Stereoselective inhibition of muscarinic receptor subtypes by the enantiomers of hexahydro-difenidol and acetylenic analogues


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1 The affinities of the (R)- and (S)-enantiomers of hexahydro-difenidol (1) and its acetylenic analogues hexbutinol (2), hexbutinol methiodide (3) and p-fluoro-hexbutinol (4) (stereochemical purity > 99.8%) for muscarinic receptors in rabbit vas deferens (M1), guinea-pig atria (M2) and guinea-pig ileum (M3) were measured by dose-ratio experiments.

2 The (R)-enantiomers consistently showed higher affinities than the (S)-isomers. The stereoselectivity ratios ([R]/[S]) were greatest with the enantiomers of 1 (vas deferens: 550; ileum: 191; atria: 17) and least with those of the p-Fluoro-analogue 4 (vas deferens: 34; ileum: 8.5; atria: 1.7).

3 The enantiomeric potency ratios for compounds 1-4 were highest in rabbit vas deferens, intermediate in guinea-pig ileum and much less in guinea-pig atria. Thus, these ratios may serve as a predictor of muscarinic receptor subtype identity.

4 (S)-p-Fluoro-hexbutinol ([S]-4) showed a novel receptor selectivity profile with preference for M3 receptors: M3 > M2 > M1.

5 These results do not conform to Pfieffer's rule that activity differences between enantiomers are greater with more potent compounds.

Introduction

A large body of evidence derived from both functional and radioligand binding studies suggests that there are at least three pharmacological muscarinic receptor subtypes (M1, M2 and M3) ([Eglen & Whiting, 1986; Mitchelson, 1988; Giraldo et al., 1989; Waelbroeck et al., 1988a; 1989; Lambrecht et al., 1989d]). This subclassification was recently confirmed by cloning, sequencing and expression of complementary DNA encoding five muscarinic receptors (m1-m5) (Kerlavage et al., 1987; Peralta et al., 1987; Akiba et al., 1988; Brann et al., 1988). The antagonist binding properties of m1-m3 and their patterns of expression in various tissues closely correspond to those of the M1, M2 and M3 receptors (Peralta et al., 1987; Akiba et al., 1988; Maeda et al., 1988; Buckley et al., 1989). The selective muscarinic antagonists pirenzepine (Hammer et al., 1980; Lambrecht et al., 1988a; Waelbroeck et al., 1988a, 1989), methoctramine (Melchiorre et al., 1987; Wess et al., 1988), AF-DX 116 (Giachetti et al., 1986), hexahydro-sila-difenidol (Mutschler & Lambrecht, 1984; Lambrecht et al., 1988a; 1989d; Waelbroeck et al., 1988a; 1989d; Waelbroeck et al., 1989d) and p-fluoro-hexahydro-sila-difenidol (Lambrecht et al., 1988a; 1989b,d) have proved to be useful tools in this sub-classification.

Among these selective muscarinic antagonists, racemic hexahydro-difenidol ([R,S]-1; Figure 1) has been shown to have high affinity for M1 receptors in neuronal tissues as well as for M3 receptors in exocrine glands and smooth muscles, but a much lower affinity for cardiac M2 receptors (Mutschler & Lambrecht, 1984; Eltze et al., 1988; Lambrecht et al., 1988c; Waelbroeck et al., 1989). The main aim of the present study was to characterize the structural demands, including stereocchemical aspects, for potency and selectivity of some chiral acetylenic analogues of hexahydro-difenidol (1) (Figure 1). Since a conformationally rigid acetylenic moiety is present in some selective muscarinic agonists such as McN-A-343 (Roszkowski, 1961; Lambrecht et al., 1986; Eltze et al., 1988; Wess et al., 1988) and arecaidine propargyl ester (Mutschler & Hultzsch, 1973; Mutschler & Lambrecht, 1984; Moese et al., 1989), it would be of interest to investigate whether the acetylenic analogues of hexahydro-difenidol are also selective for muscarinic receptor subtypes.

In the last few years, data have accumulated that muscarinic receptors can be differentiated on the basis of their stereoselectivity to chiral antagonists such as procyclidine (Lambrecht & Mutschler, 1986; Tacke et al., 1986; Waelbroeck et al., 1988b), trihexyphenidyl (Lambrecht et al., 1988a, 1989d), phenyltiramide (Lambrecht et al., 1989a), biperiden (Elzle & Figala, 1988) and telenzepine (Eveleigh et al., 1989). Hexahydro-difenidol (1) and its analogues hexbutinol (2), hexbutinol methiodide (3) and p-fluoro-hexbutinol (4) (Figure 1) possess a centre of chirality and therefore exist in two enantiomers. We took advantage of this by determining the antagonist affinities of the individual enantiomers of these compounds at muscarinic receptor subtypes. The results were compared with those obtained for the selective reference drugs hexahydro-sila-difenidol (M3 > M1 > M2) and p-fluoro-hexahydro-sila-difenidol (M2 > M1 > M3). The receptors studied were presynaptic M1 heteroreceptors in rabbit vas deferens (Elzle, 1988; Elzle et al., 1988; Lambrecht et al., 1988a,b), cardiac M2 receptors present in guinea-pig atria and smooth muscle M3 receptors present in guinea-pig ileum.

Some of the present results have been briefly presented elsewhere (Feifel et al., 1988; Lambrecht et al., 1989c; Tacke et al., 1989).

Methods

Rabbit isolated vas deferens

Experiments on rabbit isolated vas deferens were carried out according to Elzle (1988) and Elzle et al. (1988). Male New Zealand white rabbits (2.5-3.0 kg) were killed by i.v. injection of 120 mg kg-1 pentobarbital sodium. Vasa deferentia were excised, dissected free of connective tissues and divided into four segments of approximately 1.5 cm length. The preparations were set up in 7 ml organ baths containing modified...
Electively inhibited by the M

(4-Cl-McN-A-343) (Eltze CaCl₂ 2.5, ester (Mutschler

dislocation. The organs required were removed and set up in

displacement transducer connected to a

The contractions were measured isometrically by a

stimulation

a Rikadenki polygraph. These effects were

isometric twitch contractions were elicited by electrical field

was applied and after a

30min period of initial equilibration

were paced electrically (2 Hz, 3 ms, 5 V) by means of platinum

electrodes. Negative inotropic effects to the agonist were mea-

sured as changes in isometric tension. Responses of ileal longi-

tudinal muscle strips (Paton & Zar, 1968) to arecaidine propargyl ester were measured as isometric contractions. The effects in atria and ileum were recorded as with the rabbit isolated vas deferens.

Antagonist affinities

After a 1 h equilibration period, concentration-response curves were constructed by adding doses of the agonists cumula-

tively, according to the method of Van Rossum (1963). When these responses were constant, concentration-response curves were repeated in the presence of antagonists. At least three concentra-

tions of antagonists with log intervals of 0.5 were tested 3 to 5 times (see Table 1) in the three tissues. Each concentration of antagonist was allowed to equilibrate for 15 to 30 min (ileum) and 30 min (atrium) in guinea-pig pre-

parations, respectively, and 30 [(S)-isomers] to 60 min [(R)-

isomers] in rabbit vas deferens. Preliminary experiments indi-

cated that these intervals were sufficient for equilibration of the antagonist concentrations used. No preparation was exposed to more than three concentrations of antagonists. EC₅₀ values of agonists in the absence and presence of antagonists were determined graphically for calculation of dose-ratios. The slopes of the Arunlakshana-Schild plots (Arunlakshana & Schild, 1959) were determined by linear regression by the method of least squares. pA₂ values were estimated as the intercept on the abscissa scale by fitting to the data the best straight line with a slope of unity (Tallarida et al., 1979).

Data analysis

All data are presented as means ± s.e.mean of 9–17 experi-

ments. Differences between mean values were tested for sta-

tistical significance by Student's t test; P < 0.05 was accepted

as being significant. Linear regression analyses were carried

out by the method of least squares (Tallarida et al., 1979).

Drugs

Pirenzepine dihydrochloride was obtained from Boehringer Ingelheim (F.R.G.). 4-(4-Chlorophenylcarbamoyloxy)-2-

butynitrilmethylammonium iodide (4-Cl-McN-A-343) (Nelson et al., 1976), arecaidine propargyl ester (Mutschler &

Hültzsch, 1973), racemic hexahydro-sila-difenidol hydro-

chloride (Tacke et al., 1985), (R)- and (S)-hexahydro-sila-

hydrochloride ([R]-/HC] and ([S]-/HC] (Tacke et al., 1989)
as well as (R)- and (S)-hexbutinol [(R)-2 and (S)-2] (Tacke et al., 1985) were synthesized in our laboratories ac-

cording to the literature. The enantiomeric excess (ee) of the enantiomers of hexahydro-difenidol and hexbutinol was

> 99.8%, determined by calorimetric analysis as described by

Tacke et al. (1987). Racemic p-fluoro-hexahydro-sila-difenidol hydrochloride was prepared by analogy to the synthesis of

hexahydro-sila-difenidol (Tacke et al., unpublished results). The enantiomers of p-fluoro-hexbutinol [(R]-4 and (S)-4;

enantiomeric purity: ee > 99.6%; calorimetric analysis) were synthesized by analogy to (R)- and (S)-hexbutinol (Tacke et al., 1989). The other chemicals not described under 'Synthetic

chemistry' were of reagent grade and were used as purchased.

Synthetic chemistry

The enantiomers of hexbutinol methiodide [(R)-3 and (S)-3] were prepared as follows:

Freshly distilled methyl iodide (18 mmol) was added under an atmosphere of dried nitrogen to a solution of (R)- or (S)-

hexbutinol (2) (8 mmol) in dry ethanol (50 ml). The reaction mixture was stirred for 3 h at 30°C, dry n-pentane (100 ml) was added, and the mixture was stirred for 1 h at 20°C. Thereafter, the precipitate was collected by filtration and then recr-

Polations.
Table 1 pA2 values and slopes of Arunakshanha-Schild plots (in parentheses) for muscarinic antagonists at M1, M2, and M3 receptors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rabbit vas deferens (M1)</th>
<th>Guinea-pig atria (M2)</th>
<th>Guinea-pig ileum (M3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHSID*</td>
<td>7.92 ± 0.07</td>
<td>6.53 ± 0.05</td>
<td>7.96 ± 0.03</td>
</tr>
<tr>
<td>p-F-HHSID*</td>
<td>6.68 ± 0.12</td>
<td>6.01 ± 0.06</td>
<td>7.84 ± 0.03</td>
</tr>
<tr>
<td>Hexahydro-difenidol (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R)-1</td>
<td>8.71 ± 0.05</td>
<td>7.03 ± 0.06*</td>
<td>8.35 ± 0.04*</td>
</tr>
<tr>
<td>(S)-1</td>
<td>5.97 ± 0.04</td>
<td>5.80 ± 0.07*</td>
<td>6.07 ± 0.05*</td>
</tr>
<tr>
<td>Hexahydro-difenidol methiodide (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R)-2</td>
<td>8.78 ± 0.05</td>
<td>7.77 ± 0.04*</td>
<td>8.78 ± 0.04*</td>
</tr>
<tr>
<td>(S)-2</td>
<td>6.75 ± 0.07</td>
<td>6.84 ± 0.05*</td>
<td>7.14 ± 0.04*</td>
</tr>
<tr>
<td>Hexahydro-hexbutinol methiodide (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R)-J</td>
<td>9.43 ± 0.06</td>
<td>8.62 ± 0.02</td>
<td>8.85 ± 0.04</td>
</tr>
<tr>
<td>(S)-J</td>
<td>7.83 ± 0.05</td>
<td>7.40 ± 0.02</td>
<td>7.26 ± 0.02</td>
</tr>
<tr>
<td>p-Fluoro-hexbutinol (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R)-4</td>
<td>8.08 ± 0.06</td>
<td>6.97 ± 0.04</td>
<td>8.50 ± 0.04</td>
</tr>
<tr>
<td>(S)-4</td>
<td>6.55 ± 0.08</td>
<td>6.75 ± 0.02</td>
<td>7.57 ± 0.04</td>
</tr>
</tbody>
</table>

* Data taken from Lambrecht et al. (1988a).

Table 2 Receptor selectivity and stereoselectivity [(R)/(S)] ratios for chiral muscarinic antagonists

<table>
<thead>
<tr>
<th>Receptor selectivity</th>
<th>Stereoselectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1/M2</td>
<td>M3/M2</td>
</tr>
<tr>
<td>Hexahydro-difenidol (1)</td>
<td></td>
</tr>
<tr>
<td>(R)-J</td>
<td>48</td>
</tr>
<tr>
<td>(S)-J</td>
<td>1.5</td>
</tr>
<tr>
<td>Hexahydro-difenidol methiodide (2)</td>
<td></td>
</tr>
<tr>
<td>(R)-2</td>
<td>10</td>
</tr>
<tr>
<td>(S)-2</td>
<td>0.81</td>
</tr>
<tr>
<td>Hexahydro-hexbutinol methiodide (3)</td>
<td></td>
</tr>
<tr>
<td>(R)-3</td>
<td>6.5</td>
</tr>
<tr>
<td>(S)-3</td>
<td>2.7</td>
</tr>
<tr>
<td>p-Fluoro-hexbutinol (4)</td>
<td></td>
</tr>
<tr>
<td>(R)-4</td>
<td>13</td>
</tr>
<tr>
<td>(S)-4</td>
<td>0.63</td>
</tr>
</tbody>
</table>

The values shown represent the antilogs of the differences between corresponding mean pA2 values (Table 1) determined at M1 receptors in rabbit vas deferens as well as at atrial M3 and ileal M1 receptors of guinea-pigs.

The parameters shown represent the mean ± s.e.mean. The slopes of Arunakshanha-Schild plots were determined by linear regression analysis (Tallarida et al., 1979). pA2 values were obtained after the unity constraint had been imposed. The numbers of total data points (n) and tissues used are given in square parentheses. The slopes shown are not significantly different from unity (P > 0.05), except those for (R)-I and (R)-4 at M1 receptors (marked with an asterisk). HHSID = racemic hexahydro-sila-difenidol; p-F-HHSID = racemic p-fluoro-hexahydro-sila-difenidol.

The numbers of total data points (n) and tissues used are given in parentheses for muscarinic antagonists at M1, M2, and M3 receptors. The parameters shown represent the mean ± s.e.mean. The slopes of Arunakshanha-Schild plots were determined by linear regression analysis (Tallarida et al., 1979). pA2 values were obtained after the unity constraint had been imposed. The numbers of total data points (n) and tissues used are given in square parentheses. The slopes shown are not significantly different from unity (P > 0.05), except those for (R)-I and (R)-4 at M1 receptors (marked with an asterisk). HHSID = racemic hexahydro-sila-difenidol; p-F-HHSID = racemic p-fluoro-hexahydro-sila-difenidol.

![Figure 2](image_url)  
**Figure 2** Affinity profiles of the enantiomers of compounds 1-4 at muscarinic M1 receptors in rabbit vas deferens (solid columns), M2 receptors in guinea-pig atria (diagonally-hatched columns) and M3 receptors in guinea-pig ileum (cross-hatched columns).
hexahydro-difenidol (I) (-hexbutinol, 2), quaternionization of 2 (-hexbutinol methiodide, 3) and p-fluoro-substitution of the phenyl ring of 2 (-p-fluoro-hexbutinol, 4) changed the affinities of these compounds for $M_1$, $M_2$ and $M_3$ receptors differently. Thus compounds with qualitatively and/or quantitatively different receptor selectivity profiles were obtained (Table 2 and Figure 2).

At each of the three muscarinic receptor subtypes, the (R)-enantiomer of compounds 1–4 was more potent than the (S)-configured isomers. The differences in affinities between the enantiomers of compounds 1–4 was greatest at the $M_3$, least at $M_1$ and least at $M_2$ receptors (Table 2). The degree of stereoselectivity (Table 2) was also dependent on the structure of the compounds ($M_1$: 1 $> 2 > 3 > 4$; $M_2$: 1 $= 3 > 2 > 4$).

Discussion

Structural variations in the (R)- and (S)-hexahydro-difenidol (I) molecules led to muscarinic antagonists that exhibited a qualitatively or quantitatively different spectrum of receptor selectivity to the parent stereoisomers (R)-I and (S)-I (Table 2, Figure 2). These observed selectivities did not appear to be associated in general with high affinity and absolute configuration (Figure 2). For example, (S)-p-fluoro-hexbutinol [(S)-4] was a relatively weak compound but it had a novel receptor selectivity profile: $M_3 > M_2 > M_1$.

The results of the present study confirm and extend previous findings that rabbit vas deferens (M 1 receptors) as well as guinea-pig ileum, whereas its affinity for guinea-pig atria was lower by factors of 48 and 21, respectively (Table 1). For example, (S)-p-fluoro-hexbutinol [(S)-4] exhibited a relatively higher affinity for M 1 receptors in rabbit ileum (8.78) whereas the affinity for ileal M 3 receptors was nearly unchanged (Table 1). Thus the receptor selectivity profile of (S)-hexahydro-sila-difenidol methiodide [(S)-J] is slightly different from that obtained for the (R)-enantiomer (Figure 2).

The influence of p-fluoro-substitution on potency and selectivity is demonstrated by comparison of (S)-hexahydro-difenidol [(S)-2] and (S)-p-fluoro-hexbutinol [(S)-J] (Table 1). Compared to (S)-2 the fluoro derivative [(S)-4] exhibited a relatively higher affinity (pA 2: 7.57) for M 1 receptors, whereas its antimuscarinic potency at M 1 and M 3 receptors was lower by factors of 6.5 and 6.6, respectively (Table 1). Thus, fluoro-substitution in the para-position of the phenyl ring of (S)-hexbutinol [(S)-E] enhanced its $M_3$-selectivity. The receptor selectivity profile of (S)-p-fluoro-hexbutinol (M 3 > M 1 > M 2) is also different from that of the corresponding (R)-enantiomer (M 3 > M 1 > M 2) and of p-fluoro-hexahydro-sila-difenidol (M 1 > M 2 > M 3) (Table 1).

Stereoselectivity of muscarinic receptors

It has been suggested (Pfeiffer, 1956) that, with greater affinities of drugs, larger differences in pharmacological effects will be seen between the enantiomers of chiral compounds. The results obtained in this study (Table 1 and 2) do not substantiate the above suggestion and its implications (Lehmann, 1986). For example, at M 1 receptors the affinity constants (pA 2 values) of the more potent (R)-enantiomers decrease in the order (R)-hexbutinol methiodide (9.43) > (R)-hexbutinol (8.78) > (R)-hexahydro-difenidol (8.71) > (R)-p-fluoro-hexbutinol (8.08), whereas the stereoselectivity ratios [(R)/(S)] of these chiral compounds are in the order hexahydro-difenidol (1550) > hexbutinol (107) > hexahydro-difenidol (40) > p-fluoro-hexbutinol (34). Thus, in this series of compounds the stereoselectivity ratio at, e.g. M 1 receptors,
was higher by more than one order of magnitude for hexahydro-difenidol (1) than for hexbutinol methiodide (2), although compound (R)-3 was 5 fold more potent than (R)-1. A similar lack of correlation between potency of the eutomer and stereoselectivity ratios of enantiomers was obtained at M₂ and M₃ receptors (Tables 1 and 2). The findings of the present study confirm and extend previous results obtained with the enantiomers of biperiden (Elzé & Figula, 1988), trihexyphenidyl (Lambrecht et al., 1988b; 1989d) and phenyltiramidine (Lambrecht et al., 1989a).

In 1982, Robert et al. stated a corollary of Pfeiffer's rule: "When different receptor subtypes interact with the enantiomers of chiral drugs, their stereoselectivity should increase as a function of affinity of the more potent enantiomer (= eutomer; Lehmann, 1986) for the respective subtypes". However, when the magnitude of receptor subtype stereoselectivity (difference in pA₂ value of the (R)- and (S)-enantiomers) was plotted against the pA₂ value of the more potent isomer for that particular receptor subtype, a strong correlation (correlation coefficient r = 0.995) was only observed for hexahydro-difenidol (Figure 3). The stereoselectivity and the affinity of the eutomer of hexahydro-difenidol was greatest at M₁, intermediate at M₂ and lowest at M₃ receptors. On the other hand the enantiomers of compounds 2-4 did not fulfill the predictions made by Robert et al. (1982). However, the interesting finding of this study is that the stereoselectivity ratios of all the chiral compounds 1-4 consistently show the same order: M₂ > M₃ > M₁. This implies that the biochemical demands made by the muscarinic receptor subtypes are different for the enantiomers of compounds 1-4 being most stringent at M₁ receptors. Similar results have been obtained with the enantiomers of telenzepine (Eveleigh et al., 1989), biperiden (Elzé & Figula, 1988), trihexyphenidyl and its methiodide (Lambrecht et al., 1988b) and procyclidine (Lambrecht & Mutschler, 1986; Waelbroeck et al., 1988b).

In conclusion, the present study shows that the anti-muscarinic potencies and receptor subtype selectivities of the enantiomers of hexahydro-difenidol (1) and the acetylenic analogues 2-4 (Figure 1) depend on different structural parameters including absolute configuration. The M₁, M₂ and M₃ receptors make qualitatively and quantitatively different stereoselective demands for the (R)- and (S)-enantiomers, resulting in different receptor selectivity profiles. It is interesting to note that, of the enantiomers investigated in this study, (S)-fluro-hexbutinol shows a novel receptor selectivity profile: M₂ > M₃ > M₁ (Figure 2). There was a variation in stereoselectivity ratios on the three receptor subtypes (Table 2): M₁ > M₂ > M₃. These results indicate that stereoselectivity ratios can be successfully used as a parameter to characterize muscarinic receptor subtypes providing information that racemates cannot give. However, the stereoselectivity ratios do not conform to the predictions of the 'classical' Pfeiffer's rule (Pfeiffer, 1956), and that of its corollary (Robert et al., 1982).

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