Available online at www.sciencedirect.com
SCIENCE DIRECT®
IL FARMACO 59 (2004) 971–980
IL FARMACO
http://france.elsevier.com/direct/FARMAC/

Molecular modulation of muscarinic antagonists. Synthesis and affinity profile of 2,2-diphenyl-2-ethylthio-acetic acid esters designed to probe the binding site cavity

Serena Scapecchi [a,*], Elisabetta Martini [a], Cristina Bellucci [a], Michela Buccioni [b], Silvia Dei [a], Luca Guandalini [a], Dina Manetti [a], Cecilia Martelli [a], Gabriella Marucci [b], Rosanna Matucci [c]

[a] *Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via Ugo Schiff 6, 50019 Sesto Fiorentino, Firenze, Italy*
[b] *Dipartimento di Scienze Chimiche, Università di Camerino, Via S. Agostino 1, 62032 Camerino, Italy*
[c] *Dipartimento di Farmacologia Preclinica e Clinica, Università di Firenze, Viale Pieraccini 6, 50139 Firenze, Italy*

Received 30 June 2004; accepted 5 August 2004

Available online 30 September 2004

Abstract

The synthesis and preliminary pharmacological profile of a new series of muscarinic antagonists, derived from previously studied 2,2-diphenyl-2-ethylthio-acetic acid esters, are reported. The parent molecules were decorated with linkers of different length, carrying an amino group to catch a putative anionic function outside the recognition site of the receptor. It was hoped that the interception of this function would give molecules with higher potency and selectivity. The attempt has not been successful, but a new series of compounds with a peculiar pharmacological profile has been identified.

Keywords: Muscarinic antagonists; Affinity; Functional activity

1. Introduction

Muscarinic receptors are found in the central and peripheral nervous system. They play an important role in several vital functions such as control of movement, cognition, smooth muscle tone, and glandular secretion [1]. As a consequence of this vast array of actions, muscarinic receptor agonists and antagonists have potential for therapeutic use in a variety of pathologies such as smooth muscle hyperactivity and neurodegenerative diseases [2–4]. However, non selective compounds, because of the large distribution of the receptors in the body and the existence of several subtypes, may produce several, severe, side effects.

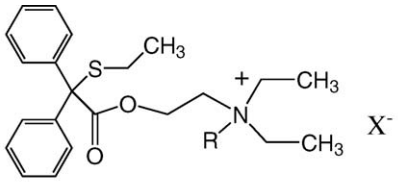
Five distinct subtypes of muscarinic receptors have been cloned (M_1–M_5) but only four have been characterized pharmacologically (M_1–M_4) [5]. Fundamental information on muscarinic receptor subtypes has been collected from a variety of more or less selective synthetic ligands [6,7], transgenic mice lacking genes encoding each of the muscarinic receptor subtypes [8] and muscarinic toxins from snake venoms [9]. As a matter of fact, several synthetic ligands (mainly antagonists) do show some subtype selectivity [2], but none is so selective as to display at least 100-fold selectivity for one subtype over all the others. This can be due to the high degree of sequence identity in the binding sites of the five subtypes [10,11], even if it has to be considered that a single amino acid difference in GPCR subtypes may yield significant differences in ligand binding affinity [12]. As a consequence, the number of muscarinic agonists and antagonists that have been introduced in therapy are relatively small [2].

Given the situation described above, it is not surprising that research on muscarinic ligands is still very active [6,7] in particular on antagonists [13–21]. Indeed, selective compounds are badly needed both for further characterization of subtypes and for therapeutic applications.

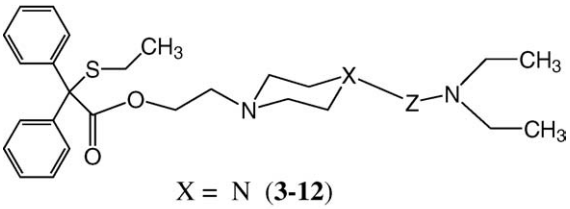
In the past decade, we have been engaged in the synthesis and study of a series of muscarinic antagonists that are amino esters of 2,2-diphenyl-2-ethylthio-acetic acid such as **1** and **2**

* Corresponding author.
E-mail address: serena.scapecchi@unifi.it (S. Scapecchi).

0014-827X/$ - see front matter © 2004 Elsevier SAS. All rights reserved.
doi:10.1016/j.farmac.2004.08.003



R = H; X = Cl (**1**)
R = CH_3; X = I (**2**)



X = N (**3-12**)
X = CH (**13-20**)
Z = linker (see Table 1)

Chart 1.

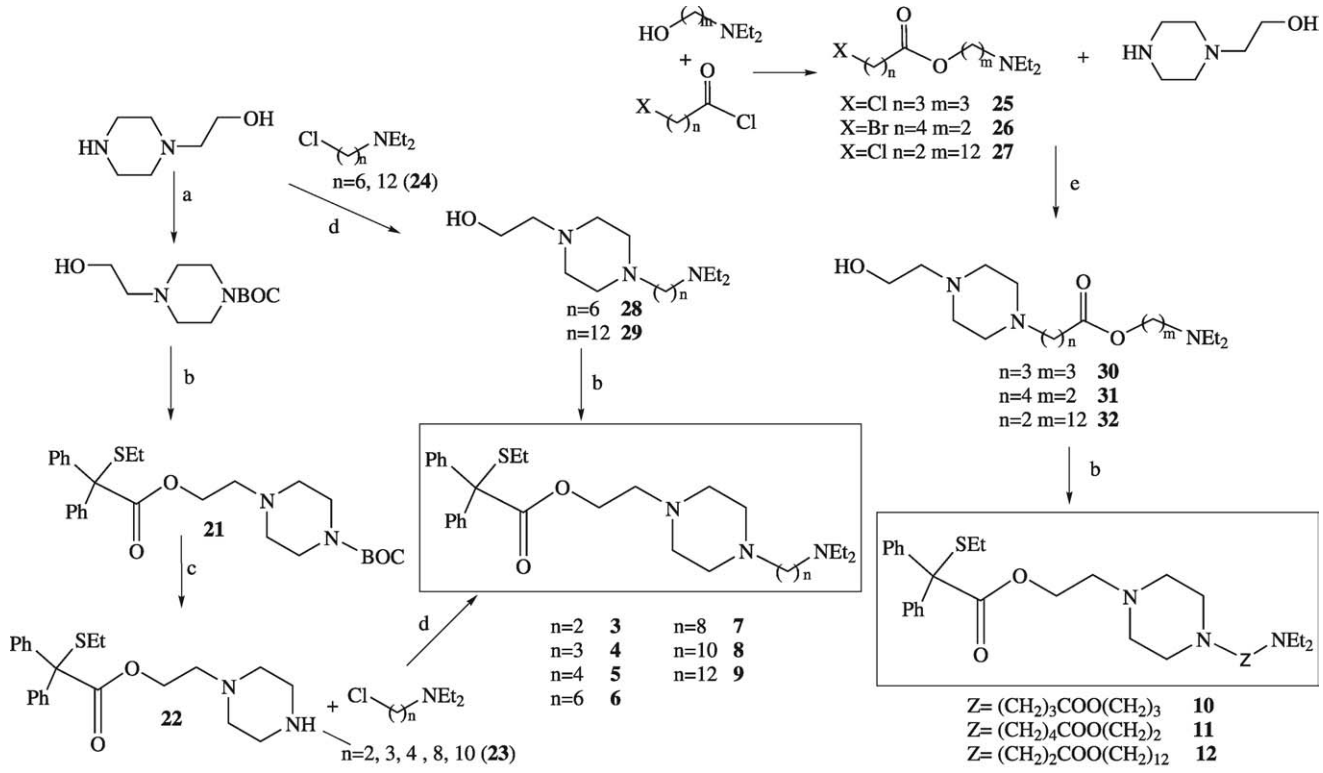
(Chart 1) [22–26]. Such compounds, while being functionally very potent and selective on M_1 and M_2 with respect to the M_3 subtypes [25], failed to show selectivity in binding studies [26].

Aiming to modulate the affinity and potency of the series, we reasoned that the high degree of sequence identity in the binding sites of the five subtypes could be circumvented by ligands designed according to the approach suggested by Jacobson et al. [27]. In brief, the approach consists in the decoration of known ligands with suitably tethered basic or acidic functions, to explore the space vicinal to the receptor recognition site which should be less conserved in the different subtypes. It is predicted that when the linker allows interaction with a complementary acid or basic function, a sudden increase of the affinity shows up.

Therefore, we have designed the series of compounds (**3–20**) shown in Chart 1, where 2,2-diphenyl-2-ethylthioacetic acid has been esterified by *N′*-piperazine ethanol and *N*-piperidine ethanol moieties carrying an *N,N*-diethylamino group linked by methylene or oxygenated chains of varying lengths. Such compounds would represent probes to check the presence of anionic binding sites in the proximity of the recognition site of the receptor, exploiting possible differences in this region of the muscarinic receptor subtypes.

2. Chemistry

The reaction pathways used to synthesize compounds **3–9** are reported in Scheme 1. Compounds **3, 4, 5, 7, 8** were obtained starting from 2-hydroxyethylpiperazine whose nitrogen was quantitatively protected as *tert*-butoxycarbonyl (BOC) derivative, and then reacted with 2,2-diphenyl-2-ethylthio-acetylchloride [25] to afford **21**. The protecting group was quantitatively removed with HCl 6N to give compound **22** that was then reacted with the appropriate chlora-



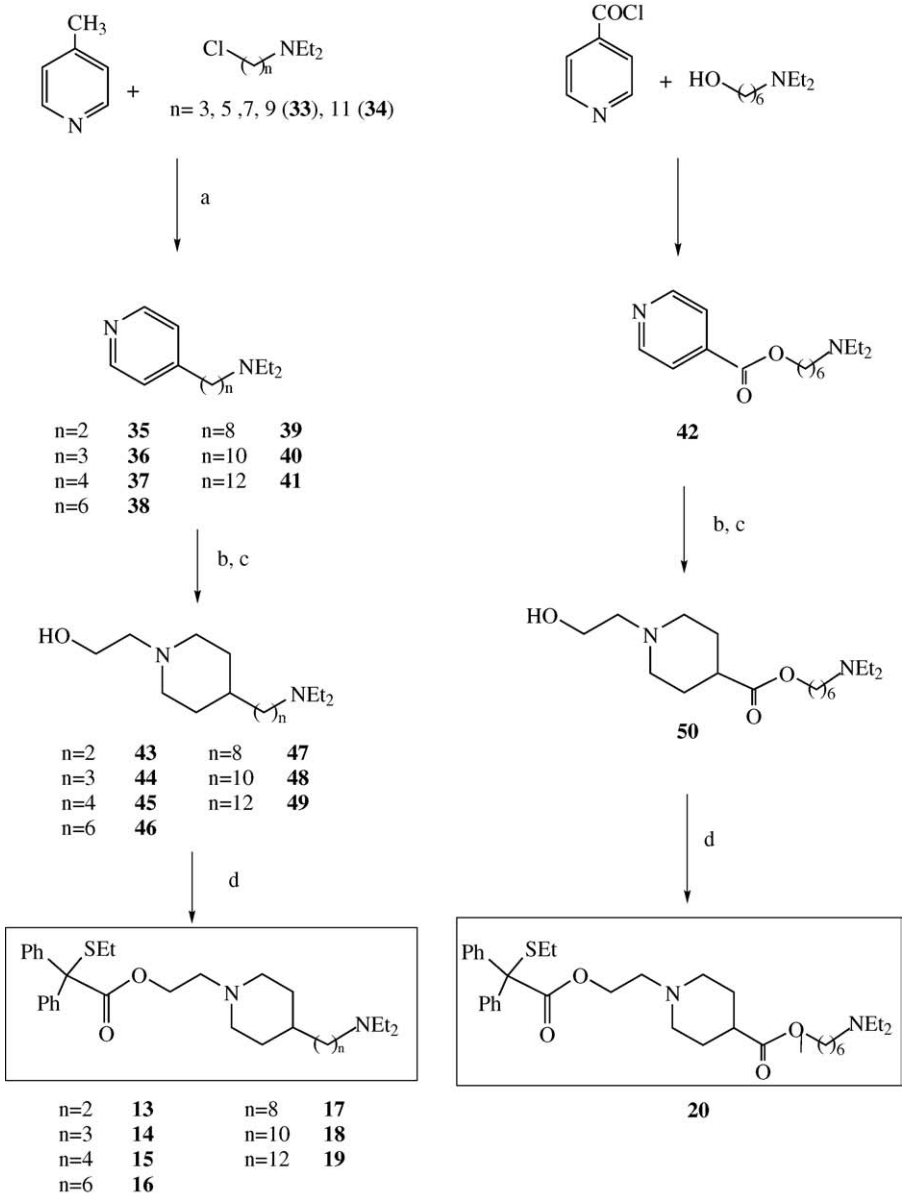
a) di-tert-butyldicarbonate, NEt_3, THF; b) 2,2-diphenyl-2-ethyltioacetylchloride, CH_2Cl_2; c) HCl 6N, AcOEt; d) Abs. EtOH, NEt_3; e) Toluene, K_2CO_3.

Scheme 1.

mines. 2-Chloroethylamine is commercially available, while other chloramines (n = 3 [28], 4 [29], 8 [30], 10 (**23**)) were obtained by treating the corresponding amino alcohols with $SOCl_2$ in $CHCl_3$ stabilized with amylene. In turn, the amino alcohols (n = 3 [31], 4 [32], 8 [30], 10 [33]) were synthesized from the corresponding commercially available chloro or bromoalcohols and diethylamine in anhydrous CH_3CN in presence of K_2CO_3 according to a previously reported procedure [34]. Compounds **6** and **9** were obtained by an alternative pathway treating 2-hydroxyethylpiperazine with the proper chloramines (n = 6 [35], 12 (**24**)) and then coupling the obtained compounds **28** and **29** with 2,2-diphenyl-2-ethylthio-acetylchloride.

In the same scheme, the synthesis of compounds **10–12** is also reported. Halo esters **25–27** were obtained from commercially available 4-chlorobutyryl chloride and 3-chloropropionyl chloride, respectively, with 3-diethylpropanolamine (**25**) and 12-diethyldodecanolamine (**27**)[33] and from the reaction of 5-bromopentanoyl chloride [36] and 2-diethyletanolamine (**26**). Their reaction with 2-hydroxyethylpiperazine gave **30–32**, which, after reaction with 2,2-diphenyl-2-ethylthio-acetylchloride, afforded compounds **10–12**.

The key intermediates in the synthesis of the piperidine compounds **13–20** (Scheme 2) were pyridine amines **35–42**. Compounds **35** [37], **36** [38] and **37** [38] have already been described, and compounds **38–41** were synthesized with the method reported in literature [38], using the proper chloramines (n = 5 [39], 7 [40], 9 (**33**), 11 (**34**)). Compound **42** was obtained from the reaction of isonicotinoyl chloride [41] with



n=2 **35** n=8 **39**
n=3 **36** n=10 **40**
n=4 **37** n=12 **41**
n=6 **38**

n=2 **43** n=8 **47**
n=3 **44** n=10 **48**
n=4 **45** n=12 **49**
n=6 **46**

n=2 **13** n=8 **17**
n=3 **14** n=10 **18**
n=4 **15** n=12 **19**
n=6 **16**

a) LDA, THF; b) H_2/PtO_2; c) $BrCH_2CH_2OH$; d) 2,2-diphenyl-2-ethyltioacetyl chloride, CH_2Cl_2.

Scheme 2.

the appropriate amino alcohol. The pyridine ring of compounds **35–42** was then hydrogenated at 48 psi for 48 h using PtO_2 as a catalyst and a mixture of MeOH and HCl conc. as a solvent. Reaction with bromoethanol in anhydrous CH_3CN gave the corresponding alcohols **43–50** that were then reacted with 2,2-diphenyl-2-ethylthio-acetyl chloride affording compounds **13–20**.

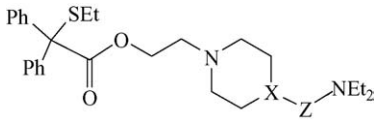
3. Results and discussion

Binding affinity of the synthesized compounds **3–20** and of the parent compounds **1, 2** was measured on CHO-expressed human muscarinic receptor subtypes (hM_1–hM_5) and the results are reported in Table 1. Compared to the parent compounds, none of the newly synthesized molecules showed the sharp increase in affinity expected in the case of a successful ionic interaction of the introduced amine function. There is indeed a small increase of affinity for piperazinic compounds with n = 8 (**7**), 10 (**8**), 12 (**9**) and for piperidinic compounds with n = 6 (**16**), 8 (**17**) but, very likely, this increase is due to the contribution of the methylene groups rather than to new ionic interactions. This is somehow confirmed by the fact that the increase in affinity is similar in all subtypes and the new molecules completely lack selectivity.

Since, as discussed in the Introduction, the parent compounds **1** and **2** show a fairly good potency and selectivity in functional assays, we decided to check the functional activity of selected compounds **7** and **17**, which are among those showing an increase in affinity. The results are reported in Table 2. Unlike the parent compounds, **7** and **17** are only modest functional antagonists and do not show any subtype selectivity, confirming that our attempt was unsuccessful.

However, these two compounds do show some peculiar features that recall those of a related series of 2,2-diphenyl-2-ethylthio-acetic acid esters described in a previous work

Table 1
Binding affinity of compounds **1–20** on CHO expressed human muscarinic receptors [a]



N	*X*	*Z*	pK_i					
			N	HM_1	HM_2	HM_3	HM_4	HM_5
1	–	–	–	6.47 ± 0.08	6.25 ± 0.10	6.44 ± 0.08	5.95 ± 0.11	6.25 ± 0.14
2	–	–	–	6.56 ± 0.04	6.48 ± 0.06	6.43 ± 0.07	6.04 ± 0.16	6.28 ± 0.10
3	N	$(CH_2)_n$	2	5.82 ± 0.39	5.79 ± 0.30	5.93 ± 0.62	5.51 ± 0.43	5.73 ± 0.39
4	N	$(CH_2)_n$	3	6.03 ± 0.19	6.39 ± 0.14	5.72 ± 0.42	5.32 ± 0.82	5.39 ± 0.85
5	N	$(CH_2)_n$	4	6.64 ± 0.06	6.68 ± 0.04	6.38 ± 0.08	6.14 ± 0.07	6.31 ± 0.09
6	N	$(CH_2)_n$	6	6.07 ± 0.19	5.73 ± 0.57	5.95 ± 0.15	5.59 ± 0.35	5.80 ± 0.30
7	N	$(CH_2)_n$	8	7.17 ± 0.01	6.59 ± 0.06	6.46 ± 0.04	6.84 ± 0.01	6.82 ± 0.03
8	N	$(CH_2)_n$	10	7.30 ± 0.01	7.00 ± 0.02	6.46 ± 0.04	7.18 ± 0.01	6.77 ± 0.04
9	N	$(CH_2)_n$	12	7.19 ± 0.02	7.07 ± 0.02	6.79 ± 0.04	7.29 ± 0.01	6.95 ± 0.03
10	N	$(CH_2)_3COO(CH_2)_3$	–	6.23 ± 0.01	6.46 ± 0.10	5.67 ± 0.23	5.34 ± 0.68	5.71 ± 0.40
11	N	$(CH_2)_4COO(CH_2)_2$	–	5.92 ± 0.20	5.65 ± 0.65	5.16 ± 1.10	5.36 ± 0.66	5.44 ± 0.77
12	N	$(CH_2)_2COO(CH_2)_{12}$	–	6.61 ± 0.08	6.47 ± 0.07	6.63 ± 0.07	6.80 ± 0.04	6.58 ± 0.07
13	CH	$(CH_2)_n$	2	6.10 ± 0.12	6.29 ± 0.09	5.82 ± 0.20	5.70 ± 0.26	5.90 ± 0.24
14	CH	$(CH_2)_n$	3	6.13 ± 0.11	6.67 ± 0.05	5.90 ± 0.11	5.86 ± 0.17	5.98 ± 0.21
15	CH	$(CH_2)_n$	4	6.49 ± 0.05	6.63 ± 0.07	6.39 ± 0.06	6.31 ± 0.06	6.54 ± 0.05
16	CH	$(CH_2)_n$	6	7.07 ± 0.02	7.08 ± 0.02	6.46 ± 0.07	6.94 ± 0.01	6.69 ± 0.04
17	CH	$(CH_2)_n$	8	7.00 ± 0.03	6.60 ± 0.07	6.12 ± 0.14	6.72 ± 0.03	6.31 ± 0.13
18	CH	$(CH_2)_n$	10	6.85 ± 0.04	6.83 ± 0.03	6.38 ± 0.10	6.89 ± 0.02	6.41 ± 0.01
19	CH	$(CH_2)_n$	12	6.43 ± 0.09	6.23 ± 0.13	6.40 ± 0.10	6.27 ± 0.09	6.35 ± 0.12
20	CH	$COO(CH_2)_6$	–	7.03 ± 0.02	6.58 ± 0.10	6.27 ± 0.07	6.53 ± 0.04	6.63 ± 0.05

[a] 3–6 Experiments in duplicate.

Table 2
Functional assays of compounds **1, 2, 7, 17** on muscarinic receptor subtypes

N	pK_b ± SE (pA_2 ± SE)			
	M_1 Rabbit vas deferens	M_2 Guinea pig atrium	M_3 Guinea pig ileum	M_4 Guinea pig lung
1	(7.79 ± 0.01)	(7.93 ± 0.03)	(5.88 ± 0.09)	–
2	(8.97 ± 0.06)	(9.12 ± 0.03)	(6.50 ± 0.07)	–
7[a]	6.47 ± 0.09	7.17 ± 0.09	5.94 ± 0.01	5.58 ± 0.01
17[a]	<5.52	6.04±0.08	<5.52	5.40 ± 0.12

[a] Compounds **7** and **17** behave also as putative muscarinic agonists on M_1 and M_2 receptors. See text for details.

[42]. In fact, compounds **7** and **17** behave also as putative muscarinic agonists on M_1 and M_2 receptors since, after 1 h incubation at 10^{-5} M, they strongly reduce the twitch induced by electrical stimulation of rabbit vas deferens and guinea pig heart, as classical muscarinic agonists do, even if they, like the compounds described previously, are characterized by large size structures typical of muscarinic antagonists. The reasons for such behavior are under investigation.

4. Experimental section

4.1. Chemistry

All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin–Elmer 681 or a Perkin–Elmer Spectrum RX I FT-IR spectrophotometer in Nujol mull for solids and neat for liquids. Unless otherwise stated, NMR spectra were recorded on a Gemini 200 spectrometer, and chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063–0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm; Merck). Although the IR spectra data are not always included (because of lack of unusual features), they were obtained for all reported compounds and are consistent with the assigned structures. Yields are given after purification, unless otherwise stated. Where analyses are indicated by symbols, the analytical results are within ±0.4% of the theoretical values. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom (version 2.1), a software for systematic names in organic chemistry. When reactions were performed in anhydrous conditions, the mixtures were maintained under nitrogen.

4.2. 4-[2-(2-Ethylsulfanyl-2,2-diphenylacetoxy)ethyl] piperazine-1-carboxylic acid tert-*butyl ester (**21**)*

A total of 1.92 g (9 mmol) of (*tert*-BOC)hydroxyethylpiperazine were reacted with 2.62 g (9 mmol) of 2,2-diphenyl-2-ethylthioacetylchloride [25] in anhydrous CH_2Cl_2 at r.t. for 2 h. The solution was then basified with 10% NaOH and extracted with $CHCl_3$. The crude product was purified by flash chromatography using abs. EtOH:CH_2Cl_2:Et_2O:petroleum ether = 180:360:360:900 as eluting system. The oily compound, 39% yield, was used as such in the next reaction.

^{1}H-NMR ($CDCl_3$) δ: 1.10 (*t, J* = 6.67 Hz, 3H, SCH_2CH_3); 1.45 (*s*, 9H, $C(CH_3)_3$); 2.16–2.26 (*m*, 4H, *4 CH* piperazine); 2.34 (*q, J* = 6.67 Hz, 2H, SCH_2CH_3); 2.53 (*t, J* =3.33 Hz, 2H, $COOCH_2CH_2N$); 3.21–3.35 (*m*, 4H, *4 CH* piperazine); 4.28 (*t, J* =3.33 Hz, 2H, $COOCH_2$); 7.15–7.45 (*m*, 10H, aromatics).

*4.3. Ethylsulfanyldiphenylacetic acid 2-piperazin-1-ylethyl ester (**22**)*

A total of 0.28 g (0,7 mmol) of **21** was dissolved in 10 ml of AcOEt added with 6 ml of 6N HCl. The reaction was stirred at r.t. for 12 h; the mixture was then treated with 10% NaOH and the product extracted in $CHCl_3$. The organic phase was anhydrified over Na_2SO_4, the solvent removed under vacuum and the title compound, obtained quantitatively as an oil, used as such for the next reaction.

^{1}H-NMR ($CDCl_3$) δ: 1.10 (*t, J* = 3.33 Hz, 3H, SCH_2CH_3); 2.25–2.45 (*m*, 6H, *4 CH* piperazine; SCH_2CH_3); 2.55 (*t, J* = 1.67 Hz, 2H, $COOCH_2CH_2N$); 2.73–2.83 (*m*, 4H, *4 CH* piperazine); 4.30 (*t, J* =1.67 Hz, 2H, $COOCH_2$); 7.20–7.50 (*m*, 10H, aromatics).

*4.4. 12-(Chlorododecyl)diethylamine (**24**)*

One equivalent of 12-diethylaminododecanol [33] was dissolved in $CHCl_3$ stabilized with amylene, added with an excess of $SOCl_2$, and refluxed for 2 h. The excess of $SOCl_2$ was eliminated under vacuum, the crude mixture basified with Na_2CO_3 and the chlorododecyldiethylamine (**24**) extracted with $CHCl_3$. The organic phase was dried over Na_2SO_4 and the solvent removed under vacuum. The title compound was obtained as oil. Yield 73%. ^{1}H-NMR ($CDCl_3$) *δ*: 1.02 (*t, J* = 7.14 Hz, 6H, $2NCH_2CH_3$); 1.22–1.50 (*m*, 18 H); 1.76 (*m*, 2H); 2.40 (*t, J* = 7.69 Hz, 2H, CH_2CH_2N); 2.68 (*q, J* = 7.14 Hz, 4H, $2NCH_2CH_3$); 3.53 (*t, J* = 6.78 Hz, 2H, CH_2Cl,). Anal. ($C_{16}H_{34}ClN$) C,H,N.

Compounds **23, 33** and **34** were obtained in the same way and their spectroscopic characteristics are consistent with the proposed structures.

*4.5. 4-Chloro-butyric acid 3-diethylamino-propyl ester (**25**)*

One equivalent of 3-diethylaminopropanol was dissolved in anhydrous CH_2Cl_2, cooled at 0 °C and added dropwise with 1 equivalent of 4-chlorobutirrylchloride; the solution was stirred at r.t. for 48 h. The mixture was basified with a 10% NaOH solution and extracted with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and the solvent removed under vacuum afforded compound **25** as oil. Yield 100%. ^{1}H-NMR ($CDCl_3$) δ: 0.99 (*t, J* = 7.10 Hz, 6H, NCH_2CH_3); 1.68–1.82 (*m*, 2H, $ClCH_2CH_2CH_2CO$); 2.00–2.14 (*m*, 2H, $OCH_2CH_2CH_2N$); 2.43–2.54 (*m*, 8H, CH_2COO, CH_2NEt_2, $2NCH_2CH_3$); 3.58 (*t, J* = 6.60 Hz, 2H, $ClCH_2$); 4.11 (*t, J* = 6.60 Hz, 2H, $COOCH_2$). Anal. ($C_{11}H_{22}ClNO_2$) C,H,N.

Compounds **26** and **27** were obtained in the same way and their spectroscopic characteristics are consistent with the proposed structures.

*4.6. 2-[4-(6-Diethylaminohexyl)piperazin-1-yl]ethanol (**28**)*

One equivalent of 6-chlorohexyldiethylamine dissolved in abs. EtOH was added with *N*-2-hydroxyethylpiperazine (1 equivalent) and with anhydrous NEt_3 (2 equivalents) and the mixture refluxed for 24 h. The solvent was removed under reduced pressure and the resulting thick oil was heated at 80 °C for 20 h; 20 ml of a saturated solution of Na_2CO_3 were

added and the resulting suspension extracted with CH_2Cl_2. After anhydrification over Na_2SO_4, the solvent was removed under vacuum and the mixture purified by flash chromatography using NH_4OH:abs. EtOH:CH_2Cl_2:petroleum ether = 45:225:600:90 as eluting system. Oil; yield 16%. ^{1}H-NMR ($CDCl_3$) δ: 1.02 (*t, J* = 6.96 Hz, 6H, 2NCH$_2$*CH$_3$*); 1.28–1.38 (*m*, 4H); 1.42–1.55 (*m*, 4H); 2.28–2.58 (*m*, 18H, HOCH$_2$*CH$_2$*N, *CH$_2$*NEt$_2$, Npip-*CH$_2$*, N*CH$_2$*CH$_3$, 8H piperazine); 2.95 (*bs*, 1H, *OH*); 3.6 (*t, J* = 5.49 Hz, 2H, *CH$_2$*OH). Anal. ($C_{16}H_{35}N_3O$) C,H,N.

Compound **29** was obtained in the same way and its spectroscopic characteristics are consistent with the proposed structure.

*4.7. 4-[4-(2-Hydroxyethyl)piperazin-1-yl]butyric acid 3-diethylaminopropyl ester (**30**)*

Compound **25** (1 equivalent) was dissolved in toluene and 2-hydroxyethylpiperazine (1 equivalent) and an excess of K_2CO_3 were added. The mixture was heated at 100 °C under stirring for 2 days, then K_2CO_3 was filtered off, the solvent evaporated under reduced pressure and the oily product purified by flash chromatography using NH_4OH:abs. EtOH:CH_2Cl_2:petroleum ether = 8:65:340:60 as eluting system. Compound **30** was obtained as oil in 100% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.01 (*t, J* = 7.10 Hz, 6H, 2NCH$_2$*CH$_3$*); 1.70–1.85 (*m*, 5H, NCH$_2$*CH$_2$*CH$_2$CO+OCH$_2$*CH$_2$*CH$_2$N+OH); 2.30–2.56 (*m*, 20H); 3.60 (*t, J* =5.30 Hz, 2H, *CH$_2$*OH); 4.11 (*t, J* = 6.20 Hz, 2H, COO*CH$_2$*,). Anal. ($C_{17}H_{35}N_3O_3$) C,H,N.

Compounds **31** and **32** were obtained in the same way and their spectroscopic characteristics are consistent with the proposed structures.

*4.8. Ethylsulfanyldiphenylacetic acid 2-[4-(6-diethylaminohexyl)piperidin-1-yl]ethyl ester (**6**)*

Compound **28** (0.04 g) was reacted with 2-ethylthio-2,2-diphenylacetyl chloride [25] (0.041 g 0.14 mmol) in 20 ml of anhydrous CH_2Cl_2 under N_2 at r.t. for 6 days. The reaction mixture was then treated with 10% NaOH solution and extracted with CH_2Cl_2; the organic phase was dried over Na_2SO_4 and CH_2Cl_2 evaporated under vacuum. The title compound was obtained as oil after flash chromatographic separation using NH_4OH:abs. EtOH:CH_2Cl_2:petroleum ether = 8:65:340:60 as eluting system. Yield 19%. ^{1}H-NMR ($CDCl_3$) *δ*: 0.99–1.18 (*m*, 9H, SCH$_2$*CH$_3$*, 2NCH$_2$*CH$_3$*); 1.25–1.35 (*m*, 4H); 1.46–1.56 (*m*, 4H); 2.25–2.65 (*m*, 20H, 8CH piperazine, S*CH$_2$*CH$_3$; 2N*CH$_2$*CH$_3$, *CH$_2$*NEt$_2$, OCH$_2$*CH$_2$*N, Npip-*CH$_2$*); 4.32 (*t, J* = 5.49 Hz 2H, COO*CH$_2$*); 7.27–7.38 (*m*, 6H, aromatics); 7.38–7.50 (*m*, 4H, aromatics). Anal. ($C_{32}H_{49}N_3O_2S$) C,H,N.

Compounds **9–12** were obtained in the same way and their spectroscopic characteristics are consistent with the proposed structures.

*4.9. Ethylsulfanyldiphenylacetic acid 2-[4-(8-diethylaminooctyl)piperazin-1-yl]ethyl ester (**7**)*

A total of 0.6 g (1.46 mmol) of **22** was dissolved in 15 ml of abs. EtOH, added with (8-chlorooctyl)d iethyl amine [30] 0.37 g (1.46 mmol) and anhydrous NEt_3 (2 equivalents, 0.4 ml) and refluxed for 3 days. The solvent was removed and the residue treated with 10% NaOH solution and extracted with $CHCl_3$; the organic phase was dried over Na_2SO_4 and $CHCl_3$ evaporated under vacuum. The title compound was obtained as oil after two column chromatographic separations using NH_4OH:abs. EtOH:CH_2Cl_2: Et_2O:petroleum ether = 9.9:180:360:360:900 and NH_4OH:abs. EtOH:CH_2Cl_2: petroleum ether = 8:65:340:60, respectively, as eluting system. Yield 22%. ^{1}H-NMR ($CDCl_3$) *δ*: 0.94–1.56 (*m*, 9H, SCH$_2$*CH$_3$*, 2NCH$_2$*CH$_3$*); 1.19–1.56 (*m*, 14H); 1.65–1.87 (*m*, 2H); 2.18–2.66 (*m*, 14H, 8CH piperazine, S*CH$_2$*CH$_3$, 2N*CH$_2$*CH$_3$); 3.52 (*t, J* = 1.67 Hz, 2H, COOCH$_2$*CH$_2$*N); 4.32 (*t, J* = 1.67 Hz 2H, COO*CH$_2$*); 7.20–7.37 (*m*, 6H, aromatics); 7.37–7.54 (*m*, 4H, aromatics). Anal. ($C_{35}H_{53}N_3O_2S$) C,H,N.

Compounds **3–5** and **8** were obtained in the same way starting from compound **22** and the appropriate chloroalkyldiethylamine (*n* = 2, 3, 4, 10) and their spectroscopic characteristics are consistent with the proposed structures.

*4.10. Diethyl(6-pyridin-4-ylhexyl)amine (**38**)*

Butyllithium in hexane (3.2 ml of a 1.6 M solution; 5 mmol) was added to 0.5 g (5 mmol) of diisopropylamine dissolved in THF (15 ml), cooled at 0 °C and the mixture stirred for 30 min. 4-Methylpyridine (0.45 g, 5 mmol) was then added at 0 °C and the mixture stirred for 30 min; then the electrofile 5-chloropentyldiethyl amine [39] was added (1.52 g, 5.5 mmol) and the mixture warmed up to r.t. and treated with a saturated solution of NH_4Cl. The solution was extracted with diethyl ether and the organic phase dried. The oily product was purified by flash chromatography using NH_4OH:abs. EtOH:$CHCl_2$:Et_2O:petroleum ether = 25:45:180:180:450 as eluting system. Yield 18%. ^{1}H-NMR ($CDCl_3$) δ: 0.84 (*t, J* = 7.30 Hz, 6H, 2NCH$_2$*CH$_3$*); 1.15–1.22 (*m*, 4H); 1.38–1.50 (*m*, 2H); 1.60–1.80 (*m*, 2H); 2.23 (*t, J* = 7.30 Hz, 2H, *CH$_2$*NEt$_2$); 2.29–2.45 (*m*, 6H, 2N*CH$_2$*CH$_3$, pyridine-*CH$_2$*,); 6.92 (*d, J* = 5.86 Hz, 2H pyridine); 8.28 (*d, J* = 5.86 Hz, 2H pyridine). Anal. ($C_{15}H_{26}N_2$) C,H,N.

Compounds **37** [38], **39, 40** and **41** were obtained in the same way, treating 4-methylpyridine with the appropriate electrofile. Their spectroscopic characteristics are consistent with the proposed structures.

*4.11. Isonicotinic acid 6-diethylaminohexyl ester (**42**)*

A total of 0.8 g (4.6 mmol) of 6-diethylaminohexanol was dissolved in $CHCl_3$, and anhydrous NEt_3 (2 equivalent, 1.3 ml) was added; the reaction was cooled at 0 °C and

isonicotinoylchloride in $CHCl_3$ (0.76 g, 4.6 mmol) was added. The mixture was stirred for 36 h at r.t., treated with 10% NaOH solution, extracted with $CHCl_3$ and the organic phase dried. The oily product was then purified by flash chromatography using NH_4OH:abs. EtOH:CH_2Cl_2:Et_2O:petroleum ether = 9.9:180:360:360:900 as eluting system. Yield 26%. ^{1}H-NMR ($CDCl_3$) δ: 1.00 (*t*, 6H, 2NCH$_2$*CH$_3$*, *J* = 7.10 Hz); 1.30–1.55 (*m*, 6H); 1.74–1.81 (*m*, 2H); 2.41 (*t, J* = 7.33 Hz, 2H, *CH$_2$*NEt$_2$,); 2.51 (*q, J* = 7.10 Hz, 4H, 2N*CH$_2$*CH$_3$); 4.34 (*t, J* = 6.59 Hz, 2H, COO*CH$_2$*); 7.83 (*d, J* = 4.76 Hz, 2H pyridine); 8.76 (*d, J* = 4.76 Hz, 2H pyridine). Anal. ($C_{16}H_{26}N_2O_2$) C,H,N.

*4.12. 2-[4-(6-Diethylaminohexyl)piperidin-1-yl]ethanol (**46**)*

A total of 1.84 g (8 mmol) of **38** were dissolved into MeOH (50 ml) and conc. HCl (2 ml), added with 0.05 g of PtO_2 and hydrogenated in a Parr apparatus for 24 h at 48 psi. At the end of the reaction, the catalyst was filtered off and the solvent removed under vacuum. The residue was treated with 10% NaOH solution, extracted with Et_2O and the organic phase dried and evaporated under reduced pressure. The oily compound was used as such for the next reaction. Yield 42%. ^{1}H-NMR ($CDCl_3$) δ: 0.98 (*t, J* = 7.10 Hz, 6H, 2NCH$_2$*CH$_3$*); 1.15–1.43 (*m*, 11H); 1.43–1.61 (*m*, 2H piperidine); 2.02–2.18 (*m*, 2H); 2.24–2.62 (*m*, 8H); 2.95–3.08 (*m*, 2H).

To 0.07 g (0,3 mmol) of diethyl(6-piperidin-4-yl)hexylamine obtained as described above, dissolved in abs. EtOH, anhydrous NEt_3 (0.084 ml, 2 equivalent) and 2-bromoethanol (0.021 ml, 1 equivalent) were added and the resulting mixture was refluxed for 24 h. The solvent was removed and the residue treated with water and extracted with $CHCl_3$. The organic phase was dried, the solvent removed and the crude product purified by column chromatography using NH_4OH:abs. EtOH:CH_2Cl_2:petroleum ether = 45:225:600:90 as eluting system. Oil, yield 35%. ^{1}H-NMR ($CDCl_3$) *δ*: 1.02 (*t, J* = 7.10 Hz, 6H, 2NCH$_2$*CH$_3$*); 1.20–1.41 (*m*, 12H); 1.43–1.62 (*m*, 2H); 1.64–1.68 (*m*, 2H); 2.01 (*t, J* = 7.69 Hz, 2H, *CH$_2$*NEt$_2$); 2.46–2.58 (*m*, 8H, 2(N*CH$_2$*CH$_3$), N*CH$_2$*CH$_2$OH, piperidine-*CH$_2$*,); 2.82–2.91 (*m*, 2H piperidine); 3.59 (*t, J* = 5.50 Hz, 2H, *CH$_2$*OH). Anal. ($C_{17}H_{36}N_2O$) C,H,N.

Compounds **43–45** and **47–50** were obtained in the same way and their spectroscopic characteristics are consistent with the proposed structures.

*4.13. Ethylsulfanyldiphenylacetic acid 2-[4-(6-diethylaminohexyl)piperidin-1-yl]ethyl ester (**16**)*

Compound **46** (0.03 g, 0.11 mmol) was refluxed for 48 h under N_2 with 2-ethylthio-2,2-diphenylacetylchloride [25] (0.031 g, 0.11 mmol) in 20 ml of anhydrous CH_2Cl_2. The reaction mixture was then treated with 10% NaOH solution and extracted with CH_2Cl_2; the organic phase was dried over Na_2SO_4 and CH_2Cl_2 evaporated under vacuum. The title compound was obtained as oil after chromatographic separation using NH_4OH:abs. EtOH:CH_2Cl_2:petroleun ether = 8:65:340:60 as eluting system. Yield 68%. ^{1}H-NMR ($CDCl_3$) *δ*: 1.03–1.18 (*m*, 9H, SCH$_2$*CH$_3$*, 2NCH$_2$*CH$_3$*); 1.20–1.28 (*m*, 10H); 1.42–1.65 (*m*, 4H); 1.81–1.96 (*m*, 2H); 2.38 (*q, J* = 7.33 Hz, 2H, S*CH$_2$*CH$_3$); 2.55–2.85 (*m*, 11H); 4.32 (*t, J* = 5.50 Hz, 2H, COO*CH$_2$*); 7.27–7.35 (*m*, 6H, aromatics); 7.35–7.45 (*m*, 4H, aromatics). Anal. ($C_{33}H_{50}N_2O_2S$) C,H,N.

Compounds **13–15** and **17–20** were obtained in the same way and their spectroscopic characteristics are consistent with the proposed structures.

5. Pharmacology

5.1. Binding

5.1.1. Cell culture and membrane preparation

Chinese hamster ovary cells stably expressing cDNA encoding human muscarinic M_1–M_5 receptors were generously provided by Prof. R. Maggio (Department of Neuroscience, University of Pisa, Italy). Growth medium consisted in Dulbecco' modified Eagle's medium supplemented with 10% fetal calf serum (Gibco, Grand Iland, N.Y.), 100 units/ml each of penicillin G and streptomicyn, 4 mM glutamine (Sigma Aldrich, Milano, Italy) and non essential amino acids (Sigma Aldrich, Milano, Italy) and 50 μg/ml of geneticin (Gibco, Grand Iland, N.Y.) in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Confluent CHO cell lines were scraped, washed with buffer (25 mM sodium phosphate containing 5 mM $MgCl_2$ at pH 7.4) and homogenized for 30 s using an Ultra-Turrax (setting 5). The pellet was sedimented 17000 × *g* for 15 min at 4 °C and the membranes were resuspended in the same buffer, rehomogenized with Ultra-Turrax and stored at –80 °C [43]. An aliquot was taken for the assessment of protein content according to the method of Bradford [44] using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Munchen, West Germany) and bovine serum albumin was used as the standard.

5.1.2. Binding assay

The radioligand binding assay was run in polypropylene 96-well plates (Sarstedt, Verona, Italy) and performed for 120 min at r.t. In a final volume of 0.25 ml in 25 mM sodium phosphate buffer containing 5 μM $MgCl_2$ at pH 7.4. Final membrane protein concentrations were 30 μg/ml (M_1), 70 μg/ml (M_2), 25 μg/ml (M_3), 50 μg/ml (M_4) and 25 μg/ml (M_5).

In homologous competition curves, [^{3}H]-NMS was present at 0.2 nM in tubes containing an increasing concentration of unlabeled NMS (0.03–1000 nM) and at 0.075–0.2 nM in tubes without unlabeled ligand. In heterologous competition curves, fixed concentrations of the tracer (0.2 nM) were displaced by increasing concentrations of several unlabeled

ligands (0.01–1000 μM); all measurements were obtained in duplicate. At the end of the binding reaction, free radioligand was separated from bound ligand by rapid filtration through UniFilter GF/C plates (Perkin Elmer Life Science, Boston, MA) using a FilterMate Cell Harvester (Perkin Elmer Life Science, Boston, MA); after filtration, the filters were washed several times with ice cold buffer and allowed to dry overnight at r.t. under air flow, added of 25 μl of scintillation liquid (Microscint-20, Perkin Elmer Life Science, Boston, MA) and counted by TopCount NXT Microplate Scintillation Counter (Perkin Elmer Life Science, Boston, MA). The binding data were analyzed by the weighted least-squares iterative curve fitting LIGAND [44] to obtain the affinity constant (K_i) and the binding capacity (B_{max}).

5.2. Functional tests

5.2.1. General considerations

Male guinea pigs (200–300 g) and male New Zealand white rabbits (3.0–3.5 kg) were killed by cervical dislocation. The organs required were set up rapidly under 1 g of tension in 20 ml organ baths containing physiological salt solution (PSS) maintained at an appropriate temperature (see below) and aerated with 5% CO_2 95% O_2. Dose–response curves were constructed by cumulative addition of the reference agonist. The concentration of agonist in the organ bath was increased approximately three-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Following 30 min of washing, tissues were incubated with the antagonist for 60 min, and a new dose–response curve to the agonist was obtained. In the case of the agonist following 30 min washing, a cumulative dose–response curve to the agonist under study was constructed. Responses were expressed as a percentage of the maximal response obtained in the control curve. Contractions were recorded by means of a force displacement transducer connected to the MacLab system PowerLab/800. In addition, parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity.

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

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

5.2.2. Guinea pig ileum

Two-centimeter-long portions of terminal ileum were taken at about 5 cm from the ileum–cecum junction and mounted in PSS, at 37 °C, of the following composition (mM): NaCl 118, $NaHCO_3$ 23.8, KCl 4.7, $MgSO_4{\cdot}7H_2O$ 1.18, KH_2PO_4 1.18, $CaCl_2$ 2.52, and glucose 11.7. Tension changes were recorded isotonically. Tissues were equilibrated for 30 min, and dose–response curves to arecaidine propargyl ester (APE) were obtained at 30 min intervals, the first one being discarded and the second one being taken as the control.

5.2.3. Guinea pig stimulated left atria

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

5.2.4. Guinea pig lung strips

The lungs were rapidly removed and strips of peripheral lung tissue were cut either from the body of a lower lobe with the longitudinal axis of the strip parallel to the bronchus or from the peripheral margin of the lobe. The preparations were mounted, with a preload of 0.3 g, in PSS with the following composition (mM): NaCl (118.78), KCl (4.32), $CaCl_2{\cdot}2H_2O$ (2.52), $MgSO_4{\cdot}7H_2O$ (1.18), KH_2PO_4 (1.28), $NaHCO_3$ (25), glucose (5.55). Contractions were recorded isotonically at 37 °C after tissues were equilibrated for 1 h, then two cumulative dose–response curves to APE (0.01, 0.1, 1, 10, 100 μM) were obtained at 45 min intervals, the first one being discarded and the second one being taken as the control.

5.2.5. Rabbit stimulated vas deferens

This preparation was set up according to Eltze [45]. Vasa deferentia were carefully dissected free of surrounding tissue and were divided into four segments, two prostatic portions of 1 cm and two epididymal portions of approximately 1.5 cm length. The four segments were mounted in PSS with the following composition (mM): NaCl (118.4), KCl (4.7), $CaCl_2$ (2.52), $MgCl_2$ (0.6), KH_2PO_4 (1.18), $NaHCO_3$ (25), glucose (11.1); 10^{-6} M yohimbine and 10^{-8} M tripitramine were included to block alpha$_2$-adrenoceptors and M_2 muscarinic receptors, respectively. The solution was maintained at 30 °C and tissues were stimulated through platinum electrodes by square-wave pulses (0.1 ms, 2 Hz, 10–15 V). Contractions were measured isometrically after tissues were equilibrated for 1 h, then a cumulative dose–response curve to pCl-McN-A-343 was constructed.

Acknowledgements

This research was supported by funds from the Italian Ministry of University and Scientific Research (MURST).

References

[1] M.P. Caulfield, Muscarinic receptors-characterization, coupling and function, Pharmac. Ther. 58 (1993) 319–379.
[2] R.M. Eglen, A. Choppin, N. Watson, Therapeutic opportunities from muscarinic receptor research, TiPS 22 (2001) 409–414.

[3] R.M. Eglen, A. Choppin, M.P. Dillon, S. Hedge, Muscarinic ligands and their therapeutic potential, Curr. Opin. Chem. Biol. 3 (1999) 426–432.
[4] M.F. Siddiqui, A.I. Levey, Cholinergic therapies in Alzheimer's diseases, Drug Future 24 (1999) 417–424.
[5] M.P. Caufield, N.J. Birdsall, International union of Pharmacology XVII. Classification of muscarinic acetylcholine receptors, Pharmacol. Rev. 50 (1998) 279–290.
[6] C.C. Felder, F.P. Bymaster, J. Ward, N. DeLapp, Therapeutic opportunities for muscarinic receptors in the central nervous system, J. Med. Chem. 43 (2000) 4333–4353.
[7] D.P. Zlotos, W. Bender, U. Holzgabe, Muscarinic receptor agonists and antagonists, Exp. Opin. Ther. Patents 9 (1999) 1029–1053.
[8] J. Wess, Novel insights into muscarinic acetylcholine receptor function using gene targeting technology, TiPS 24 (2003) 414–420.
[9] K.N. Bradley, Muscarinic toxins from the green mamba, Pharmacol. Ther. 85 (2000) 87–109.
[10] E.C. Hulme, Z.-L. Lu, M. Bee, C.A.M. Curtis, J. Saldanha, The conformational switch in muscarinic acetylcholine receptors, Life Sci. 68 (2001) 2495–2500.
[11] L. Shi, J.A. Javitch, The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop, Ann. Rev. Pharmacol. Toxicol. 42 (2002) 437–467.
[12] T. Kenakin, The classification of seven transmembrane receptors in recombinant expression systems, Pharmacol. Rev. 48 (1996) 413–463.
[13] J. Clader, W. Billard, H. Binch, L.Y. Chen, G. Crosby, R.A. Duffy, J. Ford, J. Kozlowski, J. Lachowicz, S. Li, C. Liu, S. McCombie, S. Vice, G. Zhou, W. Greenlee, Muscarinic M2 antagonists: anthranilamide derivatives with exceptional selectivity and in vivo activity, Bioorg. Med. Chem. 12 (2004) 319–326.
[14] Y. Ogino, N. Ohtake, K. Kobayashi, T. Kimura, T. Fujikawa, T. Hasegawa, K. Noguchi, T. Mase, Muscarinic M3 receptor antagonists with (2R)-2-((1R)-3,3-difluorocyclopentyl)-2-hydroxyphenylacetamide structure. Part 2, Bioorg. Med. Chem. Lett. 13 (2003) 2167–2172.
[15] Y. Sagara, T. Kimura, T. Fujikawa, K. Noguchi, N. Ohtake, Identification of novel muscarinic M-3 selective antagonists with a conformationally restricted hyp-pro spacer, Bioorg. Med. Chem. Lett. 13 (2003) 57–60.
[16] T.M. Bohme, C. Kelm, K. Kreutzmann, M. Linder, T. Dingermann, G. Dannhardt, E. Mutscler, G. Lambrecht, Structure–activity relationships on dimethindene derivatives as new M2-selective muscarinic receptor antagonists, J. Med. Chem. 46 (2003) 856–867.
[17] C.D. Boyle, J.E. Lachowicz, Orally active and selective benzylidene ketal M2 muscarinic receptor antagonists for the treatment of Alzheimer's disease, Drug Dev. Res. 56 (2002) 310–320.
[18] Y. Sagara, T. Sagara, T. Mase, T. Kimura, T. Numazawa, T. Fujikawa, K. Noguchi, N. Ohtake, Cyclohexylmethylpiperidinyltriphenylpropionamide: a selective muscarinic M3 antagonist discriminating against the other receptor subtypes, J. Med. Chem. 45 (2002) 984–987.
[19] T.M. Bohme, C.E. Augelli-Szafran, H. Hallak, T. Pugsley, K. Serpa, R.D. Schwarz, Synthesis and pharmacology of benzoxazines as highly selective antagonist at M4 muscarinic receptors, J. Med. Chem. 45 (2002) 3094–3102.
[20] O. Diouf, S. Gadeau, F. Chellé, M. Gelbcke, P. Talaga, B. Christophe, M. Gillard, R. Massingham, M. Guyaux, A new series of M3 muscarinic antagonists based on the 4-amino-piperidine scaffold, Bioorg. Med. Chem. Lett. 12 (2002) 2535–2539.
[21] T.M. Bohme, C.E. Augelli-Szafran, H. Hallak, R.D. Schwarz, Analogs of M4 selective synthetic muscarinic receptors antagonists: synthesis, binding and pharmacokinetic properties, Med. Chem. Res. 11 (2002) 423–433.
[22] S. Scapecchi, P. Angeli, S. Dei, C. Ghelardini, F. Gualtieri, G. Marucci, F. Paparelli, M.N. Romanelli, E. Teodori, Sar Studies on the potent and selective muscarinic antagonist 2-ethylthio-2,2-diphenylacetic acid *N,N*-diethylaminoethyl ester, Arch. Pharm. 330 (1997) 122–128.
[23] J. Hu, S.Z. Wang, S. Scapecchi, F. Gualtieri, E.E. El-Fakahany, Cardioselective derivatives of 2,2-diphenyl-2-ethylthioacetate do not discriminate between m2 and m3 muscarinic receptors expressed in CHO cells, Pharmacology 50 (1995) 273–285.
[24] S. Scapecchi, P. Angeli, S. Dei, F. Gualtieri, G. Marucci, R. Moriconi, F. Paparelli, M.N. Romanelli, E. Teodori, Dialkylaminoalkyl esters of 2,2-diphenyl-2-alkyltioacetic acids: a new class of potent and functionally selective muscarinic antagonists, Bioorg. Med. Chem. 2 (1994) 1061–1073.
[25] S. Scapecchi, P. Angeli, A. Dei, F. Gualtieri, G. Marucci, M.N. Romanelli, E. Teodori, *N,N*-diethylaminoethanol and *N*-methyl-4-piperidinol esters of 2,2-diphenyl-2-ethylthioacetic acid as potent and selective muscarinic antagonists, Pharm. Pharmacol. Lett. 2 (1993) 220–223.
[26] P. Angeli, F. Gualtieri, R. Maggio, F. Paparelli, S. Scapecchi, Affinity profile at five cloned human muscarinic receptors (m1–m5) of a new series of antimuscarinic drugs, Pharmaceut. Pharmacol. Lett. 3 (1993) 84–87.
[27] K.A. Jacobson, B. Fischer, A.M. van Rhee, Molecular probes for muscarinic receptors: functionalized congeners of selective muscarinic antagonists, Life Sci 56 (1995) 823–830.
[28] H. Gilman, D.A. Shirley, Some derivatives of phenotiazine, J. Am. Chem. Soc. 66 (1944) 888–892.
[29] F.P. Doyle, E.R. Stove, Alkylthioamine and quaternary salts, Beecham Researche Laboratories Ltd., 1958, pp. GB 800963.
[30] W.P. Wetter, C. De Witt Blanton, 8-(*o*-Aminoalkyl)quinolines as potential prophylactic antimalarials, J. Med. Chem. 17 (1974) 620–624.
[31] J. Cossy, M. Guha, Intramolecular quenching of iminium ions generated by photooxidation of aminoalcohols with ketones. A new synthesis of oxazines and oxazoles, Tetrahedron Lett. 35 (1994) 1715–1718.
[32] I.G. Farbenind, Method for manufacturing aminoalcohol1940, pp. DE 730237.
[33] A.J. Bitonti, Method of using triaryl-ethylene derivatives in the treatment and prevention of osteoporosis, 1995 US 005691384.
[34] R.L. Hudkins, R.B. Mailman, D.L. DeHaven-Hudkins, Novel (4-phenylpiperidinyl)-and(4-phenylpiperazinyl)alkyl-spaced esters of 1-phenylcyclopentanecarboxylic acids as potent sigma-selective compounds, J. Med. Chem. 37 (1994) 1964–1970.
[35] M.P. La Montagne, D. Dagli, M. Sami Khan, P. Blumbergs, Analogs of 8-[[6-(diethylamino)hexyl]amino]-6-methoxy-4-methylquinoline as candidate antileishmanial agents, J. Med. Chem. 23 (1980) 981–985.
[36] R. Merchant, J.N. Wickert, C.S. Marvel, Some bromine derivatives of pentanoic and hexanoic acids, J. Amer. Chem. Soc. 49 (1927) 1828–1831.
[37] A.P. Phillips, The addition of amines to 4-vinylpyridine, J. Am. Chem. Soc. 78 (1956) 4441–4443.
[38] V.I. Cohen, B.J. Gin, R.C. Reba, Facile and general synthesis of 2-,3-, or 4-[(dialkylamino)alkyl]pyridines and piperidines, Liebigs Ann. Chem. 7 (1993) 809–810.
[39] G. Courtois, P. Miginiac, Reaction of functional organometallic compounds with gem-amino ethers and immonium salts. III. Synthesis of *o*-functional tertiary amines, Bull. Soc. Chim. France 5 (1983) 148–152.
[40] E.Y. Borisova, T.T. Vasil'eva, L.A. Lukashova, S.V. Minaeva, E.M. Cherkasova, Synthesis of aminoamides and amino alcohols from 1,1,1,7-tetrachloroheptane, Zhur. Organic. Khim. 11 (1975) 943–947.
[41] N. Leventis, M. Rawaswdeh Abdel-Monem, G. Zhang, G. Elder, C. Sotiriou-Leventis, Tuning the redox chemistry of 4-benzoyl-*n*-methylpyridinium cations through para substitution. Hammett linear free energy relationships and the relative aptitude of the two-electron reduced forms for *h*-bonding, J. Org. Chem. 67 (2002) 7501–7510.

[42] S. Scapecchi, G. Marucci, P. Angeli, C. Bellucci, S. Dei, F. Gualtieri, D. Manetti, M.N. Romanelli, E. Teodori, Structure–activity relationships in 2,2-diphenyl-2-ethylthioacetic acid esters: unexpected agonistic activity in a series of muscarinic antagonists, Bioorg. Med. Chem. 9 (2001) 1165–1174.

[43] F. Dorje, J. Wess, G. Lambrecht, R. Tacke, E. Mutschler, M.R. Brann, Antagonist binding profiles of five cloned human muscarinic receptor subtypes, Pharmacol. Exper. Ther. 256 (1991) 727–733.

[44] M.M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein, utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[45] M. Eltze, Muscarinic M1 and M2 receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens, Eur. J. Pharmacol. 151 (1988) 205–221.