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Pharmacological characterization of MP349, a novel 5-HT_{1A}-receptor antagonist with anxiolytic-like activity, in mice and rats

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Abstract

The purpose of this study was to further characterize the pharmacological effects of MP349 (*trans*–1-(2-methoxyphenyl)-4-(4-succinimidocyclohexyl)piperazine), a new serotonin 5-HT_{1A} postsynaptic receptor antagonist, using several biochemical and behavioural assays. The silent 5-HT_{1A}-receptor antagonist WAY 100635 (*N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-(2-pyridinyl)cyclohexanecarboxamide) was used as a reference compound in in-vivo tests, and diazepam served as standard anxiolytic drug in animal models of anxiety. In this study we showed that MP349 bound with moderate affinity ($K_i = 234$ nM) for α_1-adrenoceptors, and with very low affinity ($K_i > 2600$ nM) for 5-HT_{2A}, dopamine D_1, D_2 and benzodiazepine receptors. The effects of MP349 on presynaptic 5-HT_{1A} receptors were studied in two models (mice and rats). Like WAY 100635, MP349 antagonized the hypothermia induced by the 5-HT_{1A}-receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin(8-OH-DPAT) in mice. Neither MP349 nor WAY 100635 administered alone induced hypothermia. In a rat microdialysis study, MP349 (like WAY 100635) did not affect 5-HT dialysate level in the prefrontal cortex; however, when given before 8-OH-DPAT, it inhibited the decrease in 5-HT release induced by the 5-HT_{1A} agonist. The data demonstrated that MP349 behaved like a functonal antagonist of presynaptic 5-HT_{1A} receptors. The potential anxiolytic activity of MP349 and reference drugs was examined in a conflict drinking test in rats, a plus-maze test in rats and a four-plate test in mice. MP349 and WAY 100635 produced anxiolytic-like effects, though somewhat weaker than those induced by diazepam, and only in the case of diazepam the anxiolytic-like effects were dose-dependent. Moreover, MP349 administered in doses inducing anxiolytic-like effects did not disturb the locomotor activity (open field test) or locomotor coordination (rota-rod test) of rats. These and earlier results indicated that MP349 was an antagonist of 5-HT_{1A} receptors which exhibited anxiolytic-like activity in an animal model of anxiety.

Introduction

It is now widely appreciated that multiple serotonin (5-HT) receptors exist in the central nervous system, among which the 5-HT_{1A} receptors are the ones that have been fully described and best characterized. These receptors are of particular interest in respect of their broad involvement in the control of physiological and pathological processes (Saxena 1995). Many studies with selective 5-HT_{1A}-receptor ligands have been carried out to characterize the functional role of these receptors. The development of the arylpiperazine partial agonists buspirone and gepirone, which exert anxiolytic and antidepressant actions in man (Lader 1991), has substantiated the hypothesis that the 5-HT_{1A} subtype is particularly relevant due to its implication in the regulatory processes of anxiety and depression.

Consequently, a number of compounds with a vast spectrum of chemical structures have been identified as 5-HT_{1A} receptor agonists, partial agonists or antagonists. Structures with an arylpiperazine as the source of basic nitrogen were successfully used to prepare 5-HT_{1A}-receptor ligands, which led to identification of agonists of those receptors (e.g. 4-methyl-2-{4-[4-pyrimidin-2-yl)piperazin)]-butyl}-2H,4H,-1,2,4-triazin-3,5-dione (F 11440 (Koek et al 1998)) or 2-{[4-(*o*-methoxyphenyl)piperazin-1-yl]methyl}-1,3-dioxoperhydroimidazo[1,5-a]pyridine (B-20991 (Beneytez et al 1998))), their partial

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agonists (e.g. 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)-butyl]piperazine (NAN-190) or 8-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-8-azaspiro[4.5]decane-7,9 dione (BMY-7378)) and antagonists (e.g. *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-(2-pyridinyl)cyclohexanecarboxamide (WAY 100635) or 4-[3-(benzotriazol-1-yl)propyl]-1-(2-methoxyphenyl)piperazine (MP 3022) (Schechter & Kelly 1997)). It is noteworthy that some of them e.g. F 11440 (Koek et al 1998), B-20991 (Beneytez et al 1998) or WAY 100635 (Griebel et al 2000; Canto-de-Souza et al 2002; Nunes-de-Souza et al 2002; Cao & Rodgers 1997a, b) showed anxiolytic-like activity.

For a number of years our research group has been interested in some arylpiperazine derivatives, including such NAN-190 analogues as 5-HT_{1A} receptor ligands. Systematic structure–activity studies have allowed us to establish optimal structural characteristics of the interaction with the 5-HT_{1A} receptors and to obtain the highly potent compound 1-(2-methoxyphenyl)-4-[(4-succinimido)butyl]-piperazine (MM77), a postsynaptic 5-HT_{1A} antagonist (Mokrosz et al 1994a), for which anxiolytic-like activity has been described (Griebel et al 2000). Recently, the synthesis and some pharmacological properties of the new 5-HT_{1A} ligand *trans*-1-(2-methoxyphenyl)-4-(4-succinimidocyclohexyl)-piperazine (MP349), a conformationally restricted analogue of MM77, have been described (Paluchowska et al 2002). Actually, binding and functional studies have shown that MP349 is a potent 5-HT_{1A}-receptor ligand ($K_i = 15$ nM) that antagonizes some effects (i.e. the behavioural syndrome and lower lip retraction in rats) induced by the 5-HT_{1A}-receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT); these effects are connected with stimulation of postsynaptic 5-HT_{1A} receptors (Tricklebank et al 1984; Berendsen et al 1991). Interestingly, the median effective dose (ED50) values for MP349 in those tests were extremely low (0.2–0.3 mg kg^{-1}) (Paluchowska et al 2002).

Following that study, it seemed of particular interest to find out whether MP349 may inhibit the presynaptic 5-HT_{1A} receptor-mediated response induced by 8-OH-DPAT (hypothermia in mice and a decrease in 5-HT release in rat prefrontal cortex in a microdialysis study). The potential anxiolytic-like activity of MP349 was assessed in rats (a conflict drinking test and a plus-maze test) and mice (a four-plate test). Additionally, the effect of MP349 on exploratory activity and motor coordination was evaluated. In these models, the pharmacological activity of MP349 in-vivo was compared with that of WAY 100635, a 5-HT_{1A}-receptor antagonist (Fletcher et al 1996). Diazepam was used as a standard drug in animal anxiety models.

Materials and Methods

Animals and housing

Male Albino–Swiss mice (25–30 g) and male Wistar rats (250–350 g) were used for the experiments. Groups of eight animals were caged (60×38×20 cm) at an ambient temperature of 20 ± 1 °C. The animals had free access to food (standard laboratory pellets) and water before the experiments. All experiments were conducted in the light phase on the natural light/dark cycle (September to March), between 0900 and 1600 h. The animals were used only once in each test. In mice, all injections were given in a volume of 10 mL kg^{-1}, and in rats in a volume of 2 mL kg^{-1}. Experiments were performed by an observer blind to the treatments. All experimental procedures were approved by the Animal Care and Use Committee at the Institute of Pharmacology, Polish Academy of Sciences in Kraków.

Drugs

8-Hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide (8-OH-DPAT, Research Biochemicals Inc., Natick) and *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride (WAY 100635, synthesized by Dr J. Boksa, Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland) were used as aqueous solutions. *Trans*-1-(2-methoxyphenyl)-4-[4-succinimidocyclohexyl]piperazine dihydrochloride (MP349, synthesized by Dr M. H. Paluchowska, Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland) and diazepam (Polfa, Poznań, Poland) were suspended in a 1% aqueous solution of Tween 80. 8-OH-DPAT and WAY 100635 were injected subcutaneously (s.c.), MP349 and diazepam were given intraperitoneally (i.p.).

Radioligand binding studies

K_i values were determined from at least three competition binding experiments in which 10 drug concentrations, run in triplicate, were used. The Cheng & Prusoff (1973) equation was used for K_i calculations.

5-HT_{2A} and α_1-adrenergic receptor binding assays

The affinity of the MP349 for 5-HT_{2A} and α_1-adrenergic receptors was assessed on the basis of its ability to displace [^{3}H]ketanserin (63.3 Ci mM^{-1}, NEN Chemicals, Boston) and [^{3}H]prazosin (25.0 Ci mM^{-1}, Amersham, Buckinghamshire, UK), respectively. Radioligand binding experiments were carried out in rat brain using tissues from the cortex for both 5-HT_{2A} and α_1-adrenergic receptor types, according to published procedures (Bojarski et al 1993; Mokrosz et al 1996).

Dopamine D_1 and D_2 receptor binding assays

Competition binding studies were performed in rat striatal membranes prepared according to Ossowska et al (2001). The radioligands used were [^{3}H]SCH 23390 (75.5 Ci mM^{-1}, NEN Chemicals, Boston) and [^{3}H]spiperone (15.7 Ci mM^{-1}, NEN Chemicals, Boston) for D_1 and D_2 receptors, respectively. Both assays were carried out in 96-well filter plates (containing glass fibre type C, Millipore) and all filters were pre-soaked with 100 μL ice-cold 50 mM potassium phosphate buffer (pH 7.4) and filtered using Millipore Vacuum Manifold before sample addition. For

D_2 binding assays 150-μL samples of striatal membrane preparations, 50 μL radioligand and either 50 μL buffer (for total binding assay) or 50 μL (±)-butaclamol (5 μM) to determine unspecific binding or 50 μL of the compounds to be tested were added to each well. Additionally, to prevent [^{3}H]spiperone binding to 5-HT_{2A} receptors ketanserin (50 nM) was included in the assay buffer. After incubation at 37 °C for 30 min, binding reaction was terminated by vacuum filtration and washed three times with 200 μL buffer. For D_1 binding assays, the same procedure was followed except that (±)-butaclamol was replaced by *cis*-(Z)-flupentixol (5 μM) and the plate was incubated at 30 °C for 60 min. Radioactivity was determined by liquid scintillation counting in a Beckman LS 6500 apparatus.

Benzodiazepine receptor binding assay

The competition experiments were conducted in the rat cortex. The membrane preparation and assay procedure were carried out according to Antkiewicz-Michaluk et al (1991). The radioligand [^{3}H]flunitrazepam (81 Ci mM^{-1}, Amersham, Buckinghamshire, UK) was used in a concentration of 2 nM L^{-1}. The incubation mixture (final volume 550 μL) consisted of 450 μL membrane suspension, 50 μL [^{3}H]flunitrazepam solution, and 50 μL solution of MP349. Samples were incubated during 45 min at 0 °C and then were filtered through GF/C Whatman fibreglass filters. The filters were placed in scintillation minivials and counted for radioactivity in a Beckman LS3801 scintillation counter.

In-vivo experiments

Body temperature in mice

The rectal body temperature in mice was measured with an Ellab thermometer. The thermocouple was inserted at a depth of 2.5 cm into a mouse rectum. The animals (housed individually throughout the experiment) were not restrained, and the thermocouple was inserted only at the time of the measurement. The body temperature was recorded 15, 30, 60 and 120 min after administration of MP349, 8-OH-DPAT, WAY 100635 or vehicle, given alone. In an independent experiment, the effect of MP349 or WAY 100635 on the hypothermia induced by 8-OH-DPAT was examined. MP349 and WAY 100635 were administered 45 and 30 min, respectively, before 8-OH-DPAT. The measurement of mice body temperatures was performed 15, 30, 45 and 60 min after administration of 8-OH-DPAT. The results were expressed as a change in the body temperature (Δt) with respect to the basal body temperature, as measured at the beginning of the experiment.

Microdialysis study

The rats were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and secured in a stereotaxic frame (David Kopf Instruments, Tujunga). Transverse microdialysis probes (outside diameter 0.2 mm, cut-off 50000 Da) prepared according to Imperato & DiChiara (1984) were implanted in the prefrontal cortex (parts of the cingulate, frontal and parietal cortices) with the following stereotaxic coordinates: A + 2.2 mm and H + 2.5 mm from the surface of the skull (Paxinos & Watson 1998).

On the following day, the microdialysis probes were perfused with artificial cerebrospinal fluid consisting of (in mM): NaCl 140, KCl 2.7, $CaCl_2$ 1.2, $MgCl_2$ 1, NaH_2PO_4 0.3, Na_2HPO_4 1.7, pH = 7.4 at a flow rate of 2 μL min^{-1} with a CMA/100 microinfusion pump (CMA/Microdialysis, Stockholm, Sweden). Samples were collected from freely moving animals at 15-min intervals, after a 3-h washout period. WAY 100635 or MP349 was given 15 min before 8-OH-DPAT. The collection of samples started 15 min after administration of 8-OH-DPAT and were taken at 15-min intervals for the following 120 min. Dialysates were injected into an HPLC system (BAS 480; Bioanalytical Systems Inc., W. Lafayette, IN) equipped with Inertsil ODS-3 (3 μm, 4.6 × 100 mm) column (Varian, Inc., US) and a glassy carbon electrode set at a potential of +650 mV vs Ag/AgCl reference electrode. The mobile phase consisted of 0.1 M monochloracetic acid, pH = 3.8, 25 mg L^{-1} 1-octanesulfonic acid sodium salt, 0.4 mM EDTA, 6% methanol and 3% acetonitrile. The column temperature was set at 26 °C and flow rate was 0.9 mL min^{-1}. Data were collected and analysed using a BAS Inject V-1.27 software run on a personal computer. The in-vitro probe recovery for 5-HT was 10–15%.

At the end of the experiments brains were examined histologically for correct probe placement. Only the data from rats in which the microdialysis probes were located correctly were included in the results for calculation. Reagents for HPLC were purchased from Merck (Darmstadt, Germany) and from Sigma-Aldrich (Poznań, Poland).

Conflict drinking test (Vogel test)

A modification of the method of Vogel et al (1971) described below was used. On the first day of the experiment the rats were adapted to the test chamber for 10 min. The test chamber was a plexiglas box (27 × 27 × 50 cm), equipped with a grid floor of stainless steel bars and a drinking bottle containing tap water. After the adaptation period, the animals were deprived of water for 24 h and were then placed in the test chamber for another 10-min adaptation period, during which time they had free access to the drinking bottle. Afterwards, they were allowed a 30-min free-drinking session in their home cage. After another 24-h water deprivation period, the rats were placed in the test chamber again and were allowed to drink for 30 s. Immediately afterwards, drinking attempts were punished with an electric shock (0.5 mA). The impulses were released every 2 s (timed from the moment when a preceding shock was delivered), between the grid floor and the spout of the drinking bottle. Each shock lasted 1 s, and if a rat was drinking when an impulse was released, it received a shock. The number of shocks accepted throughout a 5-min experimental session was recorded. MP349, WAY 100635 and diazepam (or vehicle) were administered 60, 30 and 60 min, respectively, before the test.

Shock threshold and free-drinking tests

To control the possibility of drug-induced changes in the perception of a stimulus or in the thirst drive, which might

have contributed to the activity in the conflict drinking test, stimulus threshold measurements and a free-drinking experiment were carried out in independent experiments. In both cases, the rats were treated in a similar manner as described in the conflict drinking test; that procedure included two 24-h water deprivation periods, separated by 30 min of water availability.

In the shock threshold test, the rats were placed individually in the box, and electric shocks were delivered through the grid floor. The shock threshold was determined stepwise by manually increasing the current (0.1, 0.2, 0.3, 0.4, 0.5 mA; the shocks lasted 1 s) delivered through the grid-floor until a rat showed an avoidance reaction (jump, jerk or similar) to the electrical stimulus. There was a 15-s shock-free interval between the steps.

In the free-drinking test, each animal was allowed to drink from the water spout. Licking was not punished. The total amount of water (mL) consumed in 5 min was recorded for each rat.

MP349 and WAY 100635 (or vehicle) were administered 60 and 30 min, respectively, before the tests.

Elevated plus-maze test

The construction and the testing procedure of an elevated plus-maze were based on a method described by Pellow & File (1986). Each rat was placed in the centre of the plus-maze, facing one of the enclosed arms immediately after a 5-min adaptation in a wooden box ($60 \times 60 \times 35$ cm). During a 5-min test period, two experimenters, who were sitting in the same room approximately 1 m from the end of the open arms, recorded the number of entries into the closed or the open arms, as well as the time spent in each type of arm. An entry was defined as all four feet put into one arm. At the end of each trial the maze was wiped clean. MP349, WAY 100635 or diazepam (or vehicle) were administered 60, 30 or 60 min, respectively, before the test.

Four-plate test

Single mice were placed gently into the plate, and each animal was allowed to explore for 15 s. Afterwards, each time a mouse passed from one plate to another, the experimenter electrified the whole floor for 0.5 s, which evoked a visible flight reaction of the animal. If the animal continued running, it received no new shocks for the following 3 s. The number of punished crossings was counted for 60 s (Aron et al 1971). MP349, WAY 100635 or diazepam (or vehicle) were administered 60, 30 or 60 min, respectively, before the test.

Open field test

The studies were carried out with rats according to a slightly modified method of Janssen et al. (1960). The centre of the open arena (1 m in diameter), divided into six symmetrical sectors without walls, was illuminated with a 75 W electric bulb hung directly above (75 cm) it. During all the experiments the laboratory room was dark. Individual control or drug-injected animals were placed gently in the centre of the arena and were allowed to explore freely. The time of walking, ambulation (the number of crossing of sector lines) and the number of rearing and peeping episodes (looking under the edge of the arena) were recorded for 3 min. MP349 or vehicle were administered 60 min before the test.

Rota-rod test

Rats were preselected one day before the test on the rotating rod (6 cm in diameter, 6 rev min^{-1}). Those staying on the rotating rod for 2 min were placed again on the same rotating rod the next day after drug administration and were observed for 2 min. The number of animals falling from the rota-rod within 2 min was recorded. MP349 or vehicle were administered 60 min before the test.

Data analysis

The data obtained were presented as means ± s.e.m. and evaluated using one-way analysis of variance, followed by Dunnett's test or followed by pairwise Student's *t*-test with Bonferroni correction for multiple comparisons (microdialysis study). Differences between groups were considered as significant if $P < 0.05$.

Results

Radioligand binding studies

The radioligand binding data (Table 1) indicated that MP349 showed moderate affinity for α_1-adrenergic receptors ($K_i = 234$ nM). MP349 did not exhibit an affinity for 5-HT_{2A}, D_1, D_2 or benzodiazepine receptors.

In-vivo experiments

Body temperature

MP349 (0.125–0.5 mg kg^{-1}) or WAY 100635 (0.1 mg kg^{-1}), used as a reference 5-HT_{1A} antagonist, given alone did not change body temperature in mice during a 2-h measurement, whereas 8-OH-DPAT (2.5–5 mg kg^{-1}) induced a hypothermic effect (Table 2). The decrease in body temperature induced by 8-OH-DPAT was abolished by MP349 administered at the highest dose used (0.5 mg kg^{-1}); lower doses of MP349 attenuated the effect of 8-OH-DPAT, but their action was statistically non-significant. WAY 100635 (0.1 mg kg^{-1}) effectively blocked the hypothermic effect of 8-OH-DPAT (Table 3).

Table 1 Affinity of MP349 for the selected receptors.

Binding site	K_i (nM)
5-HT_{1A}	15.2 ± 3.2[a]
5-HT_{2A}	11575 ± 20
α_1	234 ± 15
D_1	3216 ± 36
D_2	2606 ± 160
Benzodiazepine	>10000

[a]Data by Paluchowska et al (2002).

Table 2 The effects of MP349, WAY 100635 and 8-OH-DPAT on body temperature in mice.

Compound	Dose (mg kg^{-1})	Δt(°C)			
		15 min	30 min	60 min	120 min
Vehicle	–	−0.3 ± 0.1	−0.3 ± 0.1	−0.2 ± 0.1	−0.3 ± 0.1
MP349	0.125	−0.2 ± 0.1	−0.1 ± 0.1	−0.1 ± 0.1	0.0 ± 0.1
	0.25	0.0 ± 0.2	−0.1 ± 0.1	0.1 ± 0.2	0.0 ± 0.2
	0.5	−0.1 ± 0.1	−0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.1
WAY 100635	0.1	0.1 ± 0.1	−0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Vehicle	–	−0.2 ± 0.1	−0.1 ± 0.1	−0.1 ± 0.1	−0.1 ± 0.1
8-OH-DPAT	2.5	−0.8 ± 0.2*	−0.6 ± 0.2*	−0.4 ± 0.1	−0.1 ± 0.1
	5	−1.4 ± 0.2**	−1.2 ± 0.1**	−0.8 ± 0.1*	−0.2 ± 0.1

MP349 (i.p.), WAY 100635 (s.c.) and 8-OH-DPAT (s.c.) were administered 30 min before the test. Absolute initial mean body temperatures were within the range 36.7 ± 0.4 °C. n = 8. *P < 0.05, **P < 0.01 compared with the respective vehicle group.

Table 3 The effects of MP349 and WAY 100635 on the hypothermia induced by 8-OH-DPAT (5 mg kg^{-1}) in mice.

Compound and dose (mg kg^{-1})	Δt(°C)			
	15 min	30 min	45 min	60 min
Vehicle + vehicle	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Vehicle + 8-OH-DPAT	−1.3 ± 0.2**	−1.1 ± 0.1**	−0.8 ± 0.1**	−0.5 ± 0.1*
MP349 0.125 + 8-OH-DPAT	−0.8 ± 0.2**	−0.8 ± 0.2*	−0.6 ± 0.1	−0.4 ± 0.1
MP349 0.25 + 8-OH-DPAT	−0.8 ± 0.2**	−0.6 ± 0.2*	−0.6 ± 0.2	−0.1 ± 0.1
MP349 0.5 + 8-OH-DPAT	−0.4 ± 0.2^{+}	−0.1 ± 0.1^{+}	0.2 ± 0.1^{+}	0.2 ± 0.1^{+}
Vehicle + vehicle	−0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Vehicle + 8-OH-DPAT	−1.2 ± 0.1**	−1.0 ± 0.1**	−0.7 ± 0.1**	−0.2 ± 0.1
WAY 100635 0.1 +8-OH-DPAT	−0.1 ± 0.1^{+}	−0.1 ± 0.1^{+}	0.1 ± 0.1^{+}	0.2 ± 0.1

MP349 (i.p.) and WAY 100635 (s.c.) were administered 45 and 30 min, respectively, before 8-OH-DPAT. Body temperature was recorded 15, 30, 45 and 60 min after administration of 8-OH-DPAT (s.c.). Absolute initial mean body temperatures were within the range 36.7 ± 0.4 °C. n = 8. *P < 0.05, **P < 0.01 compared with the respective vehicle group. ^{+}P < 0.01 compared with the respective vehicle + 8-OH-DPAT group.

Microdialysis study

Acute administration of 8-OH-DPAT (0.1 mg kg^{-1}) significantly decreased extracellular concentration of 5-HT in rat prefrontal cortex (Figure 1). The selective 5-HT$_{1A}$-receptor antagonist WAY 100635 (0.1–0.3 mg kg^{-1}) produced no effect by itself, but at a dose of 0.3 mg kg^{-1} significantly attenuated the effect of 8-OH-DPAT on 5-HT release (Figure 1). WAY 100635 at a lower dose (0.1 mg kg^{-1}) did not significantly change the effect of 8-OH-DPAT on 5-HT release (data not shown). MP 349 (0.25 and 0.5 mg kg^{-1}) did not affect 5-HT dialysate level in the prefrontal cortex, but it dose-dependently reversed the decrease in 5-HT release induced by 8-OH-DPAT (0.1 mg kg^{-1}) (Figure 2).

Conflict drinking test (Vogel test)

MP349 (0.25–0.5 mg kg^{-1}) and WAY 100635 (0.5–1 mg kg^{-1}) produced an anti-anxiety-like effect, significantly increasing the number of shocks accepted in the conflict drinking test (Table 4); however, their dose–response relationship was bell-shaped and the maximum effects (an increase by 223% and 414%, respectively) were observed after administration of 0.25 mg kg^{-1} MP349 and of 1 mg kg^{-1} WAY 100635. Diazepam (2.5–10 mg kg^{-1}), used as a reference drug, produced a dose-dependent anticonflict effect, significantly increasing the number of punished responses; the maximum effect (an increase by 594%) was observed after administration of 10 mg kg^{-1} of the drug (Table 4).

The possibility that the efficacy of MP349 (0.25–0.5 mg kg^{-1}) and WAY 100635 (0.5–1 mg kg^{-1}) was related to reduced perception of the stimulus or to an increased thirst drive may be excluded, since MP349 and WAY 100635 administered in doses effective in the conflict drinking test increased neither the threshold current nor water intake compared with vehicle administration (data not shown).

Elevated plus-maze test

Following pretreatment with the vehicle, 27–39% of the total entries (open + closed entries) made by rats were into the open arms, and 11–16% of the total time spent in the

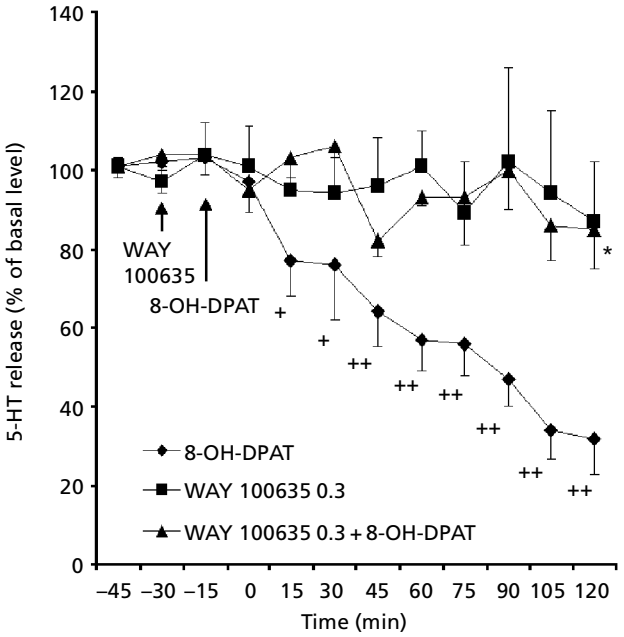

Figure 1 Reversal of the effect of 8-OH-DPAT (0.1 mg kg^{-1}) on 5-HT release by the 5-HT_{1A} antagonist WAY 100635 (0.3 mg kg^{-1}) in rat prefrontal cortex. WAY 100635 (s.c.) was given 15 min before 8-OH-DPAT (s.c.), as indicated with arrows. Basal extracellular concentrations of 5-HT in pg/20 μL of dialysate were: 3.7 ± 0.18, 4.4 ± 0.19 and 3.4 ± 0.34 for 8-OH-DPAT, WAY 100635 and WAY 100635 +8-OH-DPAT, respectively. The data expressed as a percentage of basal level are the mean ± s.e.m., n = 4. *$P < 0.01$ denotes a significant difference between the groups treated with 8-OH-DPAT alone and WAY100635+8-OH-DPAT; $^{+}P < 0.05$, $^{++}P < 0.01$ compared with the basal level.

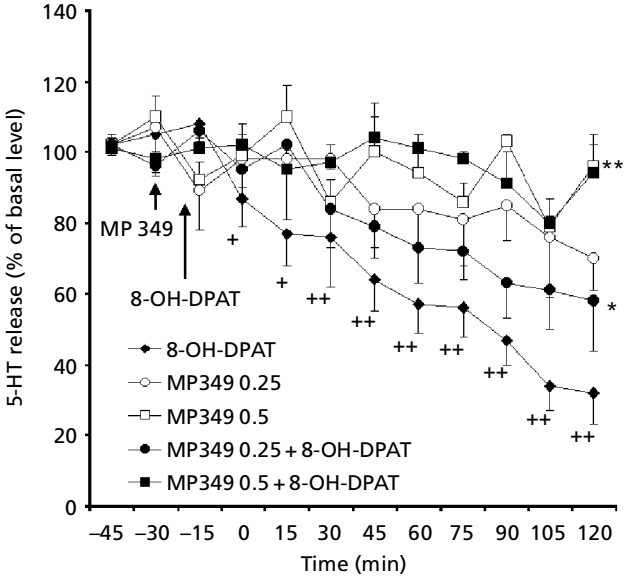

Figure 2 The effect of MP349 on the decrease in extracellular 5-HT level induced by the 5-HT_{1A}-receptor agonist 8-OH-DPAT (0.1 mg kg^{-1}) in rat prefrontal cortex. MP349 alone, or used 15 min before 8-OH-DPAT (indicated with arrows), was given in intraperitoneal doses of 0.25 and 0.5 mg kg^{-1}. Basal extracellular 5-HT concentrations in pg/20 μL of dialysate were: 3.8 ± 0.18, 3.1 ± 0.31, 3.1 ± 0.40, 4.7 ± 0.18 and 3.7 ± 0.19 for 8-OH-DPAT, MP349 0.25, MP349 0.25 + 8-OH-DPAT, MP349 0.5 and MP349 0.5 +8-OH-DPAT, respectively. The data expressed as a percentage of the basal level are the mean ± s.e.m., n = 4. *$P < 0.05$, ** $P < 0.01$ compared with groups treated with 8-OH-DPAT alone and 8-OH-DPAT + MP349; $^{+}P < 0.05$, $^{++}P < 0.01$ compared with the basal level.

arms (of either type) were spent in the open arms (Table 5). MP349 0.125 mg kg^{-1} significantly increased these percentages up to 63 and 58%, respectively. In the case of lower (0.06 mg kg^{-1}) or higher doses (0.25–0.5 mg kg^{-1}) of MP349, the observed increases in the time spent in the open arms and in the open arm entries did not reach the level of statistical significance. MP349 given at any dose tested did not change the total number of entries or the total time spent in the arms (of either type). WAY 100635 0.025 mg kg^{-1} did not change the number of entries into or the time spent in the open arms. When given at doses of 0.05 or 0.1 mg kg^{-1}, it significantly increased the percentage of the time spent in the open arms (up to 36 and 59%, respectively), and the percentage of entries into the open arms (up to 44 and 56%, respectively). WAY 100635 0.2 mg kg^{-1} increased the percentage of entries (up to 49%), but it did not affect the percentage of time spent in the open arms. The total number of entries and the total time spent in the arms were not changed by WAY 100635 (0.025–0.2 mg kg^{-1}). Diazepam (i.e. a positive standard) administered at a dose of 1.25 mg kg^{-1} was ineffective in that test; however, when given at doses of 2.5 and 5 mg kg^{-1}, it significantly increased the percentage of time spent in the open arms (up to 47 and 70%, respectively) and the number of entries into the open arms (up to 74 and 76%, respectively) (Table 5). Diazepam 5 mg kg^{-1} (but not lower) reduced (by 50%) the total number of entries (data not shown).

Four-plate test

MP349 0.125 mg kg^{-1} (but not 0.06, 0.25 or 0.5 mg kg^{-1}) significantly increased (by 68%) the number of punished crossings in a four-plate test in mice. WAY 100635 0.01 mg kg^{-1} increased the number of punished crossings by 59%; when used at the other doses tested (0.005, 0.02 or 0.1 mg kg^{-1}), its effects were statistically nonsignificant. Diazepam (i.e. a positive standard) at doses of 1 and 2 mg kg^{-1} increased the number of crossings by 59 and 100%, respectively (Table 6).

Open field test and rota-rod test

MP349 at doses of up to 2 mg kg^{-1} did not change the general locomotor activity of rats; instead, when given at a dose of 10 mg kg^{-1}, it markedly reduced the time of walking, ambulation and peeping + rearing (by 75%, 82% and 91%, respectively; $P < 0.01$) in rats (data not shown).

In the rota-rod test, MP349 at doses of 0.5 and 2 mg kg^{-1} did not disturb the motor coordination of rats on the rotating rod, but when given at a dose of 10 mg kg^{-1}, it made the rats (3/7) fall from the rotating rod (data not shown).

Table 4 The effects of MP349, WAY 100635 and diazepam in the conflict drinking test in rats.

Compound	Dose ($mg\,kg^{-1}$)	Number of shocks accepted
Vehicle	–	10.7 ± 1.5
MP349	0.125	17.3 ± 2.9
	0.25	34.6 ± 7.5**
	0.5	28.0 ± 3.7*
		$F(3,26) = 4.404$
		$P < 0.050$
Vehicle	–	7.3 ± 1.1
WAY 100635	0.1	15.9 ± 4.8
	0.5	26.1 ± 6.7*
	1	37.5 ± 5.7**
	2	12.7 ± 3.7
		$F(4,32) = 5.784$
		$P < 0.010$
Vehicle	–	7.7 ± 1.1
Diazepam	2.5	20.3 ± 3.0
	5	27.4 ± 5.1*
	10	53.5 ± 7.8**
		$F(3,25) = 21.638$
		$P < 0.001$

MP349 (i.p.), diazepam (i.p.) and WAY 100635 (s.c.) were administered 60 and 30 min, respectively, before the test. n = 6–8, $*P < 0.05$, $**P < 0.01$ compared with the respective vehicle group.

Discussion

The results of this study indicated that MP349 was a 5-HT_{1A}-receptor antagonist with potential anxiolytic-like activity.

Earlier in-vitro radioligand binding studies showed MP349 to be a potent ligand at 5-HT_{1A} receptors (K_i = 15 nM) (Paluchowska et al 2002). In this study we have demonstrated that this compound was at least 150-fold selective for 5-HT_{1A} sites relative to 5-HT_{2A}, dopamine D_1 or D_2 and benzodiazepine receptors, and was 15-fold selective relative to α_1-adrenoceptors. MP349 was less potent and less selective in respect of the binding to 5-HT_{1A} receptors than was WAY 100635; moreover, the latter was approximately 200-times more active at 5-HT_{1A} receptors than at α_1-adrenoceptors (Fletcher et al 1996; Johansson et al 1997).

In-vivo pharmacological studies with MP349 were carried out to explore its functional effects on presynaptic 5-HT_{1A} receptors, and to evaluate its preclinical efficacy as a potential anxiolytic. The hypothermic response induced by the 5-HT_{1A} agonist 8-OH-DPAT in mice was proposed as a model of presynaptic 5-HT_{1A} receptor activation (Goodwin et al 1985; Martin & Heal 1991). The decrease in mouse body temperature induced by 8-OH-DPAT was abolished by such antagonists of 5-HT_{1A} receptors as e.g. WAY 100635 (Fletcher et al 1996), WAY 100135 (Fletcher et al 1993; Przegaliński et al 1994) or MP 3022 (Mokrosz et al 1994b). In this study, MP349—like WAY 100635—abolished the hypothermic effect induced by 8-OH-DPAT in mice. At the same time, MP349—like WAY 100635—failed to induce changes in mouse body temperature. These results suggested that in this test MP349 exhibited characteristics of a 5-HT_{1A} presynaptic antagonist. A precise assessment of 5-HT_{1A} somatodendritic functional activity requires measurement of the activity of 5-HT neurons under influence of the tested compounds. In our experiment, rat prefrontal cortex was chosen to determine 5-HT neuronal activity, since it is the brain area that receives projections from the dorsal and median raphe nuclei (Azmitia & Segal 1978). The activation of presynaptic 5-HT_{1A} receptors by selective agonists has been reported to cause reduction of 5-HT synthesis and release from corresponding axon terminals (Invernizzi et al 1991; Casanovas & Artigas 1996). Systemic administration of MP349 or WAY 100635 did not change the extracellular 5-HT level, but dose-dependently inhibited the decrease in 5-HT release in the prefrontal cortex, induced by 8-OH-DPAT. In addition these results indicated that MP349 was an antagonist at 5-HT_{1A} somatodendric sites. Postsynaptic 5-HT_{1A}-receptor antagonistic properties of MP349 were described previously (Paluchowska et al 2002). We found that—like WAY 10635—MP349 administered in low doses (0.125–0.5 $mg\,kg^{-1}$) dose-dependently inhibited the 8-OH-DPAT-induced behavioural syndrome and lower lip retraction in rats, both those effects being connected with stimulation of postsynaptic 5-HT_{1A} receptors (Tricklebank et al 1984; Berendsen et al 1989, 1991). Neither MP349 nor WAY 100635 showed any intrinsic activity in those tests i.e. neither of them produced an agonist effect. The above-quoted results of functional in-vivo tests showed MP349 to be a novel 5-HT_{1A} receptor antagonist.

Preclinical evaluation of anxiolytic-like activity of MP349 was carried out using three behavioural tests: a rat conflict drinking test (Vogel et al 1971), a rat elevated plus-maze test (Pellow & File 1986) and a mouse four-plate test (Aron et al 1971). The action of MP349 in those tests was compared with the activity of WAY 100635, whose anxiolytic-like properties in rats were described by Griebel et al (2000); in those studies diazepam was used as a standard drug. As expected, diazepam was active in all the three models used, whereas the effects of MP349 and WAY 100635 varied depending on the test employed. In the conflict drinking test, MP349 produced anxiolytic-like activity, which seemed to be specific, since it was not related to the reduced perception of the stimulus or to the increased thirst drive. MP349 showed anticonflict activity at the same doses in which it exhibited 5-HT_{1A}-receptor antagonistic activity, whereas WAY 100635 was active at doses 5–10-times higher than those which produced full 5-HT_{1A} antagonistic effects. Our data on WAY 100635 were in line with the results obtained by Griebel et al (2000) who observed anticonflict activity of WAY 100635 at doses similar to those tested in our experiment. On the other hand, our data contrasted with Kennett et al (1998) in the Vogel conflict test. In their study, WAY 100635 was found to be inactive at doses of 0.1 and 0.3 $mg\,kg^{-1}$, whereas we observed anxiolytic-like effects at doses somewhat higher (0.5–1 $mg\,kg^{-1}$). The failure of

Table 5 The effects of MP349, WAY 100635 and diazepam in the plus-maze test in rats.

Compound	Dose (mg kg^{-1})	% of time in open arms	% of open arm entries
Vehicle	–	15.7 ± 4.9	33.2 ± 1.8
MP349	0.06	18.9 ± 4.3	49.0 ± 5.8
	0.125	63.2 ± 13.1**	58.4 ± 6.7**
	0.25	41.2 ± 11.3	51.1 ± 7.9
	0.5	20.0 ± 3.8	40.3 ± 3.4
		F(4,29) = 6.850	F(4,29) = 2.977
		$P < 0.001$	$P < 0.050$
Vehicle	–	11.2 ± 2.5	27.5 ± 4.6
WAY 100635	0.025	20.5 ± 4.9	34.8 ± 7.7
	0.05	36.3 ± 6.9*	44.3 ± 2.3*
	0.1	59.1 ± 10.6**	56.3 ± 6.9**
	0.2	26.6 ± 7.7	48.5 ± 3.8*
		F(4,34) = 7.266	F(4,34) = 3.810
		$P < 0.001$	$P < 0.050$
Vehicle	–	10.9 ± 1.1	38.5 ± 3.1
Diazepam	1.25	20.3 ± 5.5	45.2 ± 8.9
	2.5	47.2 ± 5.3*	73.8 ± 4.2*
	5	70.4 ± 10.9**	76.2 ± 8.8**
		F(3,22) = 14.524	F(3,22) = 5.871
		$P < 0.001$	$P < 0.010$

MP349 (i.p.), diazepam (i.p.) and WAY 100635 (s.c.) were administered 60 and 30 min, respectively, before the test. n = 6–8, *$P < 0.05$, **$P < 0.01$ compared with the respective vehicle group.

WAY 100635 at the high dose of 2 mg kg^{-1} to modify punished responses in the Vogel test is difficult to explain. Like our results, the findings of Cao & Rodgers (1997b) showed that WAY 100635 produced an anxiolytic-like effect in mice, having developed an apparent bell-shaped dose–response relationship. The latter authors suggested that the behaviourally nonselective high dose effect of WAY 100635 could be mediated by an action at α_1-adrenoceptors, since a metabolite of WAY 100635 showed high affinity for those binding sites in-vitro (Pike et al 1996). In the elevated plus-maze test in rats, MP349—like WAY 100635 (Griebel et al 2000)—exerted anxiolytic-like activity, since it increased the percentage of time spent and the number of entries into the open arms of the plus-maze; nevertheless, the obtained dose–response relationships were bell-shaped. It is noteworthy that MP349 and WAY 100635 exhibited anxiolytic-like effects in that test at doses lower than those which were active in the rat conflict drinking test. Importantly, the above effects of both those 5-HT$_{1A}$ ligands occurred at doses which did not change the total number of arm entries, which suggests that the anxiolytic-like activity did not result from motor impairment. Moreover, MP349 used in doses up to 2 mg kg^{-1} did not change the locomotor activity of rats in the open field test. Hence the lack of the anxiolytic-like activity of MP349 administered at higher doses (0.25–0.5 mg kg^{-1}) in the plus-maze test cannot be explained by competitive behaviour such as e.g. a decrease in locomotor activity. Interestingly, a decrease in locomotor activity (open field test) and disturbance in the motor coordination of rats (rota-rod test) were observed after administration of MP349 at the high dose of 10 mg kg^{-1}. We did not investigate the effect of WAY 100635 on the spontaneous locomotor activity of rats, but McCreary et al (1999) reported that WAY 100635 in doses up to 2 mg kg^{-1} did not modify the exploratory activity of rats. On the other

Table 6 The effects of MP349, WAY 100635 and diazepam in the four-plate test in mice.

Compound	Dose (mg kg^{-1})	Number of punished crossings
Vehicle	–	3.4 ± 0.2
MP349	0.06	4.4 ± 0.5
	0.125	5.7 ± 0.6**
	0.25	4.4 ± 0.6
	0.5	4.7 ± 0.6
		F(4,44) = 2.157
		ns
Vehicle	–	3.7 ± 0.3
WAY 100635	0.005	4.2 ± 0.4
	0.01	5.9 ± 0.5**
	0.02	4.3 ± 0.5
	0.1	5.2 ± 0.6
		F(4,44) = 3.502
		$P < 0.050$
Vehicle	–	3.4 ± 0.2
Diazepam	1	5.4 ± 0.5*
	2	6.8 ± 0.4**
		F(2,26) = 14.646
		$P < 0.001$

MP349 (i.p.), diazepam (i.p.) and WAY 100635 (s.c.) were administered 60 and 30 min, respectively, before the test. n = 9–10, *$P < 0.05$, **$P < 0.01$ compared with the respective group.

hand, Jackson et al (1998) observed enhancement of some parameters of rat locomotion after administration of 1.82 μmol kg^{-1} WAY 100635 (i.e. 1 mg kg^{-1}). In the four-plate test, MP349 and WAY 100635 were active in one medium dose only. The loss of anxiolytic-like activity in mice after administration of higher doses of these compounds seems to be a common feature of 5-HT$_{1A}$-receptor antagonists (Cao & Rodgers 1997a, b; this study). It is noteworthy that a number of other compounds with 5-HT$_{1A}$ receptor antagonist properties reduced the anxiety-related behaviour in the punished drinking test (SL 88.0338, *p*-MPPI and MM77 (Griebel et al 2000)), in the rodent elevated plus-maze (*S*-UH-301, *S*-WAY 100135, *p*-MPPI, and SDZ 216-525 (Moreau et al 1992; Rodgers & Cole 1994; Cao & Rodgers 1997a, b)), in the mouse light/dark exploration (*S*-UH-301 and *S*-WAY 100135 (Moreau et al 1992; Bill & Fletcher 1994)) and in the mouse anti-predator defense test (S 21187 and S 21357 (Griebel et al 1996a, b)). However, negative results have been reported also for some of those compounds (including WAY 100635) in conflict procedures (Moreau et al 1992; Charrier et al 1994; Cervo & Samanin 1995; Przegaliński et al 1995; Remy et al 1996; Dekeyne et al 2000), the rat social interaction (Cadogan et al 1994) and the elevated plus-maze test (Millan et al 1994; Bickerdike et al 1995; Collinson & Dawson 1997; Griebel et al 2000). Moreover, WAY 100635 was capable of inducing anxiogenic-like effects in the rat shock-induced ultrasonic vocalization test (Groenink et al 1995). The reasons for the above discrepancies still need to be elucidated.

Our results of preclinical studies with MP349 showed that this compound—like WAY 100635—exhibited an activity characteristic of anxiolytic drugs in the conflict drinking and plus-maze tests in rats and in the four-plate test in mice. Although the anxiolytic-like effect of MP349 (and WAY 100635) was weaker than that of diazepam, it appeared at doses which did not change the exploratory activity of rats; on the other hand, the strongest anxiolytic-like effect of diazepam was observed after administration of doses reducing the exploratory activity (5 and 10 mg kg^{-1}) (Chojnacka-Wójcik et al 1999). On the basis of the results from receptor binding studies and functional experiments it is supposed that the blockade of 5-HT$_{1A}$ receptors induced by MP 349 may be responsible for its anxiolytic-like activity. The affinity of MP349 for 5-HT$_{2A}$, benzodiazepine, D_1 and D_2 receptors is negligible, or moderate for α_1-adrenoceptors; therefore it seems that these receptors are not directly involved in its anti-anxiety effect. On the other hand this in-vitro receptor characterization of MP349 is sketchy and does not exclude involvement of other receptors in the anxiolytic-like effect of MP349. It is possible that the anxiolytic-like effects of 5-HT$_{1A}$-receptor antagonists are not exclusively of a serotonergic nature. For example, an interaction between GABA-benzodiazepine and serotonin systems in the mediation of the anxiolytic-like action of selective 5-HT$_{1A}$ agonists or partial agonists has been reported by Lopez-Rubalcava et al (1992) and Fernandez-Guasti & Lopez-Rubalcava (1998). Therefore it is very difficult to draw any definite conclusions about the mechanism of action of MP349 in an animal model of anxiety. The mechanism underlying the positive effects of silent 5-HT$_{1A}$ antagonist WAY 100635 in animal models of anxiety is unclear; the results obtained by Nunes-de-Souza et al (2002) and Canto-de-Souza et al (2002) showed that the anxiolytic-like effects of WAY 100635 in mice were connected with the blockade of 5-HT$_{1A}$ receptors localized in the median raphe nucleus and ventral hippocampus, which suggested an involvement of pre- and postsynaptic 5-HT$_{1A}$ receptors, respectively, in the effect of this 5-HT$_{1A}$ receptor antagonist. Further studies are necessary to determine the precise mechanism of the anxiolytic-like activity of 5-HT$_{1A}$ receptor antagonists.

Recent studies using 5-HT$_{1A}$ receptor knockout mice have shown that these animals display anxious-like behaviour (Heisler et al 1998; Parks et al 1998; Ramboz et al 1998). Those results differ from those obtained in this and other studies (Griebel et al 2000) in which blockade of 5-HT$_{1A}$ receptors produced an opposite action. However, acute blockade of 5-HT$_{1A}$ receptors cannot be compared with the lack of 5-HT$_{1A}$ receptors in mutant mice, as it induces developmental compensation effects.

Conclusion

These data suggested that the new 5-HT$_{1A}$ pre- and postsynaptic receptor antagonist MP349 displayed an anxiolytic-like profile in animal anxiety models, at doses significantly lower than those producing visible unfavourable motor effects. Whether agents of this class conclusively prove to be of use in the therapy of clinical anxiety remains an open question.

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