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# Antagonist Binding Profiles of Five Cloned Human Muscarinic Receptor Subtypes[1]

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## ABSTRACT

A variety of muscarinic antagonists are currently used as tools to pharmacologically subclassify muscarinic receptors into $M_1$, $M_2$ and $M_3$ subtypes. In the present study, we have determined the affinity profiles of several of these antagonists at five cloned human muscarinic receptors (m1–m5) stably expressed in Chinese hamster ovary cells (CHO-K1). At all five receptors, the (R)-enantiomers of trihexyphenidyl and hexbutinol displayed considerably higher affinities (up to 525-fold) than their corresponding (S)-isomers. The stereoselectivity ratios [inhibition constant(S)/inhibition constant(R)] for both pairs of enantiomers were lowest at m2 receptors, suggesting that less stringent configurational demands are made by this receptor subtype. The "$M_1$-selective" antagonist (R)-trihexyphenidyl displayed high affinities for m1 and m4 receptors. The "$M_2$-selective" antagonists himbacine, (±)-5,11-dihydro-11-{[(2-[(dipropylamino)methyl]-1-piperidinyl}ethyl)amino]carbonyl}-6H-pyrido(2,3-*b*)(1,4)benzodiazepine-6-one (AF-DX 384), 11-({4-[4-(diethylamino)butyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3-*b*)(1,4)benzodiazepine-6-one (AQ-RA 741) and (+)-(11-({2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-di-hydro-6H-pyrido(2,3-*b*)(1,4)benzodiazepine-6-one [AF-DX 250; the (+)-enantiomer of AF-DX 116] exhibited high affinities for m2 and m4, intermediate affinities for m1 and m3 and low affinities for m5 receptors. This selectivity profile was most prominent for AQ-RA 741, which displayed 195- and 129-fold higher affinities for m2 and m4 receptors than for m5 receptors. The "$M_3$-selective" antagonist (±)-*p*-fluoro-hexahydro-sila-difenidol hydrochloride (p-FHHsiD) exhibited high affinity for m1, m3 and m4 receptors. 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) bound with up to 7-fold higher affinities to m1, m3, m4 and m5 receptors than to m2 receptors. Although none of the tested antagonists showed more than 2-fold selectivity for one subtype over all other subtypes, each receptor displayed a unique antagonist binding profile.

By the use of selective antagonists, pharmacologists have identified at least three muscarinic receptor subtypes, designated $M_1$, $M_2$ and $M_3$. Antagonists used in this subclassification include pirenzepine ($M_1$ selective), AF-DX 116, methoctramine, himbacine ($M_2$ selective) and HHSiD, p-F-HHSiD, sila-hexocyclium and 4-DAMP ($M_1/M_3$ selective) (for reviews, see Mitchelson, 1988; Lambrecht *et al.*, 1989a; see also table 3). More recently, five muscarinic receptors have been cloned (m1–m5) (for reviews, see Hulme *et al.*, 1990; Jones *et al.*, in press) and shown to be widely expressed in the brain and in peripheral tissues (Peralta *et al.*, 1987; Buckley *et al.*, 1988; Maeda *et al.*, 1988; Weiner *et al.*, 1990). While conventional pharmacologic studies on muscarinic receptor subtypes are complicated by the fact that most tissues express a mixture of different muscarinic receptor genes (Buckley *et al.* 1988; Maeda *et al.*, 1988; Weiner *et al.*, 1990), the use of transformed cell lines individually expressing the various receptors offers the advantage that distinct subtypes can be studied in isolation. To date, only a limited characterization of the antagonist binding properties of the m1 to m5 receptors has been performed (Bonner *et al.*, 1987, 1988; Peralta *et al.*, 1987; Akiba *et al.*, 1988; Buckley *et al.*, 1989). In these studies, the antagonist binding properties of

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**ABBREVIATIONS:** HHSiD, (±)-hexahydro-sila-difenidol hydrochloride; [$^3$H]NMS, [$^3$H]*N*-methylscopolamine chloride; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; p-F-HHSiD, (±)-*p*-fluoro-hexahydro-sila-difenidol hydrochloride; AF-DX 250, (+)-[11-({2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one; AQ-RA 741, 11-({4-[4-(diethylamino)butyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one; AF-DX 384, (±)-5,11-dihydro-11-{[(2-[2-[(dipropylamino)methyl]-1-piperidinyl}ethyl)amino]carbonyl}-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one; $K_i$, inhibition constant.

the m2 and m3 receptors correlated well with those of the pharmacologically defined $M_2$ and $M_3$ subtypes (Peralta *et al.*, 1987; Akiba *et al.*, 1988; Buckley *et al.*, 1989). However, the antagonist binding properties of both m1 and m4 receptors were similar to those of $M_1$ receptors, suggesting that the putative $M_1$ receptors may be composed of a mixture of these two receptor proteins (Buckley *et al.*, 1989).

Many of the antagonists commonly used to classify muscarinic receptor subtypes have not been studied at cloned receptors. To ensure a judicious use of these compounds as pharmacologic tools for receptor subclassification and to further characterize the antagonist binding properties of the m1 to m5 receptors, we have determined the affinities of 11 muscarinic antagonists (fig. 1) for the five cloned human muscarinic receptors stably expressed in CHO-K1 cells. The enantiomers of trihexyphenidyl and hexbutinol were tested to study the stereochemical demands of the individual subtypes. These stereoisomers have been used as tools to classify $M_1$ to $M_3$ muscarinic receptor subtypes (Lambrecht *et al.*, 1988b, 1989a; Feifel *et al.*, 1990). Furthermore, we have investigated two widely used "selective" antagonists, 4-DAMP (Barlow *et al.*, 1976; Doods *et al.*, 1987) and p-F-HHSiD (Lambrecht *et al.*, 1988a, 1989a,b), as well as a series of "$M_2$-selective" antagonists including methoctramine (Melchiorre *et al.*, 1987; Waelbroeck *et al.*, 1989a), himbacine (Gilani and Cobbin 1986; Lazareno and Roberts 1989), AF-DX 384, AQ-RA 741 and AF-DX 250 [(+)-enantiomer of AF-DX 116] (Eberlein *et al.*, 1989; Engel *et al.*, 1989) (for chemical structures, see fig. 1). AF-DX 384 and AQ-RA 741 are new derivatives of the well-known antimuscarinic drug AF-DX 116 and have been found to be more potent and/or selective than the parent compound (Eberlein *et al.*, 1989).

## Materials and Methods

**Cell culture.** Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection. Except for the overnight transfection procedure, cells were incubated at 37°C in a humidified atmosphere (5% $CO_2$) as a monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), 100 units/ml each of penicillin G and streptomycin, and 4 mM glutamine (M.A. Bioproducts, Walkersville, MD).

**Transfection procedure.** The CHO-K1 cell lines stably expressing the human m2 to m5 receptors have been described previously (Buckley *et al.*, 1989). To establish a cell line expressing the human m1 receptor, CHO-K1 cells were transformed with a plasmid containing the human m1 coding sequence (Bonner *et al.*, 1988) inserted into the pcD expression vector (Okayama and Berg, 1983). Cells were transfected according to the method of Chen and Okayama (1987) using a modified calcium phosphate procedure involving the use of cotransfected pcDneo as a selectable marker. Selection with the neomycin analog G 418 (600 μg/ml; Gibco, NY) was started 72 h after transfection and continued for 2 to 3 weeks. Media were changed every 3 days. Clonal cell lines were obtained by single-cell cloning and assayed for [$^3$H]NMS binding capacity.

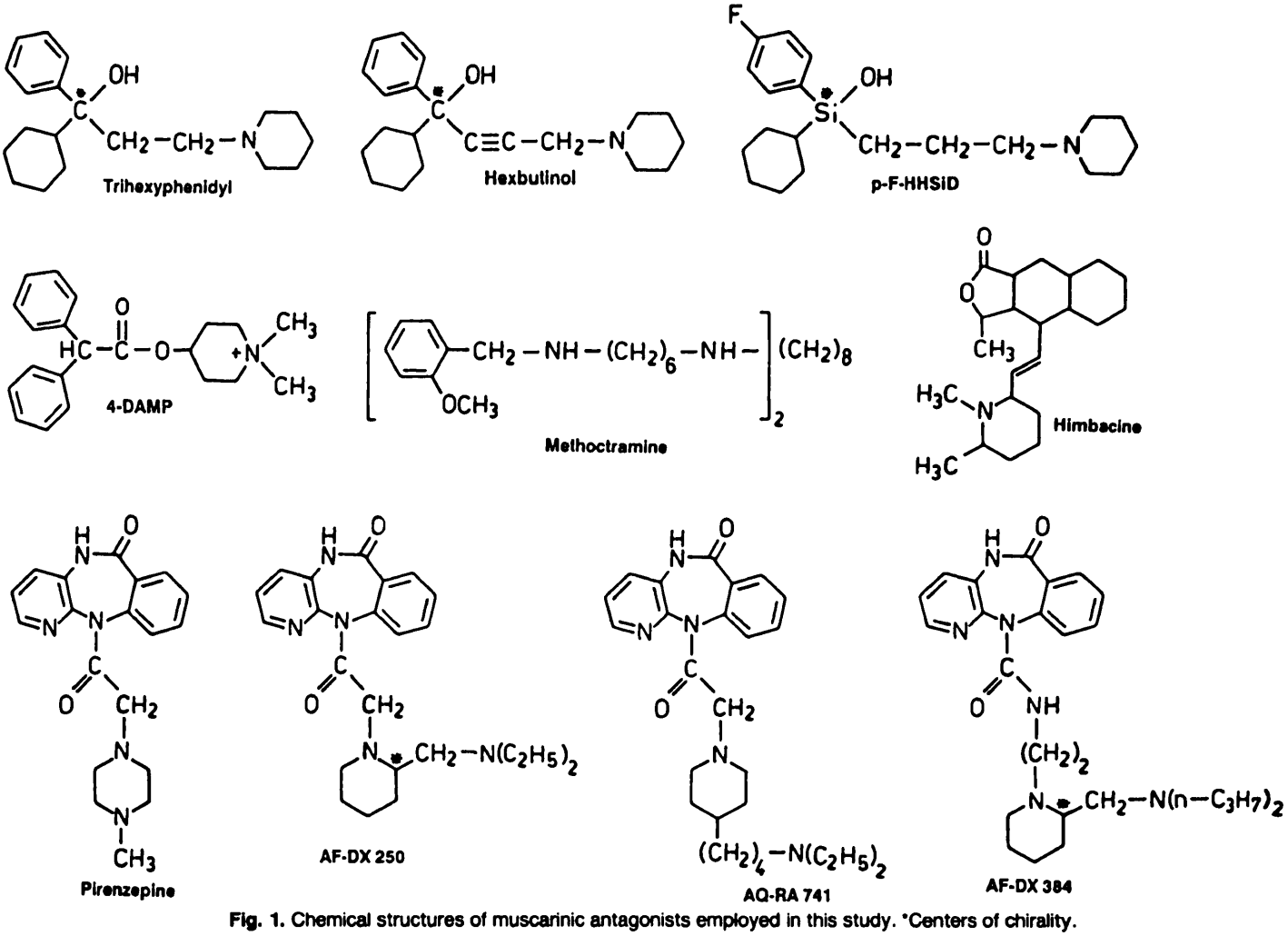


Fig. 1. Chemical structures of muscarinic antagonists employed in this study. *Centers of chirality.

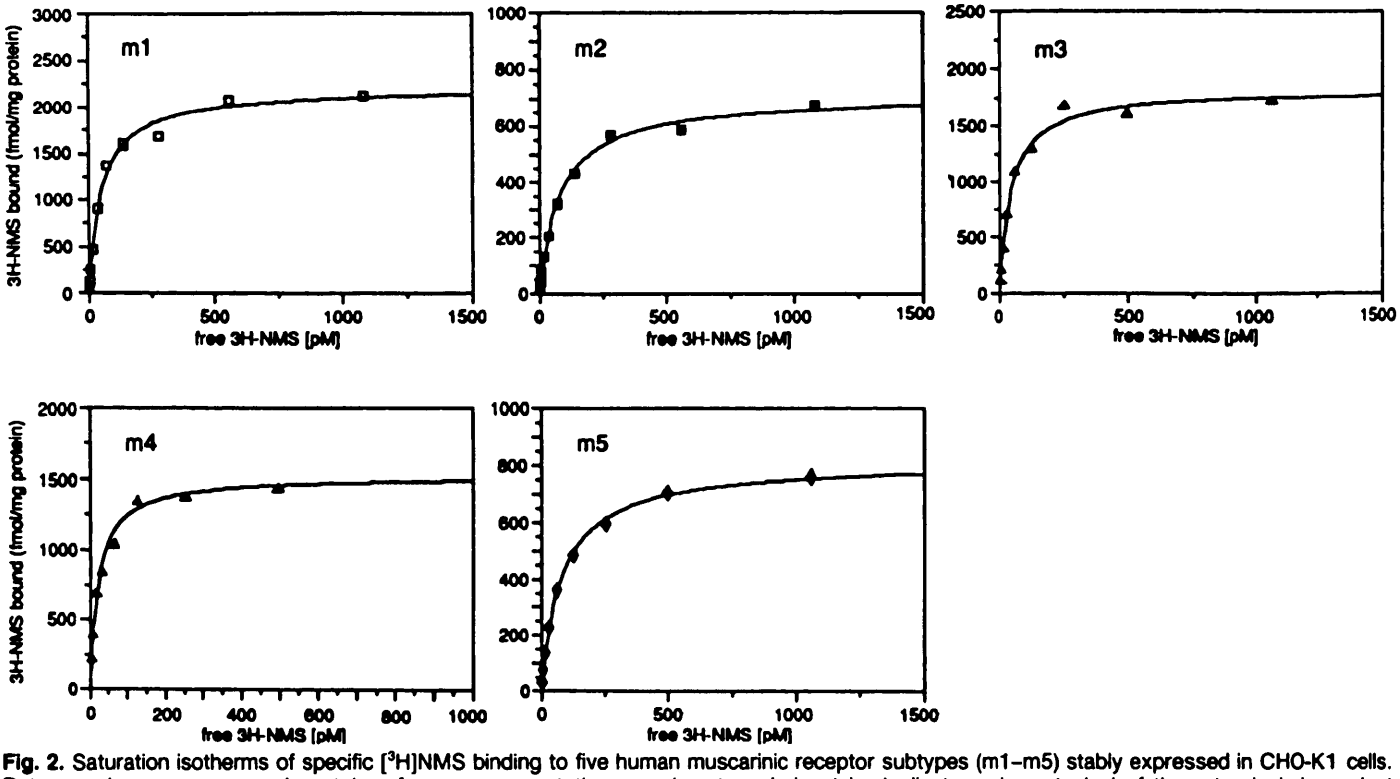


Fig. 2. Saturation isotherms of specific [$^3$H]NMS binding to five human muscarinic receptor subtypes (m1–m5) stably expressed in CHO-K1 cells. Data are shown as mean values taken from a representative experiment carried out in duplicate and are typical of three to six independent experiments.

TABLE 1

**Equilibrium dissociation constants, Hill coefficients and total number of muscarinic binding sites ($B_{max}$) in stably transformed CHO-K1 cells, derived from [$^3$H]NMS saturation experiments**

The data generated as described under "Materials and Methods" represent the mean ± S.E.M. of at least three independent experiments, each run in duplicate. Hill numbers were not significantly different from unity ($P > .05$; unpaired, one-tailed Student's $t$ test).

| Receptor | $K_D$* [$^3$H]NMS | nH* | $B_{max}$ |
|---|---|---|---|
| | pM | | fmol/mg protein |
| m1 | 54 ± 1 | 0.96 ± 0.06 | 2518 ± 125 |
| m2 | 83 ± 4 | 0.94 ± 0.06 | 747 ± 21 |
| m3 | 52 ± 2 | 0.94 ± 0.11 | 1830 ± 40 |
| m4 | 26 ± 5 | 0.95 ± 0.05 | 1778 ± 182 |
| m5 | 106 ± 11 | 0.94 ± 0.11 | 954 ± 90 |

* $K_D$, dissociation constant; nH, Hill coefficient.

**Membrane preparation.** Cells were grown to about 80% confluence, washed, scraped into ice-cold binding buffer and homogenized for 30 sec using a Brinkmann Homogenizer (setting 5). Membranes were pelleted at 16,000 × $g$ for 15 min and rehomogenized. Protein concentrations were determined according to the method of Bradford (1976) using a Bio-Rad protein assay kit. Membranes were stored frozen at −80°C before use.

**Radioligand binding studies.** Binding buffer consisted of 25 mM sodium phosphate (pH 7.4) containing 5 mM magnesium chloride. Assays were conducted in 1 ml total volume. Final membrane protein concentrations (in μg/ml) were: m1, 6; m2, 10; m3, 5; m4, 3; and m5, 4. In [$^3$H]NMS saturation experiments, 8 to 10 different concentrations of the radioligand (2–1400 pM) were employed. For displacement experiments, the concentration of [$^3$H]NMS was 150 pM and 10 different concentrations of the cold displacers were used. Specific binding was defined as the difference in [$^3$H]NMS binding in the absence and presence of 1 μM atropine. Incubations were carried out at 22°C for 3 h. Assays were terminated by filtration through a Brandell cell harvester onto Whatman GF/C filters. Membranes were washed three times with 5 ml of ice-cold binding buffer before being dried, transferred to 10 ml of scintillant (New England Nuclear Aquasol) and counted in an LKB β-counter.

**Data analysis.** Data from direct binding experiments were fitted to the equation:

$$a = (B_{max}\, x^n/k)/(1 + x^n/k)$$

to derive the Hill coefficient $n$ and to:

$$a = (B_{max}\, x/K_D)/(1 + x/K_D)$$

to obtain the dissociation constant $K_D$ and the total number of binding sites $B_{max}$ ($a$ = [$^3$H]NMS specifically bound; $x$ = [$^3$H]NMS concentration). Data from displacement experiments were fitted to the equation:

$$\%\ [^3\text{H}]\text{NMS bound} = 100 - [100x^n/k/(1 + x^n/k)]$$

to obtain the Hill number $n$ and to:

$$\%\ [^3\text{H}]\text{NMS bound} = 100 - [100x/\text{IC}_{50}/(1 + x/\text{IC}_{50})]$$

to derive the $IC_{50}$ value ($x$ = concentration of the cold inhibitor). $K_i$ values were calculated by the method of Cheng and Prusoff (1973):

$$K_i = \text{IC}_{50}/(1 + L/K_D)$$

where $L$ is the concentration of the radioligand, $IC_{50}$ is the concentration of drug causing 50% inhibition of the specific radioligand binding and $K_D$ the dissociation constant of the radioligand receptor complex. Data were analyzed by a nonlinear least-squares curve fitting procedure using the program DATAPLOT (distributed by the National Technical Information Services) run on a VAX II computer.

**Statistical evaluation.** Results are expressed as mean values ± S.E.M. of $n$ experiments. Statistical significance was assessed using Student's $t$ test or Scheffé's method (Wallenstein *et al.*, 1980); $P < .05$ was accepted as being significant.

**Drugs.** Drugs were obtained from the following sources: [$^3$H]NMS (71 Ci/mmol; New England Nuclear, Boston, MA); atropine sulfate, 4-DAMP and methoctramine tetrahydrochloride (Research Biochemi-

TABLE 2

**Binding parameters of muscarinic antagonists at five human muscarinic receptor subtypes**

The affinity estimates were derived from [$^3$H]NMS displacement experiments and represent the mean (±S.E.M.; $n$ = 3-5) for the negative logarithm of the $K_i$. The Hill coefficients are given in parentheses. Hill numbers shown are not significantly different from unity ($P > .05$; unpaired, one-tailed Student's $t$ test).

| Antagonist | m1 | m2 | m3 | m4 | m5 |
|---|---|---|---|---|---|
| (R)-Trihexyphenidyl | 9.43 ± 0.07 | 8.15 ± 0.04 | 8.61 ± 0.05 | 9.08 ± 0.04 | 8.30 ± 0.05 |
| | (0.97 ± 0.02) | (0.90 ± 0.05) | (0.90 ± 0.13) | (0.94 ± 0.11) | (0.97 ± 0.06) |
| (S)-Trihexyphenidyl | 6.91 ± 0.05 | 6.31 ± 0.15 | 5.89 ± 0.02 | 6.57 ± 0.08 | 6.17 ± 0.07 |
| | (1.02 ± 0.05) | (1.00 ± 0.07) | (1.03 ± 0.05) | (0.91 ± 0.08) | (0.96 ± 0.04) |
| (R)-Hexbutinol | 8.68 ± 0.02 | 7.68 ± 0.04 | 8.67 ± 0.06 | 8.52 ± 0.09 | 8.26 ± 0.06 |
| | (1.02 ± 0.10) | (0.92 ± 0.09) | (1.04 ± 0.13) | (1.05 ± 0.04) | (0.97 ± 0.07) |
| (S)-Hexbutinol | 7.58 ± 0.03 | 7.14 ± 0.05 | 7.34 ± 0.05 | 7.71 ± 0.06 | 7.12 ± 0.07 |
| | (1.08 ± 0.07) | (1.06 ± 0.05) | (1.08 ± 0.04) | (1.00 ± 0.04) | (1.02 ± 0.03) |
| Pirenzepine[a] | 8.20 ± 0.13 | 6.65 ± 0.05 | 6.86 ± 0.06 | 7.43 ± 0.05 | 7.05 ± 0.04 |
| | (0.94 ± 0.07) | (1.00 ± 0.04) | (1.02 ± 0.05) | (0.99 ± 0.07) | (0.92 ± 0.08) |
| p-F-HHSiD | 7.65 ± 0.03 | 6.88 ± 0.04 | 7.81 ± 0.02 | 7.50 ± 0.01 | 7.03 ± 0.04 |
| | (1.00 ± 0.02) | (1.00 ± 0.00) | (1.04 ± 0.02) | (0.94 ± 0.03) | (0.97 ± 0.09) |
| 4-DAMP | 9.24 ± 0.06 | 8.42 ± 0.07 | 9.28 ± 0.02 | 8.93 ± 0.10 | 8.98 ± 0.00 |
| | (1.00 ± 0.03) | (0.93 ± 0.05) | (0.97 ± 0.08) | (0.95 ± 0.03) | (1.05 ± 0.03) |
| Methoctramine | 7.3 ± 0.11 | 7.88 ± 0.09 | 6.67 ± 0.09 | 7.50 ± 0.04 | 6.87 ± 0.07 |
| | (1.05 ± 0.02) | (1.02 ± 0.08) | (0.95 ± 0.06) | (1.01 ± 0.04) | (1.07 ± 0.05) |
| Himbacine | 6.97 ± 0.08 | 8.00 ± 0.05 | 7.03 ± 0.03 | 7.96 ± 0.05 | 6.31 ± 0.05 |
| | (0.99 ± 0.08) | (0.97 ± 0.13) | (0.98 ± 0.05) | (0.94 ± 0.04) | (1.06 ± 0.09) |
| AF-DX 384 | 7.51 ± 0.08 | 8.22 ± 0.04 | 7.18 ± 0.07 | 8.00 ± 0.08 | 6.27 ± 0.04 |
| | (0.92 ± 0.06) | (0.95 ± 0.07) | (0.97 ± 0.04) | (0.99 ± 0.04) | (0.94 ± 0.06) |
| AQ-RA 741 | 7.54 ± 0.03 | 8.37 ± 0.04 | 7.20 ± 0.04 | 8.19 ± 0.00 | 6.08 ± 0.05 |
| | (1.07 ± 0.07) | (1.02 ± 0.08) | (1.04 ± 0.08) | (1.03 ± 0.05) | (0.94 ± 0.06) |
| AF-DX 250 | 6.37 ± 0.02 | 7.26 ± 0.03 | 6.16 ± 0.02 | 6.79 ± 0.04 | 5.52 ± 0.02 |
| | (1.01 ± 0.05) | (0.93 ± 0.06) | (0.99 ± 0.02) | (0.99 ± 0.05) | (1.02 ± 0.04) |

[a] Data taken from Wess *et al.*, 1991.

cals, Natick, MA); (R)- and (S)-trihexyphenidyl hydrochloride (enantiomeric purity >99.9%; Schjelderup *et al.*, 1987; Lambrecht *et al.*, 1988b) (Dr. A. J. Aasen, Oslo, Norway); himbacine hydrochloride (Dr. W. C. Taylor, Sydney, Australia); AF-DX 250 (enantiomeric purity >98.9%; Engel *et al.*, 1989), AQ-RA 741 and AF-DX 384 (Dr. Karl Thomae GmbH, Biberach an der Riss, Federal Republic of Germany); p-F-HHSiD and (R)- and (S)-hexbutinol (enantiomeric purity >99.8%; Tacke *et al.*, 1989) were synthesized in our laboratories (p-F-HHSiD was prepared by analogy to the synthesis of HHSiD, Tacke *et al.*, 1985).

## Results

**[$^3$H]NMS saturation experiments.** In all saturation experiments, the muscarinic antagonist [$^3$H]NMS showed binding isotherms characterized by Hill numbers not significantly different from unity (fig. 2, table 1). The obtained dissociation constant values (table 1) ranged from 26 pM for m4 to 106 pM for m5 receptors. The [$^3$H]NMS affinity for the human m1 receptor was similar to that found for the rat m1 receptor (Buckley *et al.*, 1989). Whereas the dissociation constant values determined at m3, m4 and m5 receptors closely resembled those previously reported, we observed a [$^3$H]NMS affinity for m2 receptors which was 5-fold higher than described by Buckley *et al.* (1989).

**[$^3$H]NMS displacement experiments.** The binding parameters of the tested muscarinic antagonists are given in table 2. Unlike in previous studies (Peralta *et al.*, 1987; Buckley *et al.*, 1989), all compounds displaced [$^3$H]NMS binding to all five human muscarinic receptors (m1-m5) with simple mass action isotherms (shown for (R)- and (S)-trihexyphenidyl and AQ-RA 741 in fig. 3). None of the Hill coefficients was significantly different from unity (table 2), entirely consistent with the presence of a single population of muscarinic binding sites in each cell line. At all five receptor subtypes, the (R)-enantiomers of trihexyphenidyl and hexbutinol displayed considerably higher affinities than the corresponding (S)-configurated isomers (table 2, fig. 4). The stereoselectivity ratios [$K_i$(S)/$K_i$(R)] for the enantiomers of trihexyphenidyl and hexbutinol ranged from 69 (m2) to 525 (m3) and from 5 (m2) to 21 (m3), respectively (fig. 4). The "$M_1$-selective" antagonist (R)-trihexyphenidyl (Lambrecht *et al.*, 1988b, Waelbroeck *et al.*, 1989b) exhibited high affinity to both m1 and m4 receptors. (R)-Hexbutinol displayed the same qualitative affinity profile as p-F-HHSiD.

The "$M_3$-selective" agent p-F-HHSiD (Lambrecht *et al.*, 1988a, 1989a,b) showed similar affinities for m1, m3 and m4 receptors, which were up to 9-fold higher ($P < .05$) than those found for m2 and m5 receptors. The "selective" antagonist 4-DAMP displayed similar high affinities for m1, m3, m4 and m5 receptors, whereas its affinity for m2 receptors was at least 3-fold lower ($P < .05$) than for the other subtypes. All five "$M_2$-selective" muscarinic antagonists employed (methoctramine, himbacine, AF-DX 384, AQ-RA 741 and AF-DX 250) bound with high affinities to both m2 and m4 receptors, with intermediate affinities to m1 and m3 receptors, and, with the exception of methoctramine, with even lower affinities to m5 receptors (table 2). This feature was most prominent for AQ-RA 741, which discriminated between m2 *vs.* m5 and m4 *vs.* m5 receptors by factors of 195 and 129 ($P < .05$), respectively.

## Discussion

The characterization of muscarinic receptor subtypes has been complicated by the presence of multiple muscarinic binding sites in most tissues. To overcome this difficulty, the affinity profiles of a series of selective muscarinic antagonists has been studied by the use of CHO-K1 cell lines stably transformed with the five human muscarinic receptor genes (m1-m5).

It has been suggested that muscarinic receptor subtypes can be characterized based on their stereoselective interaction with chiral antagonists (Lambrecht *et al.*, 1988b, 1989a; Eltze and Figala, 1988; Eveleigh *et al.*, 1989; Feifel *et al.*, 1990). In agree-

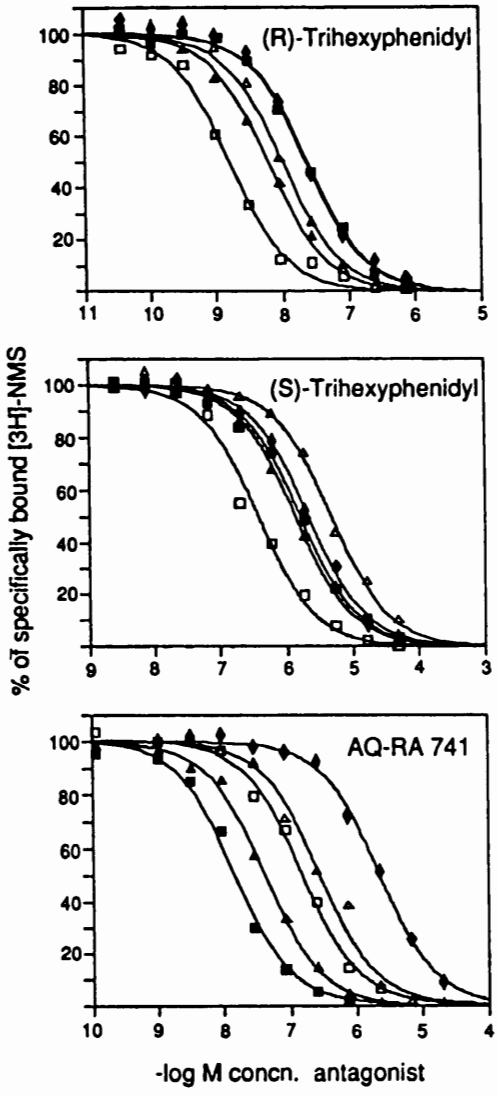


**Fig. 3.** Binding isotherms of (R)- and (S)-trihexyphenidyl and AQ-RA 741 for the displacement of specific [$^3$H]NMS binding to m1 (□), m2 (■), m3 (△), m4 (▲) and m5 (♦) muscarinic receptors. The [$^3$H]NMS concentration used was 150 pM. Specific binding was determined as that displaced by 1 μM atropine. Curves are representative of at least three independent experiments carried out in duplicate.

ment with these reports, the (R)-enantiomers of trihexyphenidyl and hexbutinol displayed considerably higher (up to 525-fold) affinities to all five subtypes than their corresponding (S)-enantiomers. Stereoselectivity ratios [($K_i$(S)/$K_i$(R)] were lowest at m2 receptors (fig. 4), suggesting that less stringent configurational demands are made by this subtype. Stereoselectivity ratios found for m1, m3, m4 and m5 receptors differed by a factor of less than 4, thus limiting the utility of the employed stereoisomers to subclassify muscarinic receptors. The "$M_1$-selective" antagonist (R)-trihexyphenidyl (Lambrecht *et al.*, 1988b; Waelbroeck *et al.*, 1989b), similar to its corresponding (S)-enantiomer, (S)-hexbutinol and pirenzepine (Wess *et al.*, 1991) displayed higher affinities for m1 and m4 than for m2, m3 and m5 receptors.

The "$M_3$-selective" antagonist p-F-HHSiD (Lambrecht *et al.*, 1988a, 1989a,b) bound with high affinities to m1, m3 and m4 receptors. Its binding profile was very similar to that obtained for (R)-hexbutinol and HHSiD (Buckley *et al.*, 1989). Although p-F-HHSiD did not show a marked selectivity for a single

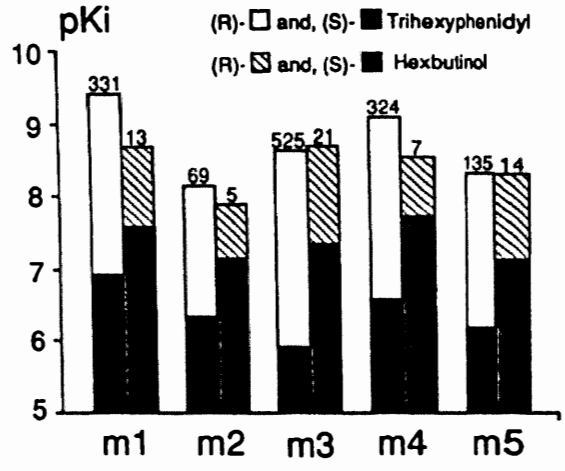


**Fig. 4.** Affinity profiles (pK$_i$ = −log inhibition constant) of the enantiomers of trihexyphenidyl and hexbutinol at five human muscarinic receptor subtypes. The stereoselectivity ratios [$K_i$(S)/$K_i$(R)] are given on top of each column.

TABLE 3

**Comparison of affinity profiles of muscarinic receptor antagonists determined at pharmacologically ($M_1$–$M_3$) and molecularly (m1–m5) characterized muscarinic receptor subtypes[a]**

| Antagonist | Affinity Profiles[b] | |
|---|---|---|
| (R)-Trihexyphenidyl[c] | $M_1 > M_3 > M_2$ | m1 ≥ m4 > m3 ≥ m5 ≥ m2 |
| (S)-Trihexyphenidyl[c] | $M_1 > M_2 \geq M_3$ | m1 ≥ m4 ≥ m2 ≥ m5 ≥ m3 |
| (R)-Hexbutinol[d] | $M_1 = M_3 > M_2$ | m1 = m3 ≥ m4 ≥ m5 ≥ m2 |
| (S)-Hexbutinol[d] | $M_3 \geq M_2 \geq M_1$ | m4 ≥ m1 ≥ m3 ≥ m2 ≈ m5 |
| Pirenzepine[e] | $M_1 > M_3 \geq M_2$ | m1 > m4 ≥ m5 ≥ m3 ≥ m2 |
| p-F-HHSiD[e] | $M_3 > M_1 > M_2$ | m3 ≥ m1 ≥ m4 > m5 ≥ m2 |
| 4-DAMP[f] | $M_1 \geq M_3 > M_2$ | m3 ≈ m1 ≥ m5 ≈ m4 > m2 |
| Methoctramine[g] | $M_2 > M_1 > M_3$ | m2 ≥ m4 ≥ m1 ≥ m5 ≥ m3 |
| Himbacine[f] | $M_1 \geq M_2 > M_3$ | m2 ≈ m4 > m3 ≈ m1 > m5 |
| AF-DX 384[h] | $M_2 > M_3$ | m2 ≥ m4 > m1 ≥ m3 > m5 |
| AQ-RA 741[h] | $M_2 > M_3$ | m2 ≥ m4 > m1 ≥ m3 > m5 |
| AF-DX 250[f] | $M_2 \geq M_1 > M_3$ | m2 ≥ m4 ≥ m1 ≥ m3 > m5 |

[a] Affinity profiles at pharmacologically defined subtypes were determined in functional studies on rabbit vas deferens ($M_1$), guinea pig atria ($M_2$) and guinea pig longitudinal smooth muscle of the ileum ($M_3$). In case of AF-DX 384 and AQ-RA 741, data from functional studies on $M_1$ receptors were not available.

[b] Differences in antagonist affinities for cloned muscarinic receptors by a factor of ≥3 are indicated by > and proved to be significant ($P < .05$).

[c,d,e,f,g,h] Affinity profiles for $M_1$, $M_2$ and $M_3$ muscarinic receptors according to references: [c] Lambrecht *et al.*, 1988b; [d] Feifel *et al.*, 1990; [e] Lambrecht *et al.*, 1988a; [f] Dörje *et al.*, 1990 [it should be noted that himbacine shows about 10-fold higher affinity for $M_1$ receptors in the rabbit vas deferens than for rat cortical $M_1$ receptors (Lazareno and Roberts, 1989)]; [g] Lambrecht *et al.*, 1989b; [h] Eberlein *et al.*, 1989.

subtype as previously reported (Lambrecht *et al.*, 1988, 1989a,b; Eglen *et al.*, 1990), it displayed up to 9-fold lower affinities for m2 and m5 than for m1, m3 and m4 receptors.

The "selective" muscarinic antagonist 4-DAMP (Barlow *et al.*, 1976; Doods *et al.*, 1987) is widely used in both functional and radioligand binding studies to discriminate between $M_1$ and $M_3$ *vs.* $M_2$ receptors. In our study, this compound exhibited similar high affinities for m1, m3, m4 and m5 but up to 7-fold lower affinity for m2 receptors. This binding profile is shared by the pirenzepine derivative UH-AH 37 (Wess *et al.*, 1991) and sila-hexocyclium (Buckley *et al.*, 1989).

Methoctramine (Melchiorre *et al.*, 1987; Waelbroeck *et al.*, 1989a), himbacine (Gilani and Cobbin, 1986; Lazareno and Roberts, 1989), AF-DX 250 [the (+)-enantimer of AF-DX 116; Engel *et al.*, 1989], AF-DX 384 and AQ-RA 741 (Eberlein *et al.*, 1989) are classified as "$M_2$-selective" antagonists. We found, however, that all five antagonists possess similarly high affinities to both m2 and m4 receptors (table 2). Nevertheless,

methoctramine, himbacine and the derivatives of AF-DX 116 represent valuable tools to distinguish between m2 and m4 receptors *vs.* all other subtypes. For example, all five compounds clearly discriminate between m2 (m4) and m3 receptors with affinity differences ranging from 9-(himbacine) to 16-fold (methoctramine). With the exception of himbacine, which showed about a 10-fold higher affinity for m2 (m4) than for m1 receptors, the ability of these compounds to distinguish between m2 (m4) receptors and m1 receptors was less pronounced. Most remarkably, himbacine, AF-DX 384, AQ-RA 741 and AF-DX 250 bound to m5 receptors with substantially reduced affinities compared with all other subtypes (table 2). AQ-RA 741, for instance, displayed 195- and 129-fold lower affinities for m5 than for m2 and m4 receptors, respectively. The close similarity between the selectivity profiles of himbacine and the three AF-DX 116 derivatives is reflected in the chemical structures of these agents. All four antagonists are composed of a tricyclic ring system linked to a substituted piperidine ring by a short side chain (fig. 1).

Our data indicate that muscarinic antagonists can be grouped based on their receptor-selectivity profiles. The most striking examples are the group of antagonists displaying high affinities for m1 and m4 receptors, and the group of compounds exhibiting preferential binding for m2 and m4 receptors (tables 2 and 3). One might speculate that the differential binding selectivities of each group of antagonists are dependent on distinct receptor domains. For instance, residues that are identical between the m1 and m4 receptors may be important in the binding of m1/m4 selective compounds, whereas regions conserved among m2 and m4 receptors may be essential in the binding of m2/m4 selective antagonists. Sequence analysis of the receptors provides numerous candidate regions. To determine the potential importance of these domains for subtype-selective antagonist binding, we are presently preparing chimeric muscarinic receptors. It is hoped that these studies will allow a precise definition of the multiple structural determinants that apparently contribute to subtype-selective drug interaction.

Although none of the tested antagonists showed a marked selectivity for one subtype over all other subtypes, distinct selectivity profiles are apparent. In general, these profiles are consistent with the known selectivities of antagonists for muscarinic receptors that led to the $M_1/M_2/M_3$ classification (table 3). However, the present data also highlight the limitations of this pharmacologic scheme, demonstrating the necessity to determine antagonist affinities to all five muscarinic receptor subtypes. Whereas the antagonist affinities determined for cloned m2 and m3 receptors (this study) generally correlated well with those for the pharmacologically defined $M_2$ and $M_3$ subtypes, the assignment of the $M_1$ receptor is more problematic. The antagonist binding properties of both m1 and m4 receptor proteins were similar to those of the putative $M_1$ receptors. Thus, the identification of muscarinic receptors with $M_1$-like antagonist binding properties in a given tissue may be indicative of either m1 or m4 or a mixture of these two receptor subtypes.

Whereas most antagonists bound to m1 and m4 receptors with similar affinities, two compounds, pirenzepine and himbacine, are able to discriminate between these two subtypes. Pirenzepine showed 6-fold higher affinity for m1 than for m4 receptors (Wess *et al.*, 1991), whereas himbacine exhibited 10-fold selectivity for m4 receptors. Given the relatively low selectivity of pirenzepine, himbacine appears to be the most valuable tool to distinguish between m1 and m4 receptors.

The fact that the antagonists currently used to classify muscarinic receptors lack a clear subtype-selectivity may explain that only three major subtypes (putative $M_1$–$M_3$) have been identified in classic pharmacologic studies. In this study, we have shown that all five muscarinic receptors exhibit unique antagonist binding profiles. Therefore, the judicious use of a variety of selected antagonists should eventually allow the pharmacologic identification of all five muscarinic receptor subtypes.

In conclusion, our study has further substantiated the utility of cloned receptors stably expressed in mammalian cell lines to unambiguously determine the selectivity profile of muscarinic antagonists. Given the considerable therapeutic potential of subtype-selective antimuscarinic agents (Wess *et al.*, 1990), this approach should be extremely useful for the future development of novel antimuscarinic drugs.

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