ELSEVIER

European Journal of Pharmacology 286 (1995) 229–240

ejp

The (*S*)-(+)-enantiomer of dimethindene: a novel M_2-selective muscarinic receptor antagonist

Otmar Pfaff [a], Caren Hildebrandt [a], Magali Waelbroeck [b], Xue Hou [b], Ulrich Moser [a], Ernst Mutschler [a], Günter Lambrecht [a,*]

[a] *Department of Pharmacology, Biocentre Niederursel, University of Frankfurt, Marie-Curie-Straße 9, D-60439 Frankfurt, Germany*
[b] *Department of Biochemistry and Nutrition, Medical School, Free University of Brussels, B-1070 Brussels, Belgium*

Received 19 January 1995; revised 21 May 1995; accepted 21 July 1995

Abstract

The present study was designed to determine the in vitro affinity profile of (*R*)-(−)-dimethindene and (*S*)-(+)-dimethindene at muscarinic receptor subtypes using both functional and binding assays. In addition, the racemate was investigated in functional studies. The functional muscarinic receptors studied were putative M_1 receptors in rabbit vas deferens and rat duodenum, M_2 receptors in guinea-pig left atria and rabbit vas deferens, as well as M_3 receptors in guinea-pig ileum and trachea. Furthermore, the histamine H_1 antagonism by (*R*)-(−)- and (*S*)-(+)-dimethindene has been examined in guinea-pig ileum. Muscarinic binding selectivity was assessed in homogenates from human neuroblastoma NB-OK 1 cells (M_1), rat heart (M_2), pancreas (M_3) and striatum (M_4). The results demonstrate that (*S*)-(+)-dimethindene is a potent M_2-selective muscarinic receptor antagonist ($pA_2 = 7.86/7.74$; $pK_i = 7.78$) with lower affinities for the muscarinic M_1 ($pA_2 = 6.83/6.36$; $pK_i = 7.08$), the M_3 ($pA_2 = 6.92/6.96$; $pK_i = 6.70$) and the M_4 receptors ($pK_i = 7.00$), respectively. The (*S*)-(+)-enantiomer was more potent (up to 41-fold) than the (*R*)-(−)-enantiomer in all muscarinic assays. In contrast, the stereoselectivity was inverse at histamine H_1 receptors, the (*R*)-(−)-enantiomer being the eutomer ($pA_2 = 9.42$; pA_2/(*S*)-isomer = 7.48). In conclusion, (*S*)-(+)-dimethindene is a useful tool to investigate muscarinic receptor heterogeneity. In addition, this lipophilic compound might become the starting point for the development of M_2-selective muscarinic receptor antagonists useful as diagnostic tools for quantifying muscarinic M_2 receptors in the central nervous system with positron emission tomography imaging, and to test the hypothesis that muscarinic M_2 receptor antagonists show beneficial effects in the treatment of cognitive disorders.

Keywords: Dimethindene, enantiomer; Muscarinic M_2 receptor antagonist; Histamine H_1 receptor; Muscarinic receptor subtype; Enantioselectivity; Stereoselectivity, inverse; PET (positron emission tomography); Alzheimer's disease

1. Introduction

A large body of evidence derived from both functional and radioligand binding studies suggests that there are at least four pharmacological muscarinic receptor subtypes, termed M_1, M_2, M_3 and M_4 receptors. Molecular biology studies suggest that at least five genes encoding muscarinic receptor subtypes (m1–m5) are expressed in mammalian tissues. Comparison of the location, antagonist binding properties and functional coupling of the native muscarinic M_1–M_4 receptors with those of the expressed m1–m4 proteins showed a good correlation. A functional equivalent of the m5 gene product has yet to be identified. This subclassification of muscarinic receptors is based primarily on the relative sensitivities of the receptor subtypes to key muscarinic antagonists such as pirenzepine ($M_1 > M_4 > M_3 \geq M_2$), 11-[[2-[(diethylamino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido-[2,3-*b*]-[1,4]-benzodiazepin-6-one (AF-DX 116) and related compounds, methoctramine and himbacine ($M_2 \geq M_4 \geq M_1 > M_3$) as well as hexahydro-sila-difenidol and its *p*-fluoro derivative ($M_3 \geq M_1 \geq M_4 > M_2$; for recent reviews, see Hulme et al., 1990; Levine and Birdsall,

* Corresponding author. Department of Pharmacology, Biocentre Niederursel, University of Frankfurt, Marie-Curie-Straße 9, Geb. N260, D-60439 Frankfurt, Germany. Tel. 49.69.79 82 93 66, fax 49.69.79 82 93 74.

SSDI 0014-2999(95)00454-8

1993, 1995; Caulfield, 1993). In addition, muscarinic receptors may be differentiated on the basis of their stereoselectivity to chiral antagonists (Feifel et al., 1990; Waelbroeck et al., 1992; Dörje et al., 1991).

Muscarinic M_2 receptors have been reported to be depleted in post-mortem brains from patients with Alzheimer's disease (Aubert et al., 1992). This has raised the necessity of developing M_2-selective muscarinic receptor antagonists, capable of penetrating into the central nervous system, that could be useful both as diagnostic tools for quantifying the loss of muscarinic M_2 receptors with positron emission tomography imaging (Dewey et al., 1990), and as therapeutic agents (Gitler et al., 1992). The muscarinic M_2 receptor in hippocampal and cortical tissues has been shown to function as an inhibitory autoreceptor, the inhibition of which enhances acetylcholine release (Doods et al., 1993). Selective blockade of these muscarinic M_2 receptors in still existing neurons might therefore be a viable means of reversing the cholinergic hypofunction and the resulting cognitive impairments in patients with Alzheimer's disease (Gitler et al., 1992; Doods et al., 1993). However, the 'M_2-selective' key muscarinic receptor antagonists (see above) as well as compounds recently discovered such as tripitramine (Melchiorre et al., 1993; Maggio et al., 1994), imperialine (Eglen et al., 1992) and its 3-α-chloro derivative (Baumgold et al., 1994), and 5-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-10,11-dihydro-5*H*-dibenzo[*b*,*e*] [1,4]diazepin-11-one (DIBA; Gitler et al., 1992; Cohen et al., 1993) suffer from clear limitations (see Discussion).

Racemic dimethindene maleate is a widely used histamine H_1 receptor antagonist capable of crossing the blood-brain barrier (Nicholson et al., 1991; Casy et al., 1992; Cicurel et al., 1992). It contains a chiral centre which results in the existence of two enantiomers (Fig. 1). Borchard et al. (1985) reported that the (*R*)-enantiomer ($pA_2 = 9.1$) proved to be 20-fold more potent at histamine H_1 receptors in guinea-pig ileum than the (*S*)-isomer ($pA_2 = 7.8$). This has been confirmed in binding studies at peripheral and central histamine H_1 receptors (Ter Laak et al., 1993). The higher histamine H_1 receptor antagonism by (*R*)-dimethindene was subsequently confirmed in vivo, both in animal and in human studies (Nicholson et al., 1991; Leuschner et al., 1992; Cicurel et al., 1992). Antimuscarinic activity has been reported for racemic dimethindene maleate which inhibited the contractile responses to carbachol in guinea-pig ileum (Sautel et al., 1992; $pA_2 = 6.7$). In addition, competition binding studies at muscarinic receptors in bovine frontal cortex using the non-selective radioligand [^{3}H]3-quinuclidinyl benzilate showed that (*S*)-dimethindene binds with higher affinity to muscarinic receptors ($K_i = 0.9$ μM) than the (*R*)-isomer ($K_i = > 10$ μM) (Nicholson et al., 1991). However, the affinity profile of (*R*)- and (*S*)-dimethindene at clearly defined muscarinic receptor subtypes M_1–M_4 has never been investigated.

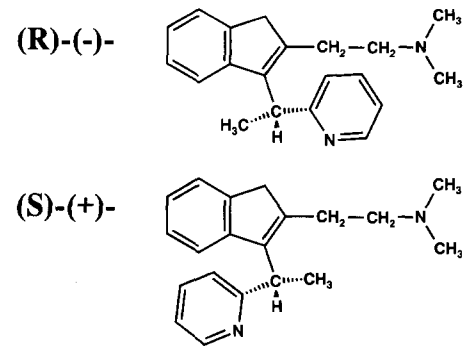

Fig. 1. Chemical structures of the enantiomers of dimethindene.

Thus, the aim of the present study was to examine the affinity of the enantiomers of dimethindene at muscarinic receptor subtypes M_1–M_4 in functional (including the racemate) as well as in binding studies. In addition, the antagonism by the enantiomers of dimethindene was studied at histamine H_1 receptors.

Preliminary accounts of these results have been published (Lambrecht et al., 1994, 1995; Pfaff et al., 1994).

2. Materials and methods

The muscarinic receptor subtypes present in the preparations used in this paper are summarized in Table 1, and the methods used have been described in detail previously (Table 1). In all functional preparations, control experiments established that there was no consistent sensitivity change to the agonists used over the normal experiment duration.

2.1. Functional studies

Rat duodenum

Experiments on intact rat duodenum were carried out according to Micheletti et al. (1987). Briefly, male Wistar rats (300–350 g) were killed by cervical dislocation and exsanguination. Rat duodenum segments of about 1.5 cm length were suspended under 1.5 g of tension in 6-ml organ baths containing a modified Tyrode buffer (see Organ bath solutions). Relaxations were induced by the M_1-selective muscarinic receptor agonist 4-(4-fluorophenylcarbamoyloxy)-2-butynyl-*N*-methylpyrrolidinium tosylate (4-F-PyMcN$^+$; Lambrecht et al., 1993) added at increasing concentrations with a 15-min interval between each. Responses were recorded isotonically using a force-displacement trans-

ducer connected to a Hellige amplifier and a Rikadenki multichannel recorder.

Rabbit vas deferens

Experiments on rabbit field-stimulated vas deferens were carried out according to Eltze (1988, 1994) and Eltze et al. (1988, 1993). Briefly, vasa deferentia from male New Zealand white rabbits (2.5–3.0 kg; killed by i.v. injection of pentobarbital sodium (120 mg/kg)) were removed, dissected free of connective tissues and divided into 5–6 segments of about 1.0–1.5 cm length. Each tissue was set up in a 6-ml organ bath containing a modified Krebs buffer (see Organ bath solutions). The resting tension was adjusted to 500 mg and twitch contractions were elicited by electrical field stimulation (0.05 Hz, 40 V, 0.5 ms) via platinum electrodes. The neurogenic responses were measured isometrically with a force-displacement transducer and recorded on a Rikadenki multichannel recorder. Antagonist affinities for prejunctional muscarinic M_1 and postjunctional muscarinic M_2 receptors were obtained from cumulative concentration-response curves either to the M_1-selective muscarinic receptor agonist 4-(4-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343; Eltze et al., 1988; Waelbroeck et al., 1994) for inhibition (muscarinic M_1 receptors) or to carbachol for potentiation (muscarinic M_2 receptors; Eltze, 1988,1994; Eltze et al., 1993) of neurogenic twitch contractions in the absence and presence of antagonists.

Guinea-pig left atria, ileum and trachea

Guinea-pigs (300–400 g) of either sex were killed by cervical dislocation and exsanguination. The organs required were removed and set up in 6-ml organ baths, under 250 (left atria divided in two) and 500 mg tension (ileum and trachea), in Tyrode solution (see Organ bath solutions).

Left atria were electrically paced (2 Hz, 3 ms, 5 V) by means of platinum electrodes, and negative inotropic effects to cumulative addition of the selective muscarinic receptor agonist arecaidine propargyl ester (Mutschler and Hultzsch, 1973; Eltze et al., 1993) were measured as changes in isometric tension and recorded as with the rabbit isolated vas deferens.

Strips of ileal longitudinal muscle (Paton and Zar, 1968) were prepared for isotonic contractions in response to cumulative administration of arecaidine propargyl ester or histamine. Responses to the agonists were recorded as with the rat duodenum.

Isolated tracheal segments (1.5 cm) were cut longitudinally, and pinned out under Tyrode solution. Thereupon, the tissues were cut in a zig-zag fashion (Emmerson and Mackay, 1979), and cumulative con-

Table 1
Preparations employed in the present study

(a) Functional studies		
Muscarinic receptor	Preparation	Reference
Putative M_1	Rabbit vas deferens [a]	Eltze (1988, 1994) Eltze et al. (1988) Waelbroeck et al. (1994)
Putative M_1	Rat duodenum [b]	Micheletti et al. (1987) Lambrecht et al. (1993) Pfaff et al. (1995)
M_2	Rabbit vas deferens [c]	Eltze (1988, 1994) Eltze et al. (1993)
M_2	Guinea-pig atria [d]	Caulfield (1993)
M_3	Guinea-pig ileum	Caulfield (1993) Eltze et al. (1993)
M_3	Guinea-pig trachea	Roffel et al. (1993) Emmerson and Mackay (1979)
Histamine receptor	Preparation	Reference
H_1	Guinea-pig ileum	Emmet et al. (1982)
(b) Radioligand binding studies [e]		
Muscarinic receptor	Preparation	Reference
M_1	NB-OK 1 cells	Waelbroeck et al. (1990a,b, 1992, 1994)
M_2	Rat heart	
M_3	Rat pancreas	
M_4	Rat striatum	

[a] Inhibition of neurogenic twitch contractions (0.05 Hz) to 4-Cl-McN-A-343. [b] Relaxations to 4-F-PyMcN$^+$. [c] Potentation of neurogenic twitch contractions (0.05 Hz) to carbachol. [d] Electrically paced left atria (2 Hz). [e] [^{3}H]*N*-Methylscopolamine as radioligand.

centration-response curves were constructed to arecaidine propargyl ester. Isotonic responses were recorded as with the rat duodenum.

Organ bath solutions

Except for the rat duodenum (37°C), the bathing fluid was maintained at 31°C and aerated with 95% O_2-5% CO_2 (pH = 7.4).

The Tyrode solution used in the experiments on guinea-pig atria, ileum and trachea as well as on the rat duodenum was of the following composition (mM): NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8 (rat duodenum = 1.0), $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and (+)-glucose 5.6.

The modified Krebs buffer used in the experiments at the rabbit vas deferens consisted of (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 1.0 (2.5 in experiments at muscarinic M_2 receptors), $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and (+)-glucose 11.1. Yohimbine (1.0 μM) was included to block α_2-adrenoceptors (Eltze, 1988).

Antagonist affinities

The tissues were allowed to equilibrate for 30 min. Then, concentration-response curves to the agonists were constructed in the absence (control) and in the presence of at least three concentrations (log interval = 0.48) of antagonists, allowing 30–60 min equilibration time. Preliminary experiments indicated that these intervals were sufficient for equilibration of the antagonist concentration used. The rat duodenum was exposed to only one concentration of antagonists. Each concentration of antagonist was tested 3–5 times and the ratios of agonist molar EC_{50} values obtained in the presence and absence of antagonists were calculated. The EC_{50} values were determined by fitting the data to a non-linear iterative curve fitting procedure (Parker and Waud, 1971; Tallarida and Murray, 1986). Curves in Fig. 2 are fitted curves. For the assessment of antagonist affinity, Schild plots were made (Arunlakshana and Schild, 1959) using linear regression by the method of least squares. pA_2 values were determined from Schild plots in which the slopes of the

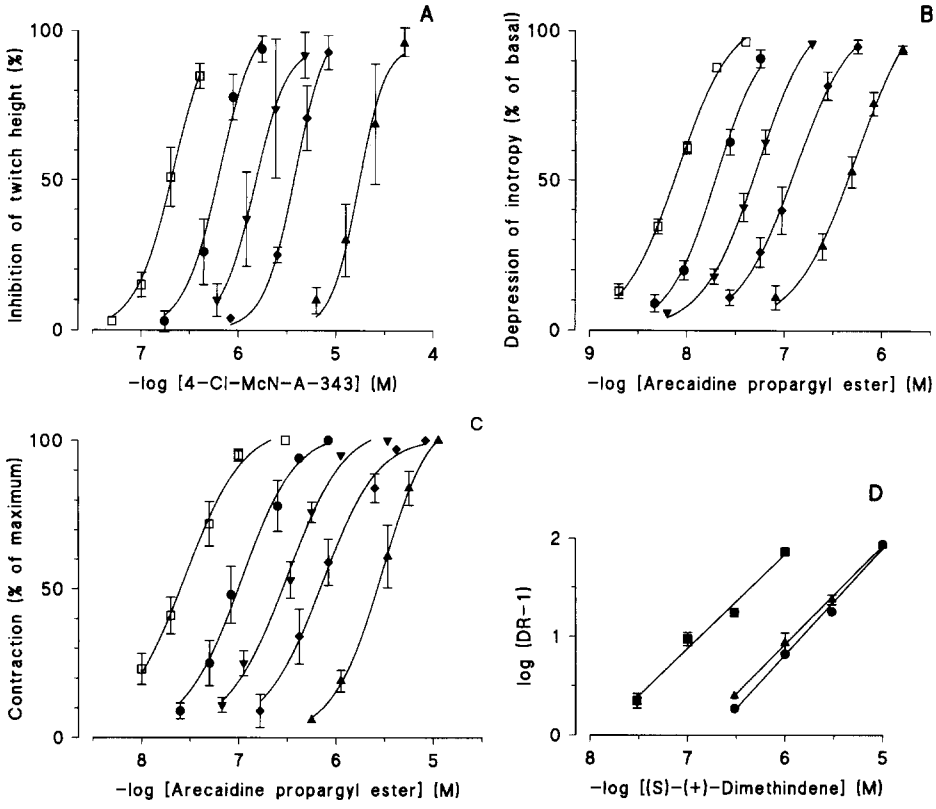

Fig. 2. Antagonism of responses to 4-Cl-McN-A-343 in rabbit vas deferens and to arecaidine propargyl ester in guinea-pig left paced atria and ileum by different concentrations of (*S*)-(+)-dimethindene. Data are means ± S.E.M. of $n = 3$–5. Error bars falling within the area covered by a symbol are not shown. (A) Concentration-response curves for 4-Cl-McN-A-343-induced inhibition of neurogenic twitch contractions in rabbit vas deferens in the absence (□) and presence of 0.3 (●), 1.0 (▼), 3.0 (◆) and 10.0 (▲) μM (*S*)-dimethindene. (B) Concentration-response curves for arecaidine propargyl ester-induced negative inotropy in guinea-pig atria in the absence (□) and presence of 0.03 (●), 0.1 (▼), 0.3 (◆) and 1.0 (▲) μM (*S*)-dimethindene. (C) Concentration-response curves for arecaidine propargyl ester-induced contractions in guinea-pig isolated ileum longitudinal smooth muscle in the absence (□) and presence of 0.3 (●), 1.0 (▼), 3.0 (◆) and 10.0 (▲) μM (*S*)-dimethindene. (D) Schild regression from experiments in rabbit vas deferens (●), guinea-pig atria (■) and guinea-pig ileum (▲).

regression lines had been constrained to 1.00. This is more consistent with the competitive theory which connects pA_2 with $-\log K_B$, since the slopes obtained in the present study (Table 2) did not differ significantly from unity (Tallarida and Murray, 1986).

2.2. Radioligand binding experiments

General considerations

Protein concentrations were determined according to Lowry et al. (1951), using bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO, USA) as standard. Male Wistar albino rats were killed by decapitation and the brain, heart or pancreas immediately removed. Human neuroblastoma NB-OK 1 cells were cultured as previously described (Waelbroeck et al., 1988,1990a,b,1992,1994) in RPMI 1640 medium enriched with 10% foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. All the homogenates were prepared at 4°C.

The binding studies were performed at 25°C, at equilibrium, in a 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$ and 1% bovine serum albumin, [^{3}H]*N*-methylscopolamine and the indicated unlabelled drug concentrations, in a total volume of 1.2 ml. 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. Control experiments in NB-OK 1 cells as well as in rat heart and striatum showed that the competition curves with (*R*)- and (*S*)-dimethindene were not significantly ($P > 0.05$) shifted to the right by inclusion of 1% bovine serum albumin into the incubation buffer (data not shown). This allowed us to perform the binding experiments in all tissues in the presence of bovine serum albumin.

The incubations were terminated by addition of 2 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.4) and filtration over glass-fiber GFC filters (Whatman, Maidstone, UK) soaked in 0.05% polyethyleneimine (Sigma Chemical Co., St. Louis, MO, USA). The filters were rinsed 3 times with the same buffer, and dried. The bound radioactivity was measured by liquid scintillation counting. Non-specific binding was estimated as binding in the presence of 1 μM atropine.

Preparation of the homogenates or crude membranes used for binding studies

For [^{3}H]*N*-methylscopolamine binding to muscarinic M_1 receptors, the NB-OK 1 cells were rinsed, harvested and centrifuged in 20 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl and 1 mM EDTA, resuspended and homogenized in 20 mM Tris/HCl buffer (pH 7.5) enriched with 5 mM $MgCl_2$ and stored in liquid nitrogen. The incubation period was 2 h, in the presence of 0.25 nM [^{3}H]*N*-methylscopolamine and about 200 μg protein per assay. The tracer concentration was equivalent to two tracers' K_D values to muscarinic M_1 sites.

The rat 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 s) followed by addition of 12.5 ml of the same buffer, seven up and down strokes with a glass-Teflon homogenizer and filtration on two layers of medical gauze. The 2-h incubation period was sufficient to allow equilibrium binding of [^{3}H]*N*-methylscopolamine and unlabelled

Table 2
Affinity estimates (pA_2 values) and slopes of Schild plots (in parentheses) for (*R*,*S*)-, (*R*)- and (*S*)-dimethindene at various functional muscarinic and histamine receptors (see Table 1)

Preparation	pA_2 values		
	(*R*,*S*)-Dimethindene	(*R*)-Dimethindene	(*S*)-Dimethindene
Vas deferens, M_1	6.61 ± 0.03	5.81 ± 0.02	6.83 ± 0.03
	(0.96 ± 0.05)	(0.98 ± 0.05)	(1.08 ± 0.04)
Duodenum, M_1	n.d.	5.49 ± 0.08	6.36 ± 0.05
		(1.12 ± 0.27)	(0.87 ± 0.10)
Left atria, M_2	7.48 ± 0.03	6.25 ± 0.05	7.86 ± 0.03
	(1.01 ± 0.05)	(0.86 ± 0.08)	(0.97 ± 0.05)
Vas deferens, M_2	n.d.	6.22 ± 0.03	7.74 ± 0.03
		(1.11 ± 0.05)	(1.00 ± 0.05)
Ileum, M_3	6.64 ± 0.03	5.61 ± 0.03	6.92 ± 0.02
	(0.99 ± 0.06)	(0.99 ± 0.06)	(1.00 ± 0.05)
Trachea, M_3	n.d.	5.59 ± 0.03	6.96 ± 0.05
		(1.03 ± 0.06)	(0.91 ± 0.08)
Ileum, H_1	n.d.	9.42 ± 0.06	7.48 ± 0.05
		(0.87 ± 0.14)	(0.87 ± 0.12)

The pA_2 values were calculated from regression lines whose slopes were constrained to 1.00 as required for the theoretical model of competitive antagonism. The results are presented as means ± S.E.M., $n = 8$–14. n.d. = not determined.

competitors to muscarinic M_2 receptors. The [^{3}H]*N*-methylscopolamine concentration used was 1.0 nM, i.e. two tracers' K_D values to muscarinic M_2 sites, and the protein concentration was 400–500 μg per assay.

The rat pancreas was minced with scissors and homogenized with a glass-Teflon homogenizer (seven up and down strokes) in 8 ml of 300 mM sucrose enriched with 0.2 mg/ml bacitracin and 500 kallikrein inhibitor units (KIU)/ml of Trasylol. The resulting homogenate was immediately filtered over two layers of medical gauze and diluted 11-fold with the incubation buffer to achieve final concentrations of 50 mM sodium phosphate buffer (pH 7.4), 2 mM $MgCl_2$ and 1% bovine serum albumin. A 4-h incubation period was necessary to allow full equilibration of [^{3}H]*N*-methylscopolamine binding at 25°C. The tracer concentration used was 0.25 nM, equivalent to two tracers' K_D values to the muscarinic M_3 sites in pancreas, and the pancreas protein concentration was 0.8–1.0 mg per assay.

The rat striatum was homogenized in 2 ml of 20 mM Tris/HCl buffer (pH 7.5), enriched with 250 mM sucrose, and stored in liquid nitrogen until use. The homogenate was diluted 20-fold with the homogenization buffer, immediately before the incubation with [^{3}H]*N*-methylscopolamine. In rat striatum homogenates, [^{3}H]*N*-methylscopolamine labels muscarinic M_1, very few M_2 and M_3, and a majority of M_4 sites. It dissociates faster from the muscarinic M_1 and M_2 sites (Waelbroeck et al., 1990a,b, 1992, 1994). The homogenate (equivalent to about 30 μg protein) was preincubated in a total volume of 1.2 ml, in the presence of [^{3}H]*N*-methylscopolamine and unlabelled drugs. A 2-h preincubation period allowed equilibrium binding. Then, 1 μM atropine was added and tracer dissociation was allowed for 35 min before filtration. This procedure allowed us to investigate tracer binding to the striatum muscarinic M_4 sites. The tracer concentration used in these experiments (0.25 nM) was equivalent to six tracers' K_D values to striatum muscarinic M_4 sites.

Analysis of binding data

All competition curves were repeated 3 times in duplicate. IC_{50} values were determined by a non-linear curve fitting program, assuming that a single receptor subtype was labelled by [^{3}H]*N*-methylscopolamine (Waelbroeck et al., 1988, 1990a,b, 1992, 1994). K_i values were calculated from IC_{50} values by the Cheng and Prusoff (1973) equation. The pK_i values shown in Table 3 correspond to $-\log K_i$ values.

2.3. Statistics

All data are presented as means (±S.E.M.) of the indicated number (n) of experiments. Differences between mean values were tested for statistical significance by means of Student's t-test, accepting $P \leq 0.05$ as being significant.

2.4. Drugs

[^{3}H]*N*-Methylscopolamine chloride (80–85 Ci/mmol) was obtained from Amersham International (Bucks, UK). Atropine sulphate, yohimbine hydrochloride and histamine dihydrochloride were from Sigma Chemical Co. (St. Louis, MO, USA). Carbachol (carbamylcholine chloride) was obtained from Merck (Darmstadt, Germany). Arecaidine propargyl ester hydrobromide (Mutschler and Hultzsch, 1973), 4-(4-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343; Nelson et al., 1976), and 4-(4-fluorophenylcarbamoyloxy)-2-butynyl-*N*-methylpyrrolidinium tosylate (4-F-PyMcN$^+$; synthesis by analogy to 4-Cl-McN-A-343) were synthesized in our laboratories according to the literature.

The enantiomers of dimethindene ((*S*)-(+)-base/(−)-tartrate and (*R*)-(−)-base/(+)-tartrate; Casy et al., 1992) were a generous gift from Zyma (Munich, Germany). There was chromatographic evidence (high-performance liquid chromatography analysis on an α_1-acid glycoprotein column; Radler and Blaschke, 1991) that the enantiomers of dimethindene were of high optical purity (ee ≥ 99%; Zyma, personal communication).

All other chemicals were of the highest grade available.

Table 3
In vitro affinity profile (pK_i values and Hill coefficients, n_H) of (*R*)- and (*S*)-dimethindene at muscarinic binding sites in various tissues (cell lines; see Table 1)

Tissue [a]	(*R*)-Dimethindene		(*S*)-Dimethindene	
	pK_i	n_H	pK_i	n_H
NB-OK 1 cells, M_1	5.96 ± 0.03	0.96 ± 0.03	7.08 ± 0.03	0.94 ± 0.04
Rat heart, M_2	6.30 ± 0.02	0.93 ± 0.03	7.78 ± 0.02	0.99 ± 0.03
Rat pancreas, M_3	5.63 ± 0.02	1.09 ± 0.02	6.70 ± 0.02	1.08 ± 0.04
Rat striatum, M_4	6.00 ± 0.03	0.94 ± 0.03	7.00 ± 0.02	0.99 ± 0.04

The pK_i and n_H values were calculated by non-linear curve fitting as explained under Materials and methods. The results are presented as means ± S.E.M. of three experiments carried out in duplicate. [a] Muscarinic binding sites were labelled using [^{3}H]*N*-methylscopolamine.

Table 4
Receptor selectivity and stereoselectivity ((*S*)/(*R*)) ratios [a] for the enantiomers of dimethindene obtained in binding studies at muscarinic M_1 (NB-OK 1 cells), M_2 (rat heart), M_3 (rat pancreas) and M_4 receptors (rat striatum). The corresponding values [a] obtained in functional experiments at muscarinic M_1 (rabbit vas deferens), M_2 (guinea-pig atria) and M_3 receptors (guinea-pig ileum) are given in parentheses

	Receptor selectivity			Stereoselectivity			
	M_2/M_1	M_2/M_3	M_2/M_4	M_1	M_2	M_3	M_4
(*R*)-Dimethindene	2	5	2				
	(3)	(4)	–				
				13	30	12	10
				(10)	(41)	(20)	–
(*S*)-Dimethindene	5	12	6				
	(11)	(9)	–				

[a] The values shown represent the antilogs of the differences between corresponding pK_i and pA_2 values (Tables 2 and 3).

3. Results

3.1. Functional studies

Racemic dimethindene and its enantiomers (0.3 nM–30 μM) did not elicit an agonist response itself but surmountably antagonized responses to the agonists in all preparations studied. There was a concentration-dependent parallel shift to the right of agonist dose-response curves without either the basal tension or the maximal responses being affected. The Schild plots were linear throughout the antagonist concentration-range studied, and slopes were not significantly different from unity ($P > 0.05$; shown for (*S*)-dimethindene in vas deferens, atria and ileum in Fig. 2). Thus, (*R,S*)-, (*R*)- as well as (*S*)-dimethindene were apparently simple competitive antagonists in the preparations studied. The affinities (pA_2 values) are shown in Table 2, and they were used to calculate stereoselectivity and receptor selectivity ratios (Table 4).

Antimuscarinic effects of racemic dimethindene and its enantiomers

Electrical field stimulation of the rabbit vas deferens elicited neurogenic twitch contractions which could be concentration dependently inhibited by the M_1-selective muscarinic receptor agonist 4-Cl-McN-A-343 (Fig. 2A; $-\log EC_{50} = 6.84 \pm 0.04$, $n = 30$). Similarly, exposure of the rat duodenum to the preferential muscarinic M_1 receptor agonist 4-F-PyMcN$^+$ elicited a concentration-dependent relaxation ($-\log EC_{50} = 6.11 \pm 0.04$, $n = 53$). Both effects were antagonized with very similar potency by pre-equilibration of the tissues with (*R*)- and (*S*)-dimethindene, the pA_2 values being 5.81/5.49 and 6.83/6.36 in vas deferens and duodenum, respectively (Table 2).

(*R*)- and (*S*)-dimethindene antagonized the negative inotropic responses to arecaidine propargyl ester in guinea-pig atria (Fig. 2B; $-\log EC_{50} = 8.05 \pm 0.02$, $n = 11$). The calculated pA_2 values were 6.25 and 7.86, respectively (Table 2). These affinity estimates were very similar to that obtained at muscarinic M_2 receptors in rabbit vas deferens (6.22 and 7.74, respectively; Table 2) using carbachol ($-\log EC_{50} = 7.35 \pm 0.04$, $n = 16$) as agonist.

Isotonic contractions of guinea-pig ileum (Fig. 2C) and trachea to cumulative administration of arecaidine propargyl ester ($-\log EC_{50}$/ileum $= 7.62 \pm 0.05$, $n =$

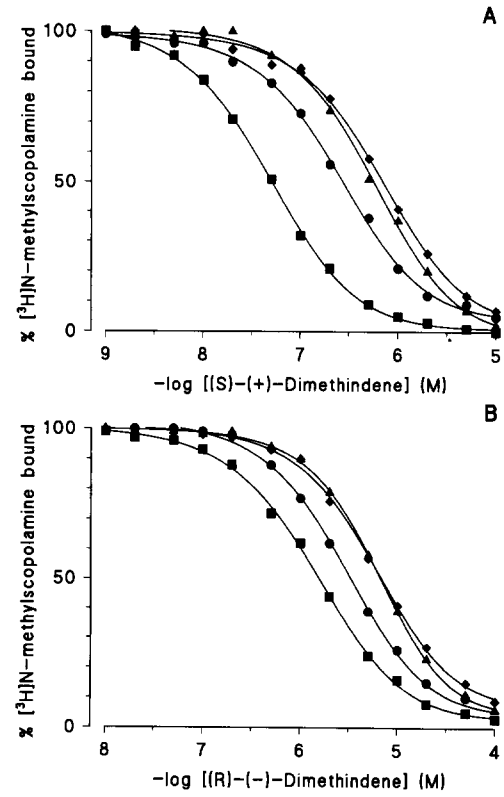

Fig. 3. [^{3}H]*N*-Methylscopolamine competition curves obtained at muscarinic M_1 (●; NB-OK 1 cells), M_2 (■; rat heart), M_3 (▲; rat pancreas) and M_4 receptors (◆; rat striatum). [^{3}H]*N*-Methylscopolamine binding was measured in the absence and presence of (*S*)- (A) and (*R*)-dimethindene (B), as described in Materials and methods. The data points represent means of three experiments performed in duplicate.

12; $-\log EC_{50}$/trachea 7.10 ± 0.05, $n = 12$) were antagonized by (*R*)- and (*S*)-dimethindene. The apparent affinities ($pA_2/(R) = 5.61/5.59$; $pA_2/(S) = 6.92/6.96$) for the two enantiomers in each of these tissues were identical.

The antimuscarinic potency of (*R,S*)-dimethindene in rabbit vas deferens ($pA_2 = 6.61$), guinea-pig atria ($pA_2 = 7.48$) and guinea-pig ileum ($pA_2 = 6.64$) is best explained by the presence of 50% of the more potent (*S*)-enantiomer (for pA_2 values, see Table 2) in the racemic mixture.

Histamine H_1 antagonism of (R)- and (S)-dimethindene

Histamine H_1 receptor antagonist activities of the enantiomers of dimethindene against histamine-induced contraction of guinea-pig ileum ($-\log EC_{50} = 6.15 \pm 0.07$; $n = 12$) are shown in Table 2. The pA_2 values obtained were 9.42 ((*R*)-isomer) and 7.48 ((*S*)-isomer), respectively, the (*R*)-enantiomer being the eutomer. These results confirm previous investigations (Borchard et al., 1985; Nicholson et al., 1991; Casy et al., 1992; Ter Laak et al., 1993).

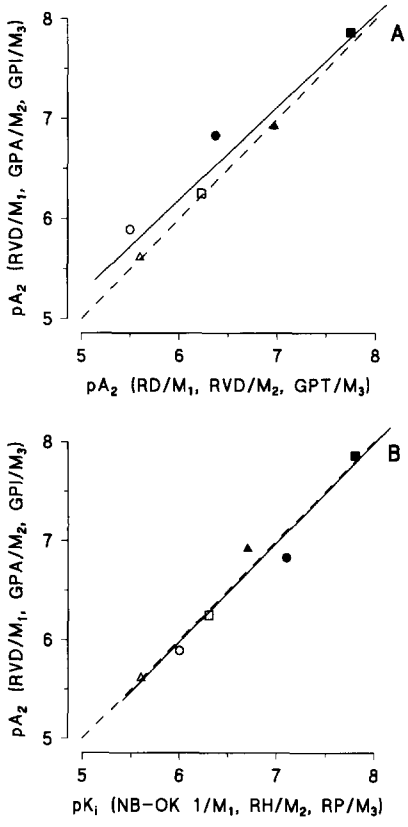

Fig. 4. (A) Relationship of mean affinity estimates (pA_2 values) from Table 2 of (*R*)- (open symbols) and (*S*)-dimethindene (solid symbols) measured in functional studies at muscarinic M_1 (rabbit vas deferens (RVD) and rat duodenum (RD); ○,●), M_2 (guinea-pig atria (GPA) and rabbit vas deferens (RVD); □,■) and M_3 receptors (guinea-pig ileum (GPI) and guinea-pig trachea (GPT); △,▲). (B) Relationship of mean affinity estimates (pA_2 values) from Table 2 of (*R*)- (open symbols) and (*S*)-dimethindene (solid symbols) measured in functional studies at muscarinic M_1 (RVD; ○,●), M_2 (GPA; □,■) and M_3 receptors (GPI; △,▲) and the mean pK_i values (Table 3) found in binding studies in NB-OK 1 cells (M_1 receptors), rat heart (RH; M_2 receptors) and rat pancreas (RP; M_3 receptors). The dotted lines represent the lines of unity. The solid lines (A: slope $= 0.93 \pm 0.12$, $r = 0.97$; B: slope $= 1.01 \pm 0.10$, $r = 0.98$) represent the normal regression lines, the slopes of which were not significantly different from unity ($P > 0.05$).

3.2. Radioligand binding studies

In all tissues tested (Table 1), (*R*)- and (*S*)-dimethindene inhibited [^{3}H]*N*-methylscopolamine binding to muscarinic receptors. The competition curves (Fig. 3) did not deviate significantly from results expected for competitive inhibition of tracer binding to a single receptor subtype. Hill coefficients (Table 3) were not significantly different from unity. The pK_i values are summarized in Table 3.

(*S*)-Dimethindene was found to prefer clearly muscarinic M_2 ($pK_i = 7.78$) over M_1 ($pK_i = 7.08$), M_3 ($pK_i = 6.70$) and M_4 ($pK_i = 7.00$) receptors, and it was considerably more potent than the (*R*)-stereoisomer (Table 4). An excellent correlation was found between the functional pA_2 values obtained in, respectively, rabbit vas deferens, guinea-pig atria or ileum and the pK_i values obtained in NB-OK 1 cells (muscarinic M_1 receptors), rat heart (M_2 receptors) and rat pancreas (M_3 receptors) (see Discussion; Fig. 4B).

4. Discussion

4.1. General considerations

It was the aim of the present study to explore the affinity of the enantiomers of dimethindene at three muscarinic receptor subtypes in functional studies on isolated organs and in binding studies with tissue homogenates containing four different muscarinic receptor subtypes. In addition, histamine H_1 affinities were determined in guinea-pig ileum.

In functional experiments, (*R*)- and (*S*)-dimethindene behaved as competitive antagonists at muscarinic M_1 (rabbit vas deferens, rat duodenum), M_2 (guinea-pig atria, rabbit vas deferens), M_3 (guinea-pig ileum and trachea) and histamine H_1 receptors (guinea-pig ileum). They produced an antagonism that was reversible and surmountable, and their apparent affinity in each tissue appeared to be independent of the concentrations used. Schild plots were linear and slopes not significantly different from unity. This functional competitive antagonism by (*R*)- and (*S*)-dimethindene could be confirmed by the results of radioligand binding experiments at muscarinic M_1 (NB-OK 1 cells), M_2 (rat heart), M_3 (rat pancreas) and M_4 receptors (rat striatum).

4.2. Correlations

In Fig. 4 A, the antimuscarinic potencies (pA_2 values; Table 2) of (*R*)- and (*S*)-dimethindene at muscarinic M_1 (mediating inhibition of neurogenic twitch contractions in rabbit vas deferens), M_2 (mediating negative inotropy in guinea-pig atria) and M_3 receptors (mediating contraction in guinea-pig ileum) were plotted against their functional potencies obtained in rat duodenum (M_1), rabbit vas deferens (M_2) and guinea-pig trachea (M_3). An excellent correlation ($r = 0.97$) was found between these affinity estimates.

In a complementary way functional antimuscarinic potencies of (*R*)- and (*S*)-dimethindene at muscarinic M_1, M_2 and M_3 receptors (Table 2) were highly correlated ($r = 0.98$; Fig. 4B) with their binding affinities (Table 3) in NB-OK 1 cells (M_1) as well as in rat heart (M_2) and pancreas (M_3), respectively.

4.3. Stereoselectivity

In general, (*S*)-dimethindene was more potent than the (*R*)-enantiomer in all muscarinic assays. However, the stereoselectivity ratios were found to be different at the four muscarinic receptor subtypes, being greatest at muscarinic M_2 receptors (30- to 41-fold; Table 4). These results provide additional evidence that stereoselectivity ratios of chiral muscarinic antagonists may be used as a parameter (besides absolute affinity data) to characterize muscarinic receptor subtypes (Feifel et al., 1990; Dörje et al., 1991; Waelbroeck et al., 1990a, 1992; Eltze et al., 1993).

The apparent affinity estimates of (*R*)- and (*S*)-dimethindene at functional histamine H_1 receptors in guinea-pig ileum ($pA_2/(R) = 9.42$; $pA_2/(S) = 7.48$) are very similar to that reported by Borchard et al. (1985) and Nicholson et al. (1991). In contrast with muscarinic receptors (Table 4) the (*R*)-enantiomer proved to be the eutomer at histamine H_1 receptors being 87-fold more potent than the (*S*)-configurated stereoisomer. Accordingly, these results demonstrate an inverse stereoselectivity and imply that the stereochemical requirements of the muscarinic receptors and histamine H_1 receptors, respectively, are different for the enantiomers of dimethindene, being most stringent at histamine H_1 receptors. Such an inverse stereoselectivity for recognition of histamine H_1 and muscarinic receptors has also been reported for chiral diphenhydramine analogues (Rekker et al., 1971) as well as for the enantiomers of *cis*-2,3-dihydro-3-(4-methyl-1-piperazinylmethyl)-2-phenyl-1,5-benzothiazepin-4(5*H*)-one (BTM-1041 and BTM-1086; Eltze et al., 1989).

Therapeutic implications

The commercially available dimethindene maleate, used therapeutically as an histamine H_1 receptor antagonist, is a racemic mixture of the (*R*)- and (*S*)-enantiomers. Although the eutomer, (*R*)-dimethindene, is about 1500-fold more potent on histamine H_1 receptors than on muscarinic M_2 receptors, the affinity of (*S*)-dimethindene for muscarinic M_2 receptors should also be taken into consideration. The H_1 ($pA_2/(R) = 9.42$) over M_2 ($pA_2/(S) = 7.86$) 'selectivity of the racemate' is still about 36-fold, so that side effects, such as tachycardia, caused by blockade of cardiac muscarinic M_2 receptors remain unlikely. Nevertheless, our results clearly illustrate the advantage of using pure enantiomers as medicines.

4.4. Muscarinic receptor subtype selectivity and receptor specificity

In both functional and binding assays (*S*)-dimethindene exhibited a higher affinity for the muscarinic M_2 receptors than for histamine H_1 receptors and for the other muscarinic receptor subtypes. It possesses the following selectivity profile: $M_2 \geq H_1 > M_1, M_3, M_4$. As far as muscarinic receptors are concerned, (*S*)-dimethindene is 5- to 30-, 9- to 12- and 6-fold selective for muscarinic M_2 versus M_1, M_3 and M_4 receptors (Tables 2–4). The affinity profile of (*R*)-dimethindene ($H_1 \gg M_2 \geq M_4 \geq M_1, M_3$) is different from that obtained for the (*S*)-isomer.

Nicholson et al. (1991) determined the binding affinities of (*R*)-and (*S*)-dimethindene for several receptors in bovine frontal cortex and striatum. No specific binding was found (up to a concentration of 10 μM) at α_2- and β-adrenoceptors as well as at benzodiazepine receptors, whereas both enantiomers showed similar affinity to α_1-adrenoceptors ($K_i = 0.33$ and 0.55 μM). (*S*)-Dimethindene ($K_i = 2.2$ and 0.3 μM) was more potent than the (*R*)-enantiomer ($K_i = > 10$ and 2.7 μM) at 5-HT_2 and dopamine D_2 receptors, but less active at 5-HT_1 receptors ($K_i/(R) = 2.8$ μM; $K_i/(S) = > 10$ μM).

As far as receptor specificity is concerned, these data show that (*S*)-dimethindene is a potent M_2-selective muscarinic receptor antagonist possessing no or very low affinity for α_2- and β-adrenoceptors, benzodiazepine receptors as well as for 5-HT_1 and 5-HT_2 receptors.

4.5. Comparison of (S)-dimethindene with other 'M_2-selective' antagonists

In contrast to (*S*)-dimethindene, other so-called 'M_2-selective' muscarinic receptor antagonists such as methoctramine, himbacine, 5,11-dihydro-11-[[(2-[2-[(dipropylamino)methyl]-1-piperidinyl]ethyl)amino]carbonyl]-6*H*-pyrido[2,3-b][1,4] benzodiazepin-6-one (AF-DX 384), 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl] acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one (AQ-RA 741) or imperialine are not able to distinguish clearly between muscarinic M_2 and M_4 re-

ceptors (see Hulme et al., 1990; Waelbroeck et al., 1990b; Dörje et al., 1991; Eglen et al., 1992; Caulfield, 1993; Maggio et al., 1994 for references). On the other hand, (*S*)-dimethindene resembles the two pirenzepine analogues, AF-DX 116 and 5,11-dihydro-8-chloro-11-[[4-[3-[(2,2-dimethyl-1-oxopentyl)ethylamino]propyl]-1-piperidinyl]acetyl]-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one (BIBN 99; Doods et al., 1993), in terms of its pharmacological and binding profile at muscarinic receptor subtypes in that it acts as an M_2-selective muscarinic receptor antagonist with respect to M_1, M_3 and M_4 sites. However, the affinity of (*S*)-dimethindene for muscarinic M_2 receptors is higher than that found for AF-DX 116 and BIBN 99. Binding studies with A9 L and CHO cells, respectively, expressing the cloned muscarinic receptor subtypes m1–m4 showed that the alkaloid derivative 3-α-chlorimperialine (Baumgold et al., 1994) and the pirenzepine analogue DIBA bound with subnanomolar affinity to m2 receptors, but with lower affinity to the other three subtypes (6-fold m2 over m4). These results obtained from radioligand binding experiments must remain provisional until confirmed in functional experiments. The methoctramine analogue tripitramine has been reported to have high affinity for native muscarinic M_2 receptors in rat heart ($pK_i = 9.54$) and to display extremely high subtype selectivity ($M_2 > M_1 = 81$-fold; $M_2 > M_3 = 2239$; $M_2 > M_4 = 41$; Melchiorre et al., 1993). However, this high muscarinic M_2 receptor selectivity of tripitramine has recently been questioned on the basis of results obtained in functional experiments (Lambrecht et al., 1995) and in binding studies at cloned muscarinic receptor subtypes m1–m4 (Maggio et al., 1994). The muscarinic receptor selectivities M_2/m2 > M_1/m1 (3.5- to 23-fold) as well as M_2/m2 > M_3/m3 (32- to 251-fold) found in these studies were much lower than that reported by Melchiorre et al. (1993). The reason for this discrepancy is unknown. Further experiments are needed to clarify this issue.

Therapeutic implications

As already mentioned in the Introduction, M_2-selective muscarinic receptor antagonists could be useful as diagnostic tools for quantifying the loss of muscarinic M_2 receptors in the brain of patients with Alzheimer's disease with positron emission tomography imaging (Dewey et al., 1990; Gitler et al., 1992; Cohen et al., 1993). In addition, such compounds might be a further option for the treatment of dementia of the Alzheimer's type (Doods et al., 1993). Although several muscarinic receptor antagonists with high affinity for M_2 receptors have been described (see above), most of these compounds suffer from clear limitations.

Antimuscarinic agents such as AF-DX 116, AQ-RA 741, AF-DX 384, himbacine, DIBA and methoctramine (probably also tripitramine) are not able to penetrate readily into the brain (Cohen et al., 1993; Doods et al., 1991, 1993). Baumgold et al. (1994) reported that 3-α-chlorimperialine is able to cross the blood-brain barrier. However, its muscarinic M_2 receptor selectivity has not been well established. In addition, 3-α-chlorimperialine has, like the parent alkaloid imperialine, a very complicated chemical structure, and both compounds are highly expensive. Recently, the pirenzepine analogue BIBN 99 has been reported to be a lipophilic M_2-selective muscarinic receptor antagonist and to exhibit central nervous system activity. Positive results were obtained in animal models of cognitive function (Doods et al., 1993). Therefore, BIBN 99 may be considered as a tool to investigate the role of muscarinic M_2 receptors in the brain.

It is noteworthy that dimethindene is a lipophilic compound, both enantiomers of which were able to penetrate readily into the brain of human healthy volunteers (Nicholson et al., 1991; Casy et al., 1992; Cicurel et al., 1992). In addition, Radler and Blaschke (1991) showed that the equal ease of (*R*)- and (*S*)-dimethindene to cross the blood-brain barrier was not influenced by differences in metabolism of the two enantiomers.

In conclusion, the experiments described in the present study demonstrate that (*S*)-dimethindene is a novel M_2-selective muscarinic receptor antagonist and a useful tool to investigate muscarinic receptor heterogeneity. In addition, it might become the starting point for the development of M_2-selective muscarinic receptor antagonists useful as diagnostic tools for quantifying muscarinic M_2 receptors in the central nervous system with positron emission tomography imaging, and to test the hypothesis that muscarinic M_2 receptor antagonists show beneficial effects in the treatment of cognitive disorders. The M_2 vs. M_1 muscarinic receptor selectivity is of special importance in terms of the potential therapeutic use of muscarinic antagonists to reverse the cholinergic hypofunction in patients with Alzheimer's disease. Such an M_2 vs. M_1 muscarinic receptor selectivity may guarantee that the compound does not counteract its acetylcholine releasing action by blockade of postsynaptic muscarinic M_1 receptors. One interesting aspect of using labelled (*S*)-dimethindene as a positron emission tomography ligand for quantifying muscarinic M_2 receptors in the central nervous system is the availability of the (*R*)-isomer, which can be used in its unlabelled form to exclude binding of labelled (*S*)-dimethindene to histamine H_1 receptors under the imaging condition.

Acknowledgements

The authors thank Dr. D. Rehn (Zyma, Munich, Germany) for the generous gift of dimethindene and

its enantiomers and the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie (Germany) as well as the Fonds National de la Recherche Scientifique (Belgium) for financial support.

References

Arunlakshana, O. and H.O. Schild, 1959, Some quantitative uses of drug antagonists, Br. J. Pharmacol. 14, 48.

Aubert, I., D.M. Araujo, D. Cecyre, Y. Robitaille, S. Gauthier and R. Quirion, 1992, Comparative alterations of nicotinic and muscarinic binding sites in Alzheimer's and Parkinson's disease, J. Neurochem. 58, 529.

Baumgold, J., R.L. Pryzbyc and R.C. Reba, 1994, 3-α-Chlorimperialine: an M_2-selective muscarinic receptor antagonist that penetrates into brain, Eur. J. Pharmacol. 251, 315.

Borchard, U., D. Hafner and R. Heise, 1985, H_1-Antagonistic actions of (+)- and (−)-dimethindene, Naunyn-Schmied. Arch. Pharmacol. 330 (Suppl.) R9.

Casy, A.F., A.F. Drake, C.R. Ganellin, A.D. Mercer and C. Upton, 1992, Stereochemical studies of chiral H-1 antagonists of histamine: the resolution, chiral analysis, and biological evaluation of four antipodal pairs, Chirality 4, 356.

Caulfield, M.P., 1993, Muscarinic receptors – characterization, coupling and function, Pharmacol. Ther. 58, 319.

Cheng, Y. and W.H. Prusoff, 1973, Relationship between the inhibition constant (K_i) and the concentration of inhibition (I_{50}) which causes a 50 percent inhibition of an enzymatic reaction, Biochem. Pharmacol. 22, 3099.

Cicurel, L., J.C. Péchadre, E. Grandjean and P. Duchêne-Marullaz, 1992, Comparison of the central and peripheral antihistamine effects of dimethindene maleate (Fenistil®) and its enantiomers, Agents Actions (Special Conference Issue), C440.

Cohen, V.I., J. Baumgold, B. Jin, R. De La Cruz, W.J. Rzeszotarski and R.C. Reba, 1993, Synthesis and structure-activity relationship of some 5-[[[(dialkylamino)alkyl]-1-piperidinyl]acetyl]-10,11-dihydro-5*H*-benzo[*b*,*e*][1,4] diazepin-11-ones as M_2-selective antimuscarinics, J. Med. Chem. 36, 162.

Dewey, S.L., N.D. Volkow, J. Logan, R.R. MacGregor, J.S. Fowler, D.J. Schlyer and B. Bendriem, 1990, Age-related decreases in muscarinic cholinergic receptor binding in the human brain measured with positron emission tomography (PET), J. Neurosci. Res. 27, 569.

Doods, H., M. Entzeroth and N. Mayer, 1991, Cardioselectivity of AQ-RA 741, a novel tricyclic antimuscarinic drug, Eur. J. Pharmacol. 192, 147.

Doods, H., M. Entzeroth, H. Ziegler, G. Schiavi, W. Engel, G. Mihm, K. Rudolf and W. Eberlein, 1993, Characterization of BIBN 99: a lipophilic and selective muscarinic M_2 receptor antagonist, Eur. J. Pharmacol. 242, 23.

Dörje, F., J. Wess, G. Lambrecht, R. Tacke, E. Mutschler and M.R. Brann, 1991, Antagonist binding profiles of five cloned human muscarinic receptor subtypes, J. Pharmacol. Exp. Ther. 256, 727.

Eglen, R.M., G.C. Harris, H. Cox, A.O. Sullivan, E. Stefanich and R.L. Whiting, 1992, Characterization of the interaction of the cervane alkaloid, imperialine, at muscarinic receptors in vitro, Naunyn-Schmied. Arch. Pharmacol. 346, 144.

Eltze, M., 1988, Muscarinic M_1- and M_2-receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens, Eur. J. Pharmacol. 151, 205.

Eltze, M., 1994, Pathways involved in muscarinic M_1 and M_2 receptor stimulation in rabbit vas deferens, Eur. J. Pharmacol. 263, 31.

Eltze, M., G. Gmelin, J. Wess, C. Strohmann, R. Tacke, E. Mutschler and G. Lambrecht, 1988, Presynaptic muscarinic receptors mediating inhibition of neurogenic contractions in rabbit vas deferens are of the ganglionic M_1-type, Eur. J. Pharmacol. 158, 233.

Eltze, M., R. Boer, E. Mutschler and G. Lambrecht, 1989, Affinity profiles of BTM-1086 and BTM-1041 at muscarinic receptor subtypes and at H_1- and α_1-receptors, Eur. J. Pharmacol. 170, 225.

Eltze, M., B. Ullrich, E. Mutschler, U. Moser, E. Bungardt, Th. Friebe, C. Gubitz, R. Tacke and G. Lambrecht, 1993, Characterization of muscarinic receptors mediating vasodilation in rat perfused kidney, Eur. J. Pharmacol. 238, 343.

Emmerson, J. and D. Mackay, 1979, The zig-zag tracheal strip, J. Pharm. Pharmacol. 31, 798.

Emmet, J.C., G.J. Durant, C.R. Ganellin, A.M. Roe and J.L. Turner, 1982, Potential histamine H_2-receptor antagonists. 4. Benzylhistamines, J. Med. Chem. 25, 1168.

Feifel, R., M. Wagner-Röder, C. Strohmann, R. Tacke, M. Waelbroeck, J. Christophe, E. Mutschler and G. Lambrecht, 1990, Stereoselective inhibition of muscarinic receptor subtypes by the enantiomers of hexahydro-difenidol and acetylenic analogues, Br. J. Pharmacol. 99, 455.

Gitler, M.S., R.C. Reba, V.I. Cohen, W.J. Rzeszotarski and J. Baumgold, 1992, A novel m2-selective muscarinic antagonist: binding characteristics and autoradiographic distribution in rat brain, Brain Res. 582, 253.

Hulme, E.C., N.J.M. Birdsall and N.J. Buckley, 1990, Muscarinic receptor subtypes, Annu. Rev. Pharmacol. Toxicol. 30, 633.

Lambrecht, G., U. Moser, U. Grimm, O. Pfaff, U. Hermanni, C. Hildebrandt, M. Waelbroeck, J. Christophe and E. Mutschler, 1993, New functionally selective muscarinic agonists, Life Sci. 52, 481.

Lambrecht, G., U. Moser, M. Waelbroeck and E. Mutschler, 1994, Selective muscarinic agonists and antagonists, Can. J. Physiol. Pharmacol. 72 (Suppl. 1), 55.

Lambrecht, G., J. Gross, U. Hacksell, U. Hermanni, C. Hildebrandt, X. Hou, U. Moser, B.M. Nilsson, O. Pfaff, M. Waelbroeck, J. Wehrle and E. Mutschler, 1995, The design and pharmacology of novel selective muscarinic agonists and antagonists, Life Sci. 56, 815.

Leuschner, J., B.W. Neumann, H. Brunnauer and D. Rehn, 1992, Evaluation of the dose-effect curves of dimethindene maleate enantiomers in various animal models, Agents Actions (Special Conference Issue), C431.

Levine, R.R. and N.J.M. Birdsall (eds.), 1993, Subtypes of muscarinic receptors, V, Life Sci. 52, 405.

Levine, R.R. and N.J.M. Birdsall (eds.), 1995, Subtypes of muscarinic receptors, VI, Life Sci. 56, 801.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

Maggio, R., P. Barbier, M.L. Bolognesi, A. Minarini, D. Tedeschi and C. Melchiorre, 1994, Binding profile of the selective muscarinic receptor antagonist tripitramine, Eur. J. Pharmacol. Mol. Pharmacol. 268, 459.

Melchiorre, C., M.L. Bolognesi, A. Chiarini, A. Minarini and S. Spampinato, 1993, Synthesis and biological activity of some methoctramine-related tetramines bearing a 11-acetyl-5,11,-dihydro-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-6-one moiety as antimuscarinics: a second generation of highly selective M_2 muscarinic receptor antagonists, J. Med. Chem. 36, 3734.

Micheletti, R., A. Schiavone and A. Giachetti, 1987, Muscarinic M_1 receptors stimulate a nonadrenergic noncholinergic inhibitory pathway in the isolated rat duodenum, J. Pharmacol. Exp. Ther. 244, 680.

Mutschler, E. and K. Hultzsch, 1973, Über Struktur-Wirkungs-Beziehungen von ungesättigten Estern des Arecaidins und Dihydroarecaidins, Arzneim.-Forsch./Drug Res. 23, 732.

Nelson, W.L., D.S. Freeman and F.F. Vincenzi, 1976, Stereochemical analogs of a muscarinic, ganglionic stimulant. 2. *Cis* and *trans* olefinic, epoxide, and cyclopropane analogs related to 4-[*N*-(3-chlorophenyl)carbamoyloxy]-2-butynyl trimethylammonium chloride (McN-A-343), J. Med. Chem. 19, 153.

Nicholson, A.N., P.A. Pascoe, C. Turner, C.R. Ganellin, P.M. Greengrass, A.F. Casy and A.D. Mercer, 1991, Sedation and histamine H_1-receptor antagonism: studies in man with the enantiomers of chlorpheniramine and dimethindene, Br. J. Pharmacol. 104, 270.

Parker, P.B. and D.R. Waud, 1971, Pharmacological estimates of drug-receptor dissociation constants. Statistical evaluation. I. Agonists, J. Pharmacol. Exp. Ther. 177, 1.

Paton, W.D.M. and M.A. Zar, 1968, The origin of acetylcholine release from guinea-pig intestine and longitudinal muscle stripes, J. Physiol. 194, 13.

Pfaff, O., M. Waelbroeck, J. Camus, M. Tastenoy, E. Mutschler and G. Lambrecht, 1994, (*S*)-Dimethindene: a novel M_2-selective muscarinic antagonist, Naunyn-Schmied. Arch. Pharmacol. 349 (Suppl.), R75.

Pfaff, O., J. Gross, M. Waelbroeck, E. Mutschler and G. Lambrecht, 1995, Functional characterization of muscarinic receptors in rat duodenum, Life Sci. 56, 1038.

Radler, S. and G. Blaschke, 1991, Enantioselective determination of dimethindene in urine after oral administration of racemic dimethindene, J. Chromatogr. Biomed. Appl. 567, 229.

Rekker, R.F., H. Timmerman, A.F. Harms and W.T. Nauta, 1971, The antihistaminic and anticholinergic activities of optically active diphenhydramine derivatives, Arzneim.-Forsch./Drug Res. 21, 688.

Roffel, A.F., C.R.S. Elzinga and J. Zaagsma, 1993, Cholinergic contraction of the guinea-pig lung strip is mediated by muscarinic M_2-like receptors, Eur. J. Pharmacol. 250, 267.

Sautel, M., R. Towart, M. Théraulaz-Lacroix and A.F. Weitsch, 1992, Effects of dimethindene maleate (Fenistil®) on histaminic, muscarinic and serotoninergic receptor system, Agents Actions (Special Conference Issue), C428.

Tallarida, R.J. and R.B. Murray, 1986, Manual of Pharmacologic Calculations with Computer Programs, 2nd edn. (Springer-Verlag, Berlin).

Ter Laak, A.M., G.M. Donné-Op den Kelder, A. Bast and H. Timmerman, 1993, Is there a difference in the affinity of histamine H_1 receptor antagonists for CNS and peripheral receptors? An in vitro study, Eur. J. Pharmacol. 232, 199.

Waelbroeck, M., J. Camus, M. Tastenoy and J. Christophe, 1988, 80% of muscarinic receptors expressed by the NB-OK 1 human neuroblastoma cell line show high affinity for pirenzepine and are comparable to rat hippocampus M_1 receptors, FEBS Lett. 226, 287.

Waelbroeck, M., J. Camus, M. Tastenoy, G. Lambrecht, E. Mutschler, R. Tacke and J. Christophe, 1990a, Stereoselectivity of procyclidine binding to muscarinic receptor subtypes M_1, M_2 and M_4, Eur. J. Pharmacol. Mol. Pharmacol. 189, 135.

Waelbroeck, M., M. Tastenoy, J. Camus and J. Christophe, 1990b, Binding of selective antagonists to four muscarinic receptors (M_1 to M_4) in rat forebrain, Mol. Pharmacol. 38, 267.

Waelbroeck, M., J. Camus, M. Tastenoy, E. Mutschler, C. Strohmann, R. Tacke, L. Schjelderup, A. Aasen, G. Lambrecht and J. Christophe, 1992, Stereoselective interaction of procyclidine, hexahydro-difenidol, hexbutinol and oxyphencyclimine, and of related antagonists, with four muscarinic receptors, Eur. J. Pharmacol. Mol. Pharmacol. 227, 33.

Waelbroeck, M., J. Camus, M. Tastenoy, R. Feifel, E. Mutschler, R. Tacke, C. Strohmann, K. Rafeiner, J.F. Rodrigues de Miranda and G. Lambrecht, 1994, Binding and functional properties of hexocyclium and sila-hexocyclium derivatives to muscarinic receptor subtypes, Br. J. Pharmacol. 112, 505.