95

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Binding affinities of hexahydro-difenidol and hexahydro-sila-difenidol analogues at four muscarinic receptor subtypes: constitutional and stereochemical aspects

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Hexahydro-sila-difenidol and eight analogues behaved as simple competitive inhibitors of [^{3}H]N-methyl-scopolamine binding to homogenates from human neuroblastoma NB-OK 1 cells (M_1 sites), rat heart (M_2 sites), rat pancreas (M_3 sites), and rat striatum 'B' sites (M_4 sites). Pyrrolidino- and hexamethyleneimino analogues showed the same selectivity profile as the parent compound. Hexahydro-sila-difenidol methiodide and the methiodide of p-fluoro-hexahydro-sila-difenidol had a higher affinity but a lower selectivity than the tertiary amines. Compounds containing a p-methoxy, p-chloro or p-fluoro substituent in the phenyl ring of hexahydro-sila-difenidol showed a qualitatively similar selectivity profile as the parent compound (i.e., $M_1 = M_3 = M_4 > M_2$), but up to 16-fold lower affinities. o-Methoxy-hexahydro-sila-difenidol has a lower affinity than hexahydro-sila-difenidol at the four binding sites. Its selectivity profile ($M_4 > M_1$, $M_3 > M_2$) was different from hexahydro-sila-difenidol.

Replacement of the central silicon atom of hexahydro-sila-difenidol, p-fluoro-hexahydro-sila-difenidol and their quaternary (N-methylated) analogues by a carbon atom did not change their binding affinities significantly. The four muscarinic receptors showed a higher affinity for the (R)- than for the (S)-enantiomers of hexahydro-difenidol, p-fluorohexahydro-difenidol and their methiodides. The stereoselectivity varied depending on the receptor subtype and drug considered.

Muscarinic receptor subtypes (M_1, M_2, M_3, and (putative) M_4); Muscarinic receptor antagonists (selective); Hexahydro-sila-difenidol analogues; p-Fluoro-hexahydro-sila-difenidol; Stereoselectivity (at muscarinic receptors)

1. Introduction

Muscarinic acetylcholine receptors are currently divided into at least three pharmacologically defined subtypes: M_1, M_2 ($M_{2\alpha}$) and M_3 ($M_{2\beta}$) receptors (for recent reviews, see Mutschler et al., 1987; 1988; Mitchelson, 1988; Levine and Birdsall, 1989). M_1 receptors are typically found in neuronal tissues (central nervous system and autonomic ganglia), M_2 receptors in lower brain areas (cerebellum) and heart, and M_3 receptors in secretory glands and smooth muscle. This classification is based mainly on the different affinities of muscarinic receptor subtypes for antagonists such as pirenzepine ($M_1 > M_3 \geqslant M_2$) (Hammer et al., 1980; Eltze et al., 1988; Waelbroeck et al., 1988), AF-DX 116 (Giachetti et al., 1986; Hammer et al., 1986; Micheletti et al., 1987), methoctramine (Melchiorre et al., 1987; Giraldo et al., 1988) ($M_2 > M_1 > M_3$) and hexahydro-

sila-difenidol (Lambrecht et al., 1987, 1989b; Waelbroeck et al., 1987a; Waelbroeck et al., 1989a; Lazareno and Roberts, 1989) ($M_3 \geqslant M_1 > M_2$). Recently a fourth subtype with high affinity for methoctramine and himbacine, as well as for hexahydro-sila-difenidol and 4-diphenylacetoxy-N-methylpiperidine methiodide has been identified in NG108-15 cells (Michel et al., 1989) and rat forebrain (Waelbroeck et al., 1990). The antagonist binding properties, or the tissue and cell line distribution of these four pharmacologically defined subtypes correspond closely to that of the recently cloned receptor proteins m1 to m4 (Akiba et al., 1988; Bonner et al., 1987; Peralta et al., 1987; Buckley et al., 1989; Dörje et al., 1990; Wess et al., in press).

Using the nonselective antagonist [^{3}H]N-methyl-scopolamine ([^{3}H]NMS) as radioligand, we previously demonstrated that over 80% of the muscarinic receptors in the human neuroblastoma NB-OK 1 cell line are of the M_1 subtype (Waelbroeck et al., 1988), that receptors in rat heart are of the M_2 subtype (Waelbroeck et al., 1987a,b), receptors in rat pancreas belong to the M_3 subtype (Waelbroeck et al., 1987a), and that 85% of the

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'B' binding sites in rat striatum (Waelbroeck et al., 1987b) can be considered as putative M_4 receptors (Waelbroeck et al., 1990).

The first aim of the present study was to examine the binding properties of eight hexahydro-sila-difenidol analogues structurally modified in the cyclic amino group and the phenyl ring (fig. 1), using the four above-mentioned test systems. The functional antimuscarinic properties of these muscarinic antagonists have been reported recently (Lambrecht et al., 1989a,b; Waelbroeck et al., 1989a). All these silicon compounds possess a center of chirality. They were used as racemates since indications were found that silanols (R_3SiOH) may racemize in aqueous solution (Tacke et al., 1987). The second goal of this study was to investigate the importance of absolute configuration for binding of hexahydro-sila-difenidol analogues to muscarinic receptors. To achieve this goal we used the configurationally stable (R)- and (S)-enantiomers of the four carbon analogues of 1b, 4b, 8b and 9b ($\rightarrow$ compounds 1a, 4a, 8a and 9a, Fig. 1). The functional antimuscarinic properties of the (R)- and (S)-enantiomers of hexahydro-difenidol (1a), p-fluoro-hexahydro-difenidol (8a) and their methiodides 4a and 9a have been reported elsewhere (Tacke et al., 1989; Feifel et al., 1990; Lambrecht et al., 1990).

2. Materials and methods

2.1. Cell and tissue preparations

Human NB-OK 1 neuroblastoma cells (a generous gift from Dr. Yanaihara, Shizuoka, Japan) were maintained in RPMI-1640 medium, enriched with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (from Gibco, Gent, Belgium). Twice a week, the cells were detached by trypsin-EDTA (Gibco, Gent, Belgium) and divided in thirds. For [^{3}H]NMS binding experiments, the cells were harvested using a 20 mM sodium phosphate buffer enriched with 1 mM EDTA and 150 mM NaCl (pH 7.4), centrifuged at 500 g for 5 min, resuspended and homogenized in 20 mM Tris-HCl buffer enriched with 5 mM $MgCl_2$ (pH 7.5) in a glass-Teflon homogenizer, and then stored in liquid nitrogen until use.

For cardiac homogenates, male Wistar albino rats (200-250 g) were decapitated, the heart immediately removed and rinsed in 150 mM NaCl. The homogenization buffer contained 20 mM Tris-HCl (pH 7.5) and 250 mM sucrose. Each heart was homogenized in 2 ml of this buffer with an Ultraturrax homogenizer (maximal speed for 5 s at 4°C) followed by further addition of 13 ml of buffer, and seven up and down strokes in a
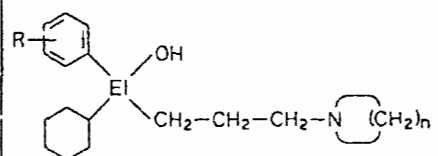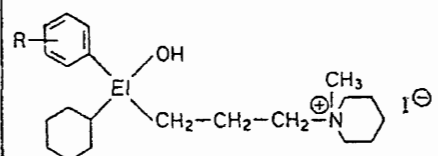
Formula	No.	El	R	n
	(R)- and (S)-1a	C	H	5
	(R/S)-1b	Si	H	5
	(R/S)-2	Si	H	4
	(R/S)-3	Si	H	6
	(R/S)-5	Si	p-CH₃O	5
	(R/S)-6	Si	o-CH₃O	5
	(R/S)-7	Si	p-Cl	5
	(R)- and (S)-8a	C	p-F	5
	(R/S)-8b	Si	p-F	5

Formula	No.	El	R
	(R)- and (S)-4a	C	H
	(R/S)-4b	Si	H
	(R)- and (S)-9a	C	F
	(R/S)-9b	Si	F

Fig. 1. Chemical structure of the carbon/silicon analogues, hexahydro-difenidol (1a), hexahydro-sila-difenidol (1b) and their derivatives (2), (3), hexahydro-difenidol methiodide (4a), hexahydro-sila-difenidol methiodide (4b), p-methoxy-hexahydro-sila-difenidol (5), o-methoxy-hexahydro-sila-difenidol (6), p-chloro-hexahydro-sila-difenidol (7); p-fluoro-hexahydro-difenidol (8a), p-fluorohexahydro-sila-difenidol (8b), p-fluoro-hexahydro-difenidol methiodide (9a) and p-fluoro-hexahydro-sila-difenidol methiodide (9b).

glass-Teflon homogenizer (at 4°C). The homogenate was filtered on two layers of medical gauze and either used fresh, or stored in liquid nitrogen until use.

For rat brain cortex and striatum homogenates the brain was immediately removed and dissected. The cortex and striatum were homogenized in 15 and 2 ml, respectively, of 20 mM Tris-HCl buffer (pH 7.5) enriched with 250 mM sucrose, with a glass-Teflon homogenizer, and stored in liquid nitrogen until use. These homogenates were diluted 20-fold with the homogenization buffer before use for [³H]NMS binding experiments.

For rat pancreas homogenates the organ was immediately removed, minced with scissors and homogenized in a glass-Teflon homogenizer (seven up and down strokes at 4°C) in a solution containing 300 mM sucrose, 0.2 mg/ml bacitracin and 500 kallikrein inhibitor U/ml of Trasylol (Bayer, Brussels, Belgium). The resulting homogenate was immediately filtered on two layers of medical gauze and diluted 11-fold with the incubation buffer made of 66 mM sodium phosphate (pH 7.4) enriched with 2.6 mM $MgCl_2$, 500 kallikrein inhibitor U/ml of Trasylol, 0.2 mg/ml bacitracin and 13 mg/ml bovine serum albumin.

2.2. [³H]NMS binding experiments

[³H]NMS binding was measured at 25°C in a total volume of 1.2 ml using the following incubation buffer: 50 mM sodium phosphate (pH 7.4) enriched with 2 mM $MgCl_2$, 1% bovine serum albumin (except when indicated) and the indicated tracer and drug concentrations. Addition of bovine serum albumin to the incubation buffer increased [³H]NMS binding very slightly (by at most 10-15%) and improved the reproducibility of duplicates in our filtration assays. In binding experiments on pancreas homogenates, we also added Trasylol and bacitracin (see above) to further inhibit proteolytic activity. Bovine serum albumin was an essential ingredient in pancreas binding studies, since the binding capacity of pancreas homogenates disappeared within 40 min at room temperature if this protein was omitted from the buffer, but was maintained over 90% for at least 4 h in its presence.

To terminate the incubation, each sample was diluted with 2 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.4) and filtered on GF/C glass-fiber filters (Whatman, Maidstone, England) presoaked in 0.05% polyethyleneimine. The filters were rinsed three times with the same filtration buffer, dried, and the radioactivity (bound tracer) counted by liquid scintillation. Nonspecific binding was defined as [³H]NMS binding in the presence of 1 µM atropine in the four systems.

For [³H]NMS binding to human NB-OK 1 cell homogenates, a 0.25 nM tracer concentration (twofold K_D in this system) was chosen with a homogenate concentration of 160-200 µg protein per assay (about 50 pM binding sites) and an incubation period of 2 h at 25°C allowing full equilibration of tracer binding.

In binding experiments on rat cardiac homogenates, a 1 nM [³H]NMS concentration (two-fold K_D in this system) was selected with a homogenate concentration of 400-500 µg protein per assay (about 250 pM binding sites) and a 2 h incubation period at 25°C allowing full equilibration of tracer binding.

In binding experiments on rat cortex or striatum homogenates, the tracer concentration was 0.25 nM and the protein concentration 30-40 µg per assay (about 50 pM binding sites). Under equilibrium conditions (2 h incubation at 25°C), [³H]NMS labelled M_1, M_3 and M_4 sites in these two brain regions. In order to analyze tracer binding to brain M_4 sites only, we chose the striatum, which possesses fewer M_1 and M_3 receptors than other forebrain areas (Waelbroeck et al., 1990). We preincubated striatum homogenates for 2 h at 25°C to allow equilibrium binding, then induced tracer dissociation by adding 1 µM atropine. [³H]NMS dissociated from its binding sites after 35 min of isotopic dilution, the residual binding being about 30% of initial binding. Since [³H]NMS dissociation from M_1 sites is faster than that from M_3 and M_4 sites, 85% of this residual [³H]NMS binding corresponded to M_4 binding sites. It is necessary to keep tracer binding below 15% of the total tracer added to avoid distorsions of the competition curves due to tracer or unlabelled drug depletion. This means that tracer binding to striatum M_4 sites in the absence of unlabelled drug must be maintained below 5% of the total tracer added (i.e., 30% of the 15% initial binding). We therefore decided to use a comparatively high [³H]NMS concentration (0.25 nM, equivalent to five-fold K_D at M_4 sites) for these experiments.

In binding experiments on rat pancreas homogenates we used 980 µl of the homogenate per 1.2 ml sample. The [³H]NMS concentration was 0.25 nM (two-fold K_D in this system) and protein concentration 800-1000 µg per assay (about 50 pM binding sites). An incubation period of 4 h was necessary to allow binding equilibrium.

Protein concentration was measured according to Lowry et al. (1951) using bovine serum albumin as standard.

2.3. Data analysis and statistics

The competition curves were analyzed using the computer program described by Richardson and Humrich (1984), and were compatible with the existence of a single receptor type. K_i values were calculated from IC_{50} values using the Cheng and Prusoff (1973) equa-

tion. The pK$_i$ value was defined as $-\log K_i$. Each experiment was repeated at least three times. The standard deviations of each IC$_{50}$ value was below 30% of the average value in all cases (corresponding to pK$_i$ standard deviations of < 0.1 log unit) and were therefore not mentioned in the tables and figures. The data are presented as means of the indicated number of experiments.

2.4. Drugs and chemicals

[^{3}H]NMS (74 Ci/mmole) was obtained from Amersham International (Bucks, England). Atropine, polyethyleneimine and bovine serum albumin (Cohn fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tissue culture material and media were obtained from Gibco (Gent, Belgium). All other chemicals were of the highest grade available.

Hexahydro-sila-difenidol (1b) and the analogues shown in fig. 1 were synthesized in our laboratories ((R)- and (S)-1a: Tacke et al., 1989; 1b: Tacke et al., 1985; 4b: Waelbroeck et al., 1989a; 2, 3, 5, 6, 7 and 8b were prepared by analogy to the synthesis of 1b, unpublished results; (R)- and (S)-8a were prepared by analogy to the synthesis of (R)- and (S)-1a, unpublished results; the quaternary ammonium compounds (R)- and (S)-4a, (R)- and (S)-9a as well as 9b were prepared by quaternization of the corresponding free amines with methyl iodide analogous to the synthesis of 4b, unpublished

results). The enantiomeric purity of the enantiomers of 1a, 4a, 8a and 9a was > 99.7% as determined on the basis of calorimetric analyses (differential scanning calorimetry) of (R)- and (S)-1a (Tacke et al., 1989) and (R)- and (S)-8a (unpublished results). The experimental procedure (Sarge and Cammenga, 1985; Tacke et al., 1987, 1989) and data evaluation (Sarge et al., 1988) followed the lines described in the literature.

3. Results

3.1. General considerations

All the competition curves obtained in this study were compatible with the existence of a single receptor subtype in the different preparations with Hill coefficients not significantly different from unity (n$_H$ varied between 0.95 and 1.10, with standard deviations below or equal to 0.05). This suggested that [^{3}H]NMS labelled in each of the tissues homogeneous binding sites which was not discriminated by the drugs used.

Competition curves with p-chloro-hexahydro-sila-difenidol (7) were shifted to the right by 0.5 to 1.0 log units in all systems in the presence of bovine serum albumin (1%). This is illustrated in fig. 2 using cortex homogenates. Almost 10-fold higher concentrations of 7 were needed to inhibit [^{3}H]NMS binding in this tissue when 'fraction V' bovine serum albumin was added to

TABLE 1

Antagonist affinities (pK$_i$ values) [a] of hexahydro-sila-difenidol (1b) and analogues 1a, 2, 3, 4a, 4b, 5-7, 8a, 8b, 9a and 9b obtained in binding studies on homogenates of human NB-OK 1 cells (M$_1$ receptors) as well as rat heart (M$_2$ receptors), pancreas (M$_3$ receptors) and striatum (M$_4$ receptors) in the presence or absence of 1% bovine serum albumin. [a]

Antagonist	Human NB-OK 1	Rat heart	Rat pancreas	Rat striatum M$_4$
(R)-1a hexahydro-difenidol	8.2	7.0	8.1	7.9
(S)-1a hexahydro-difenidol	6.1	5.8	5.9	6.0
1b hexahydro-sila-difenidol [b]	7.8	6.7	7.8	7.9
2 pyrrolidino analogue of hexahydro-sila-difenidol	8.2	6.9	7.8	7.9
3 hexamethyleneimino analogue of hexahydro-sila-difenidol	7.3	6.4	7.1	7.4
(R)-4a hexahydro-difenidol methiodide	8.6	8.2	8.1	8.5
(S)-4a hexahydro-difenidol methiodide	6.5	6.0	5.5	6.1
4b hexahydro-sila-difenidol methiodide [b]	8.8	8.0	8.2	8.6
5 p-methoxy-hexahydro-sila-difenidol	6.7	5.8	7.0	6.7
6 o-methoxy-hexahydro-sila-difenidol	6.6	6.1	6.5	7.0
7 p-chloro-hexahydro-sila-difenidol [c]	7.6	6.1	7.6 [d]	7.6
(R)-8a p-fluoro-hexahydro-difenidol [c]	7.9	6.7	–	7.9
(S)-8a p-fluoro-hexahydro-difenidol [c]	5.9	5.6	–	5.8
8b p-fluoro-hexahydro-sila-difenidol [c]	7.8	6.5	7.8	7.8 [d]
(R)-9a p-fluoro-hexahydro-difenidol methiodide [c]	8.4	7.8	–	8.2
(S)-9a p-fluoro-hexahydro-difenidol methiodide [c]	6.3	5.9	–	6.2
9b p-fluoro-hexahydro-sila-difenidol methiodide [c]	8.3	7.6	–	8.3

[a] pK$_i$ values ($-\log K_i$) were measured by competition with [^{3}H]NMS binding, as explained in Materials and methods. The numbers show the mean estimate for three experiments. The S.D. was approximately 0.1 log unit.

[b] pK$_i$ values for 1b and 4b in membranes from NB-OK 1 cells, rat heart and rat pancreas were previously published (Waelbroeck et al., 1989a).

[c] pK$_i$ values measured in the absence of bovine serum albumin (see text).

[d] The competition curves obtained in the presence of bovine serum albumin in pancreas and NB-OK 1 homogenates were superimposable, suggesting that compounds 7 and 8b had the same affinity for M$_3$ as for M$_1$ receptors.
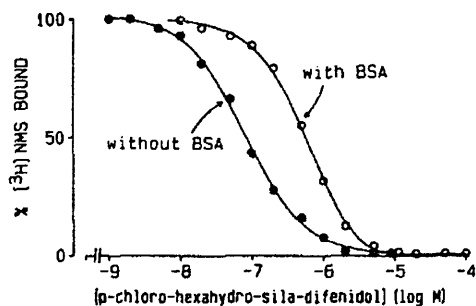
Fig. 2. p-Chloro-hexahydro-sila-difenidol (7)-[³H]NMS competition curves in rat brain cortex homogenates, in the absence (●) or presence (○) of 1% BSA (Cohn fraction V) in the incubation buffer. One experiment is representative of three performed in duplicate.

the incubation buffer. This suggested that about 90% of compound 7 was bound to bovine serum albumin or a contaminant. Similar results were obtained with p-fluoro-hexahydro-sila-difenidol (8b) and its methiodide (9b) and with the (R)-enantiomers of the corresponding carbon analogues 8a and 9a. We therefore determined the binding affinities of compounds 7, 8a, 8b, 9a and 9b (Fig. 1) to muscarinic receptors in homogenates from human NB-OK 1 cells, rat heart and striatum in the absence of bovine serum albumin. pK$_i$ values are given in table 1.

The binding properties of hexahydro-difenidol (1a), hexahydro-sila-difenidol (1b) and their analogues 2, 3, 4a, 4b, 5 and 6 (fig. 1) were hardly affected by inclusion of bovine albumin into the incubation buffer (pK$_i$ increase below 0.3 log units). This allowed us to perform binding experiments with these compounds in all tissues in the presence of bovine serum albumin (table 1).

3.2. Structural variations of the amino group

A comparison of binding affinities of (R)-hexahydro-difenidol ((R)-1a), hexahydro-sila-difenidol (1b) and compounds 2/3 and 4a/4b as well as 8a/9a and 8b/9b outlined the effect of structural variations of the cyclic amino (ammonium) group on antimuscarinic potency (table 1). Increasing the size of the amino group from pyrrolidino to piperidino (2 → 1b) decreased the affinity for M$_1$ and (to a lesser extent) M$_2$ sites. Exchange of the piperidino by the hexamethyleneimino group (1b → 3) decreased the affinities for the four binding sites. N-Methylation of the silicon compounds 1b and 8b (→ 4b and 9b) increased the affinity for the four binding sites, this increase being greatest at M$_2$ receptors. This N-methylation effect on the affinity of the silicon compounds 1b and 8b was comparable to or greater than the affinity increase observed with the corresponding (R)-configurated carbon analogues (R)-1a (→ (R)-4a) and (R)-8a(→ (R)-9a).

3.3. Substitutions on the phenyl ring

The influence of substituents in the phenyl ring of hexahydro-sila-difenidol (1b) on binding affinity can be demonstrated by comparison of the silicon compounds 1b, 5, 6, 7 and 8b, as well as compounds 4b and 9b (table 1). Introduction of a methoxy substituent in para or ortho position (compounds 5 and 6) or a p-chloro substituent (7) reduced the affinity to the muscarinic receptors up to 16-fold (table 1). The influence of a fluoro substituent in para position of hexahydro-sila-difenidol (1b → 8b) and its methiodide (4b → 9b) on affinity was moderate.

3.4. Sila substitution

It was not possible to determine exactly the effect of sila substitution on the binding properties of the carbon compounds 1a, 4a, 8a and 9a from the present results. We did indeed use the pure enantiomers of 1a, 4a, 8a and 9a in this study but investigated the binding affinities of the racemic mixtures (R/S) of the silicon analogues 1b, 4b, 8b and 9b (see introduction). The pK$_i$ values (table 1) of racemic 1b, 4b, 8b and 9b may therefore be lower by at most 0.3 log unit than the pK$_i$ values of their high-affinity enantiomers. This is due to the presence of 50% of the low-affinity enantiomers in the binding assay. If this is taken into account, it is obvious from table 1 that sila-substitution did not significantly affect the binding properties of 1a, 4a, 8a and 9a at any subtype.

3.5. Stereoselectivity

The effect of N-methylation of (R)- and (S)-hexahydro-difenidol ((R)- and (S)-1a; → (R)- and (S)-4a) and (R)- and (S)-p-fluoro-hexahydro-difenidol ((R)- and (S)-8a; → (R)- and (S)-9a) on the affinity for the muscarinic receptor depended on the receptor subtype and on the configuration of the drug (table 2). In NB-OK 1 cells (M$_1$ sites), the affinity of both enanti-

TABLE 2

Stereoselectivity ((R)/(S)) ratios at muscarinic receptor subtypes. The values shown represent the antilogs of the differences between corresponding mean pK$_i$ values of the (R)- and (S)-enantiomers (table 1) determined at M$_1$ receptors in NB-OK 1 cells, M$_2$ receptors in rat heart, as well as M$_3$ and M$_4$ receptors in rat pancreas and striatum.

	Stereoselectivity ratios			
	M$_1$	M$_2$	M$_3$	M$_4$
Hexahydro-difenidol (1a)	130	16	160	79
Hexahydro-difenidol/methiodide (4a)	130	160	400	250
p-Fluoro-hexahydro-difenidol (8a)	100	13	–	130
p-Fluoro-hexahydro-difenidol/ methiodide (9a)	130	80		100

omers of 1a and 8a was increased two- to threefold by N-methylation (→ 4a and 9a). In heart (M_2 sites), the affinities of the (R)-enantiomers of 1a and 8a were increased 10- to 20-fold by N-methylation (→ 4a and 9a) but the affinities of the (S)-enantiomers were almost unchanged. In pancreas (M_3 sites), the affinities of (R)-hexahydro-difenidol ((R)-1a) and its methiodide ((R)-4a) were identical but N-methylation decreased the affinity of the (S)-enantiomers ((S)-1a → (s)-4a). In striatum (M_4 sites), the affinities of (R)-1a, (R)-8a and (S)-8a were increased two- to fourfold by N-methylation, whereas the affinity of (S)-1a was unchanged.

p-Fluoro substitution of the enantiomers of hexahydro-difenidol (1a) and its methiodide (4a) changed only slightly (if at all) their affinities for M_1, M_2 and M_4 sites (table 1). This effect did not depend on the absolute configuration of the compounds.

4. Discussion

The present study investigated the effects of changing the size of the cyclic amino group (compounds 2 and 3), introducing substituents in the phenyl ring (compounds 5-7, 8b) and N-methylation (compounds 4b and 9b) of hexahydro-sila-difenidol (1b) as well as the effects of replacing the central silicon atom of compounds 1b, 4b, 8b and 9b by a carbon atom (compounds 1a, 4a, 8a and 9a) on muscarinic binding affinity and receptor selectivity. All the compounds tested in this work possess a center of chirality (central silicon or carbon) and therefore exist in two enantiomers. We had indications that silanols (R_3SiOH) may racemize in aqueous solution (Tacke et al., 1987). Thus compounds 1b, 2-7, 8b, 4b and 9b were studied as racemates. In contrast, the carbon compounds 1a, 4a, 8a and 9a (carbinols, R_3C OH) exist in configurationally stable enantiomers. We took advantage of this by investigating the binding properties of the individual enantiomers of these compounds at muscarinic receptor subtypes.

4.1. Binding properties of p-fluoro-hexahydro-sila-difenidol

The binding affinities (pK_i values, table 1) obtained with most of the hexahydro-sila-difenidol analogues correspond closely to their antimuscarinic potencies (pA_2 values) determined in functional experiments at M_1, M_2 and M_3 receptors (Lambrecht et al., 1988; 1989a,b; Eltze et al., 1988). We were therefore particularly interested in the binding properties of p-fluoro-hexahydro-sila-difenidol (8b) which behaved in functional experiments as a selective M_3 antagonist (M_3 >

M_1 > M_2) (Lambrecht et al., 1988, 1989a,b; Eglen et al., 1989; Whiting et al., 1989). In this study we did not find M_1 muscarinic binding sites with a low affinity for p-fluoro-hexahydro-sila-difenidol (8b) in human neuroblastoma NB-OK 1 cells (labelled by [³H]NMS; table 1) or in rat brain cortex, hippocampus and striatum homogenates labelled by [³H]pirenzepine (data not shown). In contrast, the pK_i values of p-fluoro-hexahydro-sila-difenidol (8b) obtained at cardiac M_2 (6.5) and pancreas M_3 sites (7.8) were in reasonable agreement with the pA_2 values observed in pharmacological studies using isolated guinea-pig atria (M_2; 6.0) and ileum (M_3; 7.8) preparations (Lambrecht et al., 1988, 1989b).

In fact, compound 8b showed a markedly higher affinity for M_1 binding sites in this study (pK_i = 7.8; table 1) as compared to functional M_1 receptors in rat ganglia (pA_2 = 7.2), rabbit vas deferens (pA_2 = 6.7) (Lambrecht et al., 1988, 1989b) or canine femoral (pA_2 = 7.1) and saphenous vein (pA_2 = 7.1) (Eglen et al., 1989; Whiting et al., 1989). Our results also contrast with the low affinity of 8b in binding studies using [³H]telenzepine-labelled calf superior cervical ganglia M_1 receptors (Lambrecht et al., 1989a). We do not have at present a satisfactory explanation for these differences. Since it was well known that the binding of antagonists to muscarinic M_1 receptors is sensitive to the ionic conditions in the incubation medium (Freedman et al., 1988) we repeated our binding experiments at M_1 receptors in NB OK 1 cells with p-fluoro-hexahydro-sila-difenidol (8b) using the Tris buffer which was used by Lambrecht et al. (1989a and unpublished data) in binding studies at M_1 receptors of calf superior cervical ganglia. Under these ionic conditions we again found only high-affinity binding for compound 8b (data not shown) in NB-OK 1 cells. An alternative explanation, that M_1 receptors in ganglia and rabbit vas deferens are different from M_1 receptors in rat brain and human NB-OK 1 cells seems also unlikely. All the other compounds investigated in this and other studies (including the closely related compounds 8a and 9b) had similar affinities for the M_1 binding sites of rat brain and human NB-OK 1 cells and for the functional (rabbit vas deferens and ganglia) M_1 receptors (Waelbroeck et al., 1989a; Lambrecht et al., 1988; 1989a,b; Eltze et al., 1988; Feifel et al., 1990; Lambrecht et al., unpublished results).

4.2. Structure-selectivity relationships

When comparing the structure-binding relationship of hexahydro-sila-difenidol analogues to muscarinic receptor subtypes, these muscarinic antagonists showed some quantitative and qualitative differences in receptor selectivity profiles (table 1).

The influence of the ring size of the cyclic amino group on binding affinity and selectivity can be demonstrated by comparison of compounds 1b, 2 and 3. It is obvious from the data in table 1 that the affinity to the four muscarinic receptor subtypes depends on the structure of the heterocyclic ring and varies up to fivefold. Compound 2, possessing a pyrrolidino ring shows nearly the same affinity for the four muscarinic receptors as the parent compound hexahydro-sila-difenidol (1b). In contrast, ring extension to the hexamethyleneimino analogue 3 results in a decrease in affinity, this decrease being greatest at M_3 receptors. However, the influence of the size of the cyclic amino group on receptor selectivity is moderate as compounds 2 and 3 showed about the same selectivity pattern as the parent compound 1b: $M_1 \approx M_3 \approx M_4 > M_2$.

N-Methylation of (R)-hexahydro-difenidol ((R)-1a → (R)-4a), hexahydro-sila-difenidol (1b → 4b), (R)-p-fluoro-hexahydro-difenidol [(R)-8a → (R)-9a] and p-fluoro-hexahydro-sila-difenidol (8b → 9b) increased the affinity for M_1, M_2 and M_4 receptors up to 20-fold, this increase being consistently greatest at M_2 receptors. In contrast, the affinity of compound (R)-1a for M_3 receptors in pancreas was not changed by N-methylation, and that of 1b was increased only 2.5-fold. Thus, N-methylation of the tertiary amines (R)-1a, 1b, (R)-8a and 8b changed the receptor selectivity pattern from $M_1 \approx M_3 \approx M_4 > M_2$ to $M_1 \geqslant M_4 > M_3 \approx M_2$ receptors, abolishing the selectivity between M_2 and M_3 receptors. It is interesting to note that the binding affinities of (S)-1a and (S)-8a were only slightly affected by N-methylation at all muscarinic receptor subtypes. It is generally assumed that tertiary antimuscarinic agents interact in their protonated form with these receptors (Barlow and Chan, 1982; Asselin et al., 1983). N-Methylation probably does not change the overall charge of the cationic head of the compounds studied: steric factors due to the presence of an additional N-methyl group may play an important role in the interaction of the quaternary compounds 4a, 4b, 9a and 9b with muscarinic receptors.

We also investigated in this study the effect of substitution of the phenyl ring in the para and ortho positions. The derivatives of hexahydro-sila-difenidol and hexahydro-difenidol and of their methiodides tested had equal or lower affinities than the parent compounds (table 1) at the four subtypes. The affinity decreases might be due to steric hindrance, modification of the electron distribution of the molecules, or both. It is interesting in this respect that the affinities of compounds (R)-1a/(R)-8a, (S)-1a/(S)-8a, 1b/8b, (R)-4a/(R) ≈ 9a and (S)-4a/(S)-9a were very similar, regardless of the receptor subtype (M_1, M_2, M_4) studied and of the absolute drug configuration. In contrast, 9b had a two- to threefold lower affinity than 4b for M_1, M_2 and M_4 receptors. This supports the view that the

drug position in the muscarinic binding site is adapted depending on the actual drug structure, and is not necessarily identical when comparing tertiary and quaternary analogues or carbon/silicon bioisosters.

It is noteworthy that the affinity decrease due to p-chloro substitution (1b → 7) was greatest at M_2 receptors. Thus, the p-chloro analogue 7 shows a greater selectivity (30-fold) for $M_1/M_3/M_4$ receptors over M_2 receptors than the parent compound 1b. o-Methoxy-hexahydro-sila-difenidol (6) presented a unique selectivity profile. It recognized preferentially the M_4 receptors, had an intermediate affinity for M_1 and M_3 receptors and lowest affinity for M_2 receptors.

In general, the (R)-enantiomers of compounds 1a, 4a, 8a and 9a showed higher binding affinities (up to 398-fold) for the four muscarinic receptor subtypes than the corresponding (S)-configurated isomers (table 1). However, this stereoselectivity was not the same for all receptor subtypes. The stereoselectivity ratios (table 2) for the tertiary compounds hexahydro-difenidol (1a) and its p-fluoro analogue 8a consistently show the same order: $M_1 \approx M_3 \approx M_4 > M_2$. This implies that the stereochemical requirements of the muscarinic M_2 receptors are less stringent than that of the other subtypes for the enantiomers of 1a and 8a. Similar results have been obtained with the enantiomers of other tertiary antimuscarinics such as trihexiphenidyl, hexbutinol, telenzepine or biperiden (for a recent review, see Lambrecht et al., 1989a; Waelbroeck et al, 1989b). The stereoselectivity ratios for the two quaternary compounds 4a and 9a at M_2 receptors are very similar to those of the other subtypes. This is mainly due to the stereoselective effect of N-methylation on the affinity of the (R)-enantiomers of compounds 1a and 8a.

In conclusion, this report describes structure–activity relationships (including stereochemical aspects) of muscarinic antagonists related to hexahydro-sila-difenidol (1b). All compounds behaved as competitive inhibitors of [^{3}H]-N-methylscopolamine binding at M_1 receptors in human neuroblastoma NB-OK 1 cells, M_2 receptors in rat heart as well as M_3 and M_4 receptors in rat pancreas and striatum, respectively. The binding affinity and receptor selectivity in this series of compounds was found to be controlled by the structure of the cyclic amino (ammonium) group, the substitution pattern of the phenyl moiety and the absolute configuration of the chiral carbinols 1a, 4a, 8a and 9a. There was little influence of sila-substitution (carbon/silicon exchange) on binding affinity of these chiral compounds. In contrast to pharmacological studies using isolated organs such as rabbit vas deferens, rat ganglia and canine veins as well as to binding studies in calf ganglia, we could not use p-fluoro-hexahydro-sila-difenidol (8b) to discriminate M_3 from M_1 binding sites, due to the high affinity of this antimuscarinic agent for M_1 binding sites in homogenates of NB-OK 1 cells and rat brain.

102

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