol/dichloromethane).

^{1}H NMR: 1.80 (m, 4H), 2.62 (m, 4H), 3.48 (t, 2H, J = 1.9), 4.98 (t, 2H, J = 1.9), 6.18 (s, 1H), 7.42 (m, 3H), 7.72 (m, 2H). Anal. Calcd for $C_{17}H_{18}N_2O_2$: C, 72.32; H, 6.43; N, 9.92. Found: C, 72.61; H, 6.12; N, 10.22.

To a solution of fumaric or oxalic acid (2.20 mmol) in 7 mL of 2-propanol/methanol (7:3) was added the appropriate tertiary base (1.0 mmol). The corresponding salt precipitated quantitatively on standing.

4.1.2.7. *N*,*N*-Dimethyl-4-[(5-methylisoxazol-3-yl)oxy]-but-2-yn-1-amine fumarate 6 (30a·$C_4H_4O_4$). Mp 99–101 °C (colorless prisms from 90% 2-propanol/diethyl ether). ^{1}H NMR (D_2O): 2.18 (s, 3H), 2.76 (s, 6H), 3.92 (bs, 2H), 4.80 (bs, 2H), 5.75 (s, 1H), 6.50 (s, 2H). Anal. Calcd for $C_{14}H_{18}N_2O_6$: C, 54.19; H, 5.55; N, 9.03. Found: C, 54.37; H, 5.10; N, 9.21.

4.1.2.8. 5-Methyl-3-[(4-pyrrolidin-1-ylbut-2-yn-1-yl)oxy]-isoxazole fumarate 8 (31a·$C_4H_4O_4$). Mp 107–111 °C (colorless prisms from 2-propanol). ^{1}H NMR (CD_3OD): 2.05 (m, 4H), 2.34 (s, 3H), 3.35 (m, 4H), 4.12 (t, 2H, J = 1.9), 4.92 (t, 2H, J = 1.9), 5.82 (s, 1H), 6.68 (s, 2H). Anal. Calcd for $C_{16}H_{20}N_2O_6$: C, 57.14; H, 5.99; N, 8.33. Found: C, 57.04; H, 6.13; N, 8.15.

4.1.2.9. 5-Phenyl-3-[(4-pyrrolidin-1-ylbut-2-yn-1-yl)oxy]-isoxazole fumarate 11 (31b·1/2 $C_4H_4O_4$). Mp 115–116 °C (pale yellow prisms from 90% 2-propanol/diethyl ether). ^{1}H NMR (CD_3OD): 1.98 (m, 4H), 3.12 (m, 4H), 3.92 (t, 2H, J = 1.9), 5.01 (t, 2H, J = 1.9), 6.51 (s, 1H), 6.63 (s, 1H), 7.48 (m, 3H), 7.79 (m, 2H). Anal. Calcd for $C_{19}H_{20}N_2O_4$: C, 67.05; H, 5.92; N, 8.23. Found: C, 67.29; H, 6.13; N, 8.15.

4.1.2.10. *N*,*N*-Dimethyl-4-[(5-phenylisoxazol-3-yl)oxy]but-2-yn-1-amine oxalate 10 (30b·$C_2H_2O_4$). Mp 188–189 °C (colorless prisms from 90% methanol/diethyl ether). ^{1}H NMR (CD_3OD): 2.91 (s, 6H), 4.12 (bs, 2H), 5.04 (bs, 2H), 6.51 (s, 1H), 7.48 (m, 3H), 7.78 (m, 2H). Anal. Calcd for $C_{17}H_{18}N_2O_6$: C, 58.96; H, 5.24; N, 8.09. Found: C, 58.76; H, 5.16; N, 7.97.

The following procedure is representative. A solution of **30a** (0.220 g, 1.13 mmol) in 2-propanol (5 mL) was treated with a fivefold excess of methyl iodide at rt The corresponding methiodide precipitated quantitatively upon addition of anhydrous diethyl ether.

4.1.2.11. *N*,*N*-Dimethyl-4-[(5-methylisoxazol-3-yl)oxy]but-2-yn-1-amine methiodide (7). Mp 186–188°C (colorless prisms from absolute ethanol). ^{1}H NMR (CD_3OD): 2.32 (s, 3H), 3.21 (s, 9H), 4.40 (t, 2H, J = 1.8), 5.00 (t, 2H, J = 1.8), 5.86 (s, 1H). Anal. Calcd for $C_{11}H_{17}IN_2O_2$: C, 39.30; H, 5.10; N, 8.33. Found: C, 39.55; H, 4.87; N, 8.11.

4.1.2.12. 5-Methyl-3-[(4-pyrrolidin-1-ylbut-2-yn-1-yl)oxy]isoxazole methiodide (9). Mp 72–74 °C (pale yellow prisms from 90% absolute ethanol/diethyl ether). ^{1}H NMR (D_2O): 2.09 (m, 4H), 2.00 (s, 3H), 3.01 (s, 3H), 3.40 (m, 2H), 3.50 (m, 2H), 4.19 (t, 2H, J = 1.8), 4.85 (t, 2H, J = 1.8), 5.80 (s, 1H). Anal. Calcd for $C_{13}H_{19}IN_2O_2$: C, 43.11; H, 5.29; N, 7.73. Found: C, 42.97; H, 5.48; N, 7.98.

4.1.2.13. 5-Phenyl-3-[(4-pyrrolidin-1-ylbut-2-yn-1-yl)oxy]isoxazole methiodide (12). Mp 124–127 °C (pale yellow prisms from 90% absolute ethanol/diethyl ether). ^{1}H NMR (D_2O): 2.05 (m, 4H), 3.01 (s, 3H), 3.39 (m, 2H), 3.50 (m, 2H), 4.19 (t, 2H, J = 1.9), 4.92 (t, 2H, J = 1.9), 6.42 (s, 1H), 7.41 (m, 3H), 7.69 (m, 2H). Anal. Calcd for $C_{18}H_{21}IN_2O_2$: C, 50.96; H, 4.99; N, 6.60. Found: C, 51.19; H, 5.18; N, 6.38.

4.1.3. Synthesis of compounds 13 and 14. To a solution of **32**[26] (5 g, 58.78 mmol) in acetone (50 mL) was added potassium carbonate (16 g, 115.76 mmol). The suspension was heated at reflux for 1 h and then cooled at rt 4-chlorobut-2-yn-1-ol (13 g, 125 mmol)[31] was hence added and, after heating at reflux for 18 h, the reaction mixture was filtered under vacuum. The filtrate was poured into water (500 mL), extracted with dichloromethane (3× 200 mL), and 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: 30% ethyl acetate/petroleum ether) to give derivative **33** (4.6 g, 51% yield).

4.1.3.1. 4-(Isoxazol-3-yloxy)but-2-yn-1-ol (33). Yellow oil, bp 165–170 °C/0.5 mm Hg. R_f 0.38 (10% ethyl acetate/petroleum ether). ^{1}H NMR: 2.80 (bs, 1H), 4.30 (t, 2H, J = 1.8), 4.92 (t, 2H, J = 1.8), 6.00 (d, 1H, J = 1.5), 8.13 (d, 1H, J = 1.5). Anal. Calcd for $C_7H_7NO_3$: C, 54.90; H, 4.61; N, 9.15. Found: C, 54.52; H, 4.95; N, 8.83.

To a solution of **33** (3.45 g, 22.50 mmol), 2-hydroxy-isoindole-1,3-dione (3.67 g, 22.50 mmol) and triphenylphosphine (5.90 g, 22.5 mmol) in anhydrous tetrahydrofuran (60 mL) was added dropwise a 40% toluene solution of diethyl azodicarboxylate (4.12 mL, 22.50 mmol). The mixture was stirred for 18 h at rt and then concentrated in vacuo. The residue was purified by silica gel column chromatography (eluant: 10% ethyl acetate/petroleum ether) to give derivative **34** (3.41 g, 51% yield).

4.1.3.2. 2-{[4-(Isoxazol-3-yloxy)but-2-yn-1-yl]oxy}-1*H*-isoindole-1,3(2*H*)-dione (34). Colorless needles, mp 65–67 °C (from *n*-hexane/ethyl acetate). R_f 0.31 (30% ethyl acetate/petroleum ether). ^{1}H NMR: 4.91 (t, 2H, J = 1.7), 4.94 (t, 2H, J = 1.7), 5.95 (d, 1H, J = 2.0), 7.78 (m, 2H), 7.86 (m, 2H), 8.09 (d, 1H, J = 2.0). Anal. Calcd for $C_{15}H_{10}N_2O_5$: C, 60.41; H, 3.38; N, 9.39. Found: C, 60.66; H, 3.60; N, 9.12.

To a solution of **34** (1.08 g, 3.62 mmol) in diethyl ether (5 mL) was added hydrazine monohydrate (0.18 g, 3.62 mmol). After vigorous stirring for 15 min at rt, further diethyl ether (25 mL) was added and the reaction mixture was stirred for additional 15 min. The white precipitate was filtered off under vacuum and to the filtrate was added dropwise 5 mL of a 3.5 N HCl ethereal solution. The amorphous precipitated hydrochloride of

35 was separated by decantation and used in the following step without further purification.

A solution of the *O*-alkyl-hydroxylamine hydrochloride **35** (0.71 g, 3.47 mmol) and 3-quinuclidone (0.43 g, 3.47 mmol) in methanol (50 mL) was stirred at rt for 18 h. The crude reaction mixture was concentrated at reduced pressure and the residue was dissolved in 2 N HCl (5 mL) and treated with diethyl ether (3 × 5 mL). The residual aqueous phase was made alkaline by portionwise addition of solid K_2CO_3 and extracted with ethyl acetate (3 × 5 mL). After the usual work-up, the oily residue was purified by silica gel column chromatography (eluant: 5% methanol/dichloromethane) to afford the desired oxime ether **36** (0.52 g, 54%).

4.1.3.3. Quinuclidin-3-one *O*-[4-(isoxazol-3-yloxy)but-2-yn-1-yl]oxime (36). Colorless viscous oil. R_f 0.42 (10% methanol/dichloromethane). ^{1}H NMR: 1.70 (m, 4 H), 2.52 (m, 1H), 2.67–2.94 (m, 4H), 3.52 (s, 2H), 4.58 (bs, 2H), 4.81 (bs, 2H), 5.89 (d, 1H, J = 1.9), 8.03 (d, 1H, J = 1.9). Anal. Calcd for $C_{14}H_{17}N_3O_3$: C, 61.08; H, 6.22; N, 15.26. Found: C, 60.76; H, 6.57; N, 15.45.

Fumarate **13** and methiodide **14** were prepared according to the above-described procedures.

4.1.3.4. Quinuclidin-3-one *O*-[4-(isoxazol-3-yloxy)but-2-yn-1-yl]oxime fumarate 13 (36·3/2 $C_4H_4O_4$). Mp 132–137 °C, dec (colorless prisms from 90% 2-propanol/diisopropyl ether). ^{1}H NMR (CD_3OD): 2.04 (m, 2H), 2.16 (m, 2H), 2.85 (m, 1H), 3.30–3.45 (m, 4H), 4.15 (s, 2H), 4.63 (t, 2H, J = 1.9), 4.93 (t, 2H, J = 1.9), 6.13 (d, 1H, J = 1.8), 6.61 (s, 3H), 8.40 (d, 1H, J = 1.8). Anal. Calcd for $C_{20}H_{23}N_3O_9$: C, 53.45; H, 5.16; N, 9.35. Found: C, 53.63; H, 5.22; N, 9.58.

4.1.3.5. Quinuclidin-3-one *O*-[4-(isoxazol-3-yloxy)but-2-yn-1-yl]oxime methiodide (14). Mp 160–175 °C, dec. (colorless prisms from 90% 2-propanol/methanol). ^{1}H NMR (D_2O): 1.97 (m, 2H), 2.16 (m, 2H), 2.80 (m, 1H), 3.00 (s, 3H), 3.38 (m, 2H), 3.52 (m, 2H), 4.26 (m, 2H), 4.61 (t, 2H, J = 1.6), 4.82 (t, 2H, J = 1.6), 6.09 (d, 1H, J = 1.8), 8.23 (d, 1H, J = 1.8). Anal. Calcd for $C_{15}H_{20}IN_3O_3$: C, 43.18; H, 4.83; N, 10.07. Found: C, 43.29; H, 4.60; N, 9.85.

4.1.4. Synthesis of compounds 15–20. To a solution of **37**[20] (0.6 g, 3.46 mmol) in methanol (25 mL) was added piperidine (684 μL, 6.92 mmol). After stirring at room temperature for 36 h, the solvent was removed at reduced pressure, the crude reaction mixture was dissolved in 2 N HCl (5 mL) and treated with diethyl ether (3 × 5 mL). The residual aqueous phase was made alkaline by portionwise addition of solid K_2CO_3 and extracted with ethyl acetate (3 × 5 mL). After the usual work-up, the oily residue was purified by silica gel column chromatography (eluant: 2% methanol/dichloromethane) affording the desired tertiary amine **38** (0.54 g, 70%).

4.1.4.1. 2-(4-Piperidin-1-ylbut-2-yn-1-yl)isoxazolidin-3-one (38). Colorless oil, bp 155–160 °C/0.5 mm Hg. R_f 0.64 (10% methanol/dichloromethane). ^{1}H NMR: 1.42 (m, 2H), 1.60 (m, 4H), 2.46 (m, 4H), 2.77 (t, 2H, J = 8.2), 3.26 (t, 2H, J = 1.7), 4.32 (t, 2H, J = 1.7), 4.36 (t, 2H, J = 8.2). Anal. Calcd for $C_{12}H_{18}N_2O_2$: C, 64.84; H, 8.16; N, 12.60. Found: C, 65.20; H, 7.87; N, 12.29.

According to the procedure described for **38**, derivatives **39**, **40**, and **41** were prepared by reacting **37** with indoline, 1,2,3,4-tetrahydroisoquinoline, and nortropane,[32] respectively. Compound **39** was obtained heating at reflux the reaction mixture for 24 h whereas derivative **41** was formed in the presence of cesium carbonate (equimolar with nortropane) at rt.

4.1.4.2. 2-[4-(2,3-Dihydro-1*H*-indol-1-yl)but-2-yn-1-yl]isoxazolidin-3-one (39). Thick pale yellow oil (60% yield). R_f 0.21 (20% ethyl acetate/petroleum ether). ^{1}H NMR: 2.62 (t, 2H, J = 8.1), 2.97 (t, 2H, J = 7.9), 3.39 (t, 2H, J = 7.9), 3.92 (t, 2H, J = 1.6), 4.21 (t, 2H, J = 8.1), 4.23 (t, 2H, J = 1.6), 6.58 (d, 1H, J = 7.1), 6.73 (t, 1H, J = 7.1), 7.09 (m, 2H). Anal. Calcd for $C_{15}H_{16}N_2O_2$: C, 70.29; H, 6.29; N, 10.93. Found: C, 69.95; H, 6.53; N, 11.18.

4.1.4.3. 2-[4-(3,4-Dihydroisoquinolin-2(1*H*)-yl)but-2-yn-1-yl]isoxazolidin-3-one (40). Thick yellow oil (51% yield). R_f 0.14 (40% ethyl acetate/petroleum ether). ^{1}H NMR: 2.78 (t, 2H, J = 8.1), 2.82 (t, 2H, J = 6.1), 2.94 (t, 2H, J = 6.1), 3.52 (t, 2H, J = 1.7), 3.78 (s, 2H), 4.32 (t, 2H, J = 1.7), 4.37 (t, 2H, J = 8.1), 7.02 (m, 1H), 7.11 (m, 3H). Anal. Calcd for $C_{16}H_{18}N_2O_2$: C, 71.09; H, 6.71; N, 10.36. Found: C, 71.30; H, 6.55; N, 10.08.

4.1.4.4. 2-[4-(8-Azabicyclo[3.2.1]oct-8-yl)but-2-yn-1-yl]isoxazolidin-3-one (41). Viscous colorless oil (53% yield). R_f 0.67 (20% methanol/dichloromethane). ^{1}H NMR: 1.35–1.50 (m, 4H), 1.64 (m, 2H), 1.85–2.05 (m, 4H), 2.74 (t, 2H, J = 8.1), 3.30 (t, 2H, J = 1.7), 3.38 (bs, 2H), 4.31 (t, 2H, J = 1.7), 4.38 (t, 2H, J = 8.1). Anal. Calcd for $C_{14}H_{20}N_2O_2$: C, 67.71; H, 8.12; N, 11.28. Found: C, 67.36; H, 8.41; N, 11.47.

Oxalates **15** and **18** and methiodides **16** and **19** were prepared according to the above-described procedures. Iodomethylate **17** was obtained heating at reflux an acetone solution of **39** for 6 h in the presence of a fivefold excess of iodomethane. Derivatives **17** and **19** were purified by means of a silica gel column chromatography and were obtained in 63% and 70% yield, respectively.

4.1.4.5. 2-(4-Piperidin-1-ylbut-2-yn-1-yl)isoxazolidin-3-one 15 (38·$C_2H_2O_4$). Mp 117–118 °C (colorless prisms from 90% 2-propanol/diethyl ether); ^{1}H NMR (D_2O): 1.29–1.42 (m, 1H), 1.50–1.72 (m, 3H), 1.79–1.90 (m, 2H), 2.79 (t, 2H, J = 8.2), 2.89 (dt, 2H, J = 2.6 and 12.1 and), 3.42–3.51 (m, 2H), 3.87 (t, 2H, J = 1.8), 4.33 (t, 2H, J = 1.8), 4.35 (t, 2H, J = 8.2). Anal. Calcd for $C_{14}H_{20}N_2O_6$: C, 53.84; H, 6.45; N, 8.97. Found: C, 53.56; H, 6.34; N, 8.68.

4.1.4.6. 2-(4-Piperidin-1-ylbut-2-yn-1-yl)isoxazolidin-3-one methiodide (16). Light yellow gummy solid. ^{1}H NMR (CD_3OD): 1.60–1.80 (m, 2H), 1.83–1.98 (m, 4H),

2.81 (t, 2H, $J = 8.1$), 3.17 (s, 3H), 3.30 (t, 2H, $J = 1.8$) 3.48 (m, 2H), 4.33-4.49 (m, 6H). Anal. Calcd for $C_{13}H_{21}IN_2O_2$: C, 42.87; H, 5.81; N, 7.69. Found: C, 43.22; H, 5.49; N, 7.91.

4.1.4.7. 2-[4-(2,3-Dihydro-1*H*-indol-1-yl)but-2-yn-1-yl]isoxazolidin-3-one methiodide (17). Light yellow gummy solid. R_f 0.22 (10% methanol/dichloromethane); ^{1}H NMR (CD_3OD): 2.74 (t, 2H, $J = 7.5$), 3.50 (m, 2H), 3.62 (s, 3H), 4.22 (m, 1H), 4.31 (t, 2H, $J = 7.5$), 4.35 (t, 2H, $J = 1.6$), 4.45 (m, 1H), 4.81 (m, 2H), 7.58 (m, 3H), 7.78 (d, 1H, $J = 7.6$). Anal. Calcd for $C_{16}H_{19}IN_2O_2$: C, 48.26; H, 4.81; N, 7.03. Found: C, 47.90; H, 5.10; N, 7.35.

4.1.4.8. 2-[4-(3,4-Dihydroisoquinolin-2(1*H*)-yl)but-2-yn-1-yl]isoxazolidin-3-one oxalate 18 (40·$C_2H_2O_4$). Mp 157–162 °C (colorless prisms from 10% methanol/2-propanol). ^{1}H NMR (CD_3OD): 2.79 (t, 2H, $J = 8.1$), 3.19 (t, 2H, $J = 6.3$), 3.54 (t, 2H, $J = 6.3$), 4.15 (s, 2H), 4.38 (t, 2H, $J = 8.1$), 4.40 (m, 4H), 7.15-7.35 (m, 4H). Anal. Calcd for $C_{18}H_{20}N_2O_6$: C, 59.99; H, 5.59; N, 7.77. Found: C, 59.71; H, 5.52; N, 7.61.

4.1.4.9. 2-[4-(3,4-Dihydroisoquinolin-2(1*H*)-yl)but-2-yn-1-yl]isoxazolidin-3-one methiodide (19). Pale yellow waxy compound. R_f 0.41 (10% methanol/dichloromethane). ^{1}H NMR (CD_3OD): 2.83 (t, 2H, $J = 8.1$), 3.35 (s, 3H), 3.95 (m, 4H), 4.41 (t, 2H, $J = 8.1$), 4.49 (m, 2H), 4.52 (m, 2H), 4.79 (m, 2H), 7.21–7.40 (m, 4H). Anal. Calcd for $C_{17}H_{21}IN_2O_2$: C, 49.53; H, 5.13; N, 6.80. Found: C, 49.84; H, 5.47; N, 6.53.

To a solution of **41** (0.13 g, 0.52 mmol) in diethyl ether (3 mL) was added 3 N HCl in diethyl ether (1 mL). The corresponding salt precipitated as a very hygroscopic colorless powder. After washing several times with anhydrous diethyl ether, the hydrochloride was dried under vacuum.

4.1.4.10. 2-[4-(8-Azabicyclo[3.2.1]oct-8-yl)but-2-yn-1-yl]isoxazolidin-3-one hydrochloride (20). Thick yellow oil. ^{1}H NMR (CD_3OD): 1.82 (m, 4H), 1.92–2.12 (m, 4H), 2.25 (m, 2H), 2.67 (t, 2H, $J = 7.9$), 3.99 (bs, 2H), 4.09 (bs, 2H), 4.12 (bs, 2H), 4.21 (t, 2H, $J = 7.9$).

4.1.5. Synthesis of compounds 21–27. Derivatives **43**, **44**, **45**, and **46** were prepared, according to the procedure described for **38**, by reacting mesylate **42** for 12 h at rt with two equivalents of piperidine, indoline, 1,2,3,4-tetrahydroisoquinoline, and nortropane, respectively. The tertiary base **46** was obtained in the presence of cesium carbonate (equimolar with **42**).

4.1.5.1. 1-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]piperidine (43). Colorless liquid, bp 155–160 °C/5 mm Hg (80% yield). R_f 0.64 (10% methanol/dichloromethane). ^{1}H NMR: 1.43 (m, 2H), 1.60 (m, 4H), 2.47 (m, 4H), 2.98 (t, 2H, $J = 9.5$), 3.30 (t, 2H, $J = 1.8$), 4.41 (t, 2H, $J = 9.5$), 4.79 (t, 2H, $J = 1.8$). Anal. Calcd for $C_{12}H_{18}N_2O_2$: C, 64.84; H, 8.16; N, 12.60. Found: C, 64.58; H, 8.28; N, 12.31.

4.1.5.2. 1-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]indoline (44). Thick yellow oil (81% yield). R_f 0.57 (30% ethyl acetate/petroleum ether); ^{1}H NMR: 2.95 (t, 2H, $J = 9.5$), 2.98 (t, 2H, $J = 8.1$), 3.42 (t, 2H, $J = 8.1$), 3.99 (t, 2H, $J = 1.8$), 4.40 (t, 2H, $J = 9.5$), 4.72 (t, 2H, $J = 1.8$), 6.58 (d, 1H, $J = 7.7$), 6.73 (t, 1H, $J = 7.3$), 7.10 (m, 2H). Anal. Calcd for $C_{15}H_{16}N_2O_2$: C, 70.29; H, 6.29; N, 10.93. Found: C, 69.92; H, 6.51; N, 11.23.

4.1.5.3. 2-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]-1,2,3,4-tetrahydroisoquinoline (45). Thick colorless oil (65% yield). R_f 0.63 (40% ethyl acetate/petroleum ether); ^{1}H NMR: 2.84 (t, 2H, $J = 5.9$), 2.94 (t, 2H, $J = 5.9$), 2.99 (t, 2H, $J = 9.5$), 3.56 (t, 2H, $J = 1.8$), 3.77 (s, 2H), 4.41 (t, 2H, $J = 9.5$), 4.80 (t, 2H, $J = 1.8$), 7.03-7.20 (m, 4H). Anal. Calcd for $C_{16}H_{18}N_2O_2$: C, 71.09; H, 6.71; N, 10.36. Found: C, 71.42; H, 6.32; N, 10.59.

4.1.5.4. 8-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]-8-azabicyclo[3.2.1]octane (46). Thick colorless oil (57% yield). R_f 0.71 (20% methanol/dichloromethane); ^{1}H NMR: 1.38–1.56 (m, 4H), 1.63 (m, 2H), 1.81–1.99 (m, 4H), 2.99 (t, 2H, $J = 9.5$), 3.27 (t, 2H, $J = 1.8$), 3.40 (bs, 2H), 4.41 (t, 2H, $J = 9.5$), 4.78 (t, 2H, $J = 1.8$). Anal. Calcd for $C_{14}H_{20}N_2O_2$: C, 67.71; H, 8.12; N, 11.28. Found: C, 67.78; H, 8.39; N, 11.48.

Hydrochloride **23**, oxalates **21**, **25**, and **27**, methiodides **22**, **24**, and **26** were prepared according to the above-described protocols. Derivatives **24** and **26** were purified by means of a silica gel column chromatography.

4.1.5.5. 1-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]piperidine oxalate 21 (43·$C_2H_2O_4$). Mp 131–133°C (colorless prisms from 2-propanol); ^{1}H NMR (CD_3OD): 1.62-1.73 (m, 2H,), 1.80–1.92 (m, 4H), 3.01 (t, 2H, $J = 9.8$), 3.25-3.37 (m, 4H), 4.06 (t, 2H, $J = 1.8$), 4.39 (t, 2H, $J = 9.8$), 4.87 (t, 2H, $J = 1.8$). Anal. Calcd for $C_{14}H_{20}N_2O_6$: C, 53.84; H, 6.45; N, 8.97. Found: C, 53.83; H, 6.44; N, 8.80.

4.1.5.6. 1-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]piperidine methiodide (22). Colorless gummy solid. ^{1}H NMR (CD_3OD): 1.59–1.79 (m, 2H), 1.93 (m, 4H), 3.03 (t, 2H, $J = 9.9$), 3.17 (s, 3H), 3.43–3.54 (m, 4H), 4.40 (t, 2H, $J = 9.9$), 4.42 (t, 2H, $J = 1.8$), 4.90 (t, 2H, $J = 1.8$). Anal. Calcd for $C_{13}H_{21}IN_2O_2$: C, 42.87; H, 5.81; N, 7.69. Found: C, 43.21; H, 5.59; N, 7.52.

4.1.5.7. 1-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]indoline hydrochloride (23). Mp 118–119°C (colorless prisms from 2-propanol/diethyl ether). ^{1}H NMR (CD_3OD): 2.94 (t, 2H, $J = 9.9$), 3.30 (t, 2H, $J = 7.7$), 3.92 (t, 2H, $J = 7.7$), 4.36 (t, 2H, $J = 9.9$), 4.49 (t, 2H, $J = 1.8$), 4.79 (t, 2H, $J = 1.8$), 7.26–7.43 (m, 4H). Anal. Calcd for $C_{15}H_{17}ClN_2O_2$: C, 61.54; H, 5.85; Cl, 12.11; N, 9.57. Found: C, 61.40; H, 5.97; Cl, 12.32; N, 9.38.

4.1.5.8. 1-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]indoline methiodide (24). Pale yellow waxy compound (78% yield). R_f 0.54 (10% methanol/dichloromethane). ^{1}H NMR (CD_3OD): 2.96 (t, 2H, $J = 9.5$), 3.45–3.54 (m, 2H), 3.61 (s, 3H), 4.21 (m, 1H), 4.39 (t, 2H,

J = 9.5), 4.46 (m, 1H), 4.89 (bs, 4H), 7.57 (m, 3H), 7.78 (d, 1H, J = 8.1). Anal. Calcd for $C_{16}H_{19}IN_2O_2$: C,48.26; H, 4.81; N, 7.03. Found: C, 48.56; H, 4.55; N, 6.78.

4.1.5.9. 2-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]-1,2,3,4-tetrahydroisoquinoline oxalate 25 (45·$C_2H_2O_4$). Mp 157–159 °C (colorless prisms from 2-propanol). ^{1}H NMR (CD_3OD): 3.02 (t, 2H, J = 9.5), 3.18 (t, 2H, J = 6.6), 3.56 (t, 2H, J = 6.6), 4.20 (t, 2H, J = 1.8), 4.38 (t, 2H, J = 9.5), 4.42 (s, 2H), 4.89 (t, 2H, J = 1.8), 7.25 (m, 4H). Anal. Calcd for $C_{18}H_{20}N_2O_6$: C, 59.99; H, 5.59; N, 7.77. Found: C, 59.80; H, 5.76; N, 7.52.

4.1.5.10. 2-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]-1,2,3,4-tetrahydroisoquinoline methiodide (26). Pale yellow waxy compound (85% yield). ^{1}H NMR (CD_3OD): 3.04 (t, 2H, J = 9.5), 3.27 (s, 3H), 3.33 (m, 2H), 3.78–3.94 (m, 2H), 4.40 (t, 2H, J = 9.5), 4.49 (t, 2H, J = 1.8), 4.70 (d, 1H, J = 16.0), 4.73 (d, 1H, J = 16.0), 4.93 (t, 2H, J = 1.8), 7.24 (d, 1H, J = 7.3), 7.35 (m, 3H). Anal. Calcd for $C_{17}H_{21}IN_2O_2$: C, 45.93; H, 5.13; N, 6.80. Found: C, 45.61; H, 5.40; N, 6.54.

4.1.5.11. 8-[4-(4,5-Dihydroisoxazol-3-yloxy)but-2-yn-1-yl]-8-azabicyclo[3.2.1]octane oxalate 27 (46·$C_2H_2O_4$). Colorless amorphous solid. ^{1}H NMR: 1.67 (m, 4H), 1.98 (m, 2H), 2.18 (m, 2H), 2.35 (m, 2H), 3.01 (t, 2H, J = 9.5), 3.83 (bs, 2H), 4.10 (bs, 2H), 4.43 (t, 2H, J = 9.5), 4.78 (bs, 2H). Anal. Calcd for $C_{16}H_{22}N_2O_6$: C, 56.80; H, 6.55; N, 8.28. Found: C, 56.59; H, 6.81; N, 8.47.

4.2. Pharmacology

Stable cell lines expressing the hm1–hm5 muscarinic receptors were obtained via the resources of the 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 50 mM Tris–HCl 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. Filters were covered with 6 mL of counting cocktail (Eco-Scint, Research Products International) and radioactivity was measured in a scintillation counter (Beckman) at 40% efficiency. The IC_{50} values were obtained by nonlinear curve fitting to a logistic equation (Prism, GraphPad Software, San Diego, CA). K_i values were derived from IC_{50} values using Cheng–Prusoff equation.[35]

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.[21]

4.2.1. Electrically stimulated rabbit vas deferens. According to Eltze,[36] 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, and 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-adrenoceptos stimulation. For isometric recordings, the tissues were left to equilibrate for 45 min under a passive load of 0.75 g before electrical 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, $MgSO_4.7H_2O$ 1.2, and 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. Protocols. Agonist concentration–response curves were constructed in each tissue by cumulative application of concentrations of the test compounds.[37] The agonist potency was expressed as pEC_{50} (−log EC_{50}) calculated by linear regression analysis using the least square method. Intrinsic activity (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–100 μM). Apparent affinity (pK_D) of drugs behaving as full agonists was estimated by means of the receptor inactivation technique[33] using phenoxybenzamine (1–10 μM for 20–30 min) as the alkylating agent. For partial agonists pK_D was estimated by the method of McKay[34] by comparing their concentration-response curves with those of the reference full agonist. 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.[33] All data are expressed as means of 6–8 experiments.

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