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Synthesis and In Vitro Muscarinic Activities of a Series of 3-(Pyrazol-3-yl)-1-azabicyclo[2.2.2]octanes

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Abstract—A series of 3-(pyrazol-3-yl)-1-azabicyclo[2.2.2]octane derivatives **C** (Fig. 1) was synthesized and tested for muscarinic activity in receptor binding assays using [^{3}H]-oxotremorine-M (OXO-M) and [^{3}H]-pirenzepine (PZ) as ligands. Potential muscarinic agonistic or antagonistic properties of the compounds were determined using binding studies measuring their potencies to inhibit the binding of OXO-M and PZ. Preferential inhibition of OXO-M binding was used as an indicator for potential muscarinic agonistic properties; this potential was confirmed in functional studies on isolated organs.

Introduction

Cholinergic neurons in the forebrain of patients suffering from Alzheimer's disease appear to be degenerated and these changes correlated with the reduction in cognitive function.[1] Evidence suggesting a greater loss of presynaptic rather than postsynaptic receptors[1,2] has led to the current pharmacochemical strategy in this disease based on the cholinergic deficit hypothesis[2] and is focused on the stimulation of postsynaptic receptors. The increase of the availability of acetylcholine via cholinesterase inhibitors such as tacrine[5] has been marginally successful as only some aspects of cognitive performance were found improved in association with severe side effects.[3–6] Their poor therapeutic efficacy might partly be caused by a reduction of acetylcholine release caused by stimulation of presynaptic inhibitory autoreceptors following an increase of extracellular acetylcholine after treatment with these acetylcholine esterase inhibitors. Similarly, studies involving direct stimulation of cholinergic receptors with non-selective cholinergic agonists have been marred by their side effect profile (e.g. Oxotremorine,[7] RS 86[8]).

Careful adjustment of the dose may improve certain cognitive functions without severe compromising side effects,[9,10] thus indicating the general potential of the cholinergic strategy for treatment of Alzheimer patients. Five muscarinic cholinergic receptor subtypes are known and their structure as well as their distribution have been described.[11] These receptors regulate a wide range of peripheral and central functions in addition to their role in cognition.[12] This explains why treatments involving non-selective agonists, which act at all cholinergic receptor subtypes, produce many undesirable effects. Therefore, attention has shifted to subtype selective muscarinic agonists as a means to improve the therapeutic efficacy of cholinergic agents and at the same time to reduce their side effects. Despite some potential problems with this approach,[13] attention has focused on compounds with, in particular, M_1 and M_1/M_3 agonist properties.[14] Recent animal studies indeed have shown positive results in tests of learning and memory with some of these compounds,[15,16] but definite conclusions await the outcome of clinical trials.

The aim of the present study was to design and test a series of compounds with selective effects at specific receptor subtypes in vitro, and then to investigate the central and peripheral effects in vivo including effects on memory. The ultimate object of these studies was to develop compounds with improved efficacy, preferentially affecting the central nervous system with a reduced incidence of peripheral side effects.

We chose arecoline (**A**) as a useful starting point for the design of the title series based on the observation of therapeutically beneficial effects of arecoline (**A**), a

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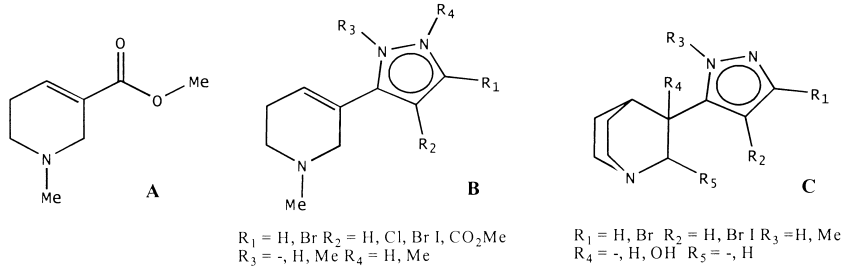


Figure 1.

compound of limited use due to its short duration of action.

In our search for a bioisosteric replacement of the ester function of arecoline (**A**) we developed the series of 3-(pyrazolyl)-1,2,5,6-tetrahydropyridine derivatives **B**,[17] a series of potent muscarinic agonists (Fig. 1). Compounds in this series produced weak agonists selective for the M_3-receptor.[18] Here we report the synthesis of the related series of 3-(pyrazol-3-yl)-1-azabicyclo-[2.2.2]octane derivatives **C** (Table 1) which was tested for muscarinic activity using [^{3}H]-oxotremorine-M (OXO-M) and [^{3}H]-pirenzepine (PZ) as ligands (Table 2) and profiled in our in vitro (Table 3) test battery.[19]

Chemistry

Synthesis of 3(pyrazol-3-yl)-1-azabicyclo[2.2.2]octane derivatives

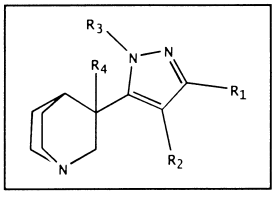
For the synthesis of the 3-(pyrazole-3-yl)-1-azabicyclo-[2.2.2]octane derivatives two approaches have been devised commencing with 1-azabicyclo[2.2.2]octane-3-one (**1**; Schemes 1 and 2).

i. Treatment of **1** with tosylmethylisocyanide (TOSMIC reagent) gave the cyanide **2**.[20] Subsequently, treatment of 3-cyano-1-azabicyclo[2.2.2]octane (**2**) with methyl lithium gave 3-acetyl-1-azabicyclo[2.2.2]octane (**3**) in good yield. Reaction of **3** with dimethylformamide dimethylacetal as reagent gave the enamine derivative **4**, unfortunately in low yield (10%). Finally, treatment of **4** with hydrazine and methylhydrazine gave the desired pyrazole derivatives **5** and **6**, respectively, in moderate yields.

ii. The second, more efficient route (Scheme 2), started with the addition of propargylaldehyde diethyl acetal to the keto function of **1.** Treatment of the adduct **7** with hydrazine gave the 1-azabicyclo[2.2.2]-octan-3-ol derivative **8**. The alcohol function of **8** was removed in a two-step procedure: (1) elimination using thionylchloride as reagent and (2) subsequent catalytic hydrogenation of **9** in a Parr apparatus (35 psi) gave **5**.

The products **5** (Scheme 3) as well as **9** (Scheme 2) were converted into the mono-bromo derivatives **10** and **11**, the mono-iodo derivative **12** and the di-bromo derivative **13** using one or two equivalents of *N*-halogen succinimide as reagent, respectively.

Table 1. Physical properties 3-(pyrazol-3-yl)-1-azabicyclo[2.2.2]octanes



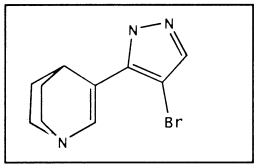
Compound	R_1	R_2	R_3	R_4	Salt	Mp (°C)	Molecular formula
5	H	H	H	H	HCl	229	$C_{10}H_{15}N_3 \cdot 2HCl$
6	H	H	Me	H	mal[a]	153	$C_{11}H_{17}N_3 \cdot C_4H_4O_4$
8	H	H	H	OH	HCl	228	$C_{10}H_{15}N_3O \cdot 2HCl$
11	H	Br	H	H	mal[a]	140	$C_{10}H_{14}BrN_3 \cdot C_4H_4O_4$
12	H	I	H	H	mal[a]	145	$C_{10}H_{14}IN_3 \cdot C_4H_4O_4$
13	Br	Br	H	H	mal[a]	163	$C_{10}H_{13}Br_2N_3 \cdot C_4H_4O_4$
							
10					mal[a]	188	$C_{10}H_{12}BrN_3 \cdot C_4H_4O_4$

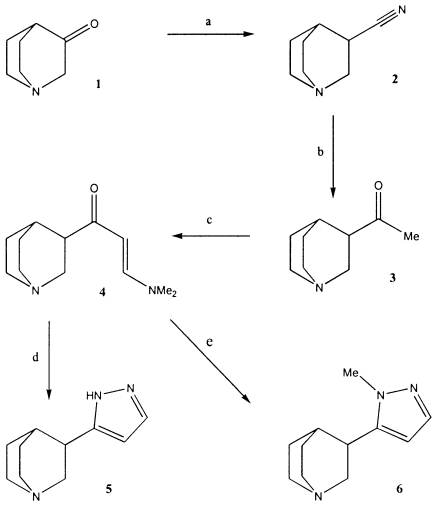
Table 2. Receptor binding affinity and affinity ratios for assays using agonist, Oxotremorine-M (OXO-M), and antagonist pirenzepine (PZ) ligands (data are mean values of 2–4 binding inhibition experiments; for further details see Experimental)

Compound	^{3}H-OXO-M (K_i in μM)	^{3}H-PZ (K_i in μM)	^{3}H-PZ/^{3}H-OXO-M ratio
Arecoline	0.0079	1.58	200
5	0.79	40	50.6
6	0.63	6.3	10.0
8	3.1	40	12.9
10	0.063	0.15	2.38
11	0.012	1.63	135
12	0.004	0.126	31.5
13	0.025	1.02	40.3

Table 3. Muscarinic cholinergic activity in guinea pig ileum (MUGI) and rat left atrium (M_2LA)

Compound	MUGI			M_2LA		
	pD_2[a]	α	pA_2[b]	pD_2	α[c]	pA_2
Arecoline	6.5	1.0				
5	4.6	1.0	—	—	—	—
10	—	—	5.7	—	—	—
11	5.7	1.0	—	< 4	0	5.9
12	6.2	0.7	5.7	6.3	0.5	6.2
13	—	—	5.2	—	—	—

[a]Agonist values, pD_2.
[b]Antagonist values, pA_2.
[c]α, intrinsic activity.

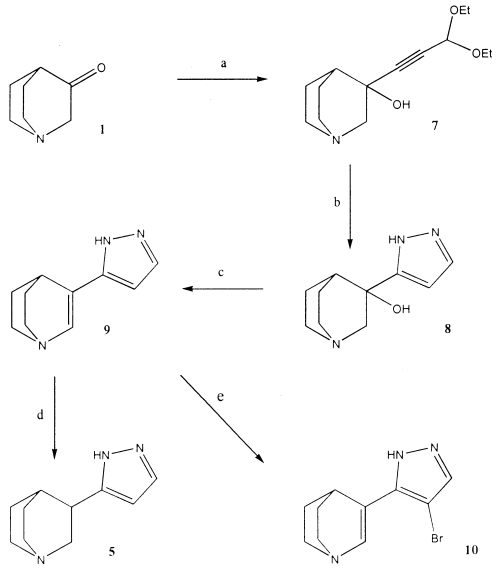


Scheme 1. (a) Tosmic, KOtBu, DME, 0–5 °C, 20 h; (b) MeLi, THF, 0 °C, 2 h; (c): DMF-dimethylacetal, DMF, 20 h, reflux; (d): $H_2NNH_2.H_2O$, MeOH, 5 h, rt; e: $MeNHNH_2$, MeOH, 20 h, rt.

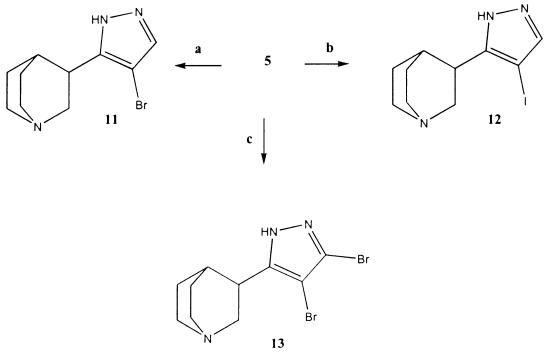
Pharmacology

Results

The physical properties of the derivatives are shown in Table 1. The effects of the compounds on cholinergic receptors measured as the inhibition of ligand binding are shown in Table 2. The affinity of the compounds as inhibitor of the binding of the radiolabeled muscarinic



Scheme 2. (a) $HC(CCH(OEt)_2$, t-BuOK, THF, 3 h, −5 °C; (b): $H_2NNH_2.H_2O$, EtOH, 18 h, reflux; (c): $SOCl_2$, benzene, 18 h, reflux; (d): H_2, 10% Pd/C, MeOH, 24 h, 30 psi; (e): NBS, DMF, 3 h, −15 °C.



Scheme 3. (a) 1 eq NBS, DMF, 1 h, −10 °C; (b) 1 eq NIS, DMF, 1 h, −10 °C; (b) 2 eq NBS, DMF, 1 h, rt.

cholinergic agonist OXO-M is compared with their potency to inhibit the binding of the radiolabeled muscarinic cholinergic antagonist pirenzepine. The ratio of antagonist/agonist binding has been suggested[14] to give an indication of agonist potential: a large ratio indicates agonistic properties of the compound (viz. Table 2, the ratio PZ/OXO-M). This was further evaluated in isolated organ preparation allowing the assessment of functional activity for muscarinic cholinergic of the subtypes M_2 and M_3 in guinea pig ileum and the isolated rat left atrium, respectively. The results show that the unsubstituted compound has a modest affinity in the binding studies. It showed full agonistic properties in the M_3 model in agreement with its binding ratio of about 50. Substitution with a methyl group enhanced the affinity in the PZ binding without affecting the OXO-M binding, thus causing a decrease in the ratio from 50 for the unsubstituted compound **5** to 10 for compound **6**. Introduction of a hydroxyl group at position R_4 decreased the affinity in OXO-M without affecting PZ binding affinity, thus reducing again the OXO-M/PZ binding ratio. Substitution with a bromine at position R_2 caused an 80-fold increase in affinity in

OXO-M binding and a smaller 25-fold increase in affinity in PZ binding. As a result a PZ/OXO-M ratio of 135 was found and this compound showed full agonistic properties in the model for M_3 receptors with a pD_2 of 5.7 and an intrinsic activity (α-value) of 1.0. In contrast, no agonistic properties were observed in the M_2 model and the compound appeared to be antagonistic with a pA_2 value of 5.9. Introduction of an iodine at position R_2 caused a further 3-fold increase in the affinity of compound **12** in OXO-M binding and a 13-fold increase in affinity in PZ binding. The compound showed a PZ/OXO-M binding ratio of 31.5 and behaved as a partial agonist ($\alpha = 0.5$–0.7) in the models used for measuring M_2 (atrium rat) and M_3 (guinea pig ileum) activity. Substitution of two bromine atoms in the pyrazole ring caused an approximate 30-fold reduction in affinity in both binding tests, leaving the PZ/OXO-M ratio roughly unchanged. However, despite a ratio of 40 this substitution resulted in loss of agonistic properties in the M_3 model (MUGI). The halo-substituted compounds **11**, **12** and **13** were also tested for potential M_1-agonistic model in the hippocampal slice preparation, but all three compounds failed to show any M_1 muscarinic agonistic properties (data not shown).

Discussion

The present series of muscarinic cholinergic ligands was evaluated for potential muscarinic cholinergic agonistic properties by assessing the PZ/OXO-M binding ratio and verification of properties in models selectively revealing muscarinic subtype selective agonistic properties. In this series the PZ/OXO-M ratio did not show a correlation with the muscarinic agonistic properties measured in isolated organs. In fact, compound **5** has the highest ratio (135) and accordingly is a full M_3 agonist, but it fails to show up as an agonist in the M_2 and M_3 models. Thus, the compounds show up as muscarinic subtype selective agonists based on their capacity to stimulate the M_2 and M_3 subtypes and their failure to stimulate M_1 receptors in hippocampal slices. However, such a profile is not favorable for their potential to correct a memory defect. Such an effect supposedly requires M_1 agonistic properties,[23] which are absent in the present series.

Experimental

Chemistry

Melting points were taken on a Büchi capillary melting point apparatus and are uncorrected**.** The elemental analyses were within 0.45 of the theoretical values. Proton magnetic resonance spectra were measured on a Bruker WP200 or AC200 instrument. Chemical shifts are reported as δ-values (parts per million) relative to Me_4Si as an internal standard. Thin layer chromatography (TLC) was carried out by using Merck precoated silicagel F-254 plates (thickness 0.25 mm). Spots were visualized with a UV hand lamp and Cl_2/tetramethylbenzidine.

Acetyl-1-azabicyclo[2.2.2]oct-2-ene (3). 3-Cyano-1-azabicyclo[2.2.2]octane **2** was prepared from 1-azabicyclo-[2.2.2]octan-3-one **1** according to the procedure described in literature.[20] Methyl lithium (76.8 mmol) was added within 1 h to a suspension of **2** (4.6 g, 26.6 mmol) in dry THF (50 mL). After 2 h stirring at 5 °C the reaction was quenched with water (30 mmol). The solvent was removed in vacuo and the residue was subjected to column chromatography (silicagel 60, eluent: MeOH:conc. $NH_4OH = 98:2$) to give the acetyl derivative **3** in 93% yield. ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.40 (d, $J = 6.4$ Hz, 1H, CH), 2.90–2.60 (m, 6H, CH_2N's), 2.25–2.20 (m, 1H, CH), 2.20 (s, 3H, CH_3), 1.75–1.60 (m, 2H), 1.50–1.35 (m, 2H).

3-(Dimethylamino)-1-azabicyclo[2.2.2.]oct-3-yl)-prop-2-en-1-one (4). Dimethylformamide dimethylacetale (2.0 mL, 14.4 mmol) was added to a solution of **3** (7.7 mmol) in 30 mL dry toluene. The reaction mixture was heated to reflux for 48 h. After cooling the mixture, the organic layer was washed three times with water. Drying of the organic layer and evaporation of the solvent gave the title compound **4** as an oil in 10% yield. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.65 (d, $J = 13.1$ Hz, 1H, HC=), 5.0 (d, $J = 13.5$ Hz, 1H, =CH), 3.70 (d, $J = 5.9$ Hz , 1H, CH), 3.20–2.70 (m, 4H, CH_2N's), 2.95 + 2.90 (s, 6H, CH_3's), 2.25 (m, 1H, CH), 1.90–1.65 (m, 3H), 1.60–1.40 (m, 1H).

3-(1H-Pyrazol-3-yl)-1-azabicyclo[2.2.2]octane (5). Hydrazine hydrate (0.5 mL) was added to a solution of the enamine **4** (0.35 g, 1.7 mmol) in 2.5 mL dry ethanol. The reaction mixture was stirred at room temperature for 15 h. After evaporation of the solvent, the pyrazole derivative was isolated as a hydrochloric acid salt in 39% yield; mp = 230 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.55 (s, 1H), 6.20 (s, 1H), 3.40–2.80 (m, 8H), 2.05 (m, 1H), 1.90–1.60 (m, 3H), 1.40 (m, 1H). Anal. ($C_{10}H_{15}N_3 \cdot 2HCl$) C, H, N. Exact mass calcd for $C_{10}H_{16}N_3$ $[M+H]^+$ 178.1344, found: 178.1328.

3-(1-Methyl-1H-pyrazol-5-yl)-1-azabicyclo[2.2.2]octane (6). A solution of **4** (0.75 g, 3.6 mmol) and methylhydrazine (0.38 mL, 7.2 mmol) in 10 mL dry methanol was stirred for 20 h at room temperature. After evaporation of the solvent the residue was subjected to column chromatography (silicagel 60, eluent MeOH: $NH_4OH = 98:2$). The title compound **6** was crystallised as a maleic acid salt in 50% yield; mp 153 °C. ^{1}H NMR (200 MHz, MeOD) δ 7.45 (s, 1H, N=CH), 6.50 (s, 1H, C–CH=), 6.25 (s, 2H, mal), 3.90 (s, 3H, CH_3), 3.90–3.40 (m, 7H), 2.30–1.70 (m, 5H). Anal. ($C_{11}H_{17}N_3 \cdot C_4H_4O_4$) C, H, N. Exact mass calcd for $C_{11}H_{18}N_3$ $[M+H]^+$ 192.1501, found: 192.1490.

3-(3,3-Diethoxyprop-1-ynyl)-1-azabicyclo[2.2.2]octan-3-ol (7). A suspension of propargylaldehyde diethylacetal (2.5 g, 19.2 mmol) and tBuOK (2.4 g, 22.5 mmol) in 40 mL anhydrous THF was stirred for 1 h at −10 °C. Then a solution of 1-azabicyclo[2.2.2]octan-3-one **1** (2.0 g, 16 mmol) in 25 mL anhydrous THF was added dropwise. After stirring for 2 h at 0 °C an aqueous acetic acid solution was added and the solvents were removed under reduced pressure.

The residue was made alkaline (2 N NaOH), and the product was extracted with ethyl acetate (3×). The combined organic layers were dried ($MgSO_4$) and evaporated to dryness to give **7** (84%). ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.95 (s, 2H), 3.80–3.50 (m, 4H), 3.30–3.00 (m, 2H), 2.85 (t, 3H), 2.10–1.40 (m, 5H), 1.25 (t, 6H).

3-(1H-Pyrazol-3-yl)-1-azabicyclo[2.2.2]octan-3-ol (8). A solution of **7** (4.05 g, 16 mmol) and hydrazine dihydrochloride (1.85 g, 17.6 mmol) in ethanol (50 mL) was heated to reflux for 18 h. The solvent was removed under reduced pressure. The residue was made alkaline using a 2 N NaOH solution, and the product was extracted with ethyl acetate (3×). The combined organic layers were dried ($MgSO_4$) and evaporated to give an oil. The hydrochloric acid salt **8** (60%); mp 228 °C. ^{1}H NMR (200 MHz, D_2O) δ 7.95 (s, 1H), 6.70 (s, 1H), 4.10 (d, J = 15 Hz, 1H), 3.60 (d, J = 15 Hz, 1H), 3.50–3.20 (m, 4H), 2.60–2.40 (m, 2H), 2.10–1.90 (m, 2H), 1.80–1.60 (m, 1H). Anal. ($C_{10}H_{15}N_3O$•2HCl) C, H, N. Exact mass calcd for $C_{10}H_{16}N_3O$ $[M+H]^+$ 194.1293, found: 194.1281.

3-(1H-Pyrazol-1-yl)-1-azabicyclo[2.2.2]oct-2-ene (9). To a suspension of **8** (4.5 g, 18.1 mmol) in anhydrous benzene (25 mL) thionyl chloride (2 mL, 27 mmol) was added. The solution was heated to reflux. After 18 h the solvent was removed under reduced pressure. The residue was made alkaline using a 2 N NaOH-solution, and the product was extracted with ethyl acetate. The combined organic layers were dried ($MgSO_4$) and evaporated to give **9** as an oil (63%).

3-(1H-Pyrazol-3-yl)-1-azabicyclo[2.2.2]octane (5). Hydrogenation of **9** (1.6 g, 10 mmol) in methanol (50 mL) over 10% Pd/C (250 mg) at 30 psi at room temperature in a Parr apparatus for 24 h gave the title compound **5**, after filtration and evaporation of the solvent, in 93% yield. The hydrochloride salt was prepared; mp 229 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.55 (s, 1H), 6.20 (s, 1H), 3.40–2.80 (m, 8H), 2.05 (m, 1H), 1.90–1.60 (m, 3H), 1.40 (m, 1H).

3-(4-Bromo-1H-pyrazol-1-yl)-1-azabicyclo[2.2.2]oct-2-ene (10). A solution of NBS (1.1 g, 6 mmol) in 15 mL dry DMF was added at −20 °C, to a solution of **9** (6.1 mmol) in 15 mL dry DMF. After 3 h stirring at −15 °C the reaction mixture was diluted with 100 mL H_2O and three times extracted with ethyl acetate. The combined organic layers were dried ($MgSO_4$) and evaporated. The residue was subjected to column chromatography (silicagel 60, eluent MeOH) to give **10** as an oil in 45% yield. The maleic acid salt was prepared: mp 188 °C. ^{1}H NMR (200 MHz, MeOD) δ 7.90 (s, 1H, N–CH=), 7.45 (s, 1H, BrC=CH), 6.25 (s, 2H, Mal), 3.95 (s (broad), 1H, =C–CH), 3.70–3.55 (m, 2H, CH_2), 3.30–3.10 (m, 2H, CH_2), 2.30–2.10 (m, 2H, CH_2), 1.95–1.70 (m, 2H, CH_2). Anal. ($C_{10}H_{12}BrN_3$•$C_4H_4O_4$) C, H, N. Exact mass calcd for $C_9H_{12}BrN_3$ $[M+H]^+$ 241.0415, found: 241.0405.

3-(4-Bromo-1H-pyrazol-1-yl)-1-azabicyclo[2.2.2]octane (11). To a solution of **5** (0.61 g, 3.7 mmol) in anhydrous DMF (10 mL) and at −10 °C a solution of *N*-bromosuccinimide (0.66 g, 3.7 mmol) in 10 mL DMF (10 mL) was added under N_2. The mixture was stirred at this temperature for 3 h and then poured into water. The aqueous layer was extracted with ethyl acetate (3×). The combined organic layers were dried ($MgSO_4$) and evaporated. The residue was subjected to column chromatography (silicagel 60, eluent MeOH) to give **11** as an oil in 42% yield. The maleic acid salt was prepared; mp 140 °C. ^{1}H NMR (200 MHz, D_2O) δ 7.70 (s, 1H), 6.30 (s, 2H, mal), 4.00 (m, 1H), 3.70–3.40 (m, 4H), 2.40 (m, 1H), 2.20–1.70 (m, 4H) 1.40 (m, 1H). Anal. ($C_{10}H_{14}BrN_3$•$C_4H_4O_4$) C, H, N. Exact mass calcd for $C_{10}H_{15}BrN_3$ $[M+H]^+$ 256.0649, found: 256.0645.

3-(4-Iodo-1H-pyrazol-1-yl)-1-azabicyclo[2.2.2]octane (12). To a solution of **5** (0.5 g, 2.8 mmol) in anhydrous DMF (20 mL) at −10 °C a solution of *N*-iodosuccinimide (0.64 g, 2.8 mmol) in DMF (10 mL) was added. The mixture was stirred under N_2 at room temperature for 20 h and then poured into water. The aqueous layer was extracted with ethyl acetate (3×). The combined organic layers were dried ($MgSO_4$) and evaporated. The maleic acid salt was prepared in 62% yield; mp 146 °C. ^{1}H NMR (200 MHz, D_2O) δ 7.70 (s, 1H), 6.30 (s, 2H, mal), 4.00 (m, 1H), 3.70–3.40 (m, 4H), 2.40 (m, 1H), 2.20–1.70 (m, 4H) 1.40 (m, 1H). Anal. ($C_{10}H_{14}IN_3$•$C_4H_4O_4$) C, H, N. Exact mass calcd for $C_{10}H_{15}IN_3$ $[M+H]^+$ 304.0311, found: 304.0301.

3-(3,4-Dibromo-1H-pyrazol-1-yl)-1-azabicyclo[2.2.2]octane (13). *N*-Bromosuccinimide (0.7 g, 9 mmol) was added all at once to a solution of **5** (0.49 g, 2.8 mmol) in 10 mL dry DMF. The reaction was strongly exothermic (60 °C). After 1 h stirring, the solvent was evaporated. The residue was subjected to column chromatography (silicagel 60, eluent MeOH:NH_4OH = 98:2) to give **13** as an oil in 32% yield. The maleic acid salt was prepared; mp 163 °C. ^{1}H NMR (D_2O, 200 MHz) δ 6.25 (s, 2H, mal), 3.70–3.20 (m, 7H), 2.40 (m, 1H, CH), 2.25–2.00 (m, 2H), 1.90–1.75 (m, 2H). Anal. ($C_{10}H_{13}Br_2N_3$•$C_4H_4O_4$) C, H, N. Exact mass calcd for $C_{10}H_{14}Br_2N_3$ $[M+H]^+$ 333.9954, found: 333.9937.

In Vitro Studies

Agonist and antagonist binding studies

Binding of [methyl-^{3}H]-oxotremorine-M acetate (^{3}H-OXO-M) in homogenates of frontal cortex. The rapid filtration method of Freedman et al.[21] was used to measure the agonist character of muscarinic cholinergic drugs in rat cerebral cortex homogenates. For routine measurements the concentration of [^{3}H]-OXO-M was 0.5 nM, tissue concentration was about 1 mg/mL original tissue and incubation was for 40 min at 30 °C. Non-specific binding was defined as the amount of binding of [^{3}H]-OXO-M in the presence of 2 mM atropine sulfate and represented about 10% of total binding.

Binding of [*N*-methyl-^{3}H]-pirenzepine [^{3}H-PZ] in homogenates of rat forebrain. The rapid filtration method of

Freedman et al.[21] was used to characterize M_1-muscarinic cholinergic properties of drugs in rat forebrain membranes. For routine measurements the concentration of [^{3}H]-PZ was 1 nM, tissue concentration was about 10 mg/mL original tissue and incubation was for 60 min at 25 °C. Non-specific binding was defined as the amount of binding of [^{3}H]-PZ in the presence of 1 mM atropine sulfate and represented about 20% of total binding.

Evaluation of the data. Displacement curves were obtained for the various compounds by measuring the specific binding in the presence of at least four different concentrations and IC_{50} values were obtained using a four-parameter fitting procedure. K_i values were obtained from the IC_{50} values by using the Chang–Prusoff equation $K_i = IC_{50}/(1 + C/K_d)$ in which C equals the radiolabelled ligand concentration and K_d equals the dissociation constant for the radiolabelled ligand. K_d values used for these calculations were as follows: [^{3}H]-OXO-M binding: $K_d = 0.7$ nM; [^{3}H]-PZ binding: $K_d = 8.3$ nM.

Interactions with muscarinic subtype mediated responses in isolated organs

M_1-mediated activity in the hippocampal slice. Muscarinic cholinergic agonists effectively suppress the electrically evoked field excitatory postsynaptic potential (fEPSP) in the rat hippocampal slice.[22] This effect is due to a decrease in release of excitatory amino acids from the Schaffer collateral/commissural fibers, probably mediated by a presynaptic M_1 muscarinic receptor.[22] Carbachol (3E-5 mol/L) causes a 100% decrease (intrinsic activity = 1.0) in the amplitude of the electrically evoked fEPSP, an effect that can be fully antagonized by the M_1 selective antagonist pirenzepine.[23]

M_2-mediated effects. Interactions with M_2-muscarinic cholinergic receptors were studied in the isolated left atrium of the rat. An automated assay was used similar to the β_1-adrenoreceptor described previously[24] but with the following adaptation. The inhibition by cholinergic agonists of the electrical stimulation evoked switch of the atrium was measured using carbachol as a reference. Cholinergic agonistic activity was compared to carbachol. Potential M_2-activity was verified via antagonism in the presence of the M_2-selective antagonist AF-DX 116. Antagonistic activity was measured as a shift to the right of the dose–response curve to carbachol in the presence of the compound as described above.

M_3-mediated effects. Interactions with M_3-muscarinic cholinergic receptors were measured in the isolated guinea pig ileum. A fully automated method was used as described previously.[23] Contractions induced with acetylcholine as an agonist (pD_2-values between 6 and 7) were used to evaluate the potency of cholinergic antagonist. Antagonistic activity was measured as described above.

References and Notes

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