Binding Profiles of a Series of 2-Arylpropionic Acid Esters on Cloned Human Muscarinic Receptors Subtypes (m1-m5) and Their

Binding Profiles of a Series of 2-Arylpropionic Acid Esters on Cloned Human Muscarinic Receptor Subtypes (m_1–m_5) and Their Relationship to Nootropic Activity

Carla Ghelardini[a], Ito Mizuma[b], Fulvio Gualtieri[c], Alessandro Bartolini[a], Nicoletta Galeotti[a], Maria Novella Romanelli[c], and Elisabetta Teodori[c]

Department of Preclinical and Clinical Pharmacology[a], University of Florence, Florence (Italy), Department of Neuropharmacology[b], University of Kyushu, Fukuoka (Japan), and Department of Pharmaceutical Sciences[c], University of Florence, Florence (Italy)

Summary

The muscarinic binding profile of a series of 2-arylpropionic acid esters on cloned human muscarinic receptor subtypes (m_1–m_5) was determined to investigate whether there is a correlation between pharmacological activity and muscarinic receptor subtype selectivity. Among the tested compounds, **1**, **7** and **9** showed the highest affinity for the m_2 and m_4 receptors. Compounds **1**, **7** and **9** show good affinity for m_4 receptors (pKi = 7.87; 7.73 and 7.10, respectively) and are able to discriminate 10–60 fold between m_4/m_1, m_4/m_3, and m_4/m_5 subtypes. Conversely, these compounds are able only to weakly discriminate between m_4/m_2. Compounds 1 (50–300 μg kg^{-1} i.p.) and **7** (1–10 μg kg^{-1} i.p.), injected 20 min before the training session, are able to prevent the amnesia induced by dicyclomine (2 mg kg^{-1} i.p.) in the mouse passive-avoidance test. Compounds 1 and **7**, at the highest antiamnesic doses, do not modify motor coordination and spontaneous motility as evaluated by the rota-rod test and Animex apparatus experiments.

Zusammenfassung

Bindungsprofile einer Reihe von 2-Arylpropionsäureestern an geklonte humane muskarinische Rezeptorsubtypen (m_1–m_5) und ihre Beziehung zur nootropischen Aktivität

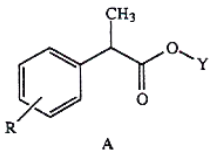
Das Profil der muskarinischen Rezeptor-Bindung von einer Reihe von 2-Arylpropionsäureestern an geklonte humane muskarinische Rezeptor-Subtypen (m_1–m_5) wurde erstellt, um zu untersuchen, ob es eine Wechselbeziehung zwischen pharmakologischer Aktivität und Selektivität des muskarinischen Rezeptorsubtypen gibt. Unter den getesteten Substanzen zeigten die Substanzen **1**, **7** und **9** die höchste Affinität für die Rezeptoren m_2 und m_4. Die Substanzen **1**, **7** und **9** haben eine gute Affinität für den m_4-Rezeptor (pKi = 7,87, 7,73 und 7,10) und sind in der Lage, 10–60fach zwischen m_4/m_1-, m_4/m_3- und m_4/m_2-Rezeptoren zu unterscheiden. Im "mouse passive-avoidance test" konnten die Substanzen **1** (50–300 μg kg^{-1} i.p.) und **7** (1–10 μg kg^{-1} i.p.), die 20 min vor der Trainingseinheit appliziert wurden, die durch Dicyclomin herbeigeführte Amnesie verhindern. Der „rota-rod test" und die „Animex apparatus"-Experimente zeigten, daß die Substanzen **1** und **7** in der höchsten gegen Amnesie wirksamen Dosis die motorische Koordinierung und die spontane Mobilität nicht beeinflussen.

Key words 2-Arylpropionic acid esters, effect on amnesia, effect on learning and memory, muscarinic binding profile · Cholinergic system · Muscarinic receptors

Arzneim.-Forsch./Drug Res. **49 (I)**, 483–488 (1999)

1. Introduction

Recently we have reported the analgesic and cognition enhancing activity of a series of compounds derived from R-(+)-hyoscyamine [1–3] among which were several ring-substituted 2-phenylpropionic acid esters with general structure A. Within this class, on the basis of its interesting pharmacological profile, compound **9** (PG 9) was singled out to be studied in more details [2].



A

R = 4-Br; Y = 3-α-tropyl; PG9 (9)

The activity of **9**, as well as that of the other compounds of the series appears to be due to a cholinergic mechanism, since it is prevented by antimuscarinic drugs such as: atropine, dicyclomine and pirenzepine, and by the cholinergic depletor hemicolinium-3 [4]. As has been shown by microdialysis studies [4], it enhances the central release of acetylcholine (ACh) which in turn is responsible for antinociceptive and antiamnesic activity. The mechanism by which ACh is released is still unclear, the simpler explanation being that of a selective blockade of presynaptic muscarinic receptors that control central ACh release.

To collect more information on this aspect of the problem, we thought it interesting to study muscarinic binding profile of the compounds of this class, to see whether there exists any correlation between pharmacological activity and muscarinic subtype selectivity. In fact, the presynaptic muscarinic receptors that modulate the release of ACh are presumed to belong to the M_2 subtype, even though the M_4 subtype may also be involved in some tissues [5–6].

2. Materials and methods

2.1. Chemistry

The synthesis of the compounds studied, together with their chemical and physical characteristics was already reported [2]. Compounds **1–3**, **8**, **21** were obtained as hydrochlorides, compounds **4**, **15**, **16**, **18**, **20**, **23** were obtained as oxalates; the remaining compounds were obtained as maleate salts.

2.1.1. Computer chemistry

CoMFA (Comparative Molecular Field Analysis) is a computational method to perform 3D-QSAR; the software is distributed by Tripos Inc. (St. Louis, MO, USA) within the Sybyl package. The sterically-allowed low-energy conformations were obtained with the module Search-Compare within InsightII (MSI, San Diego, CA, USA); the selected conformation were aligned using the phenyl ring and the nitrogen atom as fitting points. QSAR (Quantitative Structure-Activity Relationships) were performed using Statgraphics Plus (Manugistics, Inc., Rockville, MD, USA).

2.2. Pharmacology

2.2.1. Animals

Male Swiss albino mice (23–30 g) from Morini (San Polo d'Enza, Italy) breeding farm were used. Fifteen mice were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m., with food and water ad libitum. All experiments were carried out according to the guidelines of the European Community Council.

2.2.2. Binding assays

Chinese hamster ovary cells (CHO-K1), obtained from the American Type Culture Collection (ATCC), were used. Cell culture, membrane preparation and radioligand binding studies were performed according to the method of Dorje et al. [7]. Cells were transfected according to the method of Chen and Okayama [8] using a modified calcium phosphate procedure involving the use of cotransfected pcDneo as a selectable marker. Selection with neomycin analog G418 (600 μg/ml) was started 72h after transfection and continued for two to three weeks. Media were changed every three days. Clonal cell lines were obtained by single-cell cloning. Cells were grown to about 80 % confluence, washed, scraped into ice-cold binding buffer and homogenized for 30 s using a Brinkmann Homogenizer (Brinkmann, Westbury, NY, USA; Setting 5). Membranes were pelletted at 16,000 × g for 15 min and rehomogenized. In [^{3}H]N-methylscopolamine ([^{3}H]NMS) saturation experiments, 8 to 10 different concentrations of the radioligand (2–1400 pmol/l) were employed. For displacement experiments, the concentration of [^{3}H]NMS was 150 pmol/l and 10 different concentrations of the cold displacers were used. Incubation was carried out at 22 °C for 3 h. Assays were terminated by filtration through a Brandel cell harvester (Brandel, Gaithesburg, MD, USA) onto Whatman GF/C filters (Whatman, Clifton, NG, USA). Membranes were washed three times with 5 ml of ice-cold binding buffer before being dried, transferred to 10 ml of scintillant (Aquasol, New England Nuclear, Boston, MA, US) and counted in an LKB-β counter.

2.2.3. Passive-avoidance test

The test was performed according to the step-through method described by Jarvik and Kopp [2], modified by us for testing drugs endowed with analgesic properties. The apparatus consists of a two-compartment acrylic box with a lighted compartment connected to a darkened one, by a guillotine door. In the original method, mice received a punishing electrical shock as soon as they entered the dark compartment, while in our modified method, after entry into the dark compartment, mice receive a non-painful punishment, consisting of a fall into a cold water bath (10 °C). For this purpose the dark chamber was constructed with a pitfall floor. The latency times for entering the dark compartment were measured in the training test and after 24 h in the retention test. For memory disruption, mice were i.p. injected with the amnesic drug dicyclomine. All investigated drugs were injected 20 min before the training session, while dicyclomine was injected immediately after termination of the training session. The maximum entry latency allowed in the retention session was 120 s. The memory degree of received punishment (fall into cold water) was expressed as the increase in seconds between training and retention latencies.

2.2.4. Rota-rod test

The apparatus consisted of a base platform and a rotating rod of 3 cm diameter with a non-slippery surface. The rod was placed at a height of 15 cm from the base.

The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus, up to 5 mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s, according to Vaught et al. [10]. The performance time was measured before and 15, 30, and 45 min after treatment.

2.2.5. Spontaneous activity meter (Animex)

Locomotor activity in mice was quantified using an Animex activity meter Type S (LKB, Farad, Sweden) set to maximum sensitivity. Mice were placed on the top of the Animex activity meter and every mouse movement produced a signal due to variation in inductance and capacity of the apparatus resonance circuit. Signals were then automatically converted to numbers. On the day of the experiment the mice were treated and then the cage, containing 5 mice, was put on the measuring platform. Activity counts were made every 15 min for 45 min starting immediately after injection of the drug. Because of the arbitrary scale adopted to quantify movements, drug-treated mice were always compared with saline-treated ones.

2.2.7. Statistical analysis

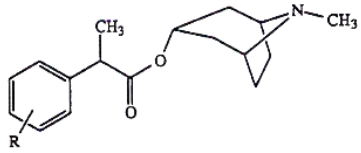
All experimental results are given as the mean ± S.E.M. Analysis of variance (ANOVA), followed by Fisher's Protected Least Significant Difference (PLSD) procedure for post-hoc comparison, was used to verify significance between two means. Data were analysed with the StatView software for the Macintosh (1992). P values of less than 0.05 were considered significant.

3. Results

Tables 1, 2 and 3 show the binding affinities (pKi), as well as some selected subtype selectivities, of the 23 compounds studied on the five human muscarinic receptor subtypes expressed in Chinese hamster ovary cells (CHO-K1). Hill coefficients ranged from 0.87 to 1.16 and were not significantly different from 1.

The compounds showed modest muscarinic antagonism on all five subtypes and did not possess any interesting subtype selectivity. There were three re-

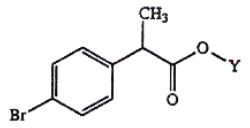
Table 1: Binding affinities and selected subtype selectivities of compounds **1–13** for the five muscarinic receptor subtypes expressed in Chinese hamster ovary cells (CHO-K1).

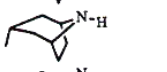
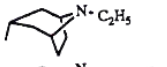
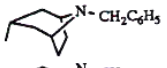
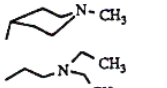
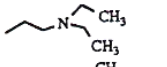
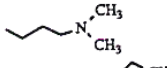
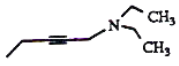


Cpd.	R	Binding (pK_i)[a]					Subtype selectivity			
		m_1	m_2	m_3	m_4	m_5	m_4/m_1	m_4/m_2	m_4/m_3	m_4/m_5
1	H	6.33 (0.04)	7.25 (0.09)	6.06 (0.09)	7.87 (0.05)	6.16 (0.07)	35	4	65	51
2	4-NO_2	6.23 (0.1)	6.51 (0.08)	6.34 (0.05)	6.75 (0.07)	6.53 (0.08)				
3	4-NH_2	6.42 (0.06)	6.05 (0.07)	6.64 (0.08)	6.79 (0.12)	5.88 (0.06)				
4	4-$N(CH_3)_2$	5.39 (0.06)	5.71 (0.06)	5.62 (0.11)	5.90 (0.04)	6.11 (0.08)				
5	4-Cl	5.98 (0.12)	6.41 (0.08)	6.15 (0.07)	6.35 (0.05)	6.77 (0.10)				
6	2-Cl	6.11 (0.09)	6.41 (0.06)	5.97 (0.10)	5.86 (0.07)	6.12 (0.06)				
7	3,4-Cl	6.37 (0.10)	7.54 (0.06)	6.17 (0.08)	7.73 (0.08)	6.44 (0.05)	23	2	36	20
8	2-Br	6.33 (0.09)	6.74 (0.05)	6.79 (0.10)	6.23 (0.07)	6.71 (0.06)				
9	4-Br	6.23 (0.05)	6.31 (0.06)	6.19 (0.11)	7.10 (0.09)	6.67 (0.05)	7	6	8	3
(−)-9	4-Br	6.20 (0.11)	6.28 (0.09)	6.32 (0.13)	7.23 (0.11)	6.73 (0.05)	11	9	8	3
(+)-9	4-Br	6.14 (0.06)	6.24 (0.10)	6.25 (0.13)	7.08 (0.08)	6.49 (0.09)	9	7	7	4
10	4-F	6.48 (0.10)	6.52 (0.09)	5.92 (0.08)	5.86 (0.05)	6.67 (0.06)				
11	4-OCH_3	6.21 (0.08)	5.86 (0.03)	6.45 (0.07)	6.66 (0.10)	6.29 (0.06)				
12	4-CF_3	7.11 (0.05)	7.03 (0.08)	6.95 (0.10)	7.10 (0.12)	6.83 (0.08)				
13	4-$CH_2CH(CH_3)_2$	5.81 (0.10)	6.18 (0.09)	5.92 (0.09)	6.61 (0.12)	5.90 (0.06)				

[a] The values represent means ± SE mean from 4 individual experiments. Hill coefficients range from 0.87 to 1.16 and are not significantly different from 1.

Table 2: Binding affinities of compounds **9** and **14–20** for the five muscarinic receptor subtypes expressed in Chinese hamster ovary cells (CHO-K1).



Cpd.	Y	Binding (pK_i[a])				
		m_1	m_2	m_3	m_4	m_5
9		6.23 (0.05)	6.31 (0.06)	6.19 (0.11)	7.10 (0.09)	6.67 (0.05)
14		7.14 (0.05)	6.95 (0.04)	6.65 (0.06)	7.12 (0.05)	6.93 (0.12)
15		5.54 (0.08)	6.14 (0.11)	6.23 (0.10)	5.97 (0.09)	6.48 (0.07)
16		6.33 (0.06)	6.91 (0.05)	6.39 (0.12)	6.77 (0.06)	6.87 (0.05)
17		5.54 (0.10)	5.78 (0.08)	6.02 (0.10)	5.56 (0.04)	5.73 (0.05)
18		6.53 (0.07)	6.33 (0.07)	6.69 (0.04)	7.05 (0.09)	6.37 (0.06)
19		7.21 (0.06)	7.11 (0.11)	6.92 (0.05)	7.18 (0.06)	6.79 (0.08)
20		6.25 (0.06)	6.83 (0.05)	5.88 (0.10)	6.62 (0.08)	6.40 (0.07)

[a] The values represent means ± SE means from 4 individual experiments. Hill coefficients range from 0.87 to 1.16 and are not significantly different from 1.

markable exceptions to this trend, namely compounds **1**, **7** and **9** that showed the highest affinity for the m_2/m_4 receptors and some weak but interesting selectivity for the m_4 (**9**) and the m_2/m_4 (**1**, **7**) subtypes.

The influence of chirality on muscarinic receptor affinity has been evaluated by testing the enantiomers of **9** ((+)-**9** and (–)-**9**). As can be seen in Table 1, the enantiomers, unlike the hyoscyamine enantiomers [3], were nearly equipotent, lakking enantioselectivity on all five muscarinic receptors.

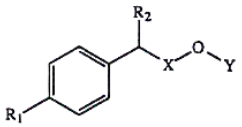
Compounds **1** (50–300 μg kg^{-1} i.p.) and **7** (1–10 μg kg^{-1} i.p.), injected 20 min before the training session, were able to prevent the amnesia induced by dicyclomine (2 mg kg^{-1} i.p.) in the mouse passive-avoidance test. Compounds **1** and **7**, at the above mentioned doses, enhanced the entrance latency up to a value comparable to that of saline-treated mice (Fig. 1, panels A and B). Compound **7**, at 1 mg kg^{-1} i.p., was completely ineffective (Fig. 1, panel B). The antiamnesic effect exerted by both compounds was comparable to that produced by **9** [11], by the cholinesterase inhibitor physostigmine (0.2 mg kg^{-1} i.p.) and the nootropic drug piracetam (30 mg kg^{-1} i.p.) (data not shown).

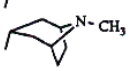
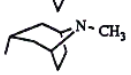
No difference among the entrance latencies of every group in the training session of the passive avoidance test was observed (Fig. 1, panel A and B).

Compounds **1** and **7** at the highest antiamnesic doses did not modify motor coordination or spontaneous motility as evaluated by experiments performed on rota-rod test (Fig. 2) and Animex apparatus (Fig. 3).

No meaningful correlation was found in a QSAR analysis correlating binding affinity (K_i) of compounds **1–13** with the electronic (σ), lipophilic (π) and steric constants (Es) of the ring-substituents of the compounds of Table 1. Moreover, there was no correlation between antiamnesic potency and the affinity for m_2 and/or m_4 subtype. Also 3D-QSAR (COMFA) analysis, performed on almost all compounds (only **13**, **15**, **17** and **21** were not included), always yielding negative cross-validated r^2 values, failed to establish any correlation between binding affinity, subtype selectivity and chemical structures.

Table 3: Binding affinities of compounds **21–23** for the five muscarinic receptor subtypes expressed in chinese hamster ovary cells (CHO-K1).



Cpd.	R_1	R_2	X	Y	Binding (pK_i)[a]				
					m_1	m_2	m_3	m_4	m_5
21	4-Cl	CH_3	C=O		6.27 (0.12)	6.49 (0.11)	6.09 (0.09)	6.63 (0.04)	6.91 (0.09)
22	H	Cl	C=O		**7.05** (0.07)	6.86 (0.05)	6.76 (0.11)	**7.11** (0.06)	6.87 (0.08)
23	4-Br	CH_3	CH_2		6.22 (0.12)	6.15 (0.06)	6.54 (0.13)	6.34 (0.07)	6.73 (0.06)

[a] The values represent means ± SE mean from 4 individual experiments. Hill coefficients range from 0.87 to 1.16 and are not significantly different from 1.

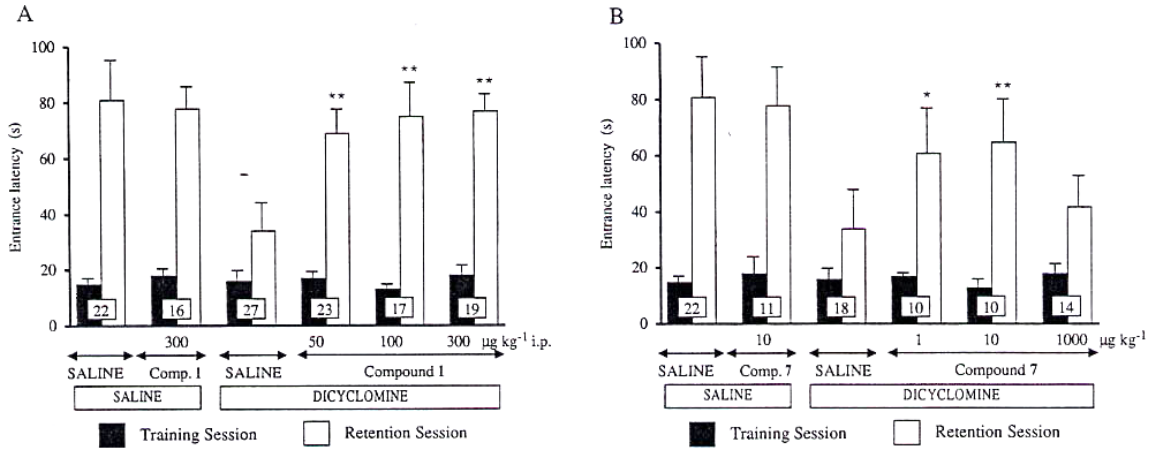


Fig. 1: Dose-response curves of compounds **1** (A) and **7** (B) on amnesia induced by dicyclomine in the mouse passive avoidance test. Compounds **1** and **7** were administered 20 min before training session while dicyclomine were injected immediately after. The number of mice is inside the column. * $p < 0.05$, ** $p < 0.01$ in comparison with dicyclomine-treated mice.

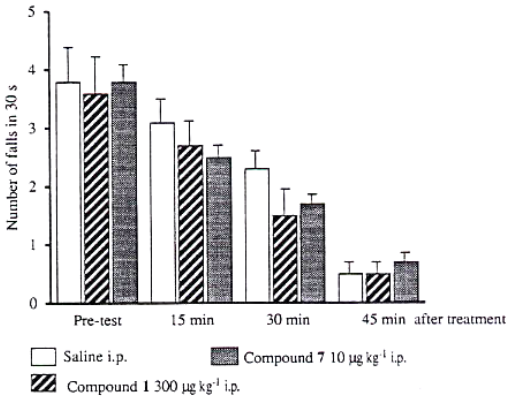


Fig. 2: Lack of effect on compounds **1** and **7** in mouse rota-rod test. Each column represents the mean of 15 mice.

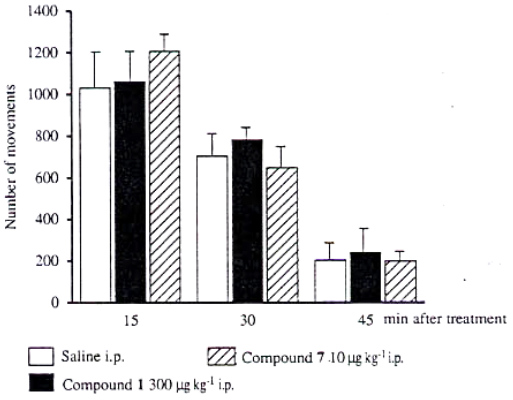


Fig. 3: Lack of effect by compounds **1** and **7** in mouse Animex test. Each column represents the mean of 15 mice.

4. Discussion

From the results obtained it is clear that the most interesting compounds are among the 3-α-tropanyl esters. Changes in the aminoalcoholic moiety or further modifications in the molecule are detrimental to both affinity and subtype selectivity.

While the failure to correlate binding affinities with chemical-physical parameters was quite unexpected and difficult to explain, the lack of correlation between m_2/m_4 subtype selectivity and antiamnesic action can be easily explained if one considers that we are attempting to relate in vitro data (K_i) with in vivo results (antiamnesic activity) that can be strongly conditioned by pharmacokinetic parameters.

Nevertheless, even if quantitative correlations cannot be established, the data shown in Table 1 do support our hypothesis on the mechanism of action of PG 9 and similar compounds [11]. It has recently been reported that PG 9 and related compounds are endowed with cholinergic antinociceptive properties [2]. Within the series reported in this work, the compounds that show the highest affinity for m_2/m_4 muscarinic subtypes are also those that are the most potent analgesics (**1**, ED_{50} = 0.05 and **7**, ED_{50} = 0.01 mg kg^{-1}, see [2]) or that show the highest analgesic efficacy (**9**, analgesic efficacy = 101 % compared to morfine, see [2], suggesting that presynaptic blockade of central muscarinic receptors might be responsible for the increased release of ACh and, as a consequence, for analgesia [12]. Since activation of the cholinergic system ameliorates memory processes [13], the enhancement of the extracellular ACh levels produced by PG 9 could also be responsible for its cognition enhancing activity.

These results prompted us to evaluate the cognition enhancing activity of compounds **1** and **7** that had been ignored because of the higher analgesic efficacy of **9** [2] which, in turn, promised to have a correspondingly high nootropic activity.

Fulfilling our expectations, both **1** and **7** resulted to be endowed with cognition enhancing properties, which were respectively about 200 and 1000 times more potent that **9** in the mouse passive avoidance test. In fact, the minimal active dose of **9** is 10 mg kg^{-1}, whereas the lowest active doses of **1** and **7** are respectively 50 and 1 μg kg^{-1}. The lack of prevention exerted by compound **7** on dicyclomine-induced amnesia at the dose of 1 mg kg^{-1} can be attributed to the fact that, at this dose, the drug antagonizes postsynaptic muscarinic receptors which belong to the M_1 subtype. This muscarinic receptor subtype is also fundamental in the enhancement of the pain threshold produced by the activation of the cholinergic system [14]. The ability of compound **7**, at the dose of 1 mg kg^{-1}, to antagonize the antinociception induced by oxotremorine and physostigmine (data not shown), confirms the hypothesis that its lack of antiamnesic effect underlies its antagonistic activity on the M_1 receptor subtype. Doses of **1** and **9** 100 times higher than those at which they exert their maximum antiamnesic effect cannot be investigated because of the appearance of behavioural side effects.

An interesting side-result of this research was the identification of three compounds with a pharmacological profile that can be of some help in the characterization of muscarinic receptor subtypes. Compound **9**, and in particular its enantiomer (−)-**9**, shows a modest (about tenfold) but interesting selectivity for the m_4 subtype, for which selective antagonists are still rare [5, 6, 15]. Compounds **1** and **7** have good affinity for m_4 receptors (pK_i = 7.87 and 7.73 respectively) and are able to discriminate between m_4/m_1, m_4/m_3, and m_4/m_5 subtypes by 20–60-fold (see Table 1) and between m_4/m_2 4-fold.

5. References

[1] Gualtieri, F., Bottalico, C., Calandrella, A., J. Med. Chem. **37,** 1712 (1994a)

[2] Gualtieri, F., Conti, G., Dei, S., J. Med. Chem. **37,** 1704 (1994b)

[3] Ghelardini, C., Gualtieri, F., Romanelli, M. N., Neuropharmacology **36,** 281 (1997)

[4] Romanelli, M. N., Bartolini, A., Bertucci, C., Chirality **8,** 225 (1996)

[5] Caufield, M. P., Pharmacol. Ther. **58,** 319 (1993)

[6] Eglen, R. M., Hegde, S. S., Watson, N., Pharmacol. Rev. **48,** 531 (1996)

[7] Dorje, F., Wess, J., Lambrecht, G., J. Pharmacol. Exp. Ther. **256,** 727 (1991)

[8] Chen, C., Okayama, H., Mol. Cel. Biol. **7,** 2745 (1987)

[9] Jarvik, M. E., Kopp, R., Psycol. Rep. **21,** 221 (1967)

[10] Vaught, J., Pelley, K., Costa, L. G., Neuropharmacology **24,** 211 (1985)

[11] Ghelardini, C., Galeotti, N., Gualtieri, F., J. Pharmacol. Exp. Ther. **284,** 806 (1998)

[12] Ghelardini, C., Giotti, A., Gualtieri, F., in: Trends in Receptors Research, P. Angeli, U. Gulini, W. Quaglia (eds.), pp. 95-114, Elsevier, Amsterdam (1992)

[13] Coyle, M. J., in: Learning and Memory Molecular Bases, L. Meyer, G. Nordeberg (eds.), pp. 11–32, Pergamon Press, London (1995)

[14] Bartolini, A., Ghelardini, C., Fantetti, L., Br. J. Pharmacol. **105,** 77 (1992)

[15] Dei, S., Bellucci, C., Ghelardini, C., Life Sci. **58,** 2147 (1996)

Acknowledgements

This research was partially supported by grants from MURST (Ministero dell'Università e della Ricerca Scientifica). The authors wish to thank Mary Forrest for linguistic revision.

Correspondence: Dr. Carla Ghelardini,
Department of Preclinical and Clinical Pharmacology,
Viale Morgagni 65, I-50134 Florence (Italy)
E-mail: ghelard@server1.pharm.unifi.it