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In vitro characterization of AR-A000002, a novel 5-hydroxytryptamine$_{1B}$ autoreceptor antagonist

Charlotte Ahlgren[a], Anders Eriksson[b], Pernilla Tellefors[c], Svante B. Ross[d], Carina Stenfors[d], Åsa Malmberg[a,*]

[a]*Department of Molecular Pharmacology, Local Discovery Research Area CNS & Pain Control, Astrazeneca R&D Södertälje S-151 85, Sweden*
[b]*Department of Molecular Sciences, Local Discovery Research Area CNS & Pain Control, Astrazeneca R&D Södertälje, Sweden*
[c]*Department of General Pharmacology, Local Discovery Research Area CNS & Pain Control, Astrazeneca R&D Södertälje, Sweden*
[d]*Department of Bioscience, Local Discovery Research Area CNS & Pain Control, Astrazeneca R&D Södertälje, Sweden*

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Abstract

The in vitro pharmacological properties of AR-A000002 ((*R*)-*N*-[5-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-2-naphthyl]-4-morpholinobenzamide), a novel 5-hydroxytryptamine (5-HT)$_{1B}$ receptor antagonist, were studied. AR-A000002 bound with high affinity to guinea pig cortex and recombinant guinea pig 5-HT$_{1B}$ receptors (K_i=0.24 and 0.47 nM) and with 10-fold lower affinity to 5-HT$_{1D}$ receptors. The compound displayed weak or no affinity for 63 other binding sites tested. In [^{35}S]GTPγS assays AR-A000002 showed 50% efficacy and inhibited 5-HT stimulation with 66% and a pA_2 value of 8.9. In slices of guinea pig cortex, AR-A000002 enhanced the outflow of [^{3}H]5-HT upon electrical stimulation. The compound blocked sumatriptan-evoked contraction of rabbit saphenous veins without inducing any contraction itself. Thus, in these two systems AR-A000002 behaved as a 5-HT$_{1B}$ receptor antagonist. It is concluded that AR-A000002 is a selective high affinity 5HT$_{1B}$ receptor ligand that shows partial agonist activity in recombinant systems. In native tissues AR-A000002 behaves as a 5-HT$_{1B}$ receptor antagonist.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); 5-HT$_{1B}$ receptor; 5-HT$_{1B}$ antagonist; GR125743

1. Introduction

5-Hydroxytryptamine (5-HT, serotonin) is one of the major neurotransmitters and its receptors are widely distributed in the brain. Dysfunction in the central serotonergic system has been implicated in the pathogenesis of many disease states such as depression, anxiety, panic and eating disorders. Thus, modulation of the serotonergic tone is believed to be an effective treatment of these disorders. The 5-HT receptor family is divided into seven distinct classes, 5-HT$_1$ to 5-HT$_7$ (Hoyer et al., 1994). All classes belong to the superfamily of G-protein coupled receptors, with the exception of 5-HT$_3$ that is a ligand-gated ion channel. Today 14 5-HT receptor subtypes have been described. The 5-HT$_{1A}$ and 5-HT$_{1B}$ subtypes have drawn particular interest because they are involved in the feedback regulation of the serotonergic neurotransmission. The observation that the terminal 5-HT autoreceptor is of the 5-HT$_{1B}$ receptor type was first made in rats (Engel et al., 1986; Maura et al., 1986). Because of small differences in the amino acid sequence, particularly between the 5-HT$_{1B}$ receptors in rodents versus most other species (Oksenberg et al., 1992), the pharmacological properties of the 5-HT$_{1B}$ receptors, particularly for antagonists, show species dependence (Hoyer and Middlemiss, 1989). The various types of species homologues are therefore classified with a species prefix (Hartig et al., 1996), e.g. h5-HT$_{1B}$, gp5-HT$_{1B}$ and r5-HT$_{1B}$ for the human, guinea pig and rat receptors, respectively. In contrast to the r5-HT$_{1B}$ receptor, the gp5-

* Corresponding author. Tel.: +46 8 55328637; fax: +46 8 55328890.
E-mail address: asa.malmberg@astrazeneca.com (A. Malmberg).

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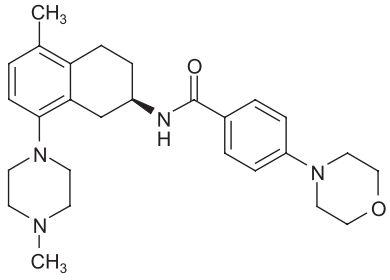

Fig. 1. Chemical structure of AR-A000002.

HT_{1B} receptor has a similar pharmacology to the h5-HT_{1B} receptor (Zgombick et al., 1997). The 5-HT_{1B} and 5-HT_{1D} receptor subtypes are pharmacologically very similar despite the relatively low amino acid homology (77% in the transmembrane domains) (Hoyer et al., 1994). Thus, it has been difficult to differentiate between these receptors in native systems (Göthert and Schlicker, 1997). The 5-HT_{1B} receptor is more highly expressed in brain tissue than the 5-HT_{1D} receptor (Hoyer et al., 1994; Bruinvels et al., 1994) and, in contrast to the 5-HT_{1D} receptor, it seems to be localized on the nerve terminals (Piñeyro et al., 1995). The first 5-$HT_{1B/1D}$ selective antagonist described was GR127935 (Skingle et al., 1993, 1996). It behaves as a partial agonist in over-expressing recombinant systems and as a silent antagonist in native tissue. This has been true for some other 5-$HT_{1B/1D}$ selective ligands identified since then (Price et al., 1997; Watson et al., 2001). SB-224289 has, however, been characterized as a 5-HT_{1B} receptor selective ligand with inverse agonist activity (Selkirk et al., 1998).

In the present study, we report the in vitro pharmacological properties of a new selective 5-HT_{1B} receptor antagonist, AR-A000002 ((*R*)-*N*-[5-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-2-naphthyl]-4-morpholinobenzamide) (Fig. 1). (Malmberg et al., 2001). It has previously been reported that this compound increase serotonergic neurotransmission in vivo in guinea pigs (Stenfors et al., 2001, 2004) and also exert behavioral effects in animal models of depression and anxiety (Hudzik et al., 2003).

2. Materials and methods

2.1. Compounds

AR-A000002 ((*R*)-*N*-[5-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-2-naphtyl]-4-morpholinobenzamide), GR127935 (2′-methyl-4′ (5-methyl-[1,2,4] oxadiazol-3-yl)-biphenyl-4-carboxylic acid [4-methoxy-3 (4 methyl-piperazin-1-yl) phenyl]-amide) and CP-135,807 ((*R*)-3-[1-methylpyrrolidin-2-ylmethyl]-5-(3-nitropyridin-2-ylamino)-1H-indole) were provided by AstraZeneca R&D Södertälje, Sweden. SB224289 (6,7-dihydro-1′-methyl-5-[[2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)[1.1′-biphenyl]-4-yl]carbonyl]-spiro-[2H-furo[2,3-*f*]indole-3(5H)]hydrochloride) and 5-hydroxytryptamine (5-HT) were purchased from Sigma, St. Louis, MO, USA. Sumatriptan was obtained from Glaxo Group Research (Ware, UK) and methiothepin and ketanserin from Research Biochemicals, Natick, MA, USA. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, penicillin/streptomycin, trypsin/EDTA and HEPES were obtained from Gibco, Paisley, Scotland, UK. Other chemicals were obtained from commercial sources and were of analytical grade.

The following radioligands were used (Ci/mmol in parenthesis): [*N*-methyl-^{3}H]GR125743 (*N*-[4-methoxy-3-(4-methylpiperazin-1-yl)]-3-methyl-4-pyridin-4-yl-benzamide) (76) and [^{35}S]GTPγS (973–991) from Amersham, Buckinghamshire, UK. [^{35}S]GTPγS (1247–1297) was also obtained from NEN, Boston, MA, USA.

2.2. Animals

Male Dunkin–Harley guinea pigs (Harlan, Germany and Charles River, Uppsala, Sweden), weighing 350–600 g, were used. The animals were housed 4 per cage and were kept under constant temperature (22 °C) and lighting (6 a.m.–6 p.m.) and were allowed free access of food (rabbit and guinea pig food K1, Lactamin, Sweden) and water.

Male New Zealand White rabbits (Charles River Sverige, Sweden and HB Lidköpings Kaninfarm, Sweden) weighing 3–5 kg were used. The animals were individually housed in polypropylene rabbit cages and were allowed free access to food (K5, Lactamin) and tap water.

The studies were approved by the local Animal Ethical Committee.

2.3. Molecular cloning of guinea pig 5-HT_{1B} and 5-HT_{1D} receptors

Molecular cloning and characterization of the coding regions of guinea pig (gp) 5-HT_{1B} and 5-HT_{1D} receptors have been described previously (Wurch et al., 1997; Zgombick et al., 1997). The sequences have been deposited in the EMBL/GeneBank databases with accession numbers U82175, U82174 and X94436. Fragments of approximately 1200 bp, were amplified by performing polymerase chain reaction (PCR) using a guinea pig liver genomic library as template and the following specific primers: 5-HT_{1B} sense 5′ GCGGGGATCCACCATGGGGAACCCTGAGGCTTCG containing a *Bam*H1 site, 5-HT_{1B} antisense 5′ GCGGGATATCTCAGGTTGTGCACTTAAAGCGTAT containing a *Eco*RV site, 5-HT_{1D} sense 5′ GCGGGAATTCACCATGTCCCCGCCAAACCAGTCAGAGGAA containing a *Eco*R1 site, 5-HT_{1D} antisense 5′ GCGGTCTAGACTAGGAGGCTTTCCGGAAATGGACAAC containing a *Xba*1 site. Initiation Methionine and stop codons are underlined. Clones containing inserts identical in sequence to those published were selected for further work. The fragments were inserted

into the episomal expression vector pE3hyg (Horlick et al., 2000).

2.4. Generation of cell lines expressing guinea pig 5-HT_{1B} and 5-HT_{1D} receptor

293-EBNA cells (Invitrogen), were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 250 μg/ml (gp5-HT_{1B}) or 200 μg/ml (gp5-HT_{1D}) of G418. Transfected cells were selected with 250 μg/ml of HygromycinB and maintained in 50 μg/ml of HygromycinB. Transfections were performed with the pE3hyg constructs using Superfect according to the manufacturer's recommendations, and selection was initiated 48 h post-transfection.

2.5. Cell culture and membrane preparation

A pool of HygromycinB-resistant cells was grown in DMEM with the above supplements plus 10 mM HEPES. For membrane preparation the cells were detached with 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline or with a cell-scraper. Upon harvesting the cells were centrifuged at 300×*g*, 4 °C for 10 min, washed and homogenized with a Dounce homogenizer in 10 mM Tris–HCl and 5 mM $MgSO_4$, pH 7.4, and then centrifuged at 48,000×*g*, 4 °C for 10 min. The final pellet was resuspended in harvesting buffer and stored in aliquots at −70 °C. On the day of the experiment, the frozen cell membranes were thawed, homogenized with an Ultra-turrax, and suspended in appropriate binding buffer. Protein concentration was determined in microtiter plates as described by Harrington (1990) using bovine serum albumin as standard.

2.6. [^{3}H]GR125743 binding studies, cerebral cortical membranes

The guinea pigs were decapitated and the cortici dissected, weighed and homogenized in 50 mM Tris–HCl, pH 7.7 with an Ultra-Turrax homogenizer, followed by centrifugation for 10 min at 48,000×*g*, 4 °C. The pellet was resuspended and recentrifuged. The final pellet was suspended in 0.32 M sucrose buffer to a concentration of 0.5 g original wet weight (w.w.) per ml, and stored at −70 °C.

[^{3}H]GR125743 saturation studies were carried out in duplicate with 3–5 mg w.w. per tube (2.8–4.6 pM receptors) in 5 ml buffer consisting of 50 mM Tris–HCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 1 mM EDTA (pH 7.7 at 30 °C) and 0.012–2 nM (8–12 concentrations) of radioligand. Non-specific binding was defined with 10 μM methiothepin. In the competition experiments 4–8 mg w.w. per tube (3.7–7.4 pM receptors) and a radioligand concentration of about 0.2 nM were used with 8–12 concentrations of the competing drug. Total volume was 5 ml. Receptor binding assays were run for 2–4 h at 30 °C, and terminated by rapid filtration through Whatman GF/B filters (pretreated with 0.3% polyethyleneimine) using a Brandel cell harvester. Bovine serum albumin (0.1%) was added to the washing buffer to reduce non-specific binding. Scintillation cocktail was added and the radioactivity determined in a Liquid Scintillation Counter (Packard 2200CA or 2500TR) with about 50% efficiency.

The K_i values (inhibition constants) of the test compounds were determined from inhibition curves using the iterative non-linear curve-fitting program Ligand (Munson and Rodbard, 1980) or PRISM 3.00 (GraphPad Software, San Diego, CA, USA). The K_d values (dissociation constants) used to calculate the K_i values were determined by saturation analyses.

2.7. [^{3}H]GR125743 binding studies, recombinant gp5-HT_{1B} and gp5-HT_{1D} receptors

[^{3}H]GR125743 saturation studies were carried out in duplicate with 30 μg protein/tube (8.9 pM receptors, for gp5-HT_{1B}) or 70–100 μg protein/tube (4.1–5.9 pM receptors, for gp5-HT_{1D}) in 2 ml buffer consisting of 50 mM Tris–HCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 1 mM EDTA (pH 7.4 at 30 °C) and 0.006–8.7 nM (10–12 concentrations) of radioligand. Non-specific binding was defined with 10 μM methiothepin. In the competition experiments 50 μg protein/tube (15 pM receptors, for gp5-HT_{1B}) or 100 μg protein/tube (5.9 pM receptors, for gp5-HT_{1D}) and a radioligand concentration of approximately 0.25 nM (gp5-HT_{1B}) or 1 nM (gp5-HT_{1D}) were used with 10–12 concentrations of the competing drug. Receptor binding experiments were incubated for 2–3 h at 30 °C, and terminated as described for the [^{3}H]GR125743 binding to guinea pig cortical membranes.

2.8. Selectivity radioligand binding studies

The selectivity studies performed at AstraZeneca were essentially done as described previously (Johansson et al., 1997). All other studies were performed at contract laboratories (MDS Pharma or Cerep) according to their standard protocols. If AR-A000002 inhibited binding (1 or 10 μM tested) more than 50%, complete concentration–response curves were generated. The IC_{50} values estimated from the one-point studies were recalculated to K_i values using the equation from Cheng and Prusoff (1973).

2.9. [^{35}S]GTPγS binding, recombinant gp5-HT_{1B}

The [^{35}S]GTPγS binding assay was performed as described previously (Malmberg et al., 1998) with some modifications. The assay was carried out in duplicate in a total volume of 0.5 ml. The buffer contained 50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.6 and 10 μM GDP. Addition of GDP keeps the G protein in a GDP-ligated form which is necessary for the measurement of drug induced responses. A GDP concentration of 10 μM gave the

best signal to noise ratio (data not shown). The [^{35}S]GTPγS binding was shown to increase with increasing protein concentrations in a linear manner (data not shown). A protein concentration of 50 μg/tube was used. Antagonist, membrane and GDP was preincubated 20 min at 30 °C before the agonist was added, and the mixture was further incubated for 30 min at 30 °C before [^{35}S]GTPγS (50–150 pM) addition. The reaction was terminated after an additional 30 min incubation by rapid filtration through Whatman GF/B filters and subsequent washing with cold buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4 at 4 °C) using a Brandel cell harvester. The radioactivity was determined in a Packard 2500TR liquid scintillation counter at about 100% efficiency.

Data were analyzed by non-linear regression analyses using PRISM 3.00 (GraphPad Software). E_{max} values are expressed as the percent of the maximal response over basal [^{35}S]GTPγS binding obtained with 5-HT. I_{max} values are expressed as percent inhibition of the 5-HT stimulation (100 nM) over basal. Schild analysis was performed as previously described (Schild, 1949).

2.10. Electrical field stimulated release of [^{3}H]5-HT from guinea pig occipital cortex

Guinea pigs were sacrificed by decapitation and occipital cortex was dissected and cut in slices (0.4×4 mm) with a McIlwain tissue chopper. The slices were preincubated twice (30 min) in 10 ml oxygenated Krebs–Heinsleit's buffer (consisting of 25 mM sodium hydrogen carbonate, 1.2 mM sodium dihydrogen phosphate monohydrate, 117 mM sodium chloride, 6 mM potassium chloride, 1.2 mM magnesium sulfate, 1.3 mM anhydrous calcium chloride, 0.003 mM disodium EDTA, 0.22 mM ascorbic acid and 11 mM glucose, pH 7.4). The buffer was aerated with carbogen gas (a mixture of O_2 and CO_2 93.5%/6.5%, v/v) at 37 °C. Next the slices were incubated 5 min in 5 ml buffer containing 5 mM pargyline chloride and a subsequent 30 min incubation with 0.1 μM [^{3}H]5-HT. The [^{3}H]5-HT preloaded brain slices were placed in the superfusion chambers (one slice per chamber; Brandel superfusion system and stimulator (Gaithersburg, USA)) and were perfused at a flow rate of 0.4 ml/min with Krebs–Heinsleit's buffer containing citalopram (2.5 μM). Following a 60-min equilibration period, 20 5-min fractions of superfusate were collected. Slices were electrically stimulated for 2 min with a train of pulses (5 Hz frequency, 2 ms duration and 30 mA current) 15 min (S_1) and 60 min (S_2) after the onset of fraction collection. AR-A000002 (0.01, 0.1 and 1 μM; n=5/concentration) was dissolved in Krebs buffer and superfused into the tissue chambers 20 min prior to S_2. 5-HT efflux was calculated as the amount of tritium in the collected fractions during a 5-min period divided with the amount in the tissue slices. This latter value was obtained after the tissue slice was solubilized in 1 ml Soluene-350 (Packard) for 60 min at 60°C and thereafter the radioactivity was counted in a scintillation counter (Beckman). The results are presented as the S_2/S_1 ratio, which is the percentage of released [^{3}H]5-HT at the second stimulation divided by that of the first stimulation. Statistical analysis was performed by Analysis of variance (ANOVA) followed by Bonferroni Post hoc test using SYSTAT version 7.0 (SPSS, Chicago, IL, USA). p-values less than 0.05 were considered significant.

2.11. Isolated rabbit saphenous vein

Rabbits were sacrificed by intravenous injection of an overdose of pentobarbitone sodium. Ring segments (4 mm) of rabbit saphenous vein were carefully suspended between two triangular metal holders in four separate organ baths (RadnotiGlass Technology, CA, USA). The baths were filled with 12 ml Krebs solution, aerated continuously with 5% CO_2 and 95% O_2 and maintained at 37 °C. One holder was connected to an isometric force–displacement transducer, connected to an amplifier (TRN001, Kent Scientific, CT, USA) and the other to a hook at the bottom of the organ bath. Segments were allowed to equilibrate for 30 min and then subjected to a tension of 1g (9.8 mN) for another 30 min. The tension was maintained by repeated adjustments and the bathing medium was replaced every 10 min (Biosystem for Tissue Bath, Buxco Electronics, CT, USA). The vessels were contracted with 1 μM 5-HT. This procedure was used to improve the tissue responsiveness. The segments of rabbit saphenous vein that failed to give a contraction >0.4 g (3.9 mN) were not included in the experimental protocol. The test compounds (AR-A000002, sumatriptan and 5-HT) were administered in a cumulative manner using the following final concentrations: 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 μM. Sufficient time (until a maximum response was obtained or relaxation started to occur) was allowed for the effects of each concentration to become fully established before adding the next concentration. AR-A000002 (0.01 or 1 μM) or methiothepin (0.01, 0.1 or 1 μM) were added to the organ baths in the experiments where their antagonistic effects were tested.

After establishment of a control response curve to sumatriptan: (a) One concentration of AR-A000002 (0.01 or 1 μM) or methiothepin (0.01, 0.1 or 1 μM) was added to the baths and left in contact with the tissue for 20 min. Thereafter a concentration–response curve for sumatriptan was repeated in the presence of AR-A000002 or methiothepin. (b) AR-A000002 was added to the baths using a cumulative concentration schedule, the same as for sumatriptan. In all experiments, washouts were carried out after each concentration–response curve and the preparation was left to recover its baseline tension before a new response curve was constructed.

Analyses of changes in the tissue tension were performed by the Slow Responses Analyser procedure (Biosystem for Tissue Bath, Buxco Electronics). Contractile responses are expressed as the absolute developed tension (1g=9.8 mN) and as a percentage of the maximum response obtained with

sumatriptan in each preparation. For the agonist effect, pD_2 values (−log of the molecular concentration of agonists causing 50% of the maximum response) were determined graphically for each preparation. The ASTUTE statistical program from AstraZeneca R&D Mölndal, Sweden and Sigma Plot graphics/statistical program from Jandel Scientific Software, Erkrath, Germany were used for the analysis and presentation of the data. Values are presented as mean±S.E.M.

3. Results

3.1. Affinities of AR-A000002 and 5-HT ligands for 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors

The receptor binding affinities of AR-A000002 for the guinea pig 5-HT$_{1B/1D}$ receptors were evaluated in membranes of guinea pig cerebral cortex, and in 293-EBNA cells expressing the cloned gp5-HT$_{1B}$ or gp5-HT$_{1D}$ receptors. The 5-HT$_{1B/1D}$ selective radioligand [^{3}H]GR125743 was used in these studies.

[^{3}H]GR125743 bound with high affinity to membranes prepared from guinea pig cerebral cortex (K_d=45±5 pM, n=6), transfected cells expressing gp5-HT$_{1B}$ (K_d=109±5 pM, n=3) and gp5-HT$_{1D}$ receptors (K_d=449±75 pM, n=3). The corresponding Bmax values were the following; 4.6±0.3 pmol/g w.w., 595±19 pmol/g of protein and 118±13 pmol/g of protein, respectively. Due to [^{3}H]GR125743 high affinity to cortex gp5-HT$_{1B/1D}$ receptors a low receptor concentration in combination with a large incubation volume (5 pM and 5 ml, respectively) and long incubation time (3 h, 30 °C) were used.

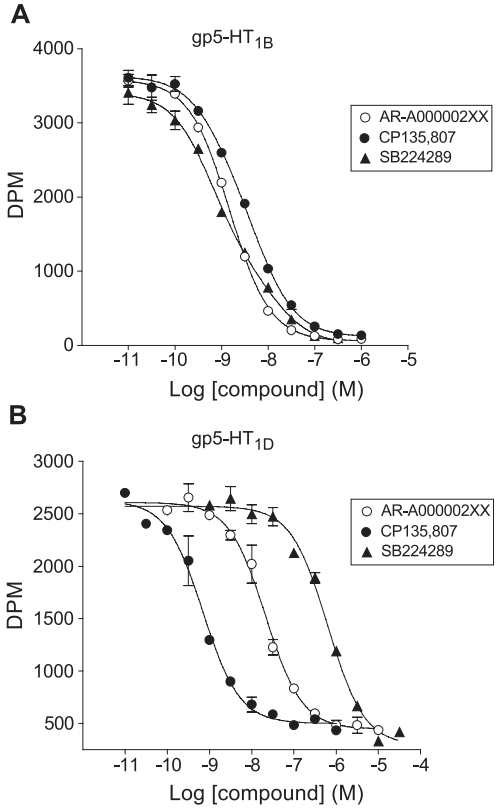

Fig. 2. Representative competition binding curves of AR-A000002, CP-135,807 and SB224289 on recombinant gp5HT$_{1B}$ (A) and gp5HT$_{1D}$ (B) receptors.

Table 1
Inhibition of [^{3}H]GR125743 binding to guinea pig (gp) cortex and cloned guinea pig 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors by various compounds

Compound	gp5-HT$_{1B}$	gp5-HT$_{1D}$	gp Cortex
	pK_i±S.E.M. (K_i, nM)	pK_i±S.E.M. (K_i, nM)	pK_i±S.E.M. (K_i, nM)
GR127935	10.43±0.07 (0.04)	9.80±0.08 (0.16)	10.26±0.02[a] (0.05)
SB224289	9.65±0.19 (0.22)	6.81±0.06 (150)	N.D.
Methiothepin	9.52±0.14[b] (0.30)	8.79±0.12 (1.6)	9.06±0.06 (0.87)
AR-A000002	9.33±0.04 (0.47)	8.30±0.07 (5.0)	9.62±0.01 (0.24)
CP-135,807	9.06±0.05[b] (0.87)	9.79±0.09 (0.16)	N.D.
5-HT	8.47±0.10 (3.4)	9.09±0.05[b] (0.82)	8.90±0.09 (1.3)
Sumatriptan	7.17±0.09 (68)	8.42±0.05 (3.8)	7.67±0.05 (21)
Ketanserin	6.37±0.04 (430)	6.98±0.20 (100)	5.88±0.03 (1300)

The pK_i values are means±S.E.M. of three independent experiments (unless otherwise stated).
N.D., not determined.
[a] n=2.
[b] n=4.

Table 2
Summary of K_i values of AR-A000002 for different serotonin binding sites (gp cortex 5-HT$_{1B/D}$, gp5-HT$_{1B}$ and gp5-HT$_{1D}$ are shown in Table 1)

Receptor	AR-A000002 K_i (nM)	Radioligand	Tissue/cells
r5-HT$_{1B/D}$[a]	20	[^{125}I]CYP	Rat cortex
5-HT$_{1A}$	3070±600	[^{3}H]8-OH-DPAT	Rat hippocampus
5-HT$_{2A}$	339±30	[^{3}H]Ketanserin	Rat cortex
5-HT$_3$[b]	>500	[^{3}H]GR65633	Rabbit ileum muscularis
5-HT$_4$[b]	>200	[^{3}H]GR113808	Guinea pig striatum
5-HT$_{5A}$[b]	>500	[^{3}H]LSD	CHO-h5HT$_{5A}$
5-HT$_6$[b]	>500	[^{3}H]LSD	Human recombinant
5-HT$_7$[b]	>500	[^{3}H]LSD	Human recombinant
5-HT uptake site	>1000	[^{3}H]citalopram	Rat cortex

The K_i values are means±S.E.M. of two independent experiments.
[a] n=1, results from Cerep.
[b] Results from MDS Pharma. Compound tested at 1 μM in duplicates and in two independent experiments.

Table 3
The effect of various compounds on the [^{35}S]GTPγS binding mediated by gp5-HT$_{1B}$ receptors

Substance	pEC$_{50}$±S.E.M. (EC$_{50}$)	E_{max} (%)±S.E.M.	n
5-HT	7.97±0.06 (11)	100	6
CP-135,807	8.38±0.13 (4.1)	78.6±7.1	3
GR127935	8.49±0.15 (3.3)	50.5±7.6	4
AR-A000002	8.35±0.11 (4.5)	48.2±7.1	4
SB224289	8.43±0.08 (3.7)	−17.1±3.1	2
Methiothepin	7.56±0.15 (28)	−22.5±0.64	3

The results are means±S.E.M. of n independent experiments. The EC$_{50}$ values in parenthesis are expressed in nM. E_{max} represents maximal stimulation over basal [^{35}S]GTPγS binding observed with 5-HT (100%) which was included in all experiments. Basal [^{35}S]GTPγS binding was 40±4 pmol/g protein.

The K_i values of AR-A000002 and seven other 5-HT ligands for cortex gp5HT$_{1B/1D}$, cloned gp5HT$_{1B}$ and gp5HT$_{1D}$ receptors are summarized in Table 1. AR-A000002 displays high affinity to [^{3}H]GR125743 binding sites in guinea pig cortex (K_i of 0.24 nM). Its affinity to cloned gp5-HT$_{1B}$ receptors was similar (0.47 nM with a Hill coefficient of 1.04±0.05), whereas it showed 10-fold lower affinity to gp5-HT$_{1D}$ receptors (5 nM).

The rank order of potency was GR127935> SB224289>methiothepin>AR-A000002>CP-135,807>5-HT>sumatriptan>ketanserin for the gp5-HT$_{1B}$ receptor, and CP-135,807=GR127935>5-HT>methiothepin> sumatriptan>AR-A000002>ketanserin>SB224289 for the gp5-HT$_{1D}$ receptor. Fig. 2 shows representative competition binding curves for AR-A000002, SB224289 and CP-135,807.

3.2. Selectivity profile of AR-A000002

Table 2 shows the affinity of AR-A000002 for other serotonin binding sites. Although the compound has high affinity for gp5HT$_{1B}$ receptors it displays >80-fold lower affinity for the rat cortical 5HT$_{1B/1D}$ receptors. The selectivity versus all other 5HT receptor binding sites is at least 400-fold or more. AR-A000002 was shown to be selective (>700-fold) against a battery of other binding sites[1] with highest affinity for dopamine D2 (330 nM) and α1-adrenoceptors (490 nM).

3.3. gp5-HT$_{1B}$ receptor mediated [^{35}S]GTPγS binding

The [^{35}S]GTPγS binding assay is a functional assay where receptor mediated G-protein activation is measured.

Table 4
Inhibition of gp5-HT$_{1B}$ mediated [^{35}S]GTPγS binding by various compounds

Substance	pIC$_{50}$±S.E.M. (IC$_{50}$)	I_{max} (%)±S.E.M.	n
GR127935	8.47±0.09 (3.4)	63.8±6.0	3
AR-A000002	7.50±0.10 (31)	66.0±6.1	4
SB224289	8.09±0.20 (8.1)	147±6.6	2
Methiothepin	7.48±0.03 (33)	128±7.5	3

I_{max} represent maximal inhibition of 5-HT-stimulation (100 nM). I_{max} values of >100% indicate inhibition of basal [^{35}S]GTPγS binding. The results are means±S.E.M. of n independent experiments. The IC$_{50}$ values in parenthesis are expressed in nM.

The intrinsic activity of the ligands tested ranged from agonists (5-HT) to inverse agonists (methiothepin) at the gp5-HT$_{1B}$ receptor. AR-A000002 and GR127935 behaved similarly in this system, i.e. as partial agonists. They stimulated [^{35}S]GTPγS binding with an E_{max} of about 50% of the maximal 5-HT stimulation (Table 3). Both AR-A000002 and GR127935 could inhibit 5-HT stimulated [^{35}S]GTPγS binding by 60–70% (Table 4). As expected the inverse agonists SB224289 and methiothepin were able to inhibit not only the 5-HT stimulated [^{35}S]GTPγS binding but also the basal binding leading to >100% inhibition (Table 4). AR-A000002 concentration-dependently induced a rightward shift of the 5-HT

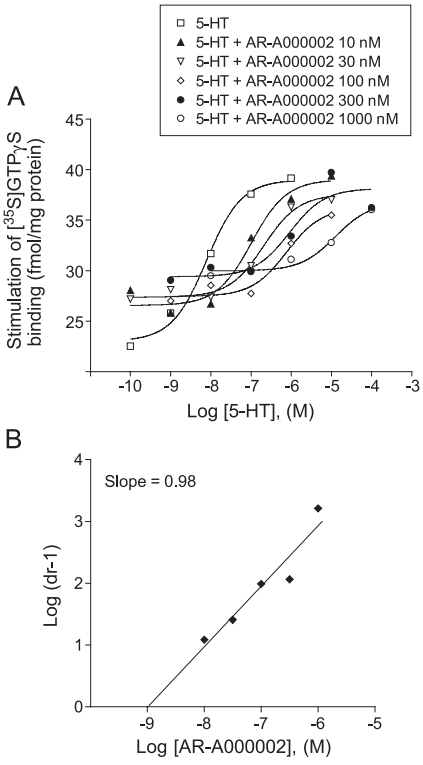

Fig. 3. Antagonist effect of AR-A000002 on 5-HT-mediated stimulation of gp5-HT$_{1B}$ receptors and [^{35}S]GTPγS binding. (A) The figure illustrates one experiment with 5-HT concentration–response curves, and (B) the corresponding Schild plot. The upward shift in the presence of AR-A000002 at low 5-HT concentrations is explained by the agonist effect of the compound.

[1] Low or no affinity for the following binding sites: adenosine A$_1$, A$_{2A}$, α$_{1A}$-, α$_{1B}$-, α$_{1D}$-, α$_{2A}$-, α$_{2B}$-, β1- and β2-adrenoceptors, Ca^{2+} channel type L (benzothiazepine-, dihydropyridine-, phenylalkylamine-site), Ca^{2+} channel type N, CCK$_1$, CCK$_2$, dopamine D$_1$, transporter, GABA$_A$, GABA$_B$, glucocorticoid, glutamate AMPA, kainate, NMDA (agonist-, glycin-, phencyclidine-, polyamine-site), glycine, histamine H$_1$ and H$_2$, K$^+$ channel [K$_A$, K$_{ATP}$, K$_V$ and SK$_{Ca}$], muscarinic M$_1$, M$_2$, M$_3$, M$_4$ and M$_5$, NPY$_1$, NPY$_2$, nicotinic ACh, opiate, sigma, somatostatin, tachykinin NK$_1$, NK$_2$, NK$_3$ and vasopressin V$_1$ binding sites.

concentration–response curves (Fig. 3). Due to the fact that AR-A000002 behaves as a partial agonist in this system the baseline at lower concentration of 5-HT are shifted upwards in the presence of the compound. The curves were therefore normalized to calculate the EC_{50} values for the Schild analysis (Schild, 1949). The slope from the Schild analysis was not significantly different from one (0.92±0.06) and the pA_2 value was 8.9±0.02 (n=2), which is in good agreement with the binding affinity for the gp5-HT_{1B} receptor.

5-HT stimulation of 5-HT_{1D} receptor mediated [^{35}S]GTPγS binding could not be established, possibly due to too low receptor expression in this system for detecting a reliable signal.

3.4. Electrical field stimulated [^{3}H]5-HT release from guinea pig brain cortical slices

An S_2/S_1 ratio (percent [^{3}H]5-HT released at the second stimulation divided by that of the first stimulation) higher than 150% is considered as a manifestation of antagonism. A statistically significant increase in the S_2/S_1 ratio could already be seen at the lowest concentration of AR-A000002 tested (0.01 μM, p<0.05) (Table 5).

3.5. Isolated rabbit saphenous vein

Cumulative administration of the 5-$HT_{1B/1D}$ receptor agonist sumatriptan and 5-HT induced concentration-dependent contractions of the isolated rabbit saphenous vein (Fig. 4A and B). The maximum vasoconstrictor response to sumatriptan corresponded to 25.2±2.6 mN (n=6) and for 5-HT to 18.0±12.1 mN. Cumulative administration of AR-A000002 alone had no effect on the basal tension of the rabbit saphenous veins, i.e. it had no agonist activity in this system. AR-A000002 at a concentration of 1 μM (n=2) antagonized the 0.3 μM sumatriptan-induced vasoconstriction while the dose 0.01 μM did not (Fig. 4B). Both the 0.1 and 0.01 μM dose of methiothepin (n=6) antagonized the 0.3 μM sumatriptan-induced vasoconstriction (Fig. 4A). Ketanserine up to a dose of 1 μM did not antagonize sumatriptan-induced vasoconstriction (data not shown).

Table 5
The mean S_2/S_1 ratios (n=5), which are the percentages of released [^{3}H]5-HT from preloaded guinea pig occipital cortex slices at the second (S_2) electrical stimulation divided by that of the first stimulation (S_1) when different concentrations of AR-A000002 was added to the superfusion buffer

Concentration of AR-A000002 (μM)	Mean S_2/S_1 ratio (±S.E.M.)
0.01	176±16*
0.1	235±21**
1	310±22***

* p<0.05.
** p<0.01.
*** p<0.001.

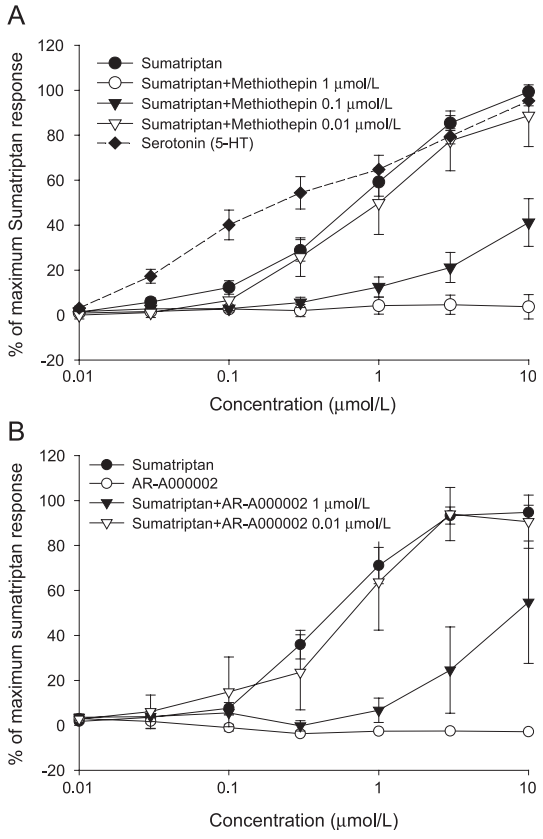

Fig. 4. Contractions of isolated rabbit saphenous vein preparations. Cumulative contractile concentration–response curves to sumatriptan alone (n=6, A and B) and in the presence of (A) methiothepin (0.01, 0.1 and 1 μM, n=6) and (B) AR-A000002 (0.01 and 1 μM, n=2). Cumulative concentrations of AR-A000002 alone displayed no contractile effects. The contractile response curve to 5-HT is also shown in (A). The contraction is expressed as the percentage of the maximum response to sumatriptan. Data are presented as mean±S.E.M.

4. Discussion

The in vitro binding experiments in the present study show that AR-A000002 has high affinity for the gp5-HT_{1B} receptor since it potently displaced the binding of the 5-$HT_{1B/1D}$ receptor antagonist [^{3}H]GR125743 to membranes prepared from guinea pig cerebral cortex, or 293-EBNA cells expressing the cloned gp5-HT_{1B} receptor. AR-A000002 displays about 10-fold selectivity for the recombinant gp5-HT_{1B} receptor versus the recombinant gp5-HT_{1D} receptor. The pharmacology of AR-A000002 was evaluated in guinea pigs since gp5-HT_{1B} receptors are similar to h5-HT_{1B} receptors (Zgombick et al., 1997). This was also evident with AR-A000002 that has about 100 times higher affinity for the guinea pig 5-HT_{1B} receptor compared to the rat 5-HT_{1B} receptor. The affinity for all other receptors examined including other 5HT receptors is at least 400-fold lower than that for the gp5-HT_{1B} receptor.

The rank order of potencies for the reference compounds tested at gp5-HT_{1B}, gp5-HT_{1D} and cortex gp5-$HT_{1B/D}$ receptors are in good agreement with the literature values for the corresponding receptors (Pauwels et al., 1998; Zgombick et al., 1997; Audinot et al., 1997). In addition, the rank order of potencies at the gp5-HT_{1B} receptor correlates very well with published values for the h5-HT_{1B} receptor (Peroutka, 1994; Doménech et al., 1997). The pharmacology of the [^{3}H]GR125743 binding in gp cortex indicate mainly labeling of the 5-HT_{1B} receptor subtype. If a substantial part of the [^{3}H]GR125743 binding was of the 5-HT_{1D} subtype we would expect to see biphasic binding with compounds such as sumatriptan (18-fold higher affinity at 5-HT_{1D} receptors), and AR-A000002 (11-fold higher affinity at 5-HT_{1B} receptors), which was not the case. High affinity compounds like GR127935, SB224289 and methiothepin are 10 to 100 times more potent in our hands than what has been reported earlier. Also, [^{3}H]GR125743 has considerably (>6-fold) higher affinity to gp5-$HT_{1B/D}$ receptors than previously reported (Audinot et al., 1997). The explanation for these discrepancies is probably the differences in assay protocols. It has been reported that [^{3}H]GR125743 has a slow and biphasic dissociation (Audinot et al., 1997) using membranes from guinea pig cortex, although this was not seen with membranes from cloned human 5-HT_{1B} receptors in HeLa cells (Doménech et al., 1997). Slow or incomplete dissociation from the 5-HT_{1B} receptor both in vitro and in vivo has also been reported for the related compound GR127936 (Skingle et al., 1996; Walsh et al., 1995). Our preliminary experiments indicated that [^{3}H]GR125743 had very high affinity for gp5-$HT_{1B/D}$ receptors. We therefore chose a higher incubation temperature (30 °C) than previously used (22 °C) in order to achieve equilibrium within reasonable time, i.e. 3 h.

In the [^{35}S]GTPγS binding assay the potencies of the reference compounds are in agreement with the literature, with exception of methiothepin that has been reported to be 10 times more potent, both on the guinea pig 5-HT_{1B} (Pauwels et al., 1998) and the human 5-HT_{1B} (Pauwels et al., 1997) receptors. On the other hand, Roberts et al. (1997) reported methiothepin to have a pEC_{50} of 7.7, which agrees with our results. Comparing EC_{50}/IC_{50} values are however difficult since assay parameters and cellular system used will affect the potencies. We used for example 10 μM GDP and 293-EBNA cell membranes expressing 0.6 pmol receptors/mg of protein, whereas Pauwels et al. (1998) used 0.3 μM GDP and C6-glial cell membranes expressing 6 pmol receptors/mg of protein. The antagonist potency (pIC_{50}, Table 4) of AR-A000002 is about 10 times lower than the agonist potency (pEC_{50}, Table 3). We do not have a good explanation to this finding and it is in contrast to the other compounds tested, GR127935, SB224289 and methiothepin, which have nearly equivalent measures of agonist or antagonist potencies. Schild analysis showed that AR-A000002 was competitive versus 5-HT with a slope not different from unity. It had a potency value (pA_2 of 8.9) that is similar to its binding affinity (pK_i of 9.3).

The enhancement of the electrical field-stimulated release of [^{3}H]5-HT from guinea pig cortical slices indicates blockade of the terminal 5-HT_{1B} autoreceptors by AR-A000002, which regulates this release. Because the 5-HT_{1B} receptors are negatively coupled to the release mechanism, the observed marked enhancement of [^{3}H]5-HT release indicates that these receptors are at least partially occupied by 5-HT in the system under the conditions used. Thus, AR-A000002 appears to work as an antagonist in the brain tissue preparation although partial agonism might be difficult to observe in this test.

An agonist at the 5-$HT_{1B/1D}$ receptors such as sumatriptan or 5-HT induces contractions of isolated rabbit saphenous vein preparations in vitro. It has been shown that rabbit and human 5-HT_{1B} receptors show significant similarities in their ligand binding profiles (Bard et al., 1996), therefore this native test system was used to show whether AR-A000002 had any agonist properties. As evident in Fig. 4B, no intrinsic activity could be detected with this compound. AR-A000002 was able to inhibit sumatriptan-induced contractions, thus showing antagonist properties. The potency as compared to methiothepin seemed however to be about 10 times lower although their affinity to the guinea pig receptor is similar. The binding affinities of the two compounds to the rabbit 5HT_{1B} receptor were not tested in our studies, which might provide an explanation for the observed differences. The conclusion that AR-A000002 is an antagonist without any agonist effect is strengthened by previously published in vivo studies where AR-A000002 blocked agonist (CP-135,807)-induced decrease in 5-HT metabolism and hypothermia in guinea pigs (Hudzik et al., 2003; Stenfors et al., 2001, 2004).

In summary, at recombinant gp5-HT_{1B} receptors, AR-A000002 is a selective and potent ligand with partial agonist activity. In vitro functional tests in guinea pig brain slices and rabbit saphenous vein show that AR-A000002 is a 5-HT_{1B} receptor antagonist, which is in accordance with our in vivo studies.

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