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Binding and functional properties of hexocyclium and sila-hexocyclium derivatives to muscarinic receptor subtypes

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1 We have compared the binding properties of several hexocyclium and sila-hexocyclium derivatives to muscarinic M_1 receptors (in rat brain, human neuroblastoma (NB-OK 1) cells and calf superior cervical ganglia), rat heart M_2 receptors, rat pancreas M_3 receptors and M_4 receptors in rat striatum, with their functional antimuscarinic properties in rabbit vas deferens (M_1/M_4-like), guinea-pig atria (M_2), and guinea-pig ileum (M_3) muscarinic receptors.

2 Sila-substitution (C/Si exchange) of hexocyclium (→ sila-hexocyclium) and demethyl-hexocyclium (→ demethyl-sila-hexocyclium) did not significantly affect their affinities for muscarinic receptors. By contrast, sila-substitution of *o*-methoxy-hexocyclium increased its affinity 2 to 3 fold for all the muscarinic receptor subtypes studied.

3 The *p*-fluoro- and *p*-chloro-derivatives of sila-hexocyclium had lower affinities than the parent compound at the four receptor subtypes, in binding and pharmacological studies.

4 In binding studies, *o*-methoxy-sila-hexocyclium ($M_1 = M_4 \geqslant M_3 \geqslant M_2$) had a much lower affinity than sila-hexocyclium for the four receptor subtypes, and discriminated the receptor subtypes more poorly than sila-hexocyclium ($M_1 = M_3 > M_4 > M_2$). This is in marked contrast with the very clear selectivity of *o*-methoxy-sila-hexocyclium for the prejunctional M_1/M_4-like heteroreceptors in rabbit vas deferens.

5 The tertiary amines demethyl-hexocyclium, demethyl-sila-hexocyclium and demethyl-*o*-methoxy-sila-hexocyclium had 10 to 30 fold lower affinities than the corresponding quaternary ammonium derivatives.

Keywords: Hexocyclium/sila-hexocyclium derivatives; *o*-methoxy-sila-hexocyclium; muscarinic receptor subtypes; structure/affinity relationships; binding/functional correlations; muscarinic receptor antagonists

Introduction

Following the discovery that pirenzepine is able to discriminate several muscarinic receptor binding sites (Hammer *et al.*, 1980), the subclassification and characterization of various muscarinic receptor subtypes has aroused increasing interest. Molecular biology studies suggest that at least five genes encoding muscarinic receptor subtypes (m1–m5) are expressed in mammalian tissues (see Hulme *et al.*, 1990; Levine & Birdsall, 1993). Functional and binding experiments revealed the existence of four different native subtypes, termed M_1–M_4. Comparison of the radioligand binding properties and functional coupling of the native M_1–M_4 receptors with those of the expressed m1–m4 proteins showed a good correlation (Hulme *et al.*, 1990; Lazareno *et al.*, 1990; Waelbroeck *et al.*, 1990; Dörje *et al.*, 1991; Levine & Birdsall, 1993).

Although several drugs have been reported to discriminate the M_1 from M_2, M_3 and M_4 receptors in binding and/or functional studies, most of these drugs possess tertiary amino groups. Their binding properties, like those of pirenzepine, might therefore be strongly pH-dependent (Barlow & Chan, 1982), a factor which may complicate the identification of receptor subtypes. It was interesting, therefore, that a quaternary ammonium compound, *o*-methoxy-sila-hexocyclium, was found to have very clear M_1 selectivity in functional studies (Lambrecht *et al.*, 1988; Boddeke *et al.*, 1989; Polidori *et al.*, 1990).

The goal of the present study was to evaluate the structural requirements for binding of the carbon/silicon analogues hexocyclium and sila-hexocyclium and related compounds (Figure 1) to M_1, cardiac M_2, glandular/smooth muscle M_3 and brain M_4 sites. To achieve this goal, we analysed the binding properties of the compounds to [^{3}H]-pirenzepine-labelled M_1 sites in rat brain cortex, [^{3}H]-telenzepine-labelled M_1 sites in calf superior cervical ganglia (Feifel *et al.*, 1991) or [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS)-labelled NB-OK 1 (M_1) sites (Waelbroeck *et al.*, 1987b; 1988), [^{3}H]-NMS-labelled rat cardiac M_2 sites (Waelbroeck *et al.*, 1987a), [^{3}H]-NMS-labelled rat pancreas M_3 sites (Waelbroeck *et al.*, 1987a) and [^{3}H]-NMS-labelled M_4 sites in rat striatum, sites with a slow dissociation rate for NMS (Waelbroeck *et al.*, 1987b; 1990).

We compared the binding affinity profiles at these four receptor subtypes with the pharmacological properties of the compounds at prejunctional muscarinic heteroreceptors in rabbit vas deferens (Eltze, 1988; Eltze *et al.*, 1988; Grimm *et al.*, 1994; Lambrecht *et al.*, 1993), M_2 receptors in guinea-pig atria, and M_3 receptors in guinea-pig ileum (see review by Caulfield, 1993).

The affinities of a number of antagonists for the prejunctional muscarinic receptors mediating inhibition of neurogenic contractions of rabbit vas deferens clearly indicate that these receptors are not the M_2, M_3 or m5 subtype (Eltze, 1988; Eltze *et al.*, 1988; 1993; Lambrecht *et al.*, 1989; Dörje *et al.*, 1991; Grimm *et al.*, 1994). The most prominent features of these prejunctional inhibitory receptors are: (i) a high affinity for the M_1-selective antagonist pirenzepine (pA_2 values = 8.08–8.52; Lambrecht *et al.*, 1989; Choo & Mitchelson, 1990; Micheletti *et al.*, 1990a; Dörje *et al.*, 1990; Eltze *et al.*, 1993; Sagrada *et al.*, 1993); (ii) a high affinity for the M_2/M_4-selective antagonist himbacine (pA_2

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values = 8.5–8.17; Dörje *et al.*, 1990; Eltze *et al.*, 1993; Sagrada *et al.*, 1993); and (iii) a low affinity for methoctramine (pA_2 value = 6.85; Lambrecht *et al.*, 1989). It is also worth noting that there is immunoreactivity to specific anti-m1 receptor antibodies, but not to anti-m4 antibodies, in rabbit vas deferens (Dörje *et al.*, 1991). Thus, we are left with arguments for and against the presence of inhibitory muscarinic M_1 and M_4 heteroreceptors in rabbit vas deferens. Perhaps the data may be reconciled by multiple, M_1 and M_4, prejunctional muscarinic receptors.

Further experiments are needed to clarify this issue. To reflect this lack of final definition of the prejunctional rabbit vas deferens receptor subtype, in this paper we will use the term M_1/M_4-like for these receptors.

Preliminary accounts of this study have been communicated to the German Society for Pharmacology and Toxicology, Hamburg, September 1988 (Mutschler *et al.*, 1988) and to the IUPHAR, Amsterdam, July 1990 (Feifel *et al.*, 1990).

Methods

Radioligand binding experiments: general considerations

Protein concentrations were determined according to Lowry *et al.* (1951), using bovine serum albumin as standard. Male Wistar albino rats were killed by decapitation and the brain, heart or pancreas immediately removed. Calf superior cervical ganglia were obtained from a regional slaughterhouse and brought into the laboratory within 70 min. NB-OK 1 cells were cultured as previously described (Waelbroeck *et al.*, 1988), in RPMI 1640 medium enriched with 10% foetal calf serum, 100 units ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin. All the following operations were performed at 4°C.

Preparation of the homogenates or crude membranes used for binding studies

M_1 receptors For [^{3}H]-NMS binding experiments, the NB-OK 1 cells were rinsed, harvested and centrifuged in 20 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl and 1 mM EDTA, resuspended and homogenized in 20 mM Tris/HCl buffer (pH 7.5) enriched with 5 mM $MgCl_2$ and stored in liquid nitrogen.

The rat brain cortex was homogenized in 15 ml of 20 mM Tris/HCl buffer (pH 7.5) enriched with 250 mM sucrose, in a glass-teflon homogenizer (7 up and down strokes). The resulting homogenate was stored in liquid nitrogen until use, and diluted 5 fold with the same buffer immediately before the experiment.

After separation of surrounding tissue, the calf superior cervical ganglia were minced with scissors and homogenized in ice-cold buffer solution (30 g tissue wet weight/100 ml of 50 mM Tris/HCl buffer (pH 7.4) enriched with 120 mM NaCl and 5 mM $MgCl_2$), with an Ultra-Turrax (maximal speed, for 2 × 30 s). The homogenate was centrifuged at 160 *g* for 10 min, and the pellet was ground a second time and processed as before. The supernatants were combined and centrifuged for 1 h at 100,000 *g*. The pellets (corresponding to 20% of the original wet weight of the original tissue) were stored at − 20°C until use. For binding studies, the pellets were resuspended in ice-cold buffer with a Potter-Elvejhem homogenizer.

M_2 receptors The rat heart was rinsed in isotonic NaCl, then homogenized in 2.5 ml of 20 mM Tris/HCl buffer (pH 7.5), enriched with 250 mM sucrose, with an Ultra-Turrax (maximal speed, for 5 s) followed by addition of 12.5 ml of the same buffer, 7 up and down strokes with a glass-teflon homogenizer and filtration on 2 layers of medical gauze.

M_3 receptors The rat pancreas was minced with scissors and homogenized with a glass-teflon homogenizer (7 up and down strokes) in 8 ml of 300 mM sucrose enriched with 0.2 mg ml^{-1} bacitracin and 500 kallikrein inhibitor units (KIU) ml^{-1} of trasylol. The resulting homogenate was immediately filtered over two layers of medical gauze and diluted 11 fold with the incubation buffer (66 mM sodium phosphate buffer, pH 7.4, enriched with 2.6 mM $MgCl_2$ and with 13 mg ml^{-1} bovine serum albumin, 0.24 mg ml^{-1} bacitracin and 600 KIU ml^{-1} of trasylol).

M_4 receptors Rat striatum homogenates were prepared in 2 ml of 20 mM Tris/HCl buffer (pH 7.5), enriched with 250 mM sucrose, and stored in liquid nitrogen until use. The homogenates were diluted 20 fold with the homogenization buffer, immediately before the incubation with [^{3}H]-NMS.

Binding studies

Most binding studies were performed at 25°C, at equilibrium, in a 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$ and 1% bovine serum albumin (and, for incubations with pancreas, 0.2 mg ml^{-1} bacitracin and 500 KIU ml^{-1} trasylol), [^{3}H]-NMS or [^{3}H]-pirenzepine and the indicated unlabelled drugs concentrations, in a total volume of 1.2 ml. In contrast, [^{3}H]-telenzepine binding studies were performed at 37°C, in a 50 mM Tris/HCl buffer enriched with 120 mM NaCl, 5 mM $MgCl_2$, [^{3}H]-telenzepine, and the indicated unlabelled drugs concentrations, in a total volume of 350 µl.

To measure [^{3}H]-pirenzepine binding to brain cortex homogenates, we used 80 µl of the homogenate, corresponding to a protein concentration of 200 µg per assay. A 2 h incubation allowed equilibrium binding. The [^{3}H]-pirenzepine concentration was 5 nM. To measure [^{3}H]-telenzepine binding to calf superior cervical ganglia, we used 250 µl of the homogenate (corresponding to 1.25 mg crude membrane wet weight). The [^{3}H]-telenzepine concentration was 0.8 nM. A 2 h incubation allowed equilibrium binding. To measure [^{3}H]-NMS binding to NB-OK 1 homogenates, we used 80 µl of homogenate, corresponding to about 200 µg protein per assay. The incubation period was 2 h to achieve equilibrium binding in the presence of 0.25 nM [^{3}H]-NMS. For incubations with rat heart homogenates, we used 80 µl of the homogenate, corresponding to 400 to 500 µg protein per assay. The 2 h incubation period was sufficient to allow equilibrium binding. The [^{3}H]-NMS concentration used was 1.0 nM. To analyse [^{3}H]-NMS binding to pancreas homogenates, we used 80 µl of homogenate, corresponding to 800 µg pancreas protein per assay. A 4 h incubation period was necessary to allow full equilibration of [^{3}H]-NMS binding at 25°C. The tracer concentration used was 0.25 nM. In rat striatum homogenates, [^{3}H]-NMS labels M_1, very few M_2 and M_3, and a majority of M_4 sites. It dissociates faster from the M_1 and M_2 sites. (Waelbroeck *et al.*, 1987b; 1990). We preincubated 80 µl of the homogenate (equivalent to about 30 µg protein) in a total volume of 1.2 ml, in the presence of [^{3}H]-NMS and unlabelled drugs. A 2 h preincubation period allowed equilibrium binding. We then added 1 µM atropine and allowed tracer dissociation for 35 min before filtration. This procedure allowed us to investigate tracer binding to the striatum M_4 sites. The tracer concentration used in these experiments was 0.25 nM.

The incubations were stopped by addition of 2 ml ice-cold filtration buffer (50 mM sodium phosphate buffer, pH 7.4). Bound and free tracer were immediately separated by filtration on glass fibre GFC filters presoaked overnight in 0.05% polyethyleneimine. The samples were rinsed three times with filtration buffer. The filters were then dried, and the bound radioactivity counted by liquid scintillation. Non specific [^{3}H]-telenzepine, [^{3}H]-NMS or [^{3}H]-pirenzepine binding was defined as tracer bound in the presence of 10 µM atropine.

For more experimental details, see Waelbroeck *et al.* (1987a,b; 1988; 1990) and Feifel *et al.* (1991).

Analysis of binding data

All competition curves were repeated three times in duplicate. IC_{50} values were determined by a computer-aided procedure described by Richardson & Humrich (1984), assuming the existence of only one receptor subtype. K_i values were determined from IC_{50} values by the Cheng & Prusoff (1973) equation, using the radioligand K_D values obtained in the same tissue. The pK_i values shown in Table 1 correspond to $-\log K_i$ values.

The standard deviations of pK_i values were equal to or below 0.1 log unit.

Pharmacological experiments

Rabbit vas deferens Male New Zealand white rabbits (2.5–3.0 kg) were killed by i.v. injection of 120 mg kg^{-1} pentobarbitone sodium. The vasa deferentia were excised, dissected free of connective tissue and divided into four segments of approximately 1.5 cm length. The preparations were set up in 7 ml organ baths containing modified Krebs buffer which consisted of (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and (+)-glucose 11.1; 1 μM yohimbine was included to block α_2-adrenoceptors. The bathing fluid was maintained at pH 7.4, 31°C, and aerated with 95% O_2/5% CO_2. A basal tension of 750 mg was applied. After a 30 min equilibration period, isometric twitch contractions were elicited by electrical field stimulation (0.05 Hz, 0.5 ms, 30 V) with platinum electrodes. These effects were concentration-dependently inhibited by the M_1 receptor agonists 4-(3-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343) and 4-(4-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343) (Eltze, 1988; Eltze *et al.*, 1988; Boddeke, 1991; Lambrecht *et al.*, 1993). The neurogenic contractions were measured by a force-displacement transducer connected to a Hellige amplifier and a Rikadenki polygraph.

Guinea-pig atria and ileum Adult guinea-pigs of either sex were killed by cervical dislocation and the organs required were removed. Left atria and strips of ileal longitudinal smooth muscle of 1.5 cm length (Paton & Zar, 1968) were set up in 6 ml organ baths, under 500 mg tension, in oxygenated (95% O_2/5% CO_2) Tyrode solution, composed of (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42 and (+)-glucose 5.6. All experiments were conducted at 32°C (pH 7.4). The agonist used was arecaidine propargyl ester (Mutschler & Lambrecht, 1984; Barlow & Weston-Smith, 1985; Lambrecht *et al.*, 1993).

Left atria were electrically paced by means of platinum electrodes (2 Hz, 3 ms duration, supramaximal voltage). Atrial responses to the agonist were measured as changes in isometric tension, and these effects were expressed as the percentage inhibition of the force of contraction. Responses of ileal longitudinal smooth muscle strips to arecaidine propargyl ester were measured as isotonic contractions. The effects in atria and ileum were recorded as with the rabbit vas deferens.

Antagonist affinities

After 1 h equilibration, concentration-response curves were obtained by cumulative addition of the agonists (Van Rossum, 1963). When these responses were constant, concentration-response curves were repeated in the presence of at least 3 concentrations (in most cases, log interval = 0.48) of antagonists, allowing 15–45 min equilibration time. Each concentration of antagonist was tested 3 to 5 times and the ratios of agonist molar EC_{50} values obtained in the presence and absence of antagonists were calculated. The slopes of the Arunlakshana-Schild plots (Arunlakshana & Schild, 1959) were determined by linear regression using the method of least squares. pA_2 values were estimated by fitting to the data the best straight line with a slope of unity (Arunlakshana & Schild, 1959; Tallarida *et al.*, 1979).

To assess whether *o*-methoxy-sila-hexocyclium (**3b**) had actions at a site in addition to that occupied by the M_1-selective agonist, McN-A-343, in rabbit vas deferens, combination antagonist studies were performed on the basis of dose-ratio analysis (Paton & Rang, 1965). The concentration-response curves to McN-A-343 in these experiments were obtained under control conditions and then in the presence of compound **3b** (50 μM) or pirenzepine (50 μM) alone or with the two antagonists combined. The contact time for pirenzepine and **3b** was always 30 min. The experimental data for individual antagonist applications were used to calculate dose-ratios (DR) expected for the combination for two models: expected single-site = $DR_1 + DR_2 - 1$ and expected independent sites = $DR_1 \times DR_2$. These dose-ratios were compared with the experimentally observed combined mean dose-ratio.

Data analysis

All data are presented as means ± s.e.mean of the indicated number (n) of experiments. Linear regression analyses were

Table 1 pK_i values obtained in binding studies on NB-OK 1 cells, rat brain cortex and calf superior cervical ganglia (CSCG; M_1 sites), rat heart (M_2 sites), rat pancreas (M_3 sites) and to the slowly-dissociating (M_4) sites in rat striatum[a]

Antagonist	*NB-OK 1*	*M_1 Cortex*	*CSCG*	*M_2 Heart*	*M_3 Pancreas*	*M_4 Striatum*
1a Hexocyclium[b]	8.8	8.9[c]		7.7	8.4	8.8
1b Sila-hexocyclium[b]	8.9	8.8[c]	8.9	7.6	8.4	8.8
2a Demethyl-hexocyclium[b]	8.0	8.0		6.7	7.4	8.0
2b Demethyl-sila-hexocyclium[b]	7.9	7.9		6.6	7.4	8.0
3a *o*-Methoxy-hexocyclium	6.8	6.8		5.9	6.2	6.8
3b *o*-Methoxy-sila-hexocyclium	7.1	7.1	6.9	6.3	6.6	7.0
4 Demethyl-*o*-methoxy-sila-hexocyclium	6.5	6.4	6.7	5.3	5.6	6.2
5 *p*-Fluoro-sila-hexocyclium	7.9	7.9	7.8	6.9	7.3	7.9
6 *p*-Chloro-sila-hexocyclium	7.4	7.5	7.4	6.4	7.0	7.1

[a]The standard deviations of the pK_i values were typically ± 0.1 log unit, always below 0.15 log unit. The Hill coefficients varied between 0.95 and 1.05 (standard deviation ± 0.1) except for the [^{3}H]-pirenzepine/**1a**/**1b** competition curves in brain cortex (Hill coefficients of 0.85 were obtained for these compounds).

[b]The pK_i values of these compounds in NB-OK 1 cells, pancreas and heart homogenates have been published previously (Waelbroeck *et al.*, 1989).

[c]The competition curves were biphasic (see text). The pK_i values indicated correspond to the major 'subclass' of binding sites (85 to 90% of tracer binding).

carried out by the method of least squares (Tallarida *et al.*, 1979). Differences between mean values were tested for statistical significance by Student's t test; $P<0.05$ was accepted as being significant.

Compounds

[^{3}H]-N-methylscopolamine methyl chloride ([^{3}H]-NMS, 80 to 85 Ci mmol^{-1}) was obtained from Amersham International (Bucks, England). [^{3}H]-pirenzepine (80 to 85 Ci mmol^{-1}) was from New England Nuclear, Boston, MA, U.S.A. Racemic [^{3}H]-telenzepine (85 Ci mmol^{-1}) was a generous gift from Byk Gulden Lomberg, Konstanz, Germany, 4-(3-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343), atropine sulphate, bovine serum albumin (Fraction V) and polyethyleneimine were from Sigma Chemical Co. (St Louis, MO, U.S.A.), and glass fibre filters GFC from Whatmann (Maidstone, England). All other reagents were of the highest grade available. Pirenzepine dihydrochloride was a generous gift from Thomae (Biberach, Germany). 4-(4-Chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343; Nelson *et al.*, 1976), arecaidine propargyl ester (Mutschler & Hultzsch, 1973), hexocyclium (**1a**; as methyl sulphate; Zaugg *et al.*, 1958), sila-hexocyclium (**1b** as methyl sulphate; Tacke *et al.*, 1989a), demethyl-hexocyclium (**2a**; Zaugg *et al.*, 1958), demethyl-sila-hexocyclium (**2b**; as dihydrochloride), *o*-methoxy-hexocyclium (**3a**; as methyl sulphate; Strohmann, 1990), *o*-methoxy-sila-hexocyclium (**3b**; as methyl sulphate; Tacke *et al.*, 1989b) and demethyl-*o*-methoxy-sila-hexocyclium (**4**; as dihydrochloride; Tacke *et al.*, 1989b) were synthesized in our laboratories according to the literature. *p*-Fluoro-sila-hexocyclium (**5**; as methyl sulphate; m.p. 108°C) and *p*-chloro-sila-hexocyclium (**6**; as methyl sulphate; m.p. 149–150°C) were prepared by analogy to the parent compound sila-hexocyclium (**1b**; Tacke *et al.*, 1989b). Compounds **5** and **6** were characterized by ^{1}H-NMR, ^{13}C-NMR and EI-MS measurements as well as by elemental analyses.

Compounds **1a/1b–3a/3b** and **4–6** (see Figure 1) possess a centre of chirality, the carbinol and silanol, respectively, carbon and silicon atom, and therefore exist in two enantiomers. In this study, all compounds were used as racemates.

Results

Radioligand binding studies

All compounds inhibited tracer binding to muscarinic receptors. Most competition curves did not deviate significantly from results expected for competitive inhibition of tracer binding to a single receptor (see for example Figures 2 and 3). The only exceptions to this rule were the [^{3}H]-pirenzepine/hexocyclium (**1a**) and [^{3}H]-pirenzepine/sila-hexocyclium (**1b**) competition curves in rat cortex (Figure 2): these two quaternary compounds inhibited [^{3}H]-pirenzepine binding to 85 or 90% of the receptors with a high affinity, and to 10 or 15% of the receptor with a very low affinity (Figure 2). However, the affinities of compounds **1a/1b–3a/3b** and **4–6** for the majority of M_1 receptors in rat cortex were very similar to that for M_1 receptors in NB-OK 1 cells and calf superior cervical ganglia (Table 1, Figures 2 and 3). The structure-affinity relationships of the hexocyclium and sila-hexocyclium derivatives at each receptor subtype are summarized in Table 1 and Figure 4.

The hexocyclium and sila-hexocyclium derivatives studied in this work, like the parent compounds hexocyclium and sila-hexocyclium, had a clear preference for M_1, M_3 and M_4 over M_2 receptors (Table 1). The same affinity profile ($M_1 \approx M_3 \approx M_4 > M_2$) has been reported for hexocyclium as well as for sila-hexocyclium at cloned muscarinic m_1–m_4 receptors expressed in CHO-K1 cells (Buckley *et al.*, 1989).

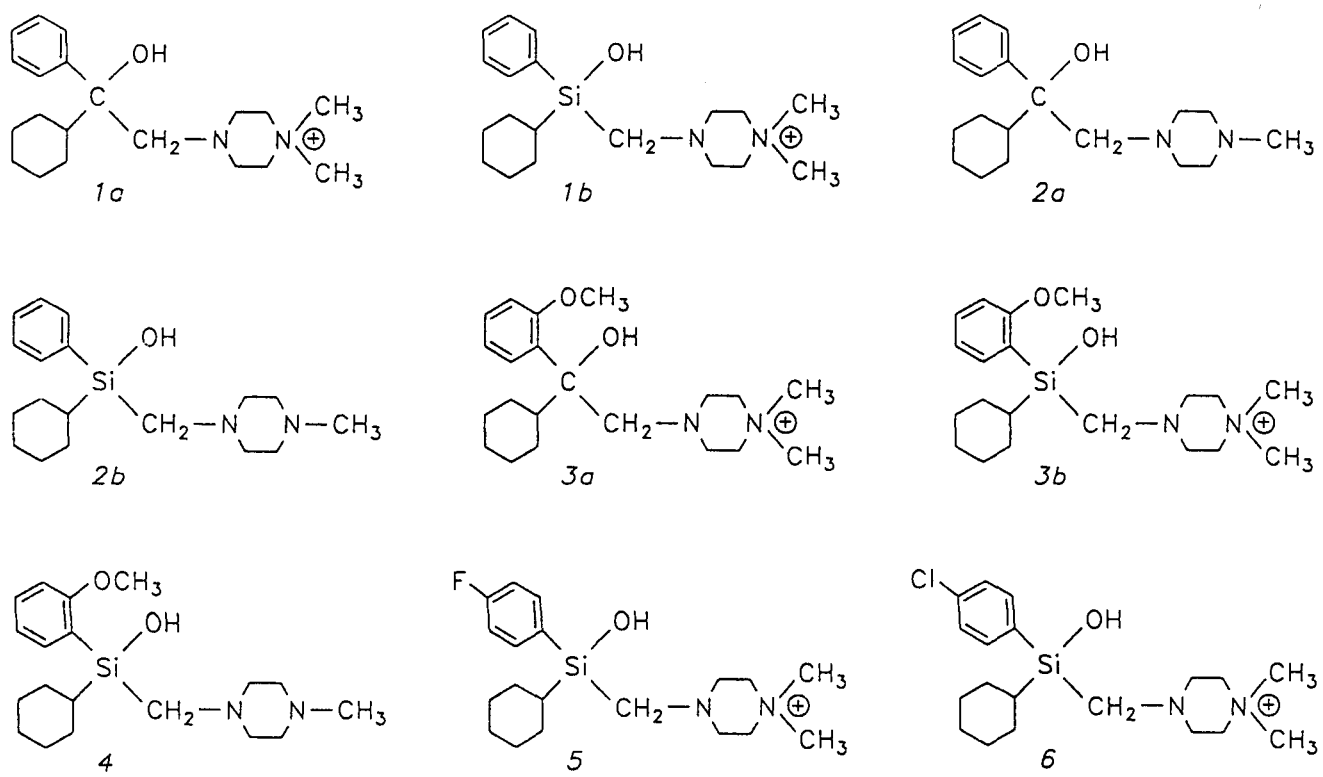


Figure 1 Structural formulas of the compounds studied: hexocyclium (**1a**) and sila-hexocyclium (**1b**), demethyl-hexocyclium (**2a**) and demethyl-sila-hexocyclium (**2b**), *o*-methoxy-hexocyclium (**3a**) and *o*-methoxy-sila-hexocyclium (**3b**), demethyl-*o*-methoxy-sila-hexocyclium (**4**), *p*-fluoro-sila-hexocyclium (**5**) and *p*-chloro-sila-hexocyclium (**6**). All compounds possess a centre of chirality and therefore exist in two enantiomers. In this study, they were used as racemates.

Pharmacological studies

All compounds antagonized the inhibition of neurogenic contractions of rabbit vas deferens by McN-A-343 or 4-Cl-McN-A-343 as well as the negative inotropic effects in electrically stimulated guinea-pig left atria and the contractions of the guinea-pig ileum to arecaidine propargyl ester. This is shown for *o*-methoxy-sila-hexocyclium (**3b**) in Figure 5. With the exception of demethyl-*o*-methoxy-sila-hexocyclium (**4**) in atria (Table 2), there was a concentration-dependent parallel shift to the right of agonist dose-response curves without either the basal tension or the maximal responses being affected. The Schild plots were linear throughout the antagonist concentration-range studied, and slopes were not significantly different from unity ($P > 0.05$), except for *o*-methoxy-sila-hexocyclium (**3b**) at M_2 receptors (Figure 5 and Table 2). The pA_2 value of **3b** in guinea-pig atria (6.41) might therefore be regarded as a purely experimental quantity. However, the binding affinity of **3b** to M_2 receptors in rat heart ($pK_i = 6.3$; Table 1) was very similar to that obtained in functional studies in guinea-pig atria. In the radioligand binding studies (Figure 3), competition curves with **3b** in rat heart homogenates did not deviate significantly from results expected for competitive inhibition of [^{3}H]-NMS binding at a single binding site. Thus, compounds **1a**/**1b**–**3a**/**3b** and **4**–**6** were apparently simple competitive antagonists in the preparations studied. The pA_2 values are shown in Table 2.

Hexocyclium (**1a**), sila-hexocyclium (**1b**) and the derivatives studied in this work had a greater affinity for the M_1/M_4-like heteroreceptors in rabbit vas deferens as compared to guinea-pig (M_2) receptors (Table 2). Most of the compounds also had a higher affinity for guinea-pig ileum M_3 receptors (Table 2). *o*-Methoxy-sila-hexocyclium (**3b**) had a different selectivity pattern: it had a 30 fold and 100 fold higher affinity for M_1/M_4-like receptors in rabbit vas deferens over M_3 receptors in guinea-pig ileum and M_2 receptors in guinea-pig atria, respectively (Table 2).

To investigate whether *o*-methoxy-sila-hexocyclium (**3b**) was interacting solely at the muscarinic receptor in rabbit vas deferens occupied by the agonist McN-A-343, combination concentration-ratio experiments were undertaken with pirenzepine using the method of Paton & Rang (1965).

The concentration-ratios, determined in separate tissues

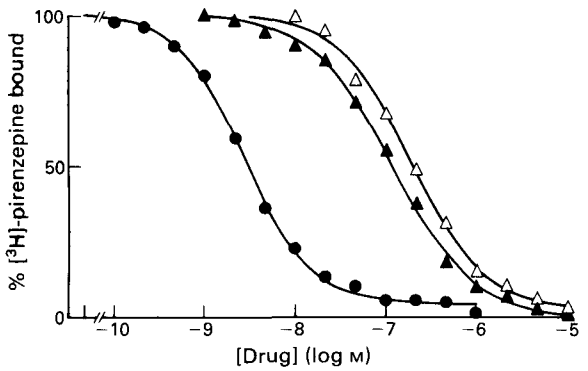


Figure 2 [^{3}H]-pirenzepine competition curves in rat cortex were obtained as explained in Methods, using **1a** or **1b** (●) (the experimental data were within 2% of the means; we therefore used a single symbol for the two compounds), **3a** (△) and **3b** (▲).

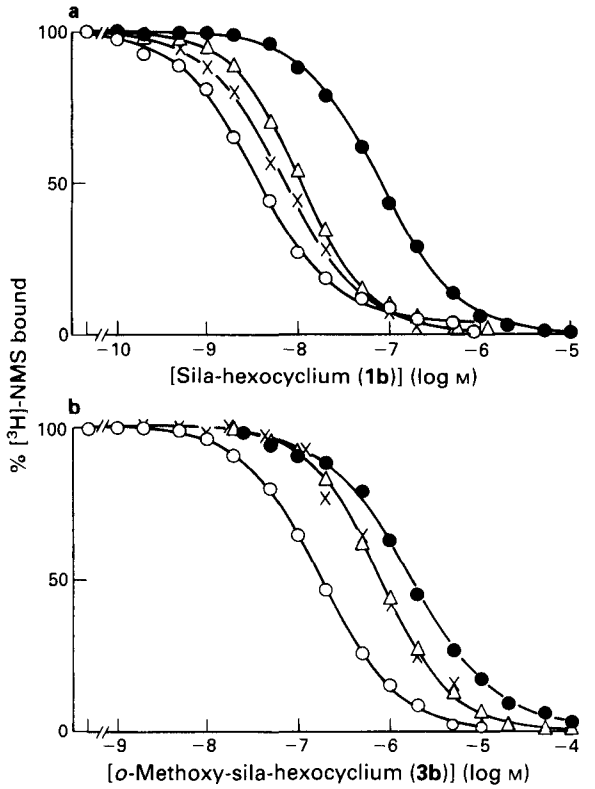


Figure 3 Competition curves obtained at four muscarinic receptor subtypes: (a) [^{3}H]-NMS/sila-hexocyclium (**1b**) competition curves were obtained as explained in Methods, using NB-OK 1 cells (○), heart (●), pancreas (△) or striatum M_4 (x) sites. (b) [^{3}H]-NMS/*o*-methoxy-sila-hexocyclium (**3b**) competition curves were obtained as explained in Methods, using NB-OK 1 cells (○), heart (●), pancreas (△), or striatum M_4 (x) sites.

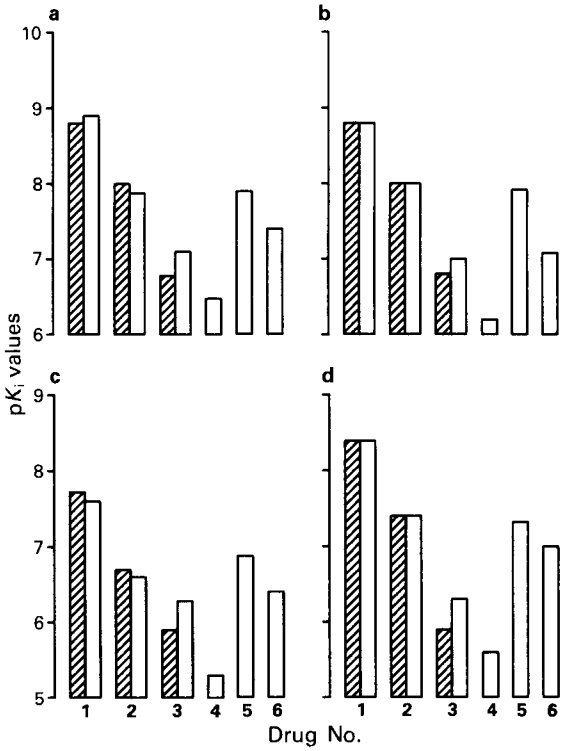


Figure 4 Structure-activity relationships for binding of compounds structurally related to hexocyclium (hatched columns) or to sila-hexocyclium (open columns). The receptors investigated were: NB-OK-1 receptors (M_1) (a); rat heart (M_2) (c); rat pancreas (M_3) (d); and M_4 in rat striatum (b). The compounds were: hexocyclium and sila-hexocyclim (**1**, open and hatched bars), demethyl-hexocyclium and demethyl-sila-hexocyclium (**2**, open and hatched bars), *o*-methoxy-hexocyclium and *o*-methoxy-sila hexocyclium (**3**, open and hatched bars), demethyl-*o*-methoxy-sila-hexocyclium (**4**), *p*-fluoro-sila-hexocyclium (**5**) and *p*-chloro-sila-hexocyclium (**6**). Their pK_i values at the four muscarinic receptor subtypes were measured as explained in Methods.

Table 2 Affinity profiles of hexocyclium, sila-hexocyclium and related muscarinic antagonists at muscarinic M_1/M_4-like receptors in rabbit vas deferens, M_2 receptors in guinea-pig atria, and M_3 receptors in guinea-pig ileum

Antagonist	*M_1/M_4-like Rabbit vas deferens*	*M_2 Guinea-pig atria*	*M_3 Guinea-pig ileum*
1a Hexocyclium	8.89 ± 0.03	7.75 ± 0.03[a]	8.49 ± 0.04[a]
	(1.03 ± 0.07)	(0.97 ± 0.06)	(1.05 ± 0.07)
1b Sila-hexocyclium	9.01 ± 0.07	7.57 ± 0.04[a]	8.78 ± 0.05[a]
	(1.04 ± 0.09)	(0.94 ± 0.07)	(1.04 ± 0.09)
2a Demethyl-hexocyclium	7.69 ± 0.06	6.51 ± 0.08[a]	7.63 ± 0.03[a]
	(0.94 ± 0.11)	(1.05 ± 0.09)	(1.08 ± 0.06)
2b Demethyl-sila-hexocyclium	7.73 ± 0.12	6.65 ± 0.04[a]	7.87 ± 0.04[a]
	(1.11 ± 0.09)	(1.02 ± 0.11)	(0.94 ± 0.08)
3a *o*-Methoxy-hexocyclium	7.23 ± 0.04	6.18 ± 0.05	6.77 ± 0.02
	(0.95 ± 0.12)	(0.89 ± 0.10)	(1.07 ± 0.04)
3b *o*-Methoxy-sila-hexocyclium	8.39 ± 0.05	6.41 ± 0.01[b]	6.96 ± 0.03[b]
	(1.01 ± 0.09)	(1.25 ± 0.06)[c]	(0.93 ± 0.06)
4 Demethyl-*o*-methoxy-sila-hexocyclium	6.44 ± 0.06	5.51 ± 0.03[d]	6.36 ± 0.04
	(1.11 ± 0.14)	–	(0.93 ± 0.06)
5 *p*-Fluoro-sila-hexocyclium	8.32 ± 0.07	7.40 ± 0.04	8.25 ± 0.06
	(1.04 ± 0.14)	(0.92 ± 0.07)	(0.99 ± 0.10)
6 *p*-Chloro-sila-hexocyclium	7.79 ± 0.04	6.93 ± 0.02	7.67 ± 0.04
	(0.98 ± 0.07)	(1.00 ± 0.03)	(1.07 ± 0.06)

pA_2 values and slopes of Schild plots (in parentheses) are presented as means ± s.e.mean ($n = 3-5$).
[a]Data taken from Waelbroeck *et al.* (1989).
[b]Data taken from Lambrecht *et al.* (1988).
[c]Significantly different from unity ($P<0.05$). [d]Only one concentration (10 μM: $n = 5$) was investigated due to the negative inotropic effects of the compound itself at higher concentrations. The pA_2 values were therefore determined from the individual dose-ratios according to Tallarida *et al.* (1979).

($n = 5$) for pirenzepine (50 nM; $DR_1 = 11.8 \pm 1.5$, $pA_2 = 8.32 \pm 0.05$) and compound **3b** (50 nM; $DR_2 = 7.4 \pm 0.6$, $pA_2 = 8.10 \pm 0.01$) were first ascertained. Again, in separate tissues ($n = 5$), the effect of compound **3b** was determined in the presence of pirenzepine ($DR = 18.3 \pm 2.4$). This combined mean dose-ratio obtained experimentally was very close to that calculated for the two antagonists acting by competing with the agonist for the receptor site ($DR_1 + DR_2 - 1 = 19.2$). The results were not consistent ($P<0.05$, unpaired t test) with a multiplication of dose-ratios ($DR_1 \times DR_2 = 87.0$) which would be expected if the two antagonists interacted at independent sites.

Discussion

The native muscarinic receptors are currently divided, in binding and functional studies, into four subtypes (M_1-M_4), the M_1 receptors being recognized by their higher affinity for pirenzepine (see, Hulme *et al.*, 1990; Waelbroeck *et al.*, 1990; Lazareno *et al.*, 1990; Dörje *et al.*, 1991; Doods *et al.*, 1993; Lazareno & Birdsall, 1993; Levine & Birdsall, 1993).

As pointed out some years ago by Barlow & Chan (1982), the fact that pirenzepine is a tertiary amine may lead to problems in determining its antimuscarinic potency. Indeed, titration of the amino group of pirenzepine by variation of the pH of the bath fluid affects markedly its affinity for muscarinic receptors in smooth muscle (Barlow & Chan, 1982). *o*-Methoxy-sila-hexocyclium (**3b**) is the first M_1-selective quaternary muscarinic antagonist in functional studies (Lambrecht *et al.*, 1988; Boddeke *et al.*, 1989; Polidori *et al.*, 1990). It has a large functional $M_1 > M_3$ selectivity, and an even greater $M_1 > M_2$ selectivity than pirenzepine. With this in mind, we decided to measure its affinity for four muscarinic receptor subtypes, and to investigate the structure-activity relationships of various tertiary and quaternary hexocyclium and sila-hexocyclium derivatives (Figure 1) at different muscarinic receptor subtypes. These studies were also performed as a part of our systematic investigations on carbon/silicon bioisosterism (Tacke & Zilch, 1986; Tacke & Linoh, 1989; Waelbroeck *et al.*, 1989).

General considerations

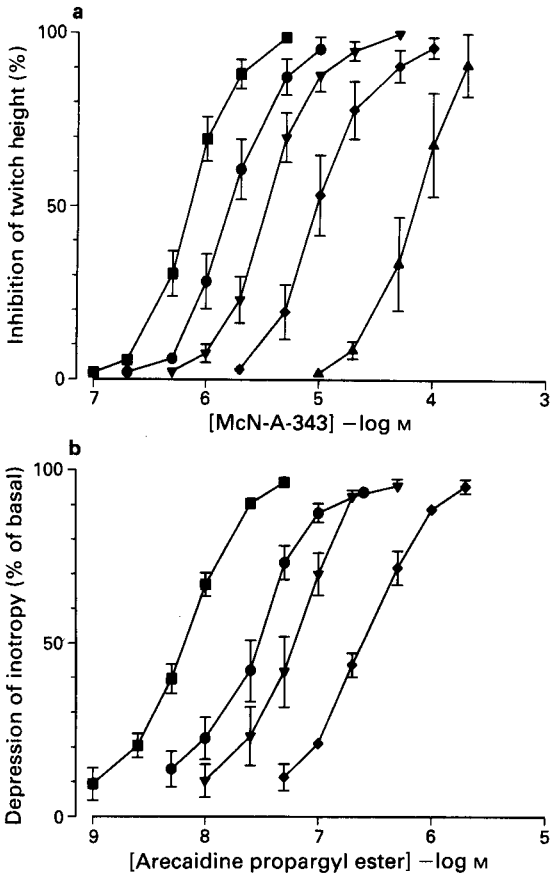
In functional experiments, hexocyclium, sila-hexocyclium and their derivatives behaved as competitive muscarinic antagonists (Figure 5 and Table 2).

In binding studies, most of the competition curves were also compatible with competitive antagonism at a single site (Figures 2 and 3). Hexocyclium and sila-hexocyclium, however, recognized a small fraction (10–15%) of the [^{3}H]-pirenzepine-labelled receptors with a very low affinity (Figure 2). We observed the same type of competition curves with other quaternary antagonists having a high affinity (results not shown), using either [^{3}H]-quinuclidinyl benzylate or [^{3}H]-pirenzepine as tracer, i.e. two tertiary amines. As demonstrated by Ellis (1988), this population of receptors with very low affinity for quaternary antagonists is not correlated with the M_1, M_2, M_3 or M_4 receptor category. We are not aware of the existence of muscarinic receptors having (in functional studies) a comparable affinity profile. We therefore believe that this reflects the existence of binding sites accessible only through a hydrophobic barrier (for instance facing inside closed vesicles) (see Ellis, 1988).

Structure-activity relationships

Sila-substitution of hexocyclium (**1a→1b**) and demethyl-hexocyclium (**2a→2b**) increased only slightly their affinities for the different muscarinic receptor subtypes (Waelbroeck *et al.*, 1989). The effect of sila-substitution on *o*-methoxy-hexocyclium (**3a→3b**) was somewhat greater (Figure 3a and Table 1). In binding studies, we observed a 2 to 3 fold greater affinity for **3b** than for **3a**, at all the muscarinic receptor subtypes (Table 1). In pharmacological studies, **3b** had a 15 fold higher affinity than **3a** for the M_1/M_4-like receptors in rabbit vas deferens (Table 2).

Demethylation of hexocyclium (**1a→2a**), sila-hexocyclium (**1b→2b**) and *o*-methoxy-sila-hexocyclium (**3b→4**) decreased affinities for all muscarinic receptor subtypes by at least 5 fold (Waelbroeck *et al.*, 1989, and this work). This very large effect cannot be explained solely by the stabilization of the positive charge on the quaternary ammonium group, and



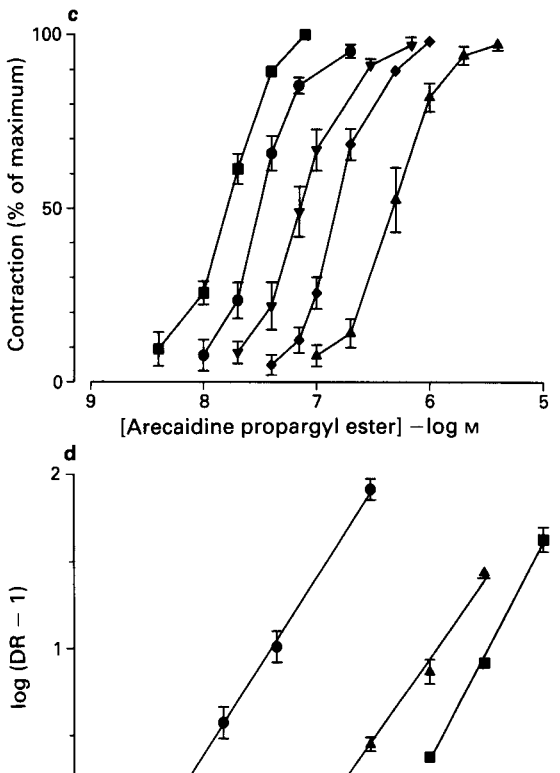


Figure 5 Antagonism of responses to McN-A-343 in rabbit vas deferens and to arecaidine propargyl ester in guinea-pig paced left atria and ileum by different concentrations of *o*-methoxy-sila-hexocyclium (**3b**). Data are means ± s.e.mean. Error bars falling within the area covered by a symbol are not shown. (a) Concentration-response curves for McN-A-343-induced inhibition of neurogenic twitch contractions in rabbit vas deferens in the absence (■; $n = 12$) and presence ($n = 4$) of 5 (●), 15 (▼), 45 (◆) and 300 (▲) nM **3b**. (b) Concentration-response curves for arecaidine propargyl ester-induced negative inotropy in guinea-pig atria in the absence (■; $n = 6$) and presence ($n = 3$) of 1 (●), 3 (▼) and 10 (◆) μM **3b**. (c) Concentration-response curves for arecaidine propargyl ester-induced contractions in guinea-pig isolated ileum longitudinal smooth muscle in the absence (■; $n = 6$) and presence ($n = 3$) of 0.1 (●), 0.3 (▼), 1 (◆) and 3 (▲) μM **3b**. (d) Schild regression from experiments in rabbit vas deferens (●), guinea-pig atria (■) and guinea-pig ileum smooth muscle (▲).

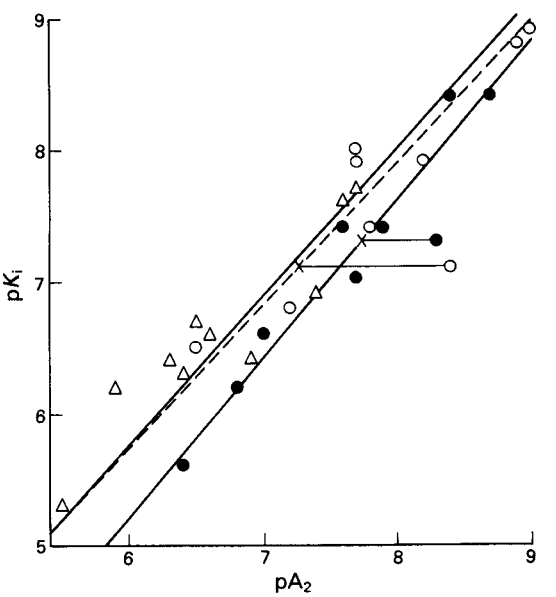


Figure 6 Comparison of the pK_i and pA_2 values of hexocyclium, sila-hexocyclium and related compounds. The pK_i values obtained in NB-OK-1 cells were compared to pA_2 values in rabbit vas deferens (○), the pK_i values obtained in rat heart with the pA_2 values in guinea-pig atria (△) and the pK_i values obtained in rat pancreas, with the pA_2 values in guinea-pig ileum (●). The lines were obtained by linear regression of the data, excluding *o*-methoxy-sila-hexocyclium (**3b**) on the M_1–M_1/M_4-like receptors, and *p*-fluoro-sila-hexocyclium (**5**) on the M_3 receptors.

therefore suggests that this ammonium group fits in a hydrophobic pocket.

Substitutions of the phenyl ring in the *para*-position (*p*-fluoro- and *p*-chloro-derivatives, **5** and **6**) and in the *ortho*-position (*o*-methoxy derivatives, **3a**, **3b** and **4**) decreased the affinities of the respective parent drugs **1a**, **1b** and **2b** at the four muscarinic receptor subtypes (Figure 4). The affinity loss due to replacement of a hydrogen by a (small) fluorine atom varied between 5 and 10 fold, depending on the receptor subtype studied. This suggested that the introduction of a fluorine atom not only induced steric repulsions between the phenyl group and the muscarinic binding site, but probably also modified the electronic properties of the whole phenyl ring (polarization), with unfavourable effects on the interaction with the muscarinic receptors.

In binding studies, the affinity loss due to introducing an *o*-methoxy group was very impressive (20 to 160 fold lower affinity). Binding to all subtypes was similarly affected by the modification (compare **1a**→**3a**, **1b**→**3b**, **2b**→**4**). In contrast, *o*-methoxy-sila-hexocyclium (**3b**) had a high potency in pharmacological experiments at M_1 receptors (pA_2 value of 8.31 in rat superior cervical ganglia, Lambrecht *et al.*, 1988; Boddeke *et al.*, 1989) and at M_1/M_4-like receptors in rabbit vas deferens (pA_2 value = 8.39; this study).

Comparison of the binding and pharmacological properties

The affinities of the compounds studied in this work for the M_2 receptors in rat heart (binding) and guinea-pig atria (functional studies) were very similar, both individually and in rank order (Figure 6). The affinities of the compounds for the M_3 receptors in rat pancreas (binding) were consistently 2–3 fold less than in guinea-pig ileum (functional studies). This might explain part of the difference between the pK_i and pA_2 values of *p*-fluoro-sila-hexocyclium, which was 10 fold less potent in binding than in pharmacological studies on M_3 receptors. The binding affinities of most of the compounds for M_1 receptors in rat cortex, human neuroblastoma (NB-OK 1) cells and calf superior cervical ganglia were very close to their potencies obtained in pharmacological assays at M_1/M_4-like heteroreceptors in rabbit vas deferens (Tables 1 and 2 and Figure 6). However, *o*-methoxy-sila-hexocyclium (**3b**) had a significantly lower affinity in binding compared to pharmacological assays (see also Lambrecht *et al.*, 1988; Boddeke *et al.*, 1989; Polidori *et al.*, 1990; Lazareno & Birdsall, 1993). This is particularly frustrating, since *o*-methoxy-sila-hexocyclium had a remarkable selectivity, in pharmacological studies, for the rabbit vas deferens and rat superior cervical ganglia receptors. It is noteworthy that the pA_2 values obtained for hexocyclium (**1a**; 8.89), sila-hexocyclium (**1b**; 9.01), demethyl-sila-hexocyclium (**2b**; 7.73) and *o*-methoxy-sila-hexocyclium (**3b**; 8.39) in rabbit vas deferens (this study) were very similar to those at M_1 receptors in rat superior cervical ganglia (Eltze *et al.*, 1988; Lambrecht *et al.*, 1988: **1a** = 8.8; **1b** = 9.6; **2b** = 7.6; **3b** = 8.3). In addition, the functional affinity of sila-hexocyclium (**1b**; $pA_2 = 8.78$) for muscarinic M_3 receptors in guinea-pig ileum (this study) was very close to reported M_3 affinity data obtained in other tissues (rat parietal cells: K_i–[^{3}H]-NMS binding = 3.2 nM; K_i–inositol phosphate production = 1.5 nM; K_i–[^{14}C]-aminopyrine accumulation = 2.7 nM; Pfeiffer *et al.*, 1990b. Human HT-29 colon carcinoma cells: K_i–[^{3}H]-NMS binding = 3.1 nM; Kopp *et al.*, 1989. Human gastric mucosa: K_i–[^{3}H]-NMS binding = 5.6 nM; Pfeiffer *et al.*, 1990a).

We checked whether there could be an additional action of *o*-methoxy-sila-hexocyclium (other than on muscarinic receptors) in rabbit vas deferens by measuring the dose-ratio for McN-A-343 in the presence either of pirenzepine, of **3b**, or of a combination of the two antagonists. The results confirmed that there is no additional action of compound **3b** which cannot be accounted for by a competitive mechanism of action. We then decided to compare its binding properties to several different M_1 binding sites of different origins (different animal species and different rat brain regions), in the hope of finding an assay system with M_1 receptors having a high affinity for **3b**. However, the binding properties of pirenzepine and of compounds **2a**, **2b**, **3a**, **3b**, **5** and **6** were identical in the three 'M_1' systems used for these studies (NB-OK 1 cells, rat brain cortex and calf superior cervical ganglia; Table 1). *o*-Methoxy-sila-hexocyclium (**3b**)/[^{3}H]-pirenzepine competition curves in three different rat brain regions (cortex, hippocampus and striatum) were also superimposable, and compatible with the existence of a single binding site with low affinity for **3b** (unpublished results).

In the hope of identifying binding sites with a high affinity for **3b**, we attempted to measure binding of racemic [^{3}H]-*o*-methoxy-sila-hexocyclium (70 Ci mmol^{-1}, lot 2423-148; developed in collaboration with Dr S. Hurt, New England Nuclear, Boston, MA, U.S.A.) to calf superior cervical ganglia. The experiments were carried out in the same way as for [^{3}H]-telenzepine binding, and revealed almost 95% non-specific binding. It was therefore impossible to estimate this tracer's ([^{3}H]-**3b**) affinity (unpublished results). It is possible that the data obtained in pharmacological studies reflect the properties of a very small receptor population, undetectable in binding studies. Further experiments are needed to clarify this issue. Here, it is noteworthy that the hexocyclium derivative N-iminomethyl-*N'*-[(2-hydroxy-2-phenyl-2-cyclohexyl)-ethyl]piperazine (DAC 5945) has also been shown to display a high degree (204 fold M_3 over M_2) of functional *in vitro* selectivity (pA_2, M_3/guinea-pig ileum = 8.56, pA_2, M_2/guinea-pig atria = 6.25, pA_2, M_1–M_4-like/rabbit vas deferens = 7.97; Micheletti *et al.*, 1990b; Lambrecht *et al.*, unpublished results), but the binding affinities of DAC 5945 (pK_i values) to muscarinic M_1 (8.3/8.3), M_2 (7.5/7.4), M_3 (8.4/7.9) and M_4 receptors (8.4/8.6) differed only 16 fold (M_4 over M_2; Doods *et al.*, 1993; Waelbroeck *et al.*, unpublished results).

In conclusion, the present study shows that sila-substitution of *o*-methoxy-hexocyclium (**3a**→**3b**) had a greater effect on the binding properties than observed for the carbon/silicon pairs **1a**/**1b** and **2a**/**2b**: this might reflect differences in the relative importance of the bonds formed between the hydroxyl and phenyl groups and muscarinic receptors. Furthermore, the presence of a quaternary ammonium group is important for binding of antagonists of the hexocyclium/sila-hexocyclium type to muscarinic receptors. Substitution of the phenyl group in the *o*- and *p*-positions of hexocyclium and sila-hexocyclium decreased affinity. Although *o*-methoxy-sila-hexocyclium was found to be remarkably M_1 (M_1/M_4-like)- selective in pharmacological studies, we were unable to confirm this property in binding studies.

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