Bioorganic & Medicinal Chemistry 16 (2008) 7311–7320

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design, synthesis, and biological evaluation of pirenzepine analogs bearing a 1,2-cyclohexanediamine and perhydroquinoxaline units in exchange for the piperazine ring as antimuscarinics

Anna Minarini [a,*], Gabriella Marucci [b], Cristina Bellucci [c], Gianluca Giorgi [d], Vincenzo Tumiatti [a], Maria Laura Bolognesi [a], Riccardo Matera [a], Michela Rosini [a], Carlo Melchiorre [a]

[a] Department of Pharmaceutical Sciences, Alma Mater Studiorum-University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy
[b] Department of Chemical Sciences, University of Camerino, Via S. Agostino 1, 62032 Camerino (MC), Italy
[c] Department of Pharmaceutical Sciences, University of Florence, Via U. Schiff 6, 50019 Sesto F.no (FI), Italy
[d] Department of Chemistry, University of Siena, Via Aldo Moro, 53100 Siena, Italy

ARTICLE INFO

Article history:
Received 9 May 2008
Revised 11 June 2008
Accepted 13 June 2008
Available online 18 June 2008

Keywords:
Pirenzepine
Cyclohexanediamine
Tripitramine
Perhydroquinoxaline
Muscarinic receptors

ABSTRACT

Pirenzepine (**2**) is one of the most selective muscarinic M_1 versus M_2 receptor antagonists known. A series of **2** analogs, in which the piperazyl moiety was replaced by a *cis*- and *trans*-cyclohexane-1,2-diamine (**3**–**6**) or a *trans*- and *cis*-perhydroquinoxaline rings (**7** and **8**) were prepared, with the aim to investigate the role of the piperazine ring of **2** in the interaction with the muscarinic receptors. The structural change leading to compounds **3**–**6** abolished in binding assays the muscarinic M_1/M_2 selectivity of **2**, due to an increased M_2 affinity. Rather, compounds **3**–**6** displayed a reversed selectivity showing more affinity at the muscarinic M_2 receptor than at all the other subtypes tested.

1. Introduction

Muscarinic receptors belong to the G-protein coupled receptor family (GPCRs) and, like other members of this family, comprise multiple subtypes.[1] Based on existing knowledge, it has been recommended that M_1, M_2, M_3, M_4, and M_5 should be used to describe the five muscarinic receptor subtypes which have been cloned and pharmacologically characterized.[2,3]

Fundamental information has been obtained by using selective chemically synthesized ligands as well as transgenic mice lacking genes encoding each of the five muscarinic receptor subtypes[4–6] and muscarinic toxins from snake venom.[7–9] Despite the widespread tissue distribution of these receptors and their involvement in a variety of physiological processes, little is known about the structural requirements that determine selectivity to one receptor subtype, rather than to another. This lack of knowledge may derive from the fact that the active binding site involves similar, if not identical, amino acids for different muscarinic receptor subtypes,[10,11] and that typically, most tissues and cell types express at least two or more muscarinic receptor subtypes. Although muscarinic receptors regulate many important physiological functions, it remains unclear in many cases which specific subtypes are involved in mediating the various muscarinic actions of ACh.[12]

Non-selective ligands exhibit many undesiderable side effects that limit their clinical usefulness. For this reason, the number of muscarinic receptor agonists and antagonists that have been introduced in therapy is modest. In particular, muscarinic receptor antagonists are used therapeutically for the treatment of smooth muscle disorders including urinary incontinence, irritable bowel syndrome, and chronic obstructive pulmonary disease.[13–17] Thus, there is an ongoing need for effective structure–activity studies based on selected muscarinic receptor subtypes.

Our research group has long been involved in designing new muscarinic receptor antagonists with the goal of developing high-affinity, site-selective ligands for subtypes of the muscarinic receptor.[18–24] These studies led to the discovering of tripitramine[21] (**1**), a potent and selective muscarinic M_2 receptor antagonist, able to discriminate also between muscarinic M_2 and M_4 receptors but not between M_1 and M_4 subtypes.

Analysis of tripitramine structure reveals that dividing in two halves the molecule and exchanging the hexamethylene chain, separating the inner from the outer nitrogen atom of the monosubstituted end of the molecule, for a cyclohexane ring, would generate a compound that can be superimposed to pirenzepine (**2**), a selective muscarinic M_1 versus muscarinic M_2 receptor antago-

* Corresponding author. Fax: +39 051 2099734.
E-mail address: anna.minarini@unibo.it (A. Minarini).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.06.025

nist.[25] It is well known that modification of the piperazyl moiety of pirenzepine may lead to muscarinic M_2 selective antagonists such as AQRA-741 and AFDX-384. It was suggested that AFDX-384 receptor binding domain overlaps partially with the M_2 muscarinic allosteric binding site, leading to muscarinic M_2selectivity.[26]

It derives that pirenzepine analogues **3**–**6** bearing a cyclohexane-1,2-diamine function in exchange for a piperazine ring would offer further opportunity to investigate the role of the piperazine ring of pirenzepine in the interaction with the receptor as the two amine functions of a cyclohexane-1,2-diamine moiety can be either *cis* or *trans* to each other. Besides *cis*/*trans*-isomers, a non-symmetrically substituted cyclohexane-1,2-diamine moiety would give rise also to additional optical stereoisomers, increasing the possibility to achieve selectivity. To further reduce the flexibility of the cyclohexane-1,2-diamine, the two amine functions were inserted in a *trans*-perhydroquinoxaline and *cis*-perhydroquinoxaline moiety, affording compounds **7** and **8**, respectively.

We describe here the synthesis and the pharmacological profile of compounds **3**–**8** in functional experiments performed on peripheral muscarinic M_2, M_3, and M_4 receptors and in binding assays carried out on human cloned muscarinic M_1, M_2, M_3, and M_4 receptors.

2. Chemistry

The design strategy for compounds **3**–**8** is shown in Figure 1.

All the compounds were synthesized by standard procedures (Schemes 1 and 2) and were characterized by IR, ^{1}H NMR, HRMS mass, and elemental analysis. Intermediate **13** · (−)-(*S*,*S*)-tartrate was also characterized by X-ray analysis.

Monoprotection with benzyl chlorocarbonate of the commercially available diamines *trans*-(1*R*,2*R*)-cyclohexanediamine, *trans*-(1*S*,2*S*)-cyclohexanediamine, and *cis*-1,2-cyclohexanediamine, and of *trans*-perhydroquinoxaline and *cis*-perhydroquinoxaline[27,28] afforded intermediates **9**-**11**, **14**, and **15**, respectively. Resolution of **11** gave the enantiomeric amines **12** and **13**.

Amines **9**, **10**, **12**, and **13** were treated with benzaldehyde, followed by reduction of the formed Schiff base with $NaBH_4$, to give the corresponding derivatives **16**–**19**.

Removal of the protecting group of **16**–**19** by hydrolysis with HBr, gave the corresponding diamines **20**–**23**. Intermediates **24**–**27**, **28**, and **29** were synthesized by Eschweiler–Clarke methylation of **20**–**23**, **14**, and **15**, respectively. Compounds **34** and **35** were obtained by hydrolysis with HBr of **28** and **29**, respectively. Removal of the benzylic group of **24**–**27** by catalytic hydrogenation gave diamines **30**–**33**.

The final compounds **3**–**8** were obtained by reaction of diamines **30**–**33**, **34**, and **35** with 11-(2-chloroacetyl)-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4]diazepin-6-one.[29]

3. X-ray analysis

A single-crystal X-ray crystallographic study was undertaken, in order to establish the structure of **13** · (−)-(*S*,*S*)-tartrate and unambiguously determine its stereochemistry (Fig. 2). In particular, atoms C(1) and C(2) have opposite configuration equal to *R* and *S*, respectively.

The structure of molecule **13** can be described according to three main planes: (a) the least squares plane defined by the cyclo-

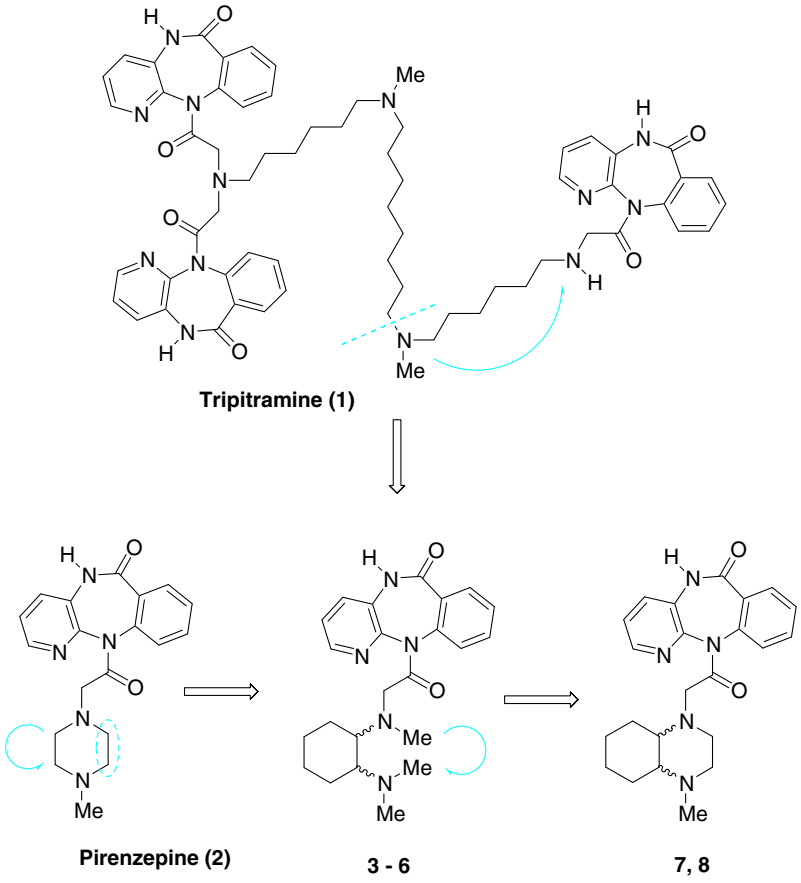
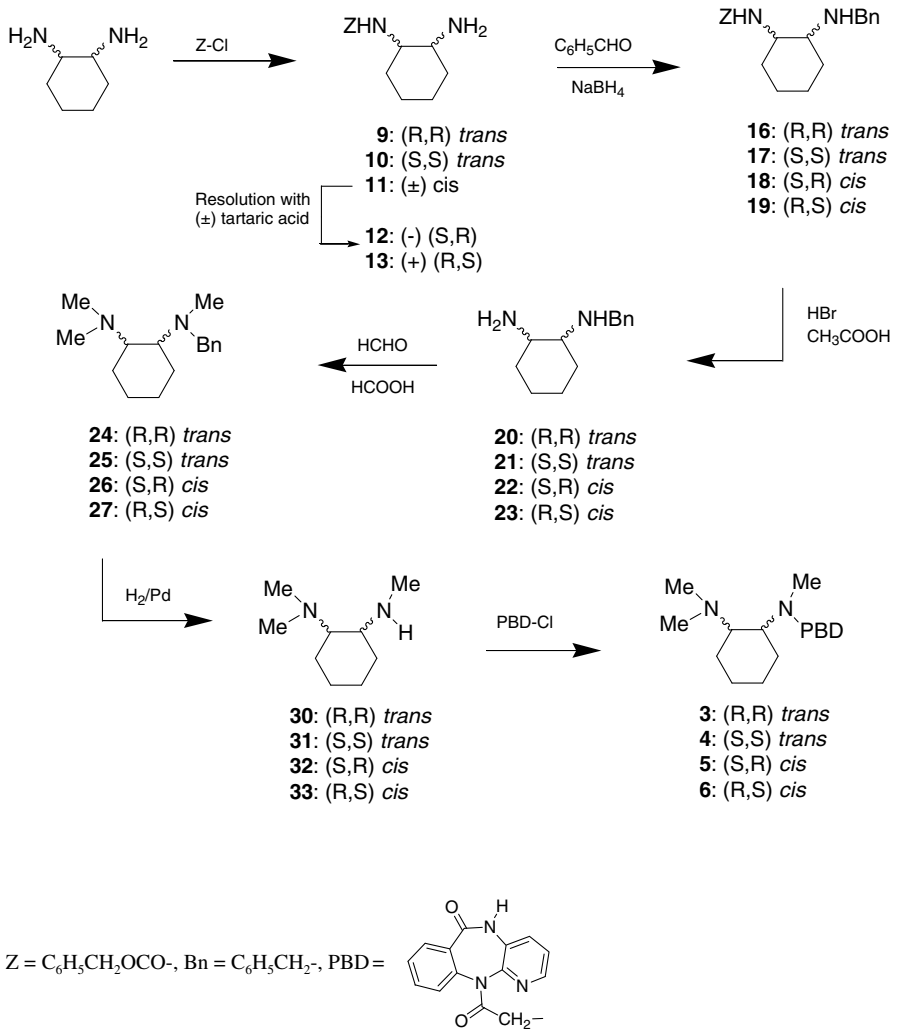


Figure 1. Design strategy of compounds **3**–**8**.



Scheme 1.

hexane ring (Φ_1); (b) another enclosing the inter ring chain formed by atoms C(1), N(7), H(7), C(8),O(9), O(10 and C(11), (Φ_2), all of them being almost coplanar, and *c*) the third plane, formed by the phenyl ring (Φ_3). The dihedral angles between these planes are $\hat{\Phi_1\Phi_2}$ = 95.2(3)°, $\hat{\Phi_1\Phi_3}$ = 93.4(3)°, and $\hat{\Phi_2\Phi_3}$ = 143.6(2)°. The cyclohexane shows a typical chair conformation, and it is almost perpendicular to the phenyl ring. The two nitrogen atoms bound to it occupy adjacent equatorial and axial positions and show different bond lengths: C(1)–N(7) and C(2)–N(12) are equal to 1.472(10) and 1.532(10) Å, respectively, in agreement with the values expected for C(sp^3)–N(amidic) and C(sp^3)–N^+(sp^3). In fact, the pendant amino group is protonated and it is involved in a network of hydrogen-bonding interactions between **13** and the tartrate anion. In particular, two strong hydrogen bonds are N(12)–H···O(22) (*x*, *y*, *z*) and N(12)–H···O(21) (−*x*, −1/2 + *y*, 2-*z*), with H···O distances equal to 1.912(6) and 1.894(6) Å, respectively.

4. Biology

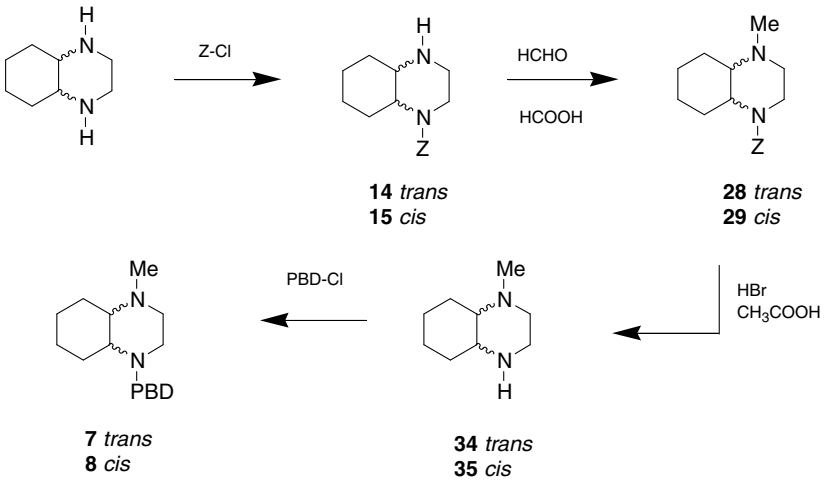
4.1. Binding experiments

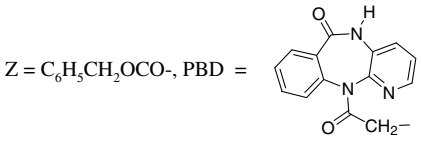
Muscarinic receptor affinity was evaluated in CHO cells expressing the four human muscarinic receptor subtypes (hm1–hm4). The results were expressed in terms of pK_i.

4.2. Functional studies

The pharmacological profile at functional muscarinic receptor subtypes of **3**–**8** was assessed in vitro on stimulated guinea pig left atria (M_2-subtype),[30] ileum (M_3-subtype),[31] and rabbit vas deferens (M_4-subtype).[32] The results were expressed in terms of pA_2.[33] For a long time, the contraction of rabbit vas deferens was referred to as an effect mediated predominantly by the muscarinic M_1 receptor subtype.[34,35] However, more recent studies attribute the same effect to an activation of the muscarinic M_4 receptor.[32] pA_2 values obtained for a series of tripitramine-related tetra-amines in functional studies using the prostatic portion of rabbit vas deferens correlated most closely with pK_i values obtained at either native or human recombinant muscarinic M_4 receptors. A very weak correlation was observed between the rabbit vas deferens site and the muscarinic M_1 receptor subtype, indicating that the two muscarinic receptors may hardly be the same. Similarly, no correlation was found comparing the antagonist potencies in vas deferens with the pK_i affinity values at native or cloned muscarinic M_2 and M_3 receptors. For this reason, in the present paper, the rabbit vas deferens will be considered an M_4-putative muscarinic receptor subtype.

In all the above experiments, to allow comparison of the results, **1** and **2** were used as the reference compounds.





Scheme 2.

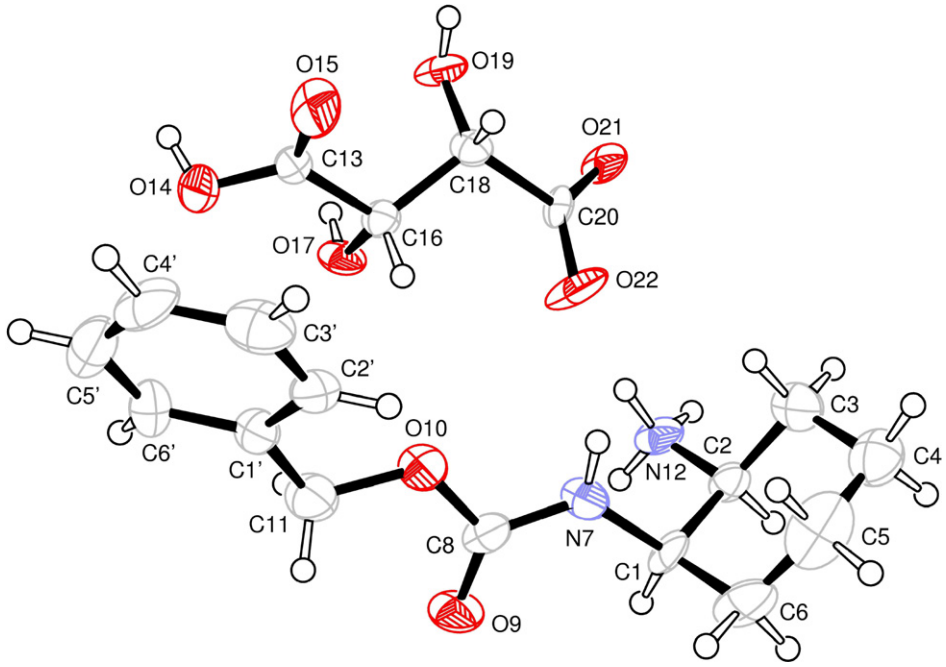


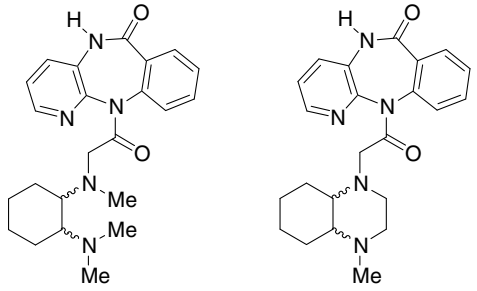
Figure 2. Crystal structure of compound **13**·(−)-(*S*,*S*)-tartrate. Ellipsoids enclose 50% probability.

5. Results and discussion

The following K_d and B_{max} were determined in cloned human muscarinic receptors in CHO cells membranes: CHO-K1 M_1 $K_d = 0.59 \pm 0.12$ nM, $B_{max} = 1987 \pm 258$ fmol mg^{-1} protein; CHO-K1 M_2 $K_d = 0.13 \pm 0.03$ nM, $B_{max} = 538 \pm 33$ fmol mg^{-1} protein; CHO-K1 M_3 $K_d = 0.16 \pm 0.04$ nM, $B_{max} = 3444 \pm 702$ fmol mg^{-1} protein; CHO-K1 M_4 $K_d = 0.14 \pm 0.02$ nM, $B_{max} = 2565 \pm 180$ fmol mg^{-1} protein.

The binding affinities of compounds **3**–**8**, expressed as pK_i values, in CHO cells expressing human cloned muscarinic M_1–M_4 receptors are shown in Table 1, in comparison with those of the standard compounds **1** and **2**. Concerning derivatives **3**–**6**, the pK_i values showed that the introduction of a cyclohexane-1,2-diamine function in exchange for a piperazine ring did not affect the affinity for M_3 and M_4 muscarinic receptor subtypes, compared to **2**. Otherwise, this structural modification on **2** led to an increased affinity almost 50-fold toward the muscarinic M_2-receptor subtype and a

Table 1
Affinity estimates of compounds **3**–**8**, expressed as pK_i values, in CHO cells expressing human cloned muscarinic M_1–M_4 receptors in comparison to pirenzepine and tripitramine



3-6 **7,8**

No.	pK_i[a]			
	M_1	M_2	M_3	M_4
3	7.65 ± 0.05	8.40 ± 0.08	7.35 ± 0.09	7.89 ± 0.07
4	7.34 ± 0.08	8.27 ± 0.07	7.40 ± 0.05	7.73 ± 0.03
5	8.20 ± 0.05	8.46 ± 0.03	7.31 ± 0.08	7.91 ± 0.13
6	7.90 ± 0.09	8.35 ± 0.14	7.39 ± 0.17	7.80 ± 0.13
7	5.64 ± 0.12	6.00 ± 0.08	<5	5.18 ± 0.05
8	5.84 ± 0.08	6.19 ± 0.09	<5	5.61 ± 0.03
2 (**Pirenzepine**)	8.55 ± 0.09	6.76 ± 0.10	7.32 ± 0.08	7.86 ± 0.12
1 (**Tripitramine**)	8.45 ± 0.13	9.52 ± 0.07	6.83 ± 0.10	7.94 ± 0.12

[a] Values are the mean ± SE of at least three separate experiments performed in duplicate. All Hill numbers (n_H) were not significantly different from unity ($p > 0.05$). Equilibrium dissociation constants (K_i) were derived from both [^{3}H]-*N*-methyl-scopolamine homologous and heterologous competition curves.

slightly reduced affinity toward the muscarinic M_1-receptor subtype, for the *trans*-derivatives **3** and **4**, abolishing the muscarinic M_1-receptor selectivity of **2** and leading to an M_2 selectivity. Although the replacement of the piperazyl moiety of pirenzepine with *cis*-cyclohexane-1,2-diamine function in **5** and **6** raised the same effect shown by **3** and **4** on muscarinic M_2 subtype, the muscarinic M_1 receptors were less sensitive to this structural modification.

It can be observed that the affinity of derivatives **3**–**6** is quantitatively similar to compound **1**, for both muscarinic M_3 and M_4-receptors. Although with reduced M_1 and M_2-receptor affinity constants, compounds **3** and **4** also maintained the same M_2/M_1 selectivity profile as that of **1**.

Besides *cis*/*trans*-isomers, the non-symmetrically substituted cyclohexane-1,2-diamine moiety afforded the two couples pairs of enantiomers **3** and **4**, and **5** and **6**, which, however, did not display enantioselectivity toward the different muscarinic receptors studied.

The insertion of the two amine functions of the cyclohexane-1,2-diamine in a more hindered *trans*- or *cis*-perhydroquinoxaline moiety, as in compounds **7** and **8**, led to reduced affinities toward all the muscarinic receptor subtypes studied, probably because of an increased steric hindrance that will not favor the interaction with the binding site.

The functional activity of derivatives **3**–**8**, expressed as pA_2 values and reported in Table 2, at peripheral muscarinic M_2, M_3, and M_4-receptor subtypes, showed that all compounds were competitive antagonists, as revealed by slope of the Schild plots, which were not significantly different from unity.

pA_2 values of **3**–**6** confirmed the improved affinity toward muscarinic M_2-receptor, relative to **2**, and the lack of enantioselectivity between the two enantiomeric pairs **3** and **4**, and **5** and **6**.

In contrast with the trend shown by compounds in *trans*-configuration **3**, **4**, and **7**, which were equiactive on muscarinic M_2 and

Table 2
Antagonist affinities of compounds **3**–**8**, expressed as pA_2 values, in the isolated guinea pig left atrium (GPLA) (M_2), and longitudinal ileum (GPLI) (M_3) and rabbit vas deferens (RVD) (M_4) muscarinic receptors, in comparison to pirenzepine and tripitramine

No.	pA_2[a]		
	M_2 (GPLA)	M_3 (GPLI)	M_4 (RVD)
3	8.55 ± 0.13	7.76 ± 0.18	8.44 ± 0.13
4	8.36 ± 0.14	7.58 ± 0.10	8.38 ± 0.12
5	8.02 ± 0.07	7.57 ± 0.06	8.85 ± 0.14
6	8.07 ± 0.12	7.64 ± 0.07	8.72 ± 0.19
7	7.06 ± 0.04	6.47 ± 0.02	7.10 ± 0.03
8	6.13 ± 0.08	6.01± 0.07	7.22 ± 0.07
2 (**Pirenzepine**)	6.47 ± 0.06	6.88 ± 0.02	8.05 ± 0.06
1 (**Tripitramine**)	9.65 ± 0.06	6.55 ± 0.04	7.95 ± 0.07

[a] pA_2 values ±SE were calculated from Schild plots,[44] constrained to slope −1.0.[33] pA_2 is the positive value of the intercept of the line derived by plotting log (DR - 1) vs log [antagonist]. The log (DR − 1) was calculated from at least three different antagonist concentrations, and each concentration was tested from four to six times. Dose-ratio (DR) values represent the ratio of the potency (EC_{50}) of the agonist arecaidine propargyl ester (M_2 and M_3) or Mc*N*-A-343 (M_4) in the presence of the antagonist and in its absence. Parallelism of concentration–response curves was checked by linear regression, and the slopes were tested for significance ($p < 0.05$).

M_4-receptors, the *cis*-derivatives **5**, **6**, and **8** were almost 10-fold more selective for muscarinic M_4-receptor than for the muscarinic M_2 subtype.

While the pA_2 values of compounds **3**–**6** overlapped the pK_i values found for muscarinic M_3 and M_4-receptor subtypes, remarkable differences in these values came out for compounds **7** and **8** (10–100-fold). We have no explanation for the discrepancy observed between functional and binding affinities.

6. Conclusion

The most intriguing result of the present study is the improved affinity toward muscarinic M_2-receptor, with respect to **2**, caused by the insertion of a cyclohexane-1,2-diamine function in exchange for a piperazine ring both in functional and binding studies. However, the higher affinity of **3**–**8** for muscarinic M_2-receptor abolished the muscarinic M_1/M_2-receptor selectivity of **2**.

7. Experimental

7.1. Chemistry

Melting points were taken in glass capillary tubes on a Buchi SMP-20 apparatus and are uncorrected. IR, HRMS mass, and ^{1}H NMR spectra were recorded on Perkin-Elmer 297, Thermo Finnigan MAT 95 XP EI, and Varian VXR 300 instruments, respectively. The optical rotations were measured on a AA-1000 Polarimeter. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Although IR spectra data are not included (because of the lack of unusual features), they were obtained for all compounds reported and were consistent with the assigned structures. The elemental compositions of the compounds agreed to within ±0.4% of the calculated value. When the elemental analysis is not included, crude compounds were used in the next step without further purification. Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063 mm; Merck), or gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed precoated silica gel plates (60 F254) that were visualized in an iodine chamber. The term "dried" refers to the use of anhydrous sodium sulfate. The enantio-

meric excess % values for compounds **12** and **13** were determined by HPLC, using Chiralcel OD (250 × 4.6 mm I.D.) chromatographic column. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom (version 2.1), a PC-integrated software package for systematic names in organic chemistry.

7.2. Benzyl *N*-[(1*R*,2*R*)-2-aminocyclohexyl]carbamate (9)

trans-(1*R*,2*R*)-Cyclohexyldiamine (2.0 g, 17.5 mmol) was dissolved in H_2O (10 mL) containing bromocresol green as indicator. Methanesulfonic acid (3.0 g, 31.0 mmol) in H_2O (10 mL) was added dropwise until pH 4 was achieved. The solution was diluted with EtOH (10 mL), vigorously stirred, and treated at 20 °C simultaneously with solutions of benzylchlorocarbonate (2.5 g, 14.7 mmol) in dimethoxyethane (6 mL) and 50% aqueous CH_3COOK 50% w/v (7 mL). After the additions were complete, the mixture was stirred at r.t. for 1 h, and solvents were removed at low temperature under vacuum. The residue was taken up in H_2O (30 mL) and filtered. The filtrate was washed with toluene (3 × 10 mL), made basic with 40% NaOH, and then extracted with toluene (5 × 20 mL). Removal of dried solvent gave **9** as a white solid that was purified by treatment with ether/petroleum ether: 35% yield. Mp 106–108 °C; ^{1}H NMR ($CDCl_3$) δ: 1.03–1.45 (m, 4H); 1.55 (s, 2H, exchangeable with D_2O); 1.67–1.84 (m, 2H); 1.85–2.12 (m, 2H); 2.28–2.48 (m, 1H); 3.10–3.31 (m, 1H); 4.65 (br s, 1H); 5.11 (s, 2H); 7.29–7.41 (m, 5H).

7.3. Benzyl *N*-[(1*S*,2*S*)-2-aminocyclohexyl]carbamate (10)

It was obtained as a white solid from *trans*-(1*S*,2*S*)-cyclohexyldiamine (2.0 g, 17.5 mmol) following the procedure described for **9**: 43% yield. Mp 106–108 °C; ^{1}H NMR ($CDCl_3$) δ: 1.03–1.45 (m, 4H); 1.55 (s, 2H, exchangeable with D_2O); 1.67–1.84 (m, 2H); 1.85–2.12 (m, 2H); 2.28–2.48 (m, 1H); 3.10–3.31 (m, 1H); 4.65 (br s, 1H); 5.11 (s, 2H); 7.29–7.41 (m, 5H).

7.4. (±) Benzyl *cis*-*N*-(2-aminocyclohexyl)carbamate (11)

It was obtained as a white solid from *cis*-cyclohexyldiamine (10.0 g, 87.6 mmol) following the procedure described for **9**: 33% yield. Mp 70–72 °C; ^{1}H NMR ($CDCl_3$) δ: 1.19 (s, 2H, exchangeable with D_2O); 1.29–1.72 (m, 8H); 2.95–3.08 (m, 1H); 3.67–3.73 (m, 1H); 5.10 (s, 2H); 5.30 (br s, 1H); 7.28–7.41 (m, 5H).

7.5. Resolution of (±) Benzyl *cis*-*N*-(2-aminocyclohexyl)carbamate (12 and 13)

A solution of racemic **11** (4.1 g, 16.4 mmol) in MeOH (50 mL) was treated with a solution of (2*R*,3*R*)-(+)-tartaric acid (2.5 g, 16.4 mmol) in EtOH (50 mL). The mixture was evaporated to dryness to give a residue that was crystallized twice with the same solvents, and other three times with water (30, 15, 10 mL, respectively) to give 1.37 g of **12** · (+) tartrate salt: [α] = +12.2 (*c* = 1.5, H_2O). The salt was dissolved in water, made basic with 2 N NaOH, and the resulting mixture extracted with chloroform (3 × 20 mL). Removal of dried solvent gave 0.76 g di (−)-**12** (**S**,**R**) as a white solid: [α] = −4.11 (*c* = 4.7, MeOH). HPLC purity: 99.7% (260 nm) eluting with i-Pr-OH/*n*-hexane (10:90).

The amine recovered by a similar alkaline treatment from the combined mother liquors of the above tartrate (2.9 g, 11.4 mmol) was dissolved in MeOH (30 mL) and treated with a solution of (2*S*,3*S*)-(−)-tartaric acid (1.7 g, 11.4 mmol) in EtOH (50 mL). The mixture was evaporated to dryness to give a residue that was crystallized twice with the same solvents, and other four times with water (40, 15, 15, 10 mL, respectively), to give 1.01 g of **13** · (−) tartrate salt: [α] = −11.78 (*c* = 1.5, H_2O). This salt was treated as described for the other enantiomer to give 0.57 g di (+)-**13** (**R**,**S**) as a white solid: [α] = +3.95 (*c* = 4.7, MeOH). HPLC purity: 99.4% (260 nm) eluting with i-Pr-OH/*n*-hexane (10:90).

7.6. Benzyl *trans*-perhydro-1-quinoxalinecarboxilate (14)

It was obtained as a solid from *trans*-perhydroquinoxaline (0.3 g, 2.14 mmol), following the procedure described for **9**: 37% yield. Mp 204–206 °C; ^{1}H NMR ($CDCl_3$) δ: 1.22–1.41 (m, 3H); 1.68–2.00 (m, 4H); 2.10–2.43 (m, 4H); 2.60–2.85 (m, 1H); 3.02–3.30 (m, 2H); 3.52–3.81 (m, 1H); 5.18 (s, 2H); 7.24–7.41 (m, 5H).

7.7. Benzyl *cis*-perhydro-1-quinoxalinecarboxilate (15)

It was obtained as an oil from *cis*-perhydroquinoxaline (0.3 g, 2.14 mmol), following the procedure described for **9**: 31% yield. ^{1}H NMR ($CDCl_3$) δ: 1.22–2.21 (m, 9H); 2.68–3.20 (m, 4H); 3.78–4.15 (m, 2H); 5.18 (s, 2H); 7.24–7.41 (m, 5H).

7.8. Benzyl *N*-[(1*R*,2*R*)-2-(benzylamino)cyclohexyl]carbamate (16)

A solution of **9** (0.5 g, 2.0 mmol) and benzaldehyde (0.26 g, 2.4 mmol) in toluene (30 mL) was refluxed and the water formed was continuously removed for 7 h. The cooled mixture was evaporated to give the corresponding Schiff base that was dissolved in EtOH (20 mL) and treated with $NaBH_4$ (0.09 g, 2.4 mmol). The mixture was stirred at room temperature for 4 h, made acidic with 2 N HCl, and the solvents were removed at reduced pressure to give a residue which was dissolved in water (10 mL). The solution was washed with ether (2× 10 mL), then it was made basic with 40% NaOH, and finally extracted with chloroform (4× 10 mL). Removal of dried solvent gave **16** as a white solid: 90% yield. Mp 68–70 °C; ^{1}H NMR ($CDCl_3$) δ: 1.01–1.45 (m, 4H); 1.56–1.87 (m, 3H); 2.01–2.20 (m, 2H); 2.2–2.36 (m, 1H); 3.28–3.49 (m, 1H); 3.79 (dd, J_1 = 10 Hz, J_2 = 46 Hz, 2H); 4.82 (br s, 1H); 5.11 (s, 2H); 7.12–7.44 (m, 10H).

7.9. Benzyl *N*-[(1*S*,2*S*)-2-(benzylamino)cyclohexyl]carbamate (17)

It was obtained from **10** (0.5 g, 2.0 mmol), following the procedure described for **16**: 97% yield. Mp: 68–70 °C; ^{1}H NMR ($CDCl_3$) δ: 1.01–1.45 (m, 4H); 1.56–1.87 (m, 3H); 2.01–2.20 (m, 2H); 2.2–2.36 (m, 1H); 3.28–3.49 (m, 1H); 3.79 (dd, J_1 = 10 Hz, J_2 = 46 Hz, 2H); 4.82 (br s, 1H); 5.11 (s, 2H); 7.12–7.44 (m, 10H).

7.10. Benzyl *N*-[(1*S*,2*R*)-2-(benzylamino)cyclohexyl]carbamate (18)

It was obtained as an oil from **12** (0.45 g, 1.81 mmol), benzaldehyde (0.23 g, 2.17 mmol), and $NaBH_4$ (0.08 g, 2.11 mmol), following the procedure described for **16**. Since, after treatment with 2 N HCl, the formed solid was slightly soluble in water, it was filtered and washed with ether (2× 10 mL), then made basic with 40% NaOH solution, and finally extracted with chloroform (4× 10 mL): 82% yield. ^{1}H NMR ($CDCl_3$) δ: 1.28–1.75 (m, 9H); 2.75–2.91 (m, 1H); 3.66–3.95 (m, 3H); 5.15 (s, 2H); 5.65 (d, *J* = 10 Hz, 1H); 7.28–7.53 (m, 10H).

7.11. Benzyl *N*-[(1*R*,2*S*)-2-(benzylamino)cyclohexyl]carbamate (19)

It was obtained as an oil from **13** (0.35 g, 1.41 mmol), benzaldehyde (0.18 g, 1.7 mmol) and $NaBH_4$ (0.06 g, 1.58 mmol), following

the procedure described for **16**: 98% yield. ^{1}H NMR ($CDCl_3$) δ: 1.28–1.75 (m, 9H); 2.75–2.91 (m, 1H); 3.66–3.95 (m, 3H); 5.15 (s, 2H); 5.65 (d, J = 10 Hz, 1H); 7.28–7.53 (m, 10H).

7.12. *N*1-Benzyl-(1*R*,2*R*)-cyclohexane-1,2-diamine (20)

A solution of 30% HBr in acetic acid (5.35 mL) was added to a solution of **16** (0.61 g, 1.78 mmol) in acetic acid (10 mL) and the resulting mixture was stirred for 2 h. Ether (50 mL) was then added yielding a solid that was dissolved in water. The solution was made basic with NaOH pellets and extracted with chloroform (3× 20 mL). Removal of the dried solvent gave in a quantitative yield **20** as a yellow oil; ^{1}H NMR ($CDCl_3$) δ: 1.82–1.38 (m, 4H); 1.43–1.95 (m, 6H); 1.95–2.12 (m, 2H); 2.14–2.45 (m, 1H); 3.81 (dd, J_1 = 10 Hz, J_2 = 46 Hz, 2H); 7.08–7.39 (m, 5H).

7.13. *N*1-Benzyl-(1*S*,2*S*)-cyclohexane-1,2-diamine (21)

It was obtained in a quantitative yield as an oil from **17** (0.66 g, 1.95 mmol) with 30% HBr in acetic acid (5.9 mL), following the procedure described for **20**. ^{1}H NMR ($CDCl_3$) δ: 1.82–1.38 (m, 4H); 1.43–1.95 (m, 6H); 1.95–2.12 (m, 2H); 2.14–2.45 (m, 1H); 3.81 (dd, J_1 = 10 Hz, J_2 = 46 Hz, 2H); 7.08–7.39 (m, 5H).

7.14. *N*1-Benzyl-(1*S*,2*R*)-cyclohexane-1,2-diamine (22)

It was obtained as a yellow oil from **18** (0.5 g, 1.48 mmol) with 30% HBr in acetic acid (4.45 mL), following the procedure described for **20**: 79% yield. ^{1}H NMR ($CDCl_3$) δ : 1.15–1.75 (m, 11H); 2.51–2.68 (m, 1H); 2.92–3.09 (m, 1H); 3.74 (d, J = 2.5 Hz, 2H); 7.15–7.41 (m, 5H).

7.15. *N*1-Benzyl-(1*R*,2*S*)-cyclohexane-1,2-diamine (23)

It was obtained as a yellow oil from **19** (0.47 g, 1.39 mmol) with 30% HBr in acetic acid (4.18 mL), following the procedure described for **20**: 77% yield. ^{1}H NMR ($CDCl_3$) δ: 1.15–1.75 (m, 11H); 2.51–2.68 (m, 1H); 2.92–3.09 (m, 1H); 3.74 (d, J = 2.5 Hz, 2H); 7.15–7.41 (m, 5H).

7.16. *N*1-Benzyl-*N*1,*N*2,*N*2-trimethyl-(1*R*,2*R*)-cyclohexane-1,2-diamine (24)

A solution of **20** (0.37 g, 1.78 mmol) in 95% HCOOH (20 mL) and aqueous 37% HCHO (18 mL) was refluxed for 12 h. The solution was made basic with 40% NaOH solution and extracted with chloroform (3× 20 mL). Removal of the dried solvent gave a residue that was dissolved in EtOH (20 mL) and treated with saturated HCl in ether. After filtration, the solid was dissolved in water, made basic with NaOH pellets, and extracted with chloroform (3× 10 mL). Removal of the dried solvent gave **24** as a yellow oil: 73% yield. $Mp_{(HCl)}$: 209–212 °C; ^{1}H NMR ($CDCl_3$) δ: 1.95–1.39 (m, 4H); 1.55–2.00 (m, 4H); 2.21 (s, 3H); 2.26 (s, 6H); 2.36–2.55 (m, 2H); 3.51–3.78 (m, 2H); 7.10–7.43 (m, 5H).

7.17. *N*1-Benzyl-*N*1,*N*2,*N*2-trimethyl-(1*S*,2*S*)-cyclohexane-1,2-diamine (25)

It was obtained as an oil from **21** (0.40 g, 1.95 mmol) as described for **24**: 97% yield. $Mp_{(HCl)}$: 209–212 °C; ^{1}H NMR ($CDCl_3$) δ: 1.94–1.38 (m, 4H); 1.56–2.02 (m, 4H); 2.22 (s, 3H); 2.25 (s, 6H); 2.37–2.55 (m, 2H); 3.68 (dd, J_1 = 12 Hz, J_2 = 22 Hz, 2H); 7.10–7.43 (m, 5H).

7.18. *N*1-Benzyl-*N*1,*N*1,*N*2-trimethyl-(1*S*,2*R*)-cyclohexane-1,2-diamine (26)

It was obtained from **22** (0.24 g, 1.17 mmol) as described for **24**. The residue was purified by gravity column. Eluting with $CHCl_3$/MeOH/aqueous 28% ammonia (9.5:0.5:0.05) afforded **26** as an yellow oil: 70% yield. ^{1}H NMR ($CDCl_3$) δ: 1.29–1.48 (m, 2H); 1.49–1.66 (m, 2H); 1.67–1.89 (m, 2H); 1.90–2.08 (m, 2H); 2.17 (s, 3H); 2.25–2.34 (m, 1H); 2.36 (s, 6H); 2.59–2.72 (m, 1H); 3.45 (d, J = 20 Hz, 1H); 4.22 (d, J = 20 Hz, 1H); 7.10–7.45 (m, 5H).

7.19. *N*1-Benzyl-*N*1,*N*1,*N*2-trimethyl-(1*R*,2*S*)cyclohexane-1,2-diamine (27)

It was obtained as an yellow oil from **23** (0.22 g, 1.08 mmol) as described for **26**: 60% yield. ^{1}H NMR ($CDCl_3$) δ: 1.29–1.48 (m, 2H); 1.49–1.66 (m, 2H); 1.67–1.89 (m, 2H); 1.90–2.08 (m, 2H); 2.17 (s, 3H); 2.25–2.34 (m, 1H); 2.36 (s, 6H); 2.59–2.72 (m, 1H); 3.45 (d, J = 20 Hz, 1H); 4.22 (d, J = 20 Hz, 1H); 7.10–7.45 (m, 5H).

7.20. Benzyl *trans*-4-methylperhydro-1-quinoxalinecarboxilate (28)

It was obtained as a solid from **14** (0.24 g, 0.87 mmol), following the procedure described for **24**: 80% yield. Mp 196–198 °C; ^{1}H NMR ($CDCl_3$) δ: 1.22–1.41 (m, 3H); 1.68–1.96 (m, 3H); 2.00–2.10 (m, 2H); 2.20 (s, 3H); 2.22–2.40 (m, 2H); 2.85–3.00 (m, 1H); 3.02–3.21 (m, 1H); 3.32–3.48 (m, 1H); 3.81–3.98 (m, 1H); 5.16 (s, 2H); 7.24–7.41 (m, 5H).

7.21. Benzyl *cis*-4-methylperhydro-1-quinoxalinecarboxilate (29)

It was obtained as an oil from **15** (0.1 g, 0.36 mmol) following the procedure described for **24**: 70% yield. ^{1}H NMR ($CDCl_3$) δ: 1.35–1.61 (m, 5H); 1.80–1.96 (m, 1H); 2.05–2.22 (m, 4H); 2.35 (s, 3H); 2.80–2.98 (m, 1H); 3.23–3.40 (m, 1H); 3.83–4.18 (m, 2H); 5.19 (s, 2H); 7.31–7.42 (m, 5H).

7.22. *N*1,*N*1,*N*2-Trimethyl-(1*R*,2*R*)-cyclohexane-1,2-diamine (30)

A solution of **24** (0.4 g, 1.62 mmol) in EtOH (20 mL), with the addition of saturated HCl in EtOH, was hydrogenated over 10% Pd on charcoal (wet, Degussa type E101 NE/W) for 2 h. Following catalyst removal, the solvent was evaporated, affording a solid that was dissolved in water. The solution was made basic with NaOH pellets and extracted with chloroform (3 × 10 mL). Removal of the dried solvent gave **30** as a yellow oil: 90% yield. ^{1}H NMR free base ($CDCl_3$) δ: 1.91–1.30 (m, 5H); 1.55–1.85 (m, 3H); 2.00–2.24 (m, 2H); 2.15 (s, 6H); 2.35 (s, 3H); 2.68–2.82 (m, 1H).

7.23. *N*1,*N*1,*N*2-Trimethyl-(1*S*,2*S*)-cyclohexane-1,2-diamine (31)

It was obtained from **25** (0.47 g, 1.91 mmol) as described for **30**: 97% yield. ^{1}H NMR free base ($CDCl_3$) δ : 1.91–1.30 (m, 5H); 1.55–1.85 (m, 3H); 2.00–2.24 (m, 2H); 2.15 (s, 6H); 2.35 (s, 3H); 2.68–2.82 (m, 1H).

7.24. *N*1,*N*1,*N*2-Trimethyl-(1*S*,2*R*)-cyclohexane-1,2-diamine (32)

It was obtained from **26** (0.32 g, 1.13 mmol) as described for **30**: 85% yield. ^{1}H NMR free base ($CDCl_3$) δ: 1.02–1.60 (m, 5H); 1.60–1.05 (m, 5H); 2.18 (s, 6H); 2.32 (s, 3H); 2.71–2.83 (m, 1H).

7.25. *N*1,*N*1,*N*2-Trimethyl-(1*R*,2*S*)-cyclohexane-1,2-diamine (33)

It was obtained from **27** (0.16 g, 0.64 mmol) as described for **30**: quantitative yield. ^{1}H NMR free base ($CDCl_3$) δ: 1.03–1.59 (m, 5H); 1.60–1.06 (m, 5H); 2.17 (s, 6H); 2.32 (s, 3H); 2.70–2.83 (m, 1H).

7.26. *trans*-1-Methylperhydroquinoxaline (34)

It was obtained as a solid from **28** (0.15 g, 0.52 mmol), following the procedure described for **20**: 90% yield. Mp 190–192 °C; ^{1}H NMR ($CDCl_3$) δ: 1.20–1.42 (m, 4H); 1.60–1.96 (m, 5H); 2.00–2.18 (m, 1H); 2.25 (s, 3H); 2.22–2.50 (m, 1H); 2.80–3.15 (m, 4H).

7.27. *cis*-1-Methylperhydroquinoxaline (35)

It was obtained as a solid from **29** (50 mg, 0.17 mmol), following the procedure described for **20**: 71% yield. Mp 188–190 °C; ^{1}H NMR ($CDCl_3$) δ: 1.22–1.42 (m, 2H); 1.50–1.73 (m, 3H); 1.81–1.98 (m, 2H); 2.01–2.08 (m, 4H); 2.16 (s, 3H); 2.52–2.68 (m, 1H); 2.87–3.18 (m, 2H), 3.62–3.78 (m, 1H).

7.28. 11-{2-[[(1*R*,2*R*)-2-(Dimethylamino)cyclohexyl](methyl) amino]acetyl}-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4] diazepin-6-one Dioxalate (3)

A mixture of **30** (0.23 g, 1.47 mmol), NEt_3 (0.26 mL), and 11-(2-chloroacetyl)-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4]diazepin-6-one (0.55 g, 1.94 mmol) in dry DMF was stirred at room temperature for 48 h. Removal of the solvent afforded a residue that was purified by flash chromatography. Eluting with $CHCl_3$/MeOH/aqueous 28% ammonia (8.0:2.0:0.2) afforded crude **3** which was converted into the dioxalate salt: 25% yield. Mp 185–190 °C (EtOH); ^{1}H NMR (CD_3OD) δ: 1.12–1.58 (m, 3H); 1.64–1.95 (m, 3H); 1.96–2.20 (m, 1H); 2.35 (br s, 1H); 2.51–2.78 (m, 4H); 2.78–3.00 (m, 3H); 3.05–3.22 (m, 1H); 3.22–3.35 (m, 3H); 3.35–3.60 (m, 1H); 3.60–3.98 (m, 1H); 7.40–7.83 (m, 5H); 7.85–8.01 (m, 1H); 8.35–8.43 (m, 1H). HRMS calcd for $C_{23}H_{29}N_5O_2$ 407.2321, found 407.2318. Anal. Calcd for $C_{27}H_{33}N_5O_{10}$: C, 55.19; H, 5.66; N, 11.92. Found: C, 55.32; H, 5.45; N, 11.81.

7.29. 11-{2-[[(1*S*,2*S*)-2-(Dimethylamino)cyclohexyl](methyl) amino]acetyl}-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4] diazepin-6-one Dioxalate (4)

It was obtained from **31** (0.29 g, 1.86 mmol), NEt_3 (0.34 mL), and 11-(2-chloroacetyl)-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4]diazepin-6-one (0.70 g, 2.4 mmol) as described for **3**. Removal of the solvent afforded a residue that was purified first by flash chromatography and then by gravity column. Eluting with a step-gradient system of $CHCl_3$/MeOH/aqueous 28% ammonia (8.0:2.0:0.2 to 9.0:1.0:0.1) and $CHCl_3$/MeOH/EtOAc/aqueous 28% ammonia (3.0:4.0:3.0:0.2) afforded **4** as a free base that was converted into the dioxalate salt: 11.8% yield. Mp 185–190 °C; ^{1}H NMR (CD_3OD) δ: 1.12–1.62 (m, 3H); 1.62–1.99 (m, 3H); 2.02–2.20 (m, 1H); 2.32 (br s, 1H); 2.52–2.80 (m, 4H); 2.80–2.98 (m, 3H); 3.12–3.25 (m, 1H); 3.25–3.35 (m, 3H); 3.35–3.64 (m, 1H); 3.65–3.95 (m, 1H); 7.43–7.83 (m, 5H); 7.84–8.00 (m, 1H); 8.32–8.42 (m, 1H). HRMS calcd for $C_{23}H_{29}N_5O_2$ 407.2321, found 407.2323. Anal. Calcd for $C_{27}H_{33}N_5O_{10}$: C, 55.19; H, 5.66; N, 11.92. Found: C, 55.26; H, 5.48; N, 11.75.

7.30. 11-{2-[[(1*S*,2*R*)-2-(Dimethylamino)cyclohexyl](methyl) amino]acetyl}-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4] diazepin-6-one Dioxalate (5)

A mixture of **32** (0.15 g, 0.96 mmol), NEt_3 (0.17 mL), and 11-(2-chloroacetyl)-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4]diazepin-6-one (0.35 g, 1.23 mmol) in dry DMF was stirred for 48 h at 100 °C. Removal of the solvent afforded a residue that was purified by gravity column. Eluting with $CHCl_3$/MeOH/aqueous 28% ammonia (8.0:2.0:0.2) afforded **5** as a free base that was converted into the dioxalate salt: 8% yield. Mp 182–185 °C; ^{1}H NMR (CD_3OD) δ: 1.35–2.05 (m, 5H); 2.35–2.62 (m, 3H); 2.74–2.99 (br s + m, 4H); 3.03–3.20 (m, 1H); 3.31 (s, 6H); 3.37–3.59 (m, 2H); 7.37–7.83 (m, 5H); 7.85–8.03 (m, 1H); 8.38 (br s, 1H). HRMS calcd for $C_{23}H_{29}N_5O_2$ 407.2321, found 407.2319. Anal. Calcd for $C_{27}H_{33}N_5O_{10}$: C, 55.19; H, 5.66; N, 11.92. Found: C, 55.13; H, 5.57; N, 11.73.

7.31. 11-{2-[[(1*R*,2*S*)-2-(Dimethylamino)cyclohexyl](methyl) amino]acetyl}-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4]diazepin-6-one Dioxalate (6)

A mixture of **33** (60 mg, 0.38 mmol), NEt_3 (0.1 mL), and 11-(2-chloroacetyl)-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4]diazepin-6-one (0.16 g, 0.56 mmol) in dry DMF was stirred for 48 h at 100 °C. Removal of the solvent afforded a residue that was purified by gravity column. Eluting with $CHCl_3$/MeOH/aqueous 28% ammonia (8.0:2.0:0.2) afforded **6** as a free base that was converted into the dioxalate salt: 11% yield. Mp 180–182 °C; ^{1}H NMR (CD_3OD) δ: 1.35–2.04 (m, 5H); 2.36–2.64 (m, 3H); 2.75–3.01 (br s + m, 4H); 3.03–3.18 (m, 1H); 3.31 (s, 6H); 3.36–3.59 (m, 2H); 7.39–7.82 (m, 5H); 7.85–8.03 (m, 1H); 8.38 (br s, 1H). HRMS calcd for $C_{23}H_{29}N_5O_2$ 407.2321, found 407.2324. Anal. Calcd for $C_{27}H_{33}N_5O_{10}$: C, 55.19; H, 5.66; N, 11.92. Found: C, 55.19; H, 5.68; N, 11.97.

7.32. 11-[2-(*trans*-4-Methylperhydro-1-quinoxalinyl)acetyl]-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4]diazepin-6-one (7)

A mixture of **34** (30 mg, 0.19 mmol), NEt_3 (0.05 mL), and 11-(2-chloroacetyl)-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4]diazepin-6-one (80 mg, 0.28 mmol) in dry DMF was stirred for 48 h at 60 °C. Removal of the solvent afforded a residue that was purified by gravity column. Eluting with $CHCl_3$/MeOH/aqueous 28% ammonia (8.0:2.0:0.2) provided **7**: 55% yield. Mp 185–190 °C; ^{1}H NMR ($CDCl_3$) δ: 1.05–1.42 (m, 8H); 1.50–1.80 (m, 2H); 1.84–2.05 (m, 2H); 2.23 (s, 3H); 2.63–2.80 (m, 1H); 2.83–2.98 (m, 1H); 3.23–3.89 (m, 1H); 4.00–4.20 (m, 1H); 7.20–7.50 (m, 3H); 7.60–7.85 (m, 2H); 7.90–8.03 (m, 1H); 8.20–8.35 (m, 1H); 10.00 (br s, 1H). HRMS calcd for $C_{23}H_{27}N_5O_2$ 405.2165, found 405.2161. Anal. Calcd for $C_{23}H_{27}N_5O_2$: C, 68.13; H, 6.71; N, 17.27. Found: C, 68.32; H, 6.51; N, 17.40.

7.33. 11-[2-(*cis*-4-Methylperhydro-1-quinoxalinyl)acetyl]-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4]diazepin-6-one (8)

A mixture of **35** (19 mg, 0.12 mmol), NEt_3 (0.03 mL), and 11-(2-chloroacetyl)-6,11-dihydro-5*H*-benzo[*e*]pyrido[3,2-*b*][1,4]diazepin-6-one (53 mg, 0.18 mmol) in dry DMF was stirred for 48 h at 60 °C. Removal of the solvent afforded a residue that was purified by gravity column. Eluting with $CHCl_3$/MeOH/aqueous 28% ammonia (9.0:1.0:0.05) afforded **8**as a wax solid: 16% yield. ^{1}H NMR ($CDCl_3$) δ: 1.10–1.42 (m, 8H); 1.50–1.90 (m, 2H); 1.84–2.20 (m, 2H); 2.03 (s, 3H); 3.25–3.34 (m, 2H); 3.80–4.00 (m, 2H); 7.23–7.87 (m, 5H); 7.90–8.03 (m, 1H); 8.20–8.35 (m, 1H); 8.80 (br s, 1H). HRMS calcd for $C_{23}H_{27}N_5O_2$ 405.2165, found 405.2169. Anal. Calcd for $C_{23}H_{27}N_5O_2$: C, 68.13; H, 6.71; N, 17.27. Found: C, 68.45; H, 6.69; N, 17.31.

8. X-ray crystal structure determination

Single crystals of **13** · (−)-(*S*,*S*)-tartrate suitable for X-ray data collection were obtained by dissolving 50 mg of powder and (−)-(2*S*,3*S*)-tartaric acid in water and by allowing it to concentrate at room temperature.

Data collection has been carried out by using a Siemens P4 four-circle diffractometer with graphite monochromated Mo-Kα radiation (λ = 0.71073 Å). The structures crystallize in an acentric space group (Monoclinic, $P2_1$), indicating its enantiomeric purity. The direct methods implemented in the SHELXS-97 program[36] were used for structure solution. Its refinement was carried out by full-matrix anisotropic least-squares on F^2 for all reflections for all non-H atoms by using the SHELXL-97 program.[37] The absolute configuration of **13** · (−)-(*S*,*S*)-tartrate has been determined by assigning the right configuration to (−)-(*S*,*S*)-tartrate. Molecular graphics were performed by using WinGX package.[38] Crystallographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 665124. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

9. Biology

9.1. Functional antagonism at guinea-pig left atria

Male guinea pigs (200–300 g) were killed by cervical dislocation. The heart was rapidly removed, and left atria were separated out and set up under 1 g of tension in 20-mL organ baths containing physiological salt solution (PSS) maintained at 30 °C and aerated with 5% CO_2–95% O_2. The left atria were mounted in PSS of the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.52; $MgSO_4 \cdot 7H_2O$, 1.18; KH_2PO_4, 1.18; $NaHCO_3$, 23.8; glucose, 11.7. Tissues were 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 concentration–response curve to arecaidin propargyl ester (APE) was constructed. Concentration–response curves were constructed by cumulative addition of the reference agonist. The concentration of agonist in the organ bath was increased approximately threefold 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 1 h, and a new dose–response curve to the agonist was obtained. 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.

9.2. Guinea pig ileum longitudinal muscle

The terminal portion of the ileum was excised after discarding the 8–10 cm nearest to the ileo-caecal junction. The tissue was cleaned, and segments 2–3 cm long of ileum longitudinal muscle were set up under 1-g tension at 37 °C in organ baths containing PSS of the following composition (mM): NaCl, (118); KCl, (4.75); $CaCl_2$, (2.54); $MgSO_4$, (1.2); $KH_2PO_4 \cdot 2H_2O$, (1.19); $NaHCO_3$, (25); glucose, (11). Tension changes were recorded isotonically. Tissues were allowed to equilibrate for at least 30 min during which time the bathing solution was changed every 10 min. Concentration–response curves to APE (0.01–0.5 μM) were obtained at 30-min intervals, the first one being discarded and the second one taken as control. Following incubation with the antagonist for 60 min, a new concentration–response curve to the agonist was obtained.

9.3. Rabbit-stimulated vas deferens

This preparation was set up according to Eltze.[39] Vas deferens 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 was included to block α_2-adrenoceptors. 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-Mc*N*-A-343 was constructed.

10. Binding experiments

10.1. Cell culture and membrane preparation

CHO cells, stably expressing the human muscarinic (M_1–M_4) receptors (provided by Prof. R. Maggio, Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy), were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Gibco, Grand Iland, N.Y.), 100 U/mL of penicillin G and streptomicyn, 4 mM glutamine (Sigma–Aldrich, Milano, Italy) and non-essential aminoacids solution 100× (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 harvested by trypsinization, followed by centrifugation (300*g* for 5 min), 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 17,000*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.[40] An aliquot was taken for the assessment of protein content,[41] using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, München, Germany). BSA was used as the standard.

10.2. Binding assay

The radioligand binding assay was run in polypropylene 96-well plates (Sarstedt, Verona, Italy) and performed for 120 min at room temperature in a final volume of 0.25 mL in 25 mM sodium phosphate buffer containing 5 mM $MgCl_2$ at pH 7.4. Final membrane protein concentrations were 30 μg/mL (M_1), 70 μg/mL (M_2), 25 μg/mL (M_3), and 50 μg/mL (M_4). In saturation experiments (homologous curves), [^{3}H]-*N*-methylscopolamine chloride ([^{3}H]-NMS) was present at 0.2 nM in tubes containing increasing concentrations 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 (0.001–1000 μM). All measurements were obtained in duplicate. After incubation, membranes were filtered through UniFilter GF/C plates (Perkin-Elmer Life and Analytical Science, Boston, MA) using a FilterMate Cell Harvester (Perkin-Elmer Life and Analytical Science, Boston, MA); filters were washed several times with ice-cold buffer and allowed to dry overnight at room temperature under air flow and 25 μL of scintillation liquid (Microscint-20, Perkin-Elmer Life and Analytical Science, Boston, MA) was added. Counting was done with TopCount NXT Microplate Scintillation Counter (Perkin-Elmer Life and Analytical Science, Boston, MA).

11. Data analysis

The binding data were evaluated quantitatively with the weighted least-squares iterative curve-fitting LIGAND program[42]; this analysis provides optimal estimates of binding parameters for the labeled ligand from the analysis of homologous curves: affinity constant (K_D), binding capacities (B_{max}), and non-specific binding (N). Moreover, data from heterologous and homologous curves were simultaneously analyzed to obtain the K_i values for the unlabeled ligands. The computer program ALLFIT[43] was used for the analysis of sigmoidal dose response curves from binding experiments to obtain the logit-log slope (''pseudo-Hill coefficient", nH). All the quoted values are means ± SEM.

In functional studies, responses were expressed as percentage of the maximal contraction observed in the agonist concentration–response curve taken as a control. Pharmacological computer programs analyzed the agonist concentration–response curves. Values were calculated according to Arunlakshana and Schild from the dose-ratios as the EC_{50} values of the agonists calculated at three antagonist concentrations.[44] Each concentration was tested five times, and Schild plots were constrained to slope −1, as required by theory.[33] When this method was applied, it was always verified that the experimental data generated a line whose derived slope was not significantly different from unity ($p < 0.05$). Student's *t*-test was used to assess the statistical significance of the difference between two means.

Acknowledgements

This research was supported by Grants from MUR, Rome (PRIN funds).

References and notes

1. Hulme, E. C.; Lu, Z. L.; Saldanha, J. W.; Bee, M. S. *Biochem. Soc. Trans.* **2003**, *31*, 29–34.
2. Caulfield, M. P. *Pharmacol. Ther.* **1993**, *58*, 319–379.
3. Eglen, R. M. *Prog. Med. Chem.* **2005**, *43*, 105–136.
4. Wess, J. *Trends Pharmacol. Sci.* **2003**, *24*, 414–420.
5. Matsui, M.; Yamada, S.; Oki, T.; Manabe, T.; Taketo, M. M.; Ehlert, F. J. *Life Sci.* **2004**, *75*, 2971–2981.
6. Wess, J.; Zhang, W.; Duttaroy, A.; Miyakawa, T.; Gomeza, J.; Cui, Y.; Basile, A. S.; Bymaster, F. P.; McKinzie, D. L.; Felder, C. C.; Deng, C.; Yamada, M. *Handb. Exp. Pharmacol.* **2004**, *159*, 65–93.
7. Gawade, S. P. *J. Toxicol. Toxin Rev.* **2004**, *23*, 37–96.
8. Bradley, K. N. *Pharmacol. Ther.* **2000**, *85*, 87–109.
9. Potter, L. T. *Life Sci.* **2001**, *68*, 2541–2547.
10. Hulme, E. C.; Lu, Z. L.; Bee, M.; Curtis, C. A.; Saldanha, J. *Life Sci.* **2001**, *68*, 2495–2500.
11. Shi, L.; Javitch, J. A. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 437–467.
12. Wess, J.; Eglen, R. M.; Gautam, D. *Nat. Rev. Drug Discov.* **2007**, *6*, 721–733.
13. Felder, C. C.; Bymaster, F. P.; Ward, J.; DeLapp, N. *J. Med. Chem.* **2000**, *43*, 4333–4353.
14. Eglen, R. M.; Choppin, A.; Watson, N. *Trends Pharmacol. Sci.* **2001**, *22*, 409–414.
15. Abrams, P.; Andersson, K. E.; Buccafusco, J. J.; Chapple, C.; de Groat, W. C.; Fryer, A. D.; Kay, G.; Laties, A.; Nathanson, N. M.; Pasricha, P. J.; Wein, A. J. *Br. J. Pharmacol.* **2006**, *148*, 565–578.
16. Hegde, S. S. *Br. J. Pharmacol.* **2006**, *147*, S80–S87.
17. Celli, B. R. *Ther. Strat. COPD* **2005**, 93–105.
18. Melchiorre, C.; Minarini, A.; Angeli, P.; Giardina, D.; Gulini, U.; Quaglia, W. *Trends Pharmacol. Sci.* **1989**, 55–59.
19. Minarini, A.; Budriesi, R.; Chiarini, A.; Melchiorre, C.; Tumiatti, V. *Farmaco* **1991**, *46*, 1167–1178.
20. Melchiorre, C.; Bolognesi, M. L.; Chiarini, A.; Minarini, A.; Spampinato, S. *J. Med. Chem.* **1993**, *36*, 3734–3737.
21. Minarini, A.; Bolognesi, M. L.; Budriesi, R.; Canossa, M.; Chiarini, A.; Spampinato, S.; Melchiorre, C. *J. Med. Chem.* **1994**, *37*, 3363–3372.
22. Melchiorre, C.; Minarini, A.; Budriesi, R.; Chiarini, A.; Spampinato, S.; Tumiatti, V. *Life Sci.* **1995**, *56*, 837–844.
23. Melchiorre, C.; Antonello, A.; Banzi, R.; Bolognesi, M. L.; Minarini, A.; Rosini, M.; Tumiatti, V. *Med. Res. Rev.* **2003**, *23*, 200–233.
24. Tumiatti, V.; Minarini, A.; Milelli, A.; Rosini, M.; Buccioni, M.; Marucci, G.; Ghelardini, C.; Bellucci, C.; Melchiorre, C. *Bioorg. Med. Chem.* **2007**, *15*, 2312–2321.
25. Wess, J.; Lambrecht, G.; Moser, U.; Mutschler, E. A. *Life Sci.* **1984**, *35*, 553–560.
26. Mohr, M.; Heller, E.; Ataie, A.; Mohr, K.; Holzgrabe, U. *J. Med. Chem.* **2004**, *47*, 3324–3327.
27. Brill, E.; Schultz, H. P. *J. Org. Chem.* **1963**, *28*, 1135–1138.
28. Brill, E.; Schultz, H. P. *J. Org. Chem.* **1964**, *29*, 579–581.
29. Eberlein, W. G.; Engel, W. W.; Trummlitz, G.; Schmidt, G.; Hammer, R. *J. Med. Chem.* **1988**, *31*, 1169–1174.
30. Kenakin, T. P.; Boselli, C. *Naunyn Schmiedebergs Arch. Pharmacol.* **1991**, *344*, 201–205.
31. Ringdahl, B. *Mol. Pharmacol.* **1987**, *31*, 351–356.
32. Budriesi, R.; Cacciaguerra, S.; Toro, R. D.; Bolognesi, M. L.; Chiarini, A.; Minarini, A.; Rosini, M.; Spampinato, S.; Tumiatti, V.; Melchiorre, C. *Br. J. Pharmacol.* **2001**, *132*, 1009–1016.
33. Tallarida, R. J.; Cowan, A.; Adler, M. W. *Life Sci.* **1979**, *25*, 637–654.
34. Eltze, M. *Eur. J. Pharmacol.* **1988**, *151*, 205–221.
35. Eltze, M.; Figala, V. *Eur. J. Pharmacol.* **1988**, *158*, 11–19.
36. Sheldrick, G. M. *SHELXS-97, Rel. 97-2, A Program for Automatic Solution of Crystal Structures*; Goettingen University, 1997.
37. Sheldrick, G. M. *SHELXL-97, Rel. 97-2, A Program for Crystal Structure Refinement*; Goettingen University, 1997.
38. Farrugia, L. J. *J. Appl. Crystallogr.* **1999**, *32*, 837–838.
39. Eltze, M. *Pharmacology* **1988**, *37*, 40–47.
40. Dei, S.; Angeli, P.; Bellucci, C.; Buccioni, M.; Gualtieri, F.; Marucci, G.; Manetti, D.; Matucci, R.; Romanelli, M. N.; Scapecchi, S.; Teodori, E. *Biochem. Pharmacol.* **2005**, *69*, 1637–1645.
41. Bradford, M. M. A. *Anal. Biochem.* **1976**, *72*, 248–254.
42. Munson, P. J.; Rodbard, D. *Anal. Biochem.* **1980**, *107*, 220–239.
43. DeLean, A.; Munson, P. J.; Rodbard, D. *Am. J. Physiol.* **1978**, *235*, E97–E102.
44. Arunlakshana, O.; Schild, H. O. *Br. J. Pharmacol. Chemother.* **1959**, *14*, 48–58.