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 M1 receptors (in rat brain, human neuroblastoma (NB-OK 1) cells and calf superior cervical ganglia), rat heart M2 receptors, rat pancreas M3 receptors and M4 receptors in rat striatum, with their functional antimuscarinic properties in rabbit vas deferens (M1/M2-like), guinea-pig atria (M3), and guinea-pig ileum (M4) 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 (M1 = M4 > M3 > M2) had a much lower affinity than sila-hexocyclium for the four receptor subtypes, and discriminated the receptor subtypes more poorly than hexocyclium (M1 = M3 > M4 > M2). This is in marked contrast with the very clear selectivity of o-methoxy-sila-hexocyclium for the prejunctional M1/M4-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 & Birdsal, 1993). Functional and binding experiments revealed the existence of four different native subtypes, termed M1–M4. Comparison of the radioligand properties and functional coupling of the native M1–M4 receptors with those of the expressed m1–m4 proteins showed a good correlation (Hulme et al., 1990; Lazarenko et al., 1990; Waelbroeck et al., 1990; Dörje et al., 1991; Levine & Birdsall, 1993).

Although several drugs have been reported to discriminate the M1 from M2, M3 and M4 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 M1 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 M1, cardiac M2, glandular/smooth muscle M3 and brain M4 sites. To achieve this goal, we analysed the binding properties of the compounds to [3H]-pirenzepine-labelled M1 sites in rat brain cortex, [3H]-telenzepine-labelled M1 sites in calf superior cervical ganglia (Feifel et al., 1991) or [3H]-N-methyl-scopolamine ([3H]-NMS)-labelled M1 sites in calf superior cervical ganglia (Feifel et al., 1990; Eltze et al., 1988), [3H]-NMS-labelled rat cardiac M2 sites (Waelbroeck et al., 1987a), [3H]-NMS-labelled rat pancreas M3 sites (Waelbroeck et al., 1987a) and [3H]-NMS-labelled M4 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., 1993; Lambrecht et al., 1993), M2 receptors in guinea-pig atria, and M3 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 M2, M3 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 M1-selective antagonist pirenzepine (pA2 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 M2/M3-selective antagonist himbacine (pA2...
values = 8.5—8.17; Dörje et al., 1990; Elte et al., 1993; Sagradaro et al., 1993; and (iii) a low affinity for methocarbamone (pA₂ 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 M₁ and M₄ heteroreceptors in rabbit vas deferens. Perhaps the data may be reconciled by multiple, M₁ and M₄, 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 M₃/M₄-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 (Fei1 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 µg ml⁻¹ streptomycin. All the following operations were performed at 4°C.

Preparation of the homogenates or crude membranes used for binding studies

M₁ receptors For [³H]-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 MgCl₂ 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. The homogenates were diluted 20 fold with the homogenization buffer, immediately before the incubation with [³H]-NMS.

M₄ 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 [³H]-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 MgCl₂ and 1% bovine serum albumin (and, for incubations with calf superior cervical ganglia, we used 50 µl ml⁻¹ bovine serum albumin, 0.24 mg ml⁻¹ bacitracin and 600 KIU ml⁻¹ trasylol). The resulting homogenate was immediately filtered over two layers of medical gauze and diluted 10 fold with the incubation buffer (66 mM sodium phosphate buffer, pH 7.4, enriched with 2.6 mM MgCl₂ and with 13 mg ml⁻¹ bovine serum albumin, 0.24 mg ml⁻¹ bacitracin and 600 KIU ml⁻¹ of trasylol).

M₄ 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 [³H]-NMS.

To measure [³H]-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 [³H]-pirenzepine concentration used was 5 nM. To measure [³H]-telenzepine binding to calf superior cervical ganglia, we used 250 µl of the homogenate (corresponding to 1.25 mg crude membrane wet weight). The [³H]-telenzepine concentration was 0.8 nM. A 2 h incubation allowed equilibrium binding. To measure [³H]-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 mM [³H]-NMS. For incubations with heart homogenates, we used 50 µl ml⁻¹ bovine serum albumin, 0.24 mg ml⁻¹ bacitracin and 600 KIU ml⁻¹ trasylol. The [³H]-NMS concentration was 0.08 nM. A 2 h incubation allowed equilibrium binding. The [³H]-NMS concentration used was 1.0 nM. To analyse [³H]-NMS binding to pancreas homogenates, we used 80 µl of homogenate, corresponding to 800 µg protein per assay. A 4 h incubation period was necessary to allow full equilibrium of [³H]-NMS binding at 25°C. The tracer concentration used was 0.25 nM. In rat striatum homogenates, [³H]-NMS labels M₁, very few M₂ and M₄, and a majority of M₅ sites. [³H]-NMS binds faster to the M₁ and M₅ sites. [³H]-Telenzepine and unlabelled drugs concentrations, in a total volume of 1.2 ml. In contrast, [³H]-telenzepine binding studies were performed at 37°C, in a 50 mM Tris/HCl buffer enriched with 120 mM NaCl, 5 mM MgCl₂, [³H]-telenzepine, and the indicated unlabelled drugs concentrations, in a total volume of 350 µl. To measure [³H]-pirenzepine binding to brain cortex homogenates, we used 80 µl of the homogenate, corresponding to about 200 µg protein per assay. The incubation period was 2 h to achieve equilibrium binding in the presence of [³H]-NMS. For incubations with heart homogenates, we used 50 µl ml⁻¹ bovine serum albumin, 0.24 mg ml⁻¹ bacitracin and 600 KIU ml⁻¹ trasylol. The [³H]-pirenzepine concentration was 5 nM. To measure [³H]-telenzepine binding to calf superior cervical ganglia, we used 250 µl of the homogenate (corresponding to 1.25 mg crude membrane wet weight). The [³H]-telenzepine concentration was 0.8 nM. A 2 h incubation allowed equilibrium binding. To measure [³H]-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 [³H]-NMS. For incubations with heart homogenates, we used 50 µl ml⁻¹ bovine serum albumin, 0.24 mg ml⁻¹ bacitracin and 600 KIU ml⁻¹ trasylol. The [³H]-NMS concentration was 0.08 nM.
For more experimental details, see Waelbroeck et al. (1987a,b; 1988; 1990) and Feipel et al. (1991).

Analysis of binding data

All competition curves were repeated three times in duplicate. IC₅₀ values were determined by a computer-aided procedure described by Richardson & Humrich (1984), assuming the existence of only one receptor subtype. Kᵣ values were determined from IC₅₀ values by the Cheng & Prusoff (1977) equation, using the radioligand Kᵣ values obtained in the same tissue. The pKᵣ values shown in Table 1 correspond to - log Kᵣ values. The standard deviations of pKᵣ 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, CaCl₂ 2.5, MgSO₄ 0.6, KH₂PO₄ 1.2, NaHCO₃ 25.0 and (+)-glucose 11.1; 1 μM yohimbine was included to block α-adrenoceptors. The bathing fluid was maintained at pH 7.4, 31℃, and aerated with 95% O₂/5% CO₂. A basal tension of 350 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 M₁ receptor agonist 4-(3-chlorophenylcarbamoyloxy)-2-butylnitrimethylammonium chloride (McN-A-343) and 4-(4-chlorophenylcarbamoyloxy)-2-butylnitrimethylammonium iodide (4-Cl-McN-A-343) (Eltz, 1988; Eltz 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% O₂/5% CO₂) Tyrode solution, composed of (mM): NaCl 137, KCl 1.8, MgCl₂ 1.05, NaHCO₃ 11.9, NaH₂PO₄ 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 EC₅₀ 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. pA₂ 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 M₁-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 = DR₁ + DR₂ - 1 and expected independent sites = DR₁ × DR₂. 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>
<thead>
<tr>
<th>Antagonist</th>
<th>NB-OK 1</th>
<th>M₁ Cortex</th>
<th>CSGC</th>
<th>M₂ Heart</th>
<th>M₃ Pancreas</th>
<th>M₄ Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a Hexocyclium</td>
<td>8.8</td>
<td>8.9³</td>
<td>8.9</td>
<td>7.7</td>
<td>8.4</td>
<td>8.8</td>
</tr>
<tr>
<td>1b Sila-hexocyclium</td>
<td>8.9</td>
<td>8.8³</td>
<td>8.9</td>
<td>7.6</td>
<td>8.4</td>
<td>8.8</td>
</tr>
<tr>
<td>2a Demethyl-hexocyclium</td>
<td>8.0</td>
<td>8.0</td>
<td>6.7</td>
<td>7.4</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>2b Demethyl-sila-hexocyclium</td>
<td>7.9</td>
<td>7.9</td>
<td>6.6</td>
<td>7.4</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>3a o-Methoxy-hexocyclium</td>
<td>6.8</td>
<td>6.8</td>
<td>6.9</td>
<td>6.2</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>3b o-Methoxy-sila-hexocyclium</td>
<td>7.1</td>
<td>7.1</td>
<td>6.9</td>
<td>6.3</td>
<td>6.6</td>
<td>7.0</td>
</tr>
<tr>
<td>4 Demethyl-o-methoxy-sila-hexocyclium</td>
<td>6.5</td>
<td>6.4</td>
<td>6.7</td>
<td>5.3</td>
<td>5.6</td>
<td>6.2</td>
</tr>
<tr>
<td>5 p-Fluro-sila-hexocyclium</td>
<td>7.9</td>
<td>7.9</td>
<td>7.8</td>
<td>6.9</td>
<td>7.3</td>
<td>7.9</td>
</tr>
<tr>
<td>6 p-Chloro-sila-hexocyclium</td>
<td>7.4</td>
<td>7.5</td>
<td>7.4</td>
<td>6.4</td>
<td>7.0</td>
<td>7.1</td>
</tr>
</tbody>
</table>

³The standard deviations of the pKᵣ 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 [³H]pirenzepine/1a/1b competition curves in brain cortex (Hill coefficients of 0.85 were obtained for these compounds).

³The pKᵣ values of these compounds in NB-OK 1 cells, pancreas and heart homogenates have been published previously (Waelbroeck et al., 1989).

³The competition curves were biphasic (see text). The pKᵣ 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, Konstanz, Germany. 4-(3-chlorophenylcarbamoyloxy)-2-butylnitrtrimethylammonium 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-butylnitrtrimethylammonium iodide ($4-CI-McN$-A-343; Nelson et al., 1976), arecaidine propargyl ester (Mutschler & Hultsch, 1973), hexocyclium (1a as methyl sulphate; Zaugg et al., 1958), sila-hexocyclium (1b as methyl sulphate; Tacke et al., 1989a), dimethyl-hexocyclium (2a; Zaugg et al., 1958), dimethyl-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 dimethyl-o-methoxy-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 (Ib; Tacke et al., 1989b). Compounds 5 and 6 were characterized by $^1$H-NMR, $^{13}$C-NMR and El-MS measurements as well as by elemental analyses.

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-OK1 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 ($M_1 \approx M_3 > M_4 > M_2$) 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).
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 M4 receptors (Figure 5 and Table 2). The pA2 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 M1 receptors in rat heart ($pK_A = 6.3$; 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 [3H]-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 pA2 values are shown in Table 2.

Hexocyclium (1a), sila-hexocyclium (1b) and the derivatives studied in this work had a greater affinity for the MdM4-like heteroreceptors in rabbit vas deferens compared to guinea-pig (M2) receptors (Table 2). Most of the compounds also had a higher affinity for guinea-pig ileum M3 receptors (Table 2). o-Methoxy-sila-hexocyclium (3b) had a different selectivity pattern: it had a 30-fold and 100-fold higher affinity for M3/M4-like receptors in rabbit vas deferens over M1 receptors in guinea-pig ileum and M2 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...
pA2 values and slopes of Schild plots (in parentheses) are presented as means ± s.e.mean (n = 3–5).

Data taken from Waelbroeck et al. (1989).

Significantly different from unity (P < 0.05).

Only one concentration (10 μM; n = 5) was investigated due to the negative inotropic effects of the compound itself at higher concentrations. The pA2 values were therefore determined from the individual dose-ratios according to Tallarida et al. (1979).

### Discussion

The native muscarinic receptors are currently divided, in binding and functional studies, into four subtypes (M1–M4). The M2 receptors being recognized by their higher affinity for pirenzepine (see, Hulme et al., 1990; Waelbroeck et al., 1990; Lazarenko et al., 1990; Dörje et al., 1991; Doods et al., 1993; Lazarenko & Birdsal, 1993; Levine & Birdsal, 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 M1-selective quaternary muscarinic antagonist in functional studies (Lambrecht et al., 1988; Boddeke et al., 1989; Polidori et al., 1990). It has a large functional M1 > M3 selectivity, and an even greater M1 > M2 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 part of our systematic investigations on carbon/silicon bioisosterism (Tacke & Zilch, 1986; Tacke & Linoh, 1989; Waelbroeck et al., 1989).

### General considerations

In experimental functions, hexocyclium, sila-hexocyclium and their derivatives behaved as competitive muscarinic antagonists (Figures 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 [3H]-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 [3H]-quinuclidinyl benzylate or [3H]-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 M1, M2, M3 or M4 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 M1/M2-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 pKᵢ and pA₂ values of hexocyclium, sila-hexocyclium and related compounds. The pKᵢ values obtained in NB-OK-1 cells were compared to pA₂ values in rabbit vas deferens (○), the pKᵢ values obtained in rat heart with the pA₂ values in guinea-pig atria (△) and the pKᵢ values obtained in rat pancreas, with the pA₂ values in guinea-pig ileum (●). The lines were obtained by linear regression of the data, excluding o-methoxy-sila-hexocyclium (3b) on the M₁-M₄/M₅-like receptors, and p-fluoro-sila-hexocyclium (5) on the M₂ 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 M₁ receptors (pA₂ value of 8.31 in rat superior cervical ganglia, Lambrecht et al., 1988; Boddeke et al., 1989) and at M₁/M₅-like receptors in rabbit vas deferens (pA₂ value = 8.39; this study).
Comparison of the binding and pharmacological properties

The affinities of the compounds studied in this work for the M₂ 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 M₁ 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 pKi and pA₂ values of p-fluoro-sila-hexocyclium, which was 10 fold less potent in binding than in pharmacological studies on M₁ receptors. The binding affinities of most of the compounds for M₁ 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 M₂/M₃-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 the o-methoxy-sila-hexocyclium (3b; 8.39) in rabbit vas deferens [Boddeke et al., 1989; Polidori et al., 1990; Lazareno & Birdsal, 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 hexocyclium (1b; 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 M₁ 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; pA₂ = 8.78) for muscarinic M₁ receptors in guinea-pig ileum (this study) was very close to reported M₁ affinity data obtained in other tissues (rat parietal cells: Kᵢ⁻[³H]-NMS binding = 3.2 nM; Kᵢ-inositol phosphate production = 1.5 nM; Kᵢ-[³H]-carnitine accumulation = 2.7 nM; Pfeiffer et al., 1990b. Human HT-29 colon carcinoma cells: Kᵢ-[³H]-NMS binding = 3.1 nM; Kopp et al., 1989. Human gastric mucosa: Kᵢ-[³H]-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 M₁ binding sites of different origins (different animal species and different rat brain regions), in the hope of finding an assay system with M₁ 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 'M₁' systems used for these studies (NB-OK 1 cells, rat brain cortex and calf superior cervical ganglia; Table 1). o-Methoxy-sila-hexocyclium (3b) [Prusoff, 1973] 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 [¹H]-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 [¹H]-telenzepine binding, and revealed almost 95% non-specific binding. It was therefore impossible to estimate the tracer's ([¹H]-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)-ethylpiperazine (DAC 5945) has also been shown to display a high degree (204 fold M₂ over M₃) of functional in vitro selectivity (pA₂[M₂/guinea-pig ileum] = 8.56; pA₂[M₂/guinea-pig atria = 6.25, pA₂(M₁-like/rabbit vas deferens = 7.97; Micheletti et al., 1990b; Lambrecht et al., unpublished results), but the binding affinities of DAC 5945 (pKi values) to muscarinic M₁ (8.3/8.3), M₂ (7.5/7.4), M₃ (8.4/7.9) and M₄ receptors (8.4/8.6) differed only 16 fold (M₂ over M₃; Doods et al., 1993; Waebroeck 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 M₁ (M₁/M₁-like)-selective in pharmacological studies, we were unable to confirm this property in binding studies.

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References

CHENG, Y. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (Kᵢ) and the concentration of inhibitor (Iₘ₈) which causes a 50 percent inhibition of an enzymatic reaction. Biochim. Pharmacol., 22, 3099–3108.


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