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Stereoselectivity of (R)- and (S)-Hexahydro-Difenidol Binding to Neuroblastoma M_1, Cardiac M_2, Pancreatic M_3, and Striatum M_4 Muscarinic Receptors

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ABSTRACT (R)-Hexahydro-difenidol has a higher affinity for M_1 receptors in NB-OK 1 cells, pancreas M_3 and striatum M_4 receptors (pK_i 7.9 to 8.3) than for cardiac M_2 receptors (pK_i 7.0). (S)-Hexahydro-difenidol, by contrast, is nonselective (pK_i 5.8 to 6.1). Our goal in the present study was to evaluate the importance of the hydrophobic phenyl, and cyclohexyl rings of hexahydro-difenidol for the stereoselectivity and receptor selectivity of hexahydro-difenidol binding to the four muscarinic receptors. Our results indicated that replacement of the phenyl ring of hexahydro-difenidol by a cyclohexyl group (→ dicyclidol) and of the cyclohexyl ring by a phenyl moiety (→ difenidol) induced a large (4- to 80-fold) decrease in binding affinity for all muscarinic receptors. Difenidol had a significant preference for M_1, M_3, and M_4 over M_2 receptors; dicyclidol, by contrast, had a greater affinity for M_1 and M_4 than for M_2 and M_3 receptors. The binding free energy decrease due to replacement of the phenyl and the cyclohexyl groups of (R)-hexahydro-difenidol by, respectively, a cyclohexyl and a phenyl moiety was almost additive in the case of M_4 (striatum) binding sites. In the case of the cardiac M_2, pancreatic M_3, or NB-OK 1 M_1 receptors the respective binding free energies were not completely additive. These results suggest that the four (R)-hexahydro-difenidol "binding moieties" (phenyl, cyclohexyl, hydroxy, and protonated amino group) cannot simultaneously form optimal interactions with the M_1, M_2, and M_3 muscarinic receptors. When each of the hydrophobic groups is modified, the position of the whole molecule, relative to the four subsites, was changed to allow an optimal overall interaction with the muscarinic receptor.

KEY WORDS: hexahydro-difenidol enantiomers, muscarinic receptor subtypes M_1, M_2, M_3, and M_4, stereoselective interaction, difenidol, dicyclidol

INTRODUCTION

At least three pharmacologically and structurally distinct muscarinic receptors coexist in mammalian tissues.[1,2] M_1 receptors, with a high affinity for pirenzepine, are typically found in neuronal tissues.[3] These receptors have a low affinity for AF-DX 116 (11-({(2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5-11-dihydro-6*H*-pyrido(2,3-*b*)(1,4)benzodiazepin-6-one) and a high affinity for 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) and hexahydro-sila-difenidol.[4–6] M_2 receptors, with a low affinity for pirenzepine and a high affinity for AF-DX 116, are typical of cardiac tissues.[7] They show a low affinity for 4-DAMP and hexahydro-sila-difenidol.[4–6,8,9] M_3 receptors have low affinities for pirenzepine and AF-DX 116, and high affinities for 4-DAMP[10] and hexahydro-sila-difenidol.[5,6,9] They are typically detected in secretory glands[11,12] and in smooth muscle.[1,2,9]

There is a candidate M_4 receptor found in rat striatum (previously called "B" sites) which differs from M_3 receptors by its high affinity for himbacine and methoctramine.[13]

Hexahydro-difenidol (Fig. 1) is well characterized as ileum-preferring muscarinic antagonist (high affinity for M_3 receptors). It recognizes M_3 receptors (in smooth muscle or secretory glands) with a 10- to 30-fold

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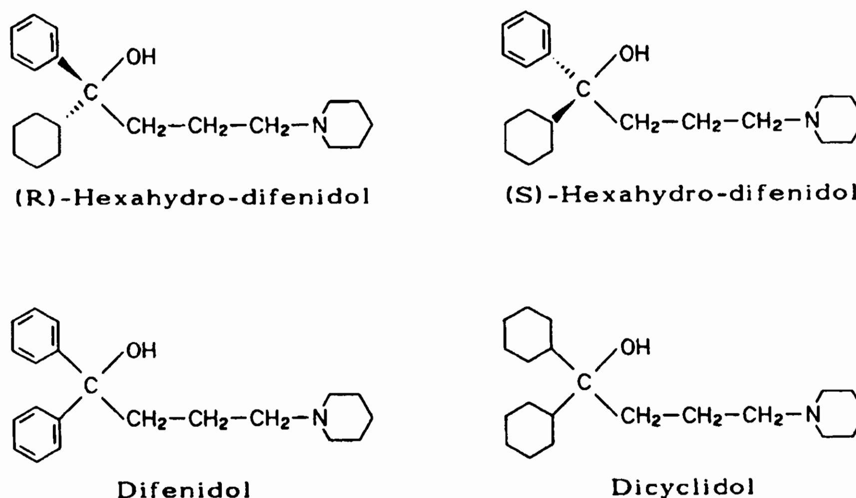

Fig. 1. Chemical structure of (R)- and (S)-hexahydro-difenidol, difenidol, and dicyclidol.

greater affinity than M_2 receptors.[5,6,9] We have already demonstrated that muscarinic receptors are capable of discriminating the two enantiomers of hexahydro-difenidol, and have a greater affinity for the (R)-enantiomer.[14,22]

In a previous study on the stereoselective binding of procyclidine enantiomers, we suggested that muscarinic receptor subtypes possess four binding subsites for antagonists.[15] One of these "subsites" is the ionic binding site recognizing the protonated amino group of procyclidine; the second, a polar site forming a hydrogen bond with the procyclidine hydroxy group, and the two hydrophobic binding sites recognizing, respectively, the phenyl or the cyclohexyl groups of (R)-procyclidine with a greater affinity. Our results further indicated that when (S)-procyclidine is brought into contact with the receptor, the ionic and hydroxy groups are in the correct position, and the phenyl and the cyclohexyl groups in contact, respectively, with the cyclohexyl-preferring and phenyl-preferring subsites.[15]

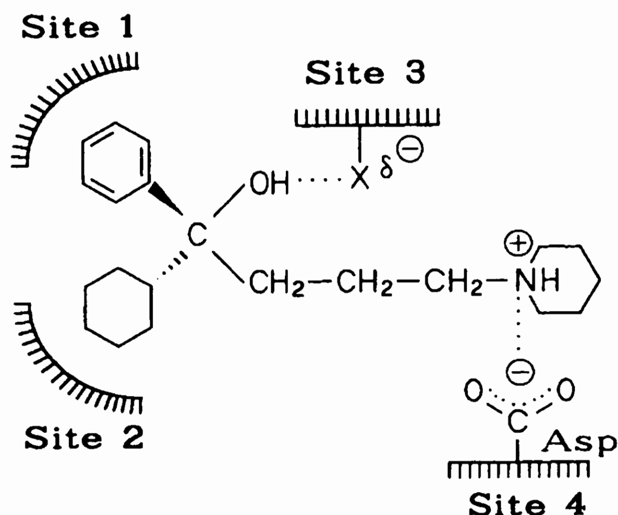

Fig. 2. Interaction of protonated (R)-hexahydro-difenidol with four subsites of the muscarinic receptor. Asp, aspartate residue.[23]

Given the great structure homology of procyclidine and hexahydro-difenidol, we expected that the procyclidine binding model should also be applicable to hexahydro-difenidol (Fig. 2). To test this hypothesis, we decided to measure the affinity of two achiral compounds structurally related to hexahydro-difenidol: difenidol (containing an additional phenyl moiety instead of the cyclohexyl group) (Fig. 1) and dicyclidol (containing an additional cyclohexyl moiety instead of the phenyl group). We hoped to measure the difference of binding energy of a phenyl versus cyclohexyl group with each hydrophobic subsite.

MATERIALS AND METHODS

Drugs

[^{3}H]NMS [1-(*N*-methyl-^{3}H)scopolamine methochloride, 80 to 85 Ci/mmol] was obtained from Amersham International (Bucks, England). Atropine sulfate and polyethyleneimine were from Sigma Chemical Co. (St. Louis, MO). All the other reagents were of the highest grade available. All antagonists tested were synthesized in our laboratories. The hexahydro-difenidol enantiomers were prepared as previously published,[16] difenidol was synthesized according to the literature,[17] and dicyclidol was obtained by analogy to the synthesis of (R)- and (S)-hexahydro-difenidol[16] starting from dicyclohexyl ketone (unpublished results). The enantiomeric excess (ee) of (R)- and (S)-hexahydro-difenidol was >99.8%, determined by calorimetric analysis.[16]

Homogenate Preparations

Human NB-OK 1 neuroblastoma cells were cultured in RPMI 1640 medium enriched with 10% foetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.[4] For [^{3}H]NMS binding experiments, the cells were rinsed, detached, and centrifuged in 20 m*M* sodium phosphate buffer (pH 7.4) containing 150 m*M* NaCl and 1 m*M* EDTA, resuspended and homogenized

in 20 mM Tris-HCl buffer (pH 7.5) enriched with 5 mM $MgCl_2$, and stored in liquid nitrogen until use.

To prepare rat tissue homogenates, male Wistar albino rats (200–250 g) were killed by decapitation and the heart, pancreas, and striatum immediately removed. Homogenate preparations were performed at 4°C. The heart was rinsed in isotonic NaCl, then homogenized in 2.5 ml of 20 mM Tris-HCl buffer (pH 7.5), enriched with 250 mM sucrose, with an Ultraturrax homogenizer (maximal speed for 5 sec) followed by addition of 12.5 ml of the same buffer, 7 up and down strokes with a glass-Teflon homogenizer, and filtration on 2 layers of medical gauze. The resulting homogenate was used immediately or stored in liquid nitrogen until use. The striatum was homogenized in 2 ml of 20 mM Tris-HCl buffer (pH 7.5) enriched with 250 mM sucrose, using a glass-Teflon homogenizer (7 up and down strokes). The resulting homogenate was stored in liquid nitrogen until use, and diluted 20-fold with the same buffer immediately before the experiment. The pancreas was minced with scissors, homogenized with a glass-Teflon homogenizer in 8 ml of 300 mM sucrose enriched with Trasylol (500 KIU/ml) and bacitracin (0.2 mg/ml). The resulting homogenate was immediately filtered on two layers of medical gauze and diluted 11-fold with an incubation buffer made of 66 mM sodium phosphate (pH 7.4), 2.6 mM $MgCl_2$, 500 KIU/ml Trasylol, 0.2 mg/ml bacitracin, and 13 mg/ml bovine serum albumin.

Receptor-Binding Studies

Binding studies were performed at 25°C in a 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$ and containing [^{3}H]NMS and the indicated unlabelled drug concentrations in a total volume of 1.2 ml. For pancreas binding studies, the incubation buffer also contained bovine serum albumin (1% w/v), bacitracin (0.2 mg/ml), and Trasylol (500 KIU/ml) to prevent receptor degradation.

To measure [^{3}H]NMS binding to human NB-OK 1 cell homogenates, we used 80 μl of homogenate, corresponding to about 200 μg protein per assay. The incubation period was 2 h in the presence of 0.25 nM [^{3}H]NMS in order to allow binding equilibrium. This [^{3}H]NMS concentration was equivalent to 2-fold the tracer's K_D value at M_1 receptors.[4] For incubation with rat heart homogenates, we used 80 μl of the homogenate, corresponding to 400–500 μg protein per assay. The 2-h incubation period was sufficient to allow equilibrium binding. The [^{3}H]NMS concentration used was 1.0 nM, i.e., 2-fold the tracer's K_D value at M_2 binding sites.[8] In rat striatum homogenates, [^{3}H]NMS labels M_1 and M_4 sites but dissociates faster from M_1 receptors.[4,8,18] We preincubated 80 μl of the homogenate (equivalent to about 30 μg protein) in a total volume of 1.2 ml in the presence of [^{3}H]NMS and unlabeled drugs. A 2-h preincubation period allowed equilibrium binding. We then added 1 μM atropine and let tracer dissociation to proceed for 35 min before filtration. This procedure permitted us to investigate tracer binding to striatum M_4 ("B") receptors only.[4,8] The tracer concentration used in these experiments (0.25 nM) was equivalent to 5-fold the tracer's K_D value at striatum M_4 receptors.[8] For incubation with rat pancreas homogenate, we added 980 μl of homogenate to 220 μl drug and tracer (in water). A 4-h incubation period was necessary to allow equilibrium binding.[11] The [^{3}H]NMS concentration used was 0.24 nM, i.e., 2-fold the tracer's K_D value at pancreas M_3 binding sites.[11] All incubations were terminated by addition of 2 ml of ice-cold filtration buffer (50 mM sodium phosphate buffer, pH 7.4). Bound and free tracer were immediately separated by filtration on GF/C glass-fiber filters (Whatman, Maidstone, England) presoaked overnight in 0.05% polyethyleneimine. The samples were rinsed three times with filtration buffer. The filters were then dried, and the bound radioactivity counted by liquid scintillation. Nonspecific [^{3}H]NMS binding was defined as tracer bound in the presence of 1 μM atropine.

Protein concentration was determined according to Lowry et al.[19] using bovine serum albumin as standard.

Analysis of Binding Data

All competition curves were repeated in duplicate, at least three times on different preparations. IC_{50} values were determined by a computer-aided procedure,[20] assuming the existence of only one receptor subtype. Indeed, experimental data points were within 3% of expected values, assuming that the molecules investigated competed with [^{3}H]NMS for binding to a single site.

K_i values were calculated from IC_{50} values, using the Cheng and Prusoff equation[21] which assumes competitive inhibition of tracer binding to a single receptor subtype. The [^{3}H]NMS K_D value for the four systems investigated was determined in separate experiments.[4,8,11] The pK_i values, summarized in Table 1, corresponds to $-\log K_i$ values.

The standard deviation of $-\log IC_{50}$ determination was equal to or below 0.1 log unit. Repeated determinations of [^{3}H]NMS K_D values were within 10% of each other. This error should be added to errors in IC_{50} determinations, since [^{3}H]NMS K_D values were used to calculate pK_i values. We therefore estimated the standard deviation of pK_i values as being of approximately 0.15 log unit (40% of K_i value).

The binding free energy (ΔG) for the formation of a ligand–receptor complex is related to its affinity constant K_a by Eq. (1):

$$\Delta G = -RT \ln K_a \qquad (1)$$

ΔG values were therefore calculated according to Eq. (2), using experimentally determined K_i values ($K_a = K_i^{-1}$):

$$\Delta G = -RT \ln 1/K_i \qquad (2)$$

RESULTS

As shown in Figures 3 and 4, the four compounds

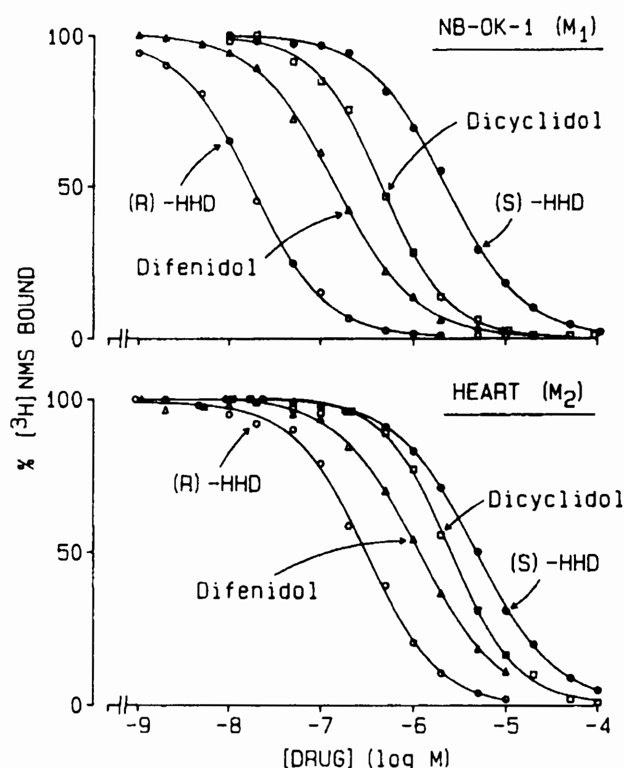

Fig. 3. [^{3}H]NMS competition curves in membranes from human neuroblastoma NB-OK 1 cells (upper panel) or rat heart (lower panel). [^{3}H]NMS binding was measured in the absence or presence of (R)-hexahydro-difenidol (○), (S)-hexahydro-difenidol (●), difenidol (△), or dicyclidol (□), as described in methods.

investigated in this study inhibited [^{3}H]NMS binding to the four muscarinic receptors in a manner consistent with competition for a single binding site (Hill coefficients were not significantly different from unity).

The affinity of the hexahydro-difenidol eutomer, (R)-hexahydro-difenidol, for M_1, M_3, and M_4 receptors was greater than its affinity for M_2 receptors. The hexahydro-difenidol distomer, (S)-hexahydro-difenidol, had a similar affinity for the four subtypes (Table 1, Figs. 3 and 4). As a result, the eudismic index [(pK_i (eutomer) $-$ pK_i (distomer)] at M_1, M_3, and M_4 receptors was greater than that at M_2 receptors (Table 1).

Difenidol and dicyclidol had lower affinities than (R)-hexahydro-difenidol and higher affinities than the (S)-enantiomer at the four subtypes (Table 1). Difenidol had a somewhat higher affinity for M_1, M_3, and M_4 sites as compared to M_2 sites (5-fold selectivity). By contrast, dicyclidol had a lower affinity for M_2 and M_3 (as compared to M_1 and M_4) sites.

By contrast with our previous results with procyclidine,[15] the affinity losses due to replacement of the phenyl ring by a cyclohexyl group and the cyclohexyl ring by a phenyl moiety in (R)-hexahydro-difenidol were not fully additive. (S)-Hexahydro-difenidol had a 2- to 5-fold higher than "expected" affinity for M_1, M_2, and M_3 receptors. Analysis of the binding properties of the 4 compounds in striatum B sites (M_4 sites) indicate that, in this system, the affinity loss was additive. Since the eudismic index of hexahydro-difenidol binding to M_1 and M_4 sites is com-

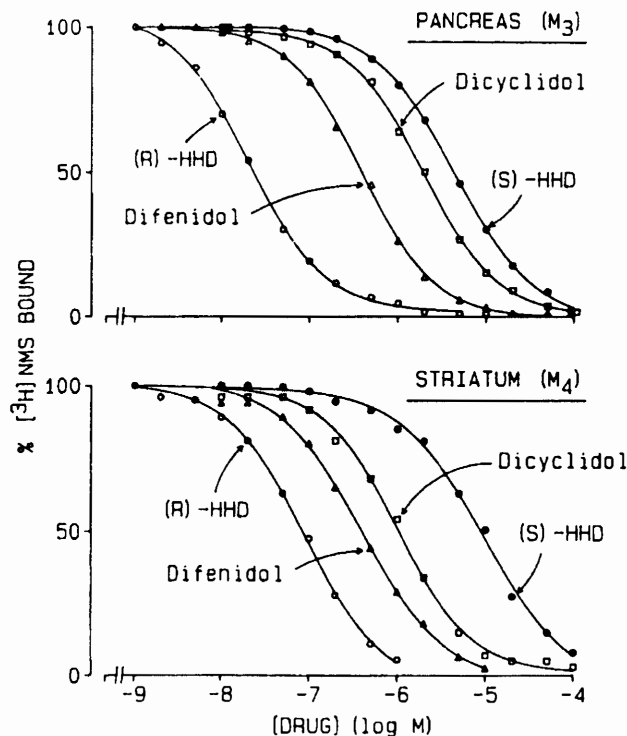

Fig. 4. As in Figure 3, using rat pancreas (upper panel) and rat striatum (lower panel).

TABLE 1. Comparison of binding affinities (pK_i values) of (R)-hexahydro-difenidol, (S)-hexahydro-difenidol, difenidol, and dicyclidol for four muscarinic receptor subtypes[a]

	M_1 (NB-OK 1)	M_2 (heart)	M_3 (pancreas)	M_4 (striatum)
(R)-Hexahydro-difenidol	8.3	7.0	8.1	7.9
Difenidol	7.1	6.4	7.1	7.2
Dicyclidol	6.8	6.1	6.2	6.8
(S)-Hexahydro-difenidol	6.1	5.8	5.9	5.9
pK_i difference (eudismic index) [(R) → (S)]	2.2	1.2	2.2	2.0
(R)-Procyclidine	8.4	7.3	—	8.1
Pyrrinol	7.5	6.9	—	7.2
Hexahydro-procyclidine	7.1	6.1	—	7.0
(S)-Procyclidine	6.3	5.8	—	6.0
pK_i difference (eudismic index) [(R) → (S)]	2.1	1.5	—	2.1

[a]The pK_i values of (R)-procyclidine, (S)-procyclidine, pyrrinol, and hexahydro-procyclidine found in a previous work[15] are shown for comparison.

parable, our results cannot be explained by contamination of (S)-hexahydro-difenidol by the (R)-enantiomer.

DISCUSSION

We have previously shown that the stereoselective binding of the enantiomers of procyclidine to muscarinic binding sites is best explained by the existence of four subsites (Fig. 2): one for the protonated amino group, one for the hydroxy group, one phenyl-preferring hydrophobic subsite, and one cyclohexyl-preferring hydrophobic subsite.[15] The relative spatial positions of these four subsites is such that (R)-procyclidine has a greater affinity than (S)-procyclidine due to a better interaction of the hydrophobic groups with their corresponding subsites. The free energy of (R)-procyclidine binding can therefore be described by Eq. (3):

$$\Delta G = \alpha\Delta G_1 + \beta\Delta G_2 + \gamma\Delta G_3 + \delta\Delta G_4 \quad (3)$$

where ΔG_1, ΔG_2, ΔG_3, and ΔG_4 represent the free energy achievable by an optimal interaction of the phenyl ring of the ligand with receptor site 1, the cyclohexyl group with receptor site 2, the hydroxy group with receptor site 3, and the protonated amino group with receptor site 4. ΔG values should be as negative as possible to obtain high affinity binding, and factors α, β, γ, and δ in Eq. (3) take into account the fact that all four groups are not of necessity simultaneously in the optimal positions to interact with receptor sites 1 to 4 (α, β, γ, and δ values probably vary between 0 and 1, provided that the corresponding group does not obstruct binding by steric hindrance).

We compared in this work the binding properties of hexahydro-difenidol enantiomers and of two achiral analogues, difenidol and dicyclidol (Fig. 1) to four muscarinic receptor subtypes. Our results confirm that the presence of a phenyl and a cyclohexyl ring in the correct spatial position [(R)-configuration] is important for the binding of hexahydro-difenidol to muscarinic receptors. Indeed, the affinity decrease due to replacement of the cyclohexyl ring by a phenyl group varied between 0.6 and 1.2 log units (corresponding to an increase of the binding free energy of 0.8 to 1.6 kcal/mol) (Table 2). The affinity decrease due to replacement of the phenyl by a cyclohexyl ring was even more impressive. It amounted to 0.9 to 1.5 log units (free energy difference: 1.2 to 2.6 kcal/mol) (Table 2).

The binding free energy decrease due to replacement of the phenyl and the cyclohexyl groups of (R)-hexahydro-difenidol by, respectively, a cyclohexyl and a phenyl moiety was additive in case of M_4 receptors (Table 2).

In contrast with our previous results, using the procyclidine enantiomers (Tables 1 and 2),[15] this substitution effect was not fully additive at M_1, M_2, and M_3 receptors: the difference of the free energy of binding of (S)- and (R)-hexahydro-difenidol to M_1, M_2, and M_3 subtypes was lower than expected by up to 1 kcal/mol. These discrepancies were subtype dependent and not correlated with receptor stereoselectivity, indicating that they did not reflect an imperfect separation of the (R)- and (S)-enantiomers. They were small, when compared with the free energy of binding of the drugs: at most 12% of the binding free energy of the (S)-enantiomer to the receptors.

TABLE 2. Differences between the free energies[a] (kcal/mol) of binding of (S)-hexahydro-difenidol, difenidol, or dicyclidol and (R)-hexahydro-difenidol for four muscarinic receptor subtypes[b]

	M_1 (NB-OK 1)	M_2 (heart)	M_3 (pancreas)	M_4 (striatum)
Difenidol	+1.64	+0.82	+1.36	+0.95
Dicyclidol	+2.05	+1.23	+2.59	+1.50
Expected[c]	+3.69	+2.05	+3.95	+2.45
(S)-Hexahydro-difenidol	+3.00	+1.64	+3.00	+2.73
Pyrrinol	+1.23	+0.55	—	+1.23
Hexahydro-procyclidine	+1.77	+1.64	—	+1.50
Expected[c]	+3.00	+2.19	—	+2.73
(S)-Procyclidine	+2.87	+2.05	—	+2.86

[a] Binding free energies were calculated according to Eq. (2), see under methods.
[b] The differences between the free energies of binding of (S)-procyclidine, pyrrinol, or hexahydro-procyclidine and (R)-procyclidine found in a previous work[15] are shown for comparison.
[c] Sum of the differences of the free energies of binding of (R)-hexahydro-difenidol and difenidol and of (R)-hexahydro-difenidol and dicyclidol.

We would like to suggest that by contrast with (R)-procyclidine, the four subgroups of hexahydro-difenidol cannot be simultaneously in the optimal position to interact with the corresponding four subsites of muscarinic M_1, M_2, and M_3 receptors. This corresponds in Eq. (3) to values of the parameters α, β, γ, and $\delta < 1.0$. The results indicate that, when the structure of (R)-hexahydro-difenidol is modified, e.g., by replacement of the phenyl ring in subsite 1 by a cyclohexyl group ($\rightarrow$ dicyclidol), the position of dicyclidol in the receptor is adjusted to increase the importance of the (good) interaction with subsites 2, 3, and 4 at the expense of the (weakened) interaction with subsite 1. Therefore, the difference of the free energy of binding of (R)-hexahydro-difenidol and, for instance, difenidol or dicyclidol does not reflect exactly the modification of ΔG_1 or ΔG_2 values. The development of an interaction model using molecular modeling methods and based on published M_1, M_2, M_3, and M_4 receptor protein sequences[23] might provide an improved rationale for the stereoselective interactions of (R)- and (S)-hexahydro-difenidol (this study), and of (R)- and (S)-procyclidine[15] with muscarinic receptor subtypes.

CONCLUSION

The muscarinic receptor stereoselectivity for the hexahydro-difenidol enantiomers is probably explained at least in part by weaker binding of the phenyl

and cyclohexyl group of the (S)-isomer. The four (S)-hexahydro-difenidol binding moieties (phenyl, cyclohexyl, OH group, and protonated amino group), however, cannot simultaneously form optimal interactions with each of the corresponding four subsites of muscarinic receptors. The results indicate that the loss of binding affinity (increase of the free energy of binding) due to replacement of the phenyl and cyclohexyl groups was not fully additive when considering the M_1, M_2, and M_3 sites.

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