

[N-methyl-³H₃]AZ10419369 Binding to the 5-HT_{1B} Receptor: In Vitro Characterization and in Vivo Receptor Occupancy

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ABSTRACT

Radiotracers suitable for positron emission tomography studies often serve as preclinical tools for in vivo receptor occupancy. The serotonin 1B receptor (5-HT_{1B}) subtype is a pharmacological target used to discover treatments for various psychiatric and neurological disorders. In psychiatry, 5-HT_{1B} antagonists may provide novel therapeutics for depression and anxiety. We report on the in vitro and in vivo evaluation of tritiated 5-methyl-8-(4-methyl-piperazin-1-yl)-4-oxo-4H-chromene-2-carboxylic acid (4-morpholin-4-yl-phenyl)-amide ([N-methyl-³H₃]AZ10419369), a potent 5-HT_{1B} radiotracer. [N-methyl-³H₃]AZ10419369 showed saturable single-site high-affinity in vitro binding (guinea pig, $K_d = 0.38$ and human, $K_d = 0.37$) to guinea pig or human 5-HT_{1B} receptors in recombinant membranes and high-affinity ($K_d = 1.9$ nM) saturable ($B_{max} = 0.099$ pmol/mg protein) binding in membranes from guinea pig striatum. When [N-methyl-³H₃]AZ10419369 was administered to guinea pigs by intravenous bolus, the measured radioactivity was up to 5-fold higher in brain areas containing the 5-HT_{1B} receptor

(striatum/globus pallidus, midbrain, hypothalamus, and frontal cortex) compared with the cerebellum, the nonspecific binding region. Specific uptake peaked 30 min after injection with slow dissociation from target regions, as suggested by the in vitro binding kinetic profile. Pretreatment with 6-fluoro-8-(4-methyl-piperazin-1-yl)-4-oxo-4H-chromene-2-carboxylic acid [4-(4-propionyl-piperazin-1-yl)-phenyl]-amide (AZD1134) and 2-aminotetralin (AR-A000002), 5-HT_{1B}-selective ligands, inhibited [N-methyl-³H₃]AZ10419369-specific binding in a dose-dependent manner. In the guinea pig striatum, AZD1134 ($ED_{50} = 0.017$ mg/kg) occupies a greater percentage of the 5-HT_{1B} receptors at a lower administered dose than AR-A000002 ($ED_{50} = 2.5$ mg/kg). In vivo receptor occupancy is an essential component to build binding-efficacy-exposure relationships and compare novel compound pharmacology. [N-methyl-³H₃]AZ10419369 is a useful preclinical tool for investigating 5-HT_{1B} receptor occupancy for novel compounds targeting this receptor.

5-Hydroxytryptamine (5-HT or serotonin) is a major neurotransmitter in the central and peripheral nervous systems. The action of 5-HT is mediated via six classes of G protein-coupled receptors (5-HT₁, 5-HT₂, 5-HT₄, 5-HT₅, 5-HT₆, and 5-HT₇) that are further divided in total to at least 14 subtypes (Westkaemper and Roth, 2006) and a family of ligand-

gated 5-HT₃ ion channels further divided into three subtypes. The 5-HT₁ class of serotonergic receptor contains five subtypes (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}) that are preferentially coupled to $G_{i/o}$ to inhibit cAMP formation (Hoyer et al., 2002). The serotonin 1B receptor (5-HT_{1B}) subtype has been implicated in normal physiological functions and behavior and in neurological and psychiatric disorders, such as diseases of locomotion, migraine, drug abuse, aggressive behavior, anxiety, and depression [Moret and Briley, 2000; Svenningsson et al., 2006; for review, see Sari (2004)]. 5-HT_{1B} receptors modulate synaptic release of sero-

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ABBREVIATIONS: 5-HT, serotonin, 5-hydroxytryptamine; 5-HT_{1B}, serotonin 1B receptor; PET, positron emission tomography; CHO, Chinese hamster ovary; AB, assay buffer; DMF, dimethylformamide; HT, hypothalamus; FC, frontal cortex; STR, striatum/globus pallidus; HEK, human embryonic kidney; HPC, hippocampus; MID, midbrain including substantia nigra; OCTx, occipital cortex; CRB, cerebellum; LC/MS, liquid chromatography mass spectroscopy; RT, room temperature; NSB, nonspecific binding; GR125743, N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl)benzamide; [N-methyl-³H₃]AZ10419369, 5-methyl-8-(4-methyl-piperazin-1-yl)-4-oxo-4H-chromene-2-carboxylic acid (4-morpholin-4-yl-phenyl)-amide; AR-A000002, 2-aminotetralin; AZD1134, 6-fluoro-8-(4-methyl-piperazin-1-yl)-4-oxo-4H-chromene-2-carboxylic acid [4-(4-propionyl-piperazin-1-yl)-phenyl]-amide; GTP γ S, guanosine 5'-3-O-(thio)triphosphate; fmol/mg, femtomole per milligram; AZ12334550, 5-methyl-N-(4-morpholinophenyl)-4-oxo-8-(piperazin-1-yl)-4H-chromene-2-carboxamide.

tonin (Hoyer et al., 2002) acting as autoreceptors on serotonergic terminals or as heteroreceptors on non-serotonergic neurons (Engel et al., 1986; Sari et al., 1999; Roberts et al., 2001; Sari, 2004). Blocking 5-HT_{1B} autoreceptors with a selective ligand increases extracellular levels of 5-HT and has an additive effect on 5-HT increase when combined with selective serotonin reuptake inhibitors, suggesting the antidepressive potential of a 5-HT_{1B} drug (Matzen et al., 2000; Smagin et al., 2003). Indeed, drugs targeting 5-HT_{1B} receptors have shown efficacy in animal models of depression and anxiety (Hudzik et al., 2003a,b; Dawson et al., 2006). In humans, selective serotonin reuptake inhibitors usually have up to 4 weeks of delayed antidepressive effect attributed to gradual down-regulation and slow desensitization of 5-HT_{1B} autoreceptors. It has been suggested that targeting the 5-HT_{1B} receptor, as adjunct therapy to selective serotonin reuptake inhibitors, may shorten the delay in onset to antidepressant efficacy by direct autoreceptor blockage (Briley and Moret, 1993; Sari, 2004).

Selective 5-HT_{1B} radiotracers are of paramount importance to the development of 5-HT_{1B} drugs to allow measurement of in vivo occupancy in small animals and for positron emission tomography (PET) ligands in monkey and human brain. In general, radiotracers that provide tools for the investigation of in vivo ligand binding require high affinity and selectivity at the target receptor are not substrates for efflux pumps and have moderate lipophilicity to improve brain penetration and reduce nonspecific binding (Passchier et al., 2002). In vitro binding studies using cloned human 5-HT_{1B} receptors found high-affinity compounds selective for the 5-HT_{1B} receptor (Doménech et al., 1997; Huang et al., 2005). Autoradiography using [³H]GR125743 (incubated with 300 nM ketanserin to block 5-HT_{1D} binding) in human brain sections shows 5-HT_{1B} receptors in the basal ganglion, substantia nigra, and neocortex corresponding to 5-HT_{1B} protein expression (Varnäs et al., 2001, 2005). The high degree of homology between the 5-HT_{1B} receptor in the guinea pig brain and the human 5-HT_{1B} receptor makes the guinea pig a suitable animal model for translational studies at the 5-HT_{1B} receptor (Zgombick et al., 1997).

Until recently (McCarthy et al., 2007; Pierson et al., 2007, 2008), bioavailable 5-HT_{1B} receptor radiotracers for analyzing in vivo occupancy during preclinical development and PET imaging have been limited. We developed AZ10419369, a compound with high affinity for the 5-HT_{1B} receptor and physical-chemical properties suitable for an in vivo radioligand. Here, we describe the in vitro and in vivo pharmacological properties of tritiated AZ10419369 and show this radioligand as a preclinical tool for comparing novel 5-HT_{1B} compounds using receptor occupancy.

Materials and Methods

Adult Dunkin-Hartley male guinea pigs (180–260 g) were purchased from Charles River Laboratories (Wilmington, MA). Guinea pigs were housed in the vivarium on a 50:50 light/dark cycle and received ad libitum food and water. Cynomolgus monkey brain tissue was purchased from Covance Research Products Inc. (Denver, PA). All procedures were approved by the Institutional Animal Care and Use Committee in accordance with *The Guide for the Care and Use of Laboratory Animals*. AR-A000002, AZD1134, AZ10419369, and citalopram were synthesized at AstraZeneca Pharmaceuticals LP (Wilmington, DE). The chemical structures for AR-A000002 (Ahlgren et al., 2004) and for AZD1134 (Horchler et al., 2007) have been published. Serotonin (H-9523) was purchased from Sigma-Aldrich (St. Louis, MO); [³H]GR125743 was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK); and methiothepin was purchased from Tocris Bioscience (Ellisville, MO). Chinese hamster ovary (CHO) cell membranes expressing human 5-HT_{1B} receptors were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Selectivity studies were performed at MDS Pharma Services (Taipei, Taiwan).

Preparation of [N-methyl-³H₃]AZ10419369

[N-methyl-³H₃]AZ10419369 (Fig. 1) was synthesized in the radiochemistry laboratory at AstraZeneca Pharmaceuticals. A solution of 1.6 mg (3.57 μmol) of AZ12334550 in 400 μl of dimethylformamide (DMF) was added to a solution of 170 mCi (85 Ci/mmol, 2.0 μmol) of ³H₃Cl in 100 μl of DMF, and the sealed reaction mixture was heated at 110°C for 50 min. The reaction was cooled to 0°C, and 5 μl (29 μmol) of diisopropylethylamine and 3 mg (13.7 μmol) of di-*tert*-butyl dicarbonate were added. The solution was stirred at 110°C for 30 min and cooled to 0°C, and a second aliquot of 5 μl (29 μmol) of diisopropylethylamine and 3 mg (13.7 μmol) of di-*tert*-butyl dicarbonate was added; the resulting mixture was stirred at 110°C for 30 min. The reaction was concentrated to near dryness and was taken up in 600 μl of EtOH to give 140 mCi. A portion (113 mCi) was purified by semipreparative high-performance liquid chromatography (10 × 250-mm Phenomenex Luna C-18(2) column, 20–60% MeCN/0.1% trifluoroacetic acid for 10 min, 3 ml/min, R_T = 8.1 min) to give 36.3 mCi (99.1% radiochemical purity determined as described above on a 4.6 × 150-mm column, 80 Ci/mmol determined by LC/MS).

Human 5-HT_{1B} Receptor Binding Assay

Frozen membrane preparations of stably transfected CHO cells expressing human 5-HT_{1B} receptors (10 mg/ml) were rapidly thawed, briefly vortexed, and diluted in assay buffer (AB; pH 7.4) containing 50 mM Tris-HCl, 4 mM MgCl₂, 4 mM CaCl₂, and 1 mM EDTA. Membranes were resuspended in AB at 125 μg/ml and dispensed at 80 μl/well for a final concentration of 10 μg/well. In saturation binding studies, [N-methyl-³H₃]AZ10419369 (0.3–12 nM diluted in AB) was incubated with membranes alone (1-h incubation at RT) or in combination with AZD1134 (3.2 μM) to define nonspecific binding. For concentration-response studies, 11 serial dilutions (10 μM to 170 pM, final concentration in dimethyl sulfoxide) of drug were added to the radioligand and membrane mixture. Final assay volumes per well were 2 μl of compound/nonspecific, 80 μl of membranes, 20 μl of

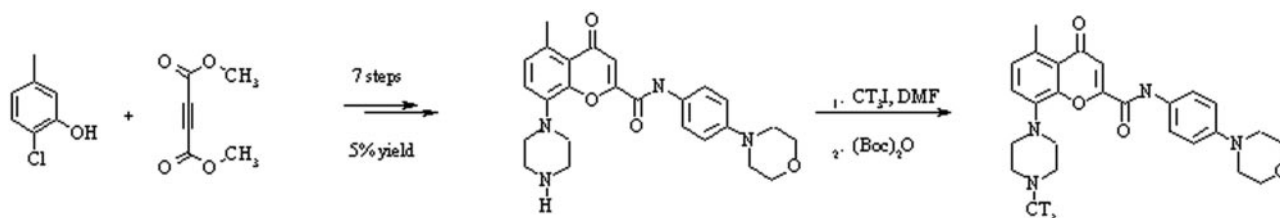


Fig. 1. Radiosynthesis of the tritiated radioligand, [N-methyl-³H₃]AZ10419369.

[*N*-methyl-³H₃]AZ10419369 (0.27 nM final concentration), and 100 µl of AB. The assay plates were incubated for 90 min at RT and then filtered through Beckman GF/B filters (soaked >2 h in 0.6% polyethylenimine) using a Packard Filtermate 196 (PerkinElmer Life and Analytical Sciences). Filters were washed two times with 1 ml of ice-cold wash buffer (5 mM Tris-HCl, pH 7.4) and dried; 40 µl of Microscint20 (PerkinElmer Life and Analytical Sciences) was added to each well, and the plates were counted on a Packard TopCount (PerkinElmer Life and Analytical Sciences).

Guinea Pig 5-HT_{1B} Receptor Binding Assay

Membranes were derived from HEK293F cells transiently transfected with the guinea pig 5-HT_{1B} receptor. Assay buffer, radioligand, filter plate procedure, and counting methods were unchanged from the human receptor binding assay. [*N*-methyl-³H₃]AZ10419369 was used at 0.001 to 3.2 nM in the saturation binding experiment and at 0.35 to 0.5 nM in the competition binding studies (1 h incubation at RT). Methiothepin (10 µM) was used to define nonspecific binding.

Guinea Pig and Monkey Membrane Preparation

Guinea pig membranes were derived from cortex, striatum/globus pallidus, and substantia nigra. Monkey membranes were derived from the cortex. Guinea pig or monkey brain tissue was homogenized in 20 mM Tris, pH 7.4, with protease inhibitors and centrifuged at 1000g for 5 min. The pellet was discarded, and the supernatant was centrifuged at 100,000g for 30 min. Pellet was resuspended in 20 mM Tris buffer, pH 7.4, and membrane protein concentration was measured by using a standard protein determination kit.

Guinea Pig and Monkey Membrane 5-HT_{1B} Receptor Binding Assay

Assay buffer, radioligand, filter plate procedure, and counting methods for the native tissue receptor assays were as described above for the recombinant receptor. In saturation binding assays, [*N*-methyl-³H₃]AZ10419369 (0.14–10 nM) was incubated (1 h at RT) alone or in competition with GR127935 (10 µM) to define nonspecific binding. Native tissue membranes were used at a final concentration of 100 µg/well in the binding assays.

[*N*-methyl-³H₃]AZ10419369 in Vivo Binding

All guinea pigs were anesthetized with isoflurane. A 2-3 French catheter was inserted into the jugular vein and terminated at the right atrium. The exterior end of the cannula was fed subcutaneously, anchored to the skin dorsal to the shoulder blades and flushed with 0.9% saline with 100 units/ml heparin to test patency. The jugular cannula was inserted 1 to 2 days before intravenous injection of the radioligand.

To establish regional uptake and dose, [*N*-methyl-³H₃]AZ10419369 was administered at a volume of 1.0 ml/guinea pig by intravenous bolus to freely moving animals via the jugular cannula 15 min before euthanasia. Dosing solutions were prepared from [*N*-methyl-³H₃]AZ10419369 stock (specific activity, 80 Ci/mmol) diluted to 2.5, 5.0, 10.0, 15.0, 25.0, or 50.0 µCi/ml with 0.9% saline. To establish the time course for in vivo binding, [*N*-methyl-³H₃]AZ10419369 (15 µCi/ml per guinea pig) was administered as an intravenous bolus and guinea pigs were euthanized 5, 15, 30, 60, or 90 min after radioligand injection. The brain was placed on a chilled plate for free-hand dissection into brain regions. Brain regions included the hypothalamus (HT), frontal cortex (FC), striatum/globus pallidus (STR), and cerebellum (CRB). Each tissue region was frozen, weighed, and transferred to scintillation vials containing tissue solubilizer, Soluene 350. Tissue was solubilized overnight, Ultima-Gold scintillation fluid was added, and total radioactivity was measured with a Tri-Carb scintillation counter (PerkinElmer Life and Analytical Sciences). Trunk blood samples were processed for scintillation count of radioactivity in the plasma. Assay conditions for [*N*-methyl-

³H₃]AZ10419369 in vivo receptor occupancy were established based on the dose-response and time course experiments.

In Vivo [*N*-methyl-³H₃]AZ10419369 Accumulation Demonstrated by Using Autoradiography

[*N*-methyl-³H₃]AZ10419369 (15 µCi/ml i.v. per guinea pig) was administered alone or 30 min after pretreatment with AR-A000002 (100 mg/kg s.c.) or AZD1134 (10 mg/kg s.c.). Guinea pigs were euthanized 30 min after radiotracer administration; the whole brain was removed and rapidly frozen for cryosectioning. Sagittal sections (20 µm thick) were thaw-mounted onto microscopy slides and dried. For regional localization of radioactivity, slides were exposed to a Fuji Imaging Plate for 5 days and processed by using the FujiFilm PhosphorImager, BAS5000 (FUJIFILM, Life Sciences, Stamford, CT).

In Vivo Receptor Occupancy Using [*N*-methyl-³H₃]AZ10419369

AZD1134 (0.001–10 mg/kg s.c.), AR-A000002 (0.01–100 mg/kg s.c.), or vehicle treatment (saline; volume, 1 ml/kg s.c.) was administered 30 min before [*N*-methyl-³H₃]AZ10419369 (15 µCi/ml i.v. per guinea pig). Guinea pigs were euthanized 30 min after radioligand injection. Brain regions were dissected, solubilized, and counted according to the method described above. Brain regions included HT, FC, STR, hippocampus (HPC), midbrain, including substantia nigra (MID), occipital cortex (OCtx), and CRB. Trunk blood samples were processed, and the compound concentration in the plasma was measured by LC/MS/MS. [*N*-methyl-³H₃]AZ10419369 binding was determined for all pretreatment groups and represented as femtomoles per milligram (fmol/mg) of tissue. In vivo receptor occupancy for unlabeled pretreatment compounds, AR-A000002 and AZD1134, was indicated by a reduction in specific binding of [*N*-methyl-³H₃]AZ10419369 and compared with vehicle controls.

Data Analysis and Statistics

In Vitro Binding. Data were analyzed by calculating percentage of control [average of (total sample binding – plate NSB)/(plate total – plate NSB) × 100%], IC₅₀, and K_i using the Kenakin correction for ligand depletion in an XLfit template: $B = [(K_D + L_T + R_T) - \{(K_D + L_T + R_T)^2 - 4R_T L_T\}^{1/2}] / 2$ and $K_i = (0.5B \times IC_{50} \times K_D) / [(L_T \times R_T) + 0.5B(R_T - L_T + 0.5B - K_D)]$. L_T and R_T are the total concentrations of the ligand and receptor, respectively (in nanomoles).

In Vivo Occupancy. Radioactivity count (cpm), scintillation counter efficiency, and brain region weight were used to calculate femtomoles per milligram of tissue. Specific binding (fmol/mg tissue) was calculated from total binding for each brain region (fmol/mg tissue) by subtracting the (fmol/mg) values for the cerebellum (region of nonspecific receptor binding). Between-treatment-group comparisons were determined by using a one-way analysis of variance (ANOVA) with a Tukey post hoc test (SigmaStat; SPSS Inc., Chicago, IL). Percentage of receptor occupancy for the unlabeled compounds was calculated from specific binding values for each brain region, with occupancy of the vehicle set at zero. Sigmoidal percentage receptor occupancy curves were fit, and nonlinear regression analysis was used to calculate the ED₅₀ values by using Prism (GraphPad Software Inc., San Diego CA).

Results

Properties and Selectivity of AZ10419369. AZ10419369 has a molecular weight of 462.55, LogD of 2.5 at pH 7.4, a lipophilicity value suggesting low nonspecific binding, and an efflux ratio of 1.2, indicating good brain penetration. In a [³⁵S]GTPγS functional assay, using CHO cell membranes expressing the recombinant human 5-HT_{1B} receptor, AZ10419369 profiles as a partial agonist in both the agonist and antagonist assay formats. AZ10419369 is able to inhibit agonist-induced

hypothermia in the guinea pig, but the data would not rule out partial agonist activity in vivo. AZ10419369 selectivity was tested at 1 μ M using radioligand in vitro binding assays for 169 targets in the SpectrumScreen Assay (MDS Pharma Services). Only seven targets showed a greater than 50% inhibition at 1 μ M. Compared with inhibition measured for the 5-HT_{1B} receptor, AZ10419369 showed 772-, 105-, 47-, 75-, 180-, 567-, and 279-fold selectivity for rat adrenergic receptor α 1b, human histamine receptor H₁, and human serotonergic receptors 5-HT_{1D}, 5-HT_{1A}, 5-HT_{2B}, 5-HT_{2C}, and 5-HT₆, respectively.

In Vitro Binding of [N-methyl-³H₃]AZ10419369. [N-methyl-³H₃]AZ10419369 synthesis was efficient and high yielding providing a reliable preclinical tool. [N-methyl-³H₃]AZ10419369 demonstrates high affinity for both the human and guinea pig 5-HT_{1B} receptors with specific binding greater than 95%. Saturation binding studies in transfected cell membranes expressing human or guinea pig 5-HT_{1B} receptors indicated that [N-methyl-³H₃]AZ10419369 is bound to a single high-affinity site (Fig. 2). [N-methyl-³H₃]AZ10419369 affinity for the human (K_d = 0.37 nM) and guinea pig receptor (K_d = 0.38 nM) and the receptor density (B_{max}) in the human (B_{max} = 8.76 pmol/mg protein) and guinea pig (B_{max} = 13.013 pmol/mg protein) membranes were similar (Table 1). These values are also comparable with the B_{max} values determined in these recombinant membrane types with the [³H]GR125743 ligand: 6.1 pmol/mg protein for the human receptor and 7.2 pmol/mg for the guinea pig receptor.

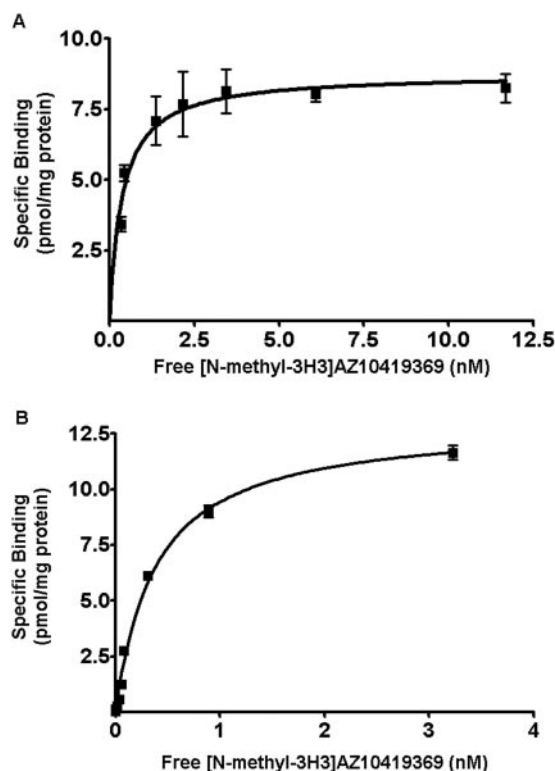


Fig. 2. Saturation binding studies in membranes from recombinant cells expressing human (A) or the guinea pig (B) 5-HT_{1B} receptors indicated that [N-methyl-³H₃]AZ10419369 bound to a single site with high affinity. The [N-methyl-³H₃]AZ10419369 affinity was similar at the human (K_d = 0.37 nM) and the guinea pig receptor (K_d = 0.38 nM). The receptor density for membranes containing the human (B_{max} = 8.76 pmol/mg protein) or the guinea pig (B_{max} = 13.013 pmol/mg protein) receptor was determined for [N-methyl-³H₃]AZ10419369.

In membranes prepared from guinea pig and monkey brain tissue, a saturation binding assay was used to evaluate [N-methyl-³H₃]AZ10419369 affinity and 5-HT_{1B} receptor density (Table 1). In native tissue, [N-methyl-³H₃]AZ10419369 affinity was reduced by ≥ 5 -fold compared with membranes containing transfected 5-HT_{1B} receptors. The affinity of [N-methyl-³H₃]AZ10419369 for the 5HT_{1B} receptors in guinea pig cortex (K_d = 8.1 nM) was two times less potent than the monkey cortex (K_d = 3.85 nM), whereas the highest affinity was seen in guinea pig striatum and substantia nigra (K_d = 1.9 and 1.5 nM, respectively). The receptor density was similar across brain regions. The receptor density in the guinea pig cortex (B_{max} = 0.349 pmol/mg protein) was ~ 2 -fold more than that in the monkey cortex (B_{max} = 0.131 pmol/mg protein). The receptor density in the guinea pig striatum (B_{max} = 0.099 pmol/mg protein) and substantia nigra (B_{max} = 0.115 pmol/mg protein) is comparable. These differences are acceptable and within typical assay variability.

Binding kinetics of [N-methyl-³H₃]AZ10419369 were determined by using transfected cell membranes expressing human or guinea pig 5-HT_{1B} receptors (Fig. 3). [N-methyl-³H₃]AZ10419369 displays association kinetics for human and guinea pig 5-HT_{1B} receptors that is relatively rapid and typical for small molecules, whereas the dissociation rate is relatively slow. The in vitro association kinetics of [N-methyl-³H₃]AZ10419369 to the human 5-HT_{1B} receptor is approximately $t_{1/2}$ of association of 5.6 min; k_{obs} of association = 0.124/min. This is comparable with the rapid association of the [³H]GR125743 ligand ($t_{1/2}$ = 2.25 min) (data not shown). The dissociation at the human 5-HT_{1B} receptor, by the addition of 5 μ M AZD1134, is relatively slow ($t_{1/2}$ of dissociation = 34 min; k = 0.020/min). After 3 h, approximately 10% of the specifically bound counts had not been displaced. Nonspecific binding was approximately 65 cpm. The k_{on} was calculated by using $k_{on} = (k_{obs} - k_{off})/[\text{radioligand}] = 0.124/\text{min} - 0.020/\text{min}/0.5 \text{ nM} = 0.208/\text{nM} \cdot \text{min}$. The K_d was calculated by using $K_d = k_{off}/k_{on} = 0.020/\text{min}/0.208/\text{nM} \cdot \text{min} = 0.096 \text{ nM}$. This calculated value is similar to the K_d determined in the saturation binding assay ($K_d \sim 0.37 \text{ nM}$). The in vitro binding of [N-methyl-³H₃]AZ10419369 to the guinea pig 5-HT_{1B} receptor is rapid (approximate $t_{1/2}$ of association of 2.3 min; k_{obs} of association = 0.303/min) and the dissociation, by the addition of 5 μ M AZD1134, was very slow ($t_{1/2}$ of dissociation = 107 min; k = 0.006/min), with approximately 30% of the counts remaining bound after 3 h.

[N-methyl-³H₃]AZ10419369 5-HT_{1B} selectivity and pharmacology were evaluated in transfected cell membranes containing the human and guinea pig 5-HT_{1B} receptors (Table 2). The slow dissociation rate of [N-methyl-³H₃]AZ10419369 does not allow for the measurement of true equilibrium binding in the time frame of the experiment, so the binding results are expressed as IC₅₀ values rather than K_i values. Although the dissociation rate of [N-methyl-³H₃]AZ10419369 is slow, a relative potency can be determined for the compounds, allowing a reasonable comparison ranking. The IC₅₀ value for AZ10419369 using [³H]GR125743 was more potent at the guinea pig receptor than at the human receptor. IC₅₀ values for AZD1134 and AR-A000002 (selective 5-HT_{1B} receptor ligands) and 5-HT using [N-methyl-³H₃]AZ10419369 were comparable with the IC₅₀ values using [³H]GR125743 for both human and guinea pig 5-HT_{1B} receptors. These

TABLE 1

In vitro saturation binding of [*N*-methyl-³H₃]AZ10419369 at the 5HT_{1B} receptor
Affinity and receptor density was determined by using [*N*-methyl-³H₃]AZ10419369 in membranes from cells expressing human (transfected CHO cell) or guinea pig (transfected HEK cell) 5-HT_{1B} receptors or from monkey (cortex) and guinea pig (cortex, striatum, and substantia nigra) brain tissue.

[<i>N</i> -methyl- ³ H ₃]AZ10419369	Human Transfected CHO Cell	Guinea Pig Transfected HEK Cell	Monkey Cortex	Guinea Pig		
				Cortex	Striatum	Substantia Nigra
<i>K_d</i> (nM)	0.37	0.38	3.85	8.1	1.9	1.5
<i>B_{max}</i> (pmol/mg protein)	8.76	13.013	0.131	0.349	0.099	0.115

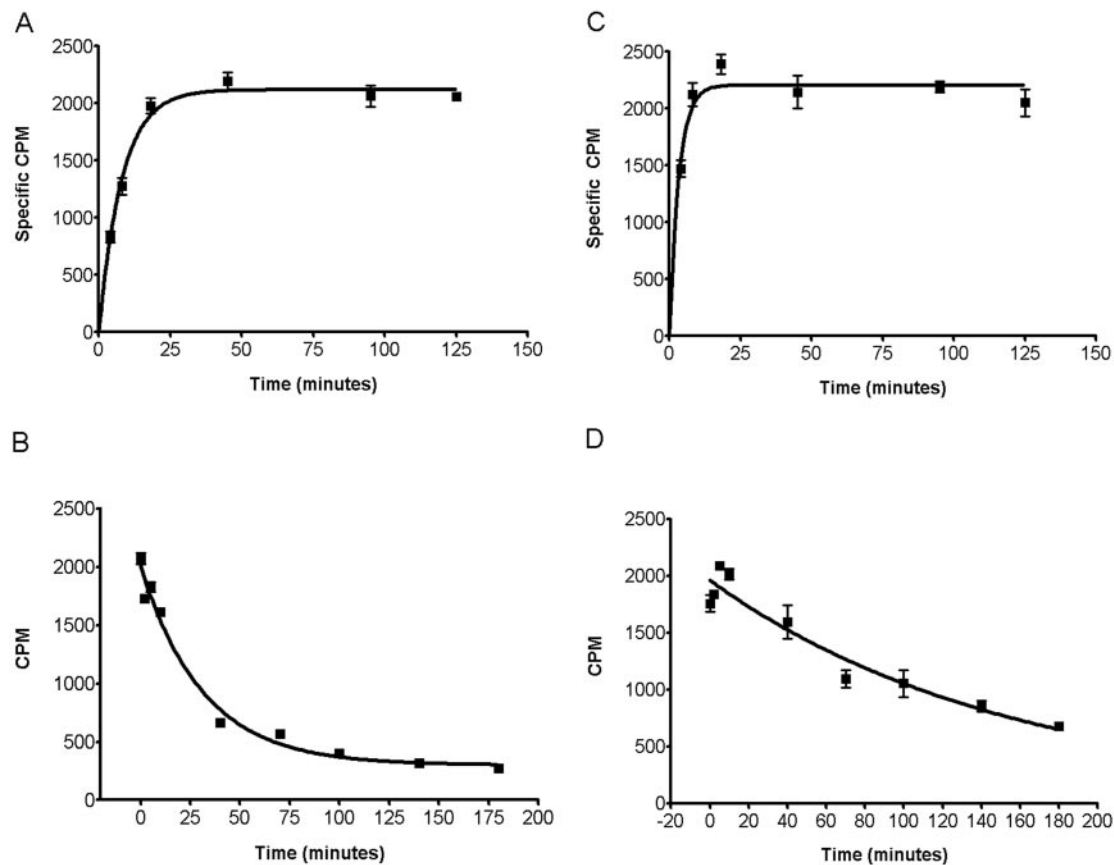


Fig. 3. In vitro binding kinetics of [*N*-methyl-³H₃]AZ10419369 were tested in membranes containing the human (A and B) and the guinea pig 5-HT_{1B} receptor (C and D). Association kinetics of [*N*-methyl-³H₃]AZ10419369 binding is relatively rapid (A and C). The dissociation kinetics, after the addition of 5 μM AZD1134, is relatively slow (B and D). After 3 h, approximately 10% (human) and 30% (guinea pig) of the specifically bound counts had not been displaced. Nonspecific binding was approximately 65 cpm.

TABLE 2

In vitro competition binding in guinea pig and human membranes
Selectivity and pharmacology of [*N*-methyl-³H₃]AZ10419369 in membranes transfected with human or guinea pig 5-HT_{1B} receptors. The IC₅₀ values were determined for AR-A000002, AZD1134, 5-HT, citalopram, and AZ10419369.

Compound	Human Receptor		Guinea Pig Receptor	
	[<i>N</i> -methyl- ³ H ₃]AZ10419369	[³ H]GR125743	[<i>N</i> -methyl- ³ H ₃]AZ10419369	[³ H]GR125743
AR-A000002	3.58×10^{-10}	9.52×10^{-9}	3.57×10^{-9}	2.65×10^{-9}
AZD1134	2.90×10^{-9}	4.4×10^{-9}	1.08×10^{-10}	3.13×10^{-10}
5-HT	1.81×10^{-8}	8.02×10^{-8}	5.08×10^{-7}	N.D.
Citalopram	Inactive	N.D.	Inactive	N.D.
AZ10419369	N.D.	1.2×10^{-9}	N.D.	8.2×10^{-11}

data indicate that the compounds can be ranked by in vitro binding potency (high to low) using the IC₅₀ values: AZ10419369 > AZD1134 > AR-A000002 > 5-HT. Citalopram did not compete with either radioligand and was labeled inactive.

In Vivo Binding of [*N*-methyl-³H₃]AZ10419369. [*N*-methyl-³H₃]AZ10419369 accumulated in the guinea pig brain

after intravenous administration (Fig. 4). Regional uptake of [*N*-methyl-³H₃]AZ10419369 was consistent with the gradient of regional distribution of 5-HT_{1B} receptors: highest in regions containing high amounts of the receptor, such as striatum/globus pallidus, and hypothalamus; and moderate, such as frontal cortex. [*N*-methyl-³H₃]AZ10419369 accumulation was dose-dependent. Peak uptake and saturation in

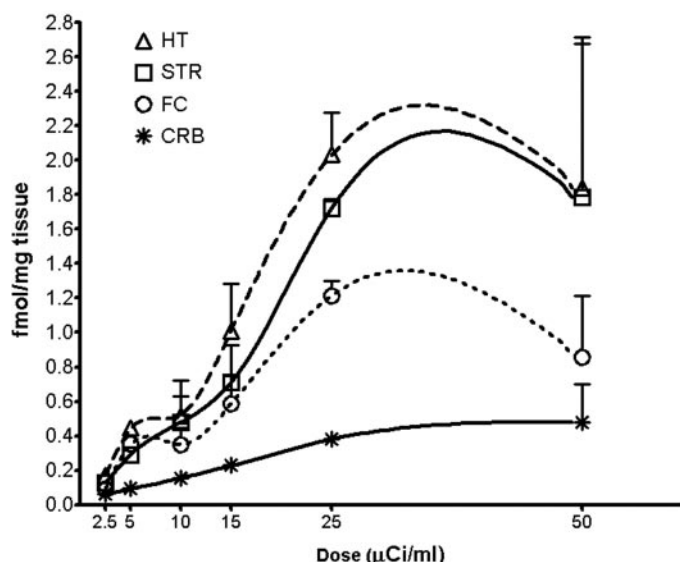


Fig. 4. [N-methyl-³H]AZ10419369 binding in the guinea pig brain was determined by measurement of radioactivity 15 min after intravenous administration. [N-methyl-³H]AZ10419369 (5, 10, 15, 25, and 50 $\mu\text{Ci/ml}$) total uptake (fmol/mg tissue, mean \pm S.E.M.) in the STR (solid line), FC (dashed line), and HT (dashed line) was greater than [N-methyl-³H]AZ10419369 uptake than the CRB (solid line).

these regions were evident at 25 $\mu\text{Ci/ml}$ per guinea pig. The cerebellum showed the lowest [N-methyl-³H]-AZ10419369 uptake at all doses and was designated as the nonspecific binding region for calculation of specific radioligand binding.

The time course study showed that [N-methyl-³H]-AZ10419369 entered the brain rapidly (Fig. 5). The uptake and distribution was similar to the dose-response data with [N-methyl-³H]AZ10419369 (15 $\mu\text{Ci/ml}$ per guinea pig) uptake in the hypothalamus > striatum/globus pallidus > frontal cortex > cerebellum. The distribution of radioactivity peaked by 30 min in all target regions. [N-methyl-³H]-AZ10419369 accumulation was not significantly different at

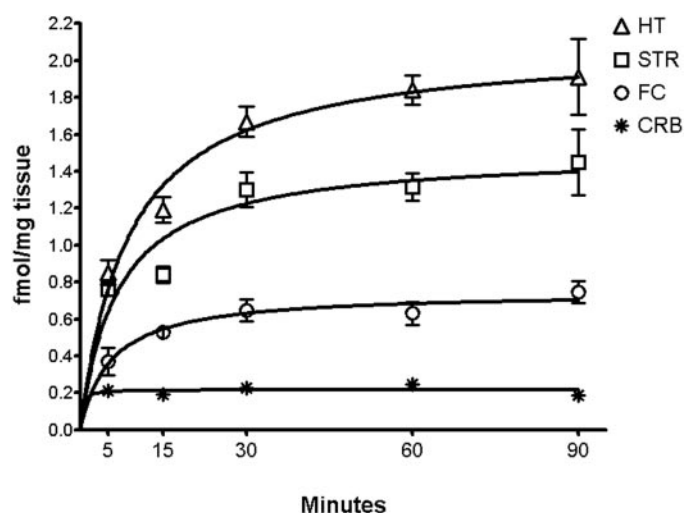


Fig. 5. [N-methyl-³H]AZ10419369 distribution (fmol/mg tissue, mean \pm S.E.M.) in the guinea pig brain was measured 5 to 90 min after administration of 15 $\mu\text{Ci/ml}$ i.v. per guinea pig. Peak accumulation in the STR, FC, and HT measured 30 min after injection of [N-methyl-³H]-AZ10419369 was complemented by low uptake in the cerebellum, resulting in an up to 5-fold signal-to-noise ratio across specific regions.

30, 60, and 90 min after intravenous injection, indicating that the binding in vivo had reached equilibrium. Radioactivity measured in the plasma from the terminal blood sample showed a dose- and time-dependent exposure of [N-methyl-³H]AZ10419369, with 1596.1 ± 86 dpm (mean \pm S.E.M.) measured at 30 min after administration of the 15 $\mu\text{Ci/ml}$ dose.

The pharmacological selectivity of [N-methyl-³H]-AZ10419369 was also demonstrated after pretreatment with a high dose of AR-A00002 (100 mg/kg s.c.) 30 min after radiotracer administration (Fig. 6). In regions with the highest [N-methyl-³H]AZ10419369 uptake, such as striatum/globus pallidus, hypothalamus, midbrain, including the substantia nigra, and in regions with moderate uptake, such as the frontal cortex, hippocampus, and occipital cortex, AR-A00002 (100 mg/kg s.c.) pretreatment significantly reduced [N-methyl-³H]AZ10419369 binding compared with saline treatment ($p < 0.05$). The pretreatment reduced [N-methyl-³H]AZ10419369 binding in all target regions to a fmol/mg level comparable with the cerebellum and nonspecific binding. In the cerebellum, [N-methyl-³H]AZ10419369 uptake was low and not significantly blocked by AR-A00002 pretreatment.

[N-methyl-³H]AZ10419369 in vivo accumulation, regional distribution, and pharmacological selectivity were also demonstrated by using autoradiography (Fig. 7). The pattern of localization for [N-methyl-³H]AZ10419369 total binding represented in the autoradiograms is consistent with the measured radioactivity (fmol/mg tissue). Inhibition of [N-methyl-³H]AZ10419369-specific binding after pretreatment with AR-A00002 in autoradiograms is similar to the dissect-and-count method. [N-methyl-³H]AZ10419369 total binding is shown 30 min after injection (15 $\mu\text{Ci/ml}$ i.v. per guinea pig). [N-methyl-³H]AZ10419369 uptake was found in regions of the brain with high 5-HT_{1B} receptor density, including the striatum, globus pallidus, hypothalamus, and midbrain (inferior colliculus, substantia nigra). Regions with moderate 5-HT_{1B} receptor density or differences in subregion density include the cerebral cortex and hippocampus. In the cerebellum, the accumulation of [N-methyl-³H]AZ10419369 was comparable with the background. AR-A00002 (100 mg/kg s.c.) and AZD1134 (10 mg/kg s.c.) pretreatment inhibited [N-methyl-³H]AZ10419369 binding in regions of the brain containing the 5-HT_{1B} receptor (Fig. 6, B and C, respectively).

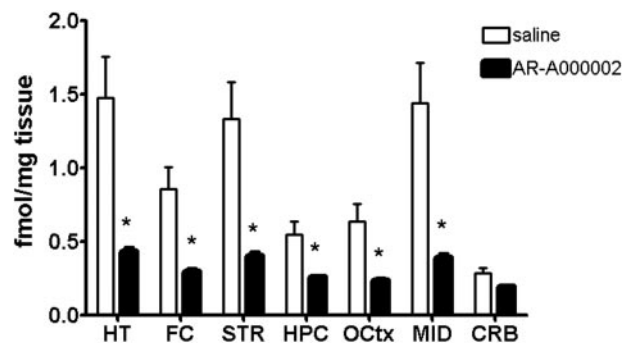


Fig. 6. [N-methyl-³H]AZ10419369 regional and pharmacological selectivity was measured (fmol/mg tissue, mean \pm S.E.M.) after pretreatment with AR-A00002 (100 mg/kg s.c.) or saline. AR-A00002 significantly blocked [N-methyl-³H]AZ10419369 binding (*, $p < 0.05$) in the HT, FC, STR, HPC, OCTx, and MID, but not in the CRB.

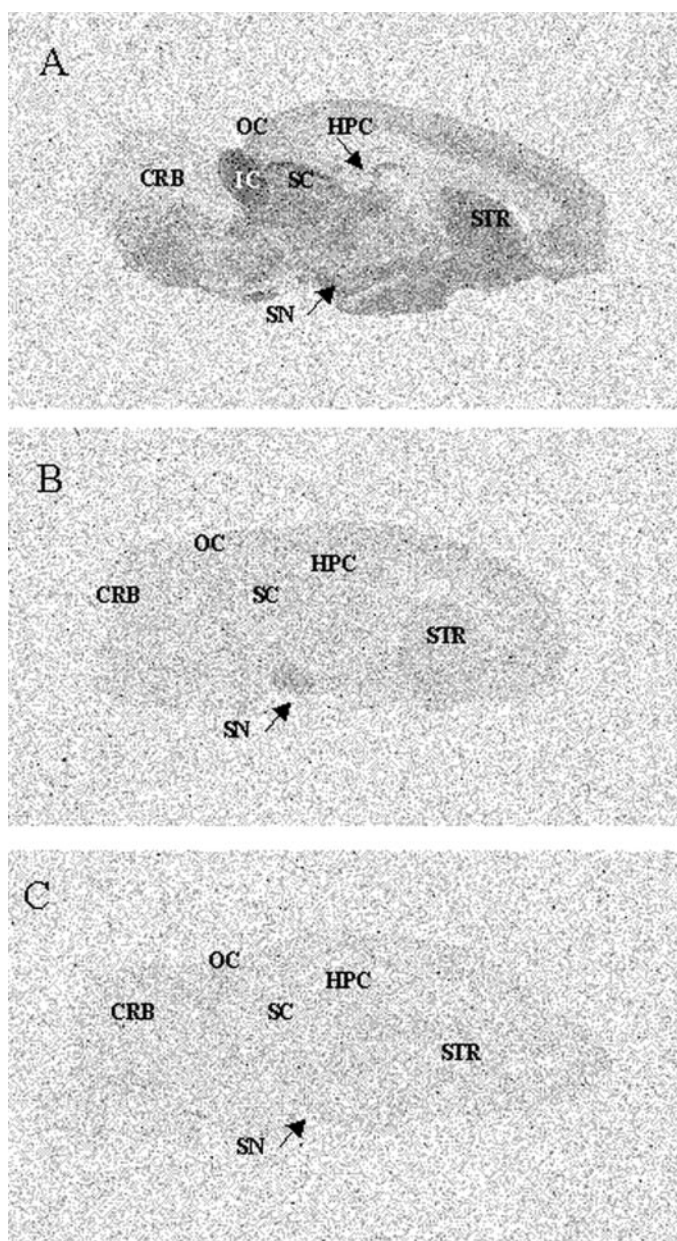


Fig. 7. Autoradiograms demonstrate in vivo brain uptake and regional selectivity for $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ in the adult guinea pig brain. A, $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ total binding 30 min after injection (15 $\mu\text{Ci/ml}$ i.v.). B, AR-A00002 (100 mg/kg s.c.) pretreatment displaced $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ binding in 5-HT_{1B} target regions. C, AZD1134 (10 mg/kg s.c.) pretreatment displaced $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ binding in 5-HT_{1B} target regions: substantia nigra (SN), superior colliculus (SC), inferior colliculus (IC), STR, HPC, cerebral cortex including the occipital cortex (OC), and CRB.

In Vivo Receptor Occupancy Using $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$. $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ binding was dose-dependently reduced after pretreatment with AZD1134 or AR-A000002 (Fig. 8) in the hypothalamus, frontal cortex, striatum, midbrain, including substantia nigra, hippocampus, and occipital cortex (target regions). One-way ANOVA for $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ binding showed a significant main effect of AR-A000002 treatment in all target regions ($p < 0.001$). Pairwise comparisons indicated that AR-A000002 significantly blocked $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ binding at 10, 25, 50, and 100 mg/kg ($p < 0.05$) compared

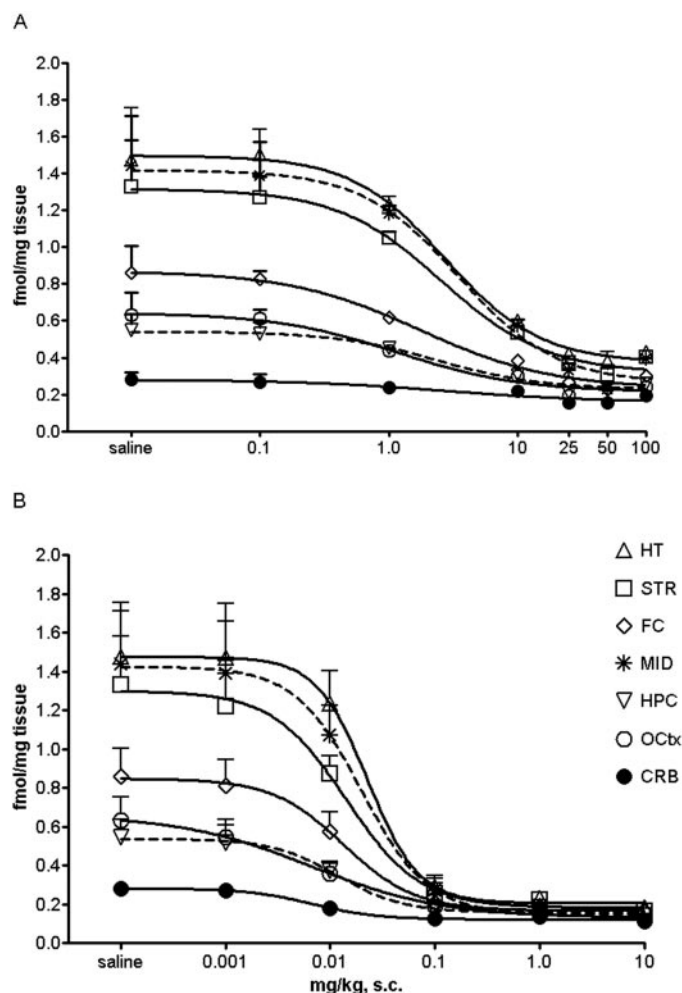


Fig. 8. $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ in vivo binding is inhibited by pretreatment with AR-A000002 and AZD1134. A, AR-A000002 (0.1–100 mg/kg s.c.) significantly blocked $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ binding at 10, 25, 50, and 100 mg/kg ($p < 0.05$) in HT, STR, FC, MID, HPC, and OCTx. B, AZD1134 (0.001–10 mg/kg s.c.) significantly blocked $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ binding at 10, 25, 50, and 100 mg/kg ($p < 0.05$) in HT, STR, FC, MID, HPC, and OCTx. Binding in the CRB was not inhibited by AR-A000002 or AZD1134. HT, STR (solid line), FC (solid line), MID (dashed line), HPC (dashed line), OCTx (solid line), and CRB (solid line). Total binding (fmol/mg tissue, mean \pm S.E.M.) inhibition curves for saline pretreatment (saline) and all administered doses (mg/kg s.c.) are shown.

with saline pretreatment in HT, FC, STR, MID, HPC, and OCTx (Fig. 8A). One-way ANOVA for $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ binding showed a significant main effect of AZD1134 treatment ($p < 0.001$) in all target regions. Pairwise comparisons indicated that AZD1134 significantly blocked $[N\text{-methyl-}^3\text{H}]\text{AZ10419369}$ binding at 0.1, 1, and 10 mg/kg ($p < 0.05$) compared with saline pretreatment in HT, FC, STR, MID, HPC, and OCTx (Fig. 8B).

Exposure in the plasma measured 1 h after subcutaneous administration of AR-A000002 or AZD1134 showed comparable mean plasma values for the 0.1, 1.0, and 10 mg/kg doses (2.62, 34.70, 261.82 nM free for AR-A000002 and 3.80, 23.56, 93.85 nM free for AZD1134, respectively). Although the 10 mg/kg dose was 100-fold higher than the saturating occupancy dose (0.1 mg/kg) for AZD1134, the 10 mg/kg dose was the lowest significant dose for AR-A000002. However, the difference in plasma concentration at 10 mg/kg was less than 2.7-fold. Similar percentage free values in guinea pig plasma

for AR-A000002 (33%) and AZD1134 (48%) suggest comparable percentage free exposure in the brain.

Receptor occupancy of AR-A000002 and AZD1134 was calculated from specific binding of [N-methyl-³H₃]AZ10419369 and represented as a percentage of the vehicle occupancy (Fig. 9). In the hypothalamus, frontal cortex, striatum, midbrain, including substantia nigra, hippocampus, and occipital cortex (target regions), the percentage receptor occupancy for AZD1134 was greater than AR-A000002 at lower administered doses. For both AZD1134 and AR-R000002, percentage receptor occupancy curves for all target regions were aligned, with only slight variations across the dose range. Occupancy curves were fit, and nonlinear regression analysis was used to calculate the ED₅₀ values for each compound tested (Table 3). The ED₅₀ values represent 50% receptor occupancy. In target regions with the highest [N-methyl-³H₃]AZ10419369 binding, such as the hypothalamus, striatum, and midbrain, the variability in ED₅₀ was less than 1.5-fold for both AR-R000002 and AZD1134. For further comparison, we combined all of the regions sampled and determined that the median ED₅₀ for AR-R000002 is 2.5 mg/kg s.c., the median ED₅₀ for AZD1134 is 0.0175 mg/kg s.c., AR-A000002 EC₅₀ = 240 nM (79.2 nM free), and AZD1134 EC₅₀ = 1.6 nM (0.77 nM free) at 50% receptor occupancy.

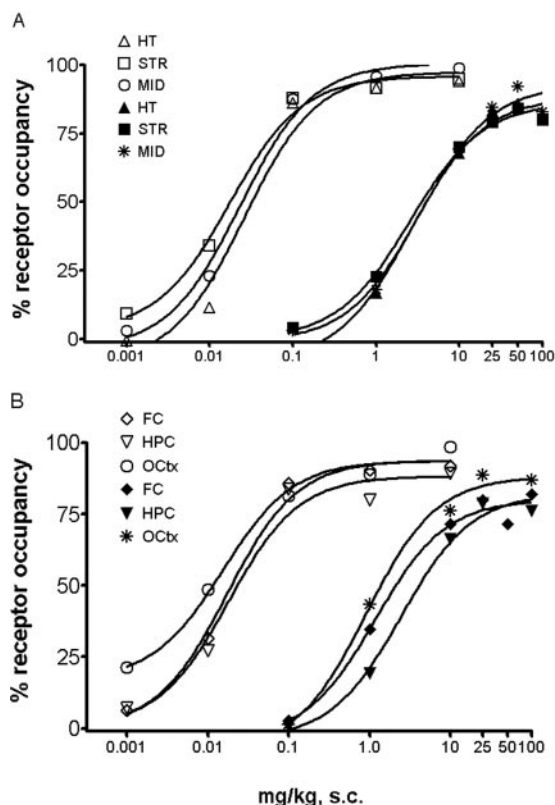


Fig. 9. In vivo occupancy for AZD1134 and AR-A000002 in the guinea pig brain using [N-methyl-³H₃]AZ10419369. A, percentage of receptor occupancy in the STR, HT, and MID shows a right shift in occupancy for AZD1134 (open symbols) compared with AR-R000002 (solid symbols). B, percentage of receptor occupancy in the FC, HPC, and OCTx shows a right shift in occupancy for AZD1134 (open symbols) compared with AR-R000002 (solid symbols).

Discussion

[N-methyl-³H₃]AZ10419369 demonstrates high-affinity specific binding at the human, monkey, and guinea pig 5-HT_{1B} receptors. In vitro binding studies demonstrated that [N-methyl-³H₃]AZ10419369 was suitable for in vivo occupancy characterization. After bolus intravenous administration, [N-methyl-³H₃]AZ10419369 enters the brain and binds selectively to regions containing the 5-HT_{1B} receptor in the guinea pig brain. Regional uptake of [N-methyl-³H₃]AZ10419369 is inhibited by pretreatment with 5-HT_{1B} ligands, AR-A000002 and AZD1134, further supporting selectivity of the radioligand. The in vivo occupancy for AR-A000002 and AZD1134 mirrors the potency and compound differentiation determined by using in vitro competition binding experiments in recombinant membranes, suggesting that [N-methyl-³H₃]AZ10419369 is a suitable tool for profiling a novel compound with 5-HT_{1B} pharmacology.

Saturation binding studies indicated that [N-methyl-³H₃]AZ10419369 bound to a single site on membranes expressing recombinant human or guinea pig 5-HT_{1B} receptors. The affinity of [N-methyl-³H₃]AZ10419369 determined from saturation binding studies was similar for the human and guinea pig receptors ($K_d \sim 0.37$ nM). The B_{max} values for [N-methyl-³H₃]AZ10419369 are similar to those determined with the [³H]GR125743 ligand and ranged from 8.76 (human 5-HT_{1B}) to 13.013 pmol/mg protein (guinea pig 5-HT_{1B}). In native tissue, [N-methyl-³H₃]AZ10419369 showed high-affinity binding with a B_{max} value of 0.131 pmol/mg in monkey cortex and 0.349 to 0.099 pmol/mg in guinea pig brain. The receptor densities determined in these brain regions are in line with those reported for [³H]GR125743 and [³H]5-carboxytryptamine (Audinot et al., 1997) in frontal cortex and with ligands known to bind to additional receptors (5-HT_{1D} and 5-HT₇).

According to Passchier et al. (2002), the B_{max} should be at least four times greater than the K_d to ensure a good signal-to-noise ratio for radiotracers used to measure drug receptor occupancy. The [N-methyl-³H₃]AZ10419369 ratio is >10 in all of the regions tested, suggesting that this criterion has been achieved. In vivo the dose-response data for [N-methyl-³H₃]AZ10419369 brain uptake at 15 min after injection show a linear uptake and saturation at ≥ 25 μ Ci/ml. [N-methyl-³H₃]AZ10419369-specific uptake in all target regions at 30 min with the 15 μ Ci/ml dose was greater than two times (and up to 5-fold) the uptake in the cerebellum, the nontarget region, providing further support for in vivo selectivity of this radiotracer.

The slow dissociation kinetics for [N-methyl-³H₃]AZ10419369 shown in vitro were reflected in the in vivo time-binding curves generated after intravenous administration. Slow dissociation did not interfere with regional differentiation of [N-methyl-³H₃]AZ10419369 uptake or specific binding of [N-methyl-³H₃]AZ10419369. Specific binding in 5-HT_{1B} receptor dense regions was greater than binding in the cerebellum at all of the time points measured. [N-methyl-³H₃]AZ10419369 dissociation kinetics did not disrupt our ability to determine AR-A000002 or AZD1134 occupancy at in vivo saturation (30 min after intravenous injection). In addition, slow dissociation in vitro or in vivo did not hinder the development of [N-methyl-³H₃]AZ10419369 as a PET ligand for primates and humans (Pierson et al., 2007).

TABLE 3

In vivo occupancy of AR-A000002 and AZD1134 by using [*N*-methyl-³H₃]AZ10419369
ED₅₀ values for AR-A000002 and AZD1134 in vivo occupancy in the guinea pig brain are shown.

Compounds	ED ₅₀					
	HT	FC	STR	MID	HPC	OCtx
	mg/kg					
AR-A000002	2.617	1.166	2.547	3.362	2.431	0.8963
AZD1134	0.02580	0.01707	0.01726	0.0230	0.01790	0.01525

[*N*-methyl-³H₃]AZ10419369 selectivity was evaluated in vitro by using selective 5-HT_{1B} receptor ligands, AR-A000002 and AZD1134, 5-HT, and citalopram. Although the dissociation rate of [*N*-methyl-³H₃]AZ10419369 is slow, a relative potency can be determined for the compounds, allowing a reasonable comparison ranking. The IC₅₀ values for these 5-HT_{1B}-selective compounds indicate the rank order of potency to be AZ10419369 > AZD1134 > AR-A000002 > 5-HT. Citalopram did not compete with either radioligand. The rank-order potency we observed with [*N*-methyl-³H₃]AZ10419369 correlated well with our results for both human and guinea pig 5-HT_{1B} receptors using [³H]GR125743, the high-affinity 5-HT_{1B/1D} antagonist radioligand tool. Consistent with our results using unlabeled AZ10419369 and [³H]GR125743, [*N*-methyl-³H₃]AZ10419369 has subnanomolar affinity for both the guinea pig and human 5-HT_{1B} receptor.

[*N*-methyl-³H₃]AZ10419369 in vivo regional distribution and pharmacological selectivity for the 5-HT_{1B} receptor were shown in autoradiograms and by measurement of radioactivity in dissected regions of the guinea pig brain. The 5-HT_{1B} binding pattern for [*N*-methyl-³H₃]AZ10419369 was consistent with localization of the protein in the guinea pig (Audinot et al., 1997; Bonaventure et al., 1998) and was inhibited by pretreatment with 5-HT_{1B} ligands. The highest binding pattern of [*N*-methyl-³H₃]AZ10419369 in striatopallidal and striatonigral GABAergic system is in accord with the diverse functional role of the 5-HT_{1B} receptor as heteroreceptor on nonserotonergic fiber terminals (Sari, 2004; Descarries et al., 2006). Similarities to the binding pattern of [*N*-methyl-³H₃]AZ10419369 in the macaque monkey (Pierson et al., 2008) were found in the guinea pig brain and support the preclinical and translational value of [*N*-methyl-³H₃]AZ10419369.

The results of our in vitro competition binding studies by using [*N*-methyl-³H₃]AZ10419369 were confirmed by in vivo binding studies. [*N*-methyl-³H₃]AZ10419369 in vivo binding was blocked by 5-HT_{1B} ligands, AZD1134 and AR-A000002, when measured in 5-HT_{1B}-containing regions (HT, FC, STR, MID, HPC, and OCtx), with ~100-fold relative difference in the ED₅₀ and EC₅₀ between AZD1134 and AR-A000002. The in vivo occupancy for AR-A000002 and AZD1134 mimics the relative order for the in vitro compound potency for binding at the guinea pig receptor and published efficacious doses.

AR-A000002 is a high-affinity 5-HT_{1B}-selective ligand in cloned guinea pig 5-HT_{1B} and guinea pig cortical membranes (Ahlgren et al., 2004). In a [³⁵S]GTPγS functional assay, AR-A000002 partially inhibited 5-HT-induced coupling of the 5-HT_{1B} receptor to G-proteins and alone partially induced coupling, suggesting partial antagonist and partial agonist activity in vitro. AR-A000002 (1–10 mg/kg s.c.) significantly attenuated 5-HT_{1D/B} agonist (CP135,807)-induced hypother-

mic effects in the guinea pig (Stenfors et al., 2004) and was efficacious in animal models of depression (Hudzik et al., 2003b) at doses consistent with our in vivo receptor occupancy ED₅₀ values and pretreatment doses that produce significant inhibition of [*N*-methyl-³H₃]AZ10419369-specific binding. The pharmacokinetics of AR-A000002 exposure in the brain tracks parallel the plasma exposure, with maximal brain concentration ~1 h after 2.2 mg/kg s.c. administration, and the percentage free in guinea pig plasma = 33%. In the guinea pig, AR-A000002 (1.0 mg/kg s.c.) reduced escape failures in a learned helplessness model and, at 30 mg/kg s.c., significantly increased the number of learned responses in a differential reinforcement of low rates test without reducing the number of responses emitted, consistent with antidepressant activity (Hudzik et al., 2003b).

AZD1134 is a high-affinity 5-HT_{1B} antagonist (Horchler et al., 2007). In an in vitro [³⁵S]GTPγS functional assay, AZD1134 inhibits 5-HT-induced coupling of the 5-HT_{1B} receptor to G-proteins with no intrinsic activity when tested alone, supporting full antagonist activity for AZD1134. In the guinea pig, AZD1134 (0.1 mg/kg s.c.) significantly attenuates 5-HT_{1D/B} agonist (CP135,807)-induced hypothermic effects. AZD1134 was efficacious in animal models of anxiety and depression (Hudzik et al., 2003a) at in vivo receptor occupancy ED₅₀ values and pretreatment doses that result in significant inhibition of [*N*-methyl-³H₃]AZ10419369-specific binding. In the guinea pig, AZD1134 (0.1–1.0 mg/kg) decreased escape failures in a learned helplessness model (Hudzik et al., 2003a). The pharmacokinetics of AZD1134 exposure in the brain tracks parallel the plasma exposure, with brain concentration in excess of free plasma concentration in plasma (guinea pig % free = 48%). Using [*N*-methyl-³H₃]AZ10419369, we have been able to differentiate candidate drugs and provide receptor occupancy data preclinically to facilitate compound selection and dose prediction.

Compounds that inhibit the 5-HT_{1B} autoreceptor, such as AR-A000002, enhance serotonin release (Stenfors et al., 2004). Radioligands have been used to measure changes in brain levels of endogenous neurotransmitters in the serotonin (Udo de Haes et al., 2005) and dopamine systems (Marengo et al., 2004; Seneca et al., 2006). Because the radioligand competes with the endogenous neurotransmitter for receptor binding sites, an increase the endogenous neurotransmitter would reduce radioligand binding. Thus, inhibition in [*N*-methyl-³H₃]AZ10419369 binding for 5-HT_{1B} ligands may, in fact, be a combination of compound occupancy at the 5-HT_{1B} receptor and binding from released endogenous serotonin. For compounds like citalopram that do not compete with [*N*-methyl-³H₃]AZ10419369, the in vivo occupancy using [*N*-methyl-³H₃]AZ10419369 would only represent an increase in endogenous serotonin. Further investigation is required to differentiate 5-HT_{1B} ligand receptor occupancy from mea-

sured changes in functional levels of endogenous 5-HT by using [N-methyl-³H₃]AZ10419369.

Our in vitro and in vivo characterization of a novel 5-HT_{1B} radioligand tool indicates that [N-methyl-³H₃]AZ10419369 is a high-affinity preclinical tool for investigating novel compound in vivo occupancy at the 5-HT_{1B} receptor in the guinea pig brain. PET imaging studies in the cynomolgus monkey using [¹¹C]AZ10419369 (Pierson et al., 2007) add further support for the translational value of this radioligand in drug discovery.

Acknowledgments

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References

- Ahlgren C, Eriksson A, Tellefors P, Ross SB, Stenfors C, and Malmberg A (2004) In vitro characterization of AR-A000002, a novel 5-hydroxytryptamine_{1B} autoreceptor antagonist. *Eur J Pharmacol* **499**:67–75.
- Audinat V, Lochon S, Newman-Tancredi A, Lavielle G, and Millan MJ (1997) Binding profile of the novel 5HT_{1B/1D} receptor antagonist, [³H]GR125,743, in guinea pig brain: a comparison with [³H]5-carboxamidotryptamine. *Eur J Pharmacol* **327**:247–256.
- Bonaventure P, Voorn P, Luyten WH, Jurzak M, Schotte A, and Leysen JE (1998) Detailed mapping of serotonin 5-HT_{1B} and 5-HT_{1D} receptor messenger RNA and ligand binding sites in guinea-pig brain and trigeminal ganglion: clues for function. *Neuroscience* **82**:469–484.
- Briley M and Moret C (1993) Neurobiological mechanisms involved in antidepressant therapies. *Clin Neuropharmacol* **16**:387–400.
- Dawson LA, Hughes ZA, Starr KR, Storey JD, Bettelini L, Bacchi F, Arban R, Poffe A, Melotto S, Hagan JJ, et al. (2006) Characterisation of the selective 5-HT_{1B} receptor antagonist SB-616234-A (1-[6-(*cis*-3,5-dimethylpiperazin-1-yl)-2,3-dihydro-5-methoxyindol-1-yl]-1-[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) biphenyl-4-yl]methanone hydrochloride): in vivo neurochemical and behavioural evidence of anxiolytic/antidepressant activity. *Neuropharmacology* **50**:975–983.
- Descarries L, Cornea-Hebert V, and Riad M (2006) Cellular and subcellular localization of serotonin receptors in the central nervous system, in *The Serotonin Receptors: From Mol Pharmacol to Human Therapeutics* (Roth BL ed) pp 39–58, Humana Press, Totowa, NJ.
- Doménech T, Beleta J, and Palacios JM (1997) Characterization of human serotonin 1D and 1B receptors using [³H]GR-125743, a novel radiolabeled serotonin 5HT_{1B/1D} receptor antagonist. *Naunyn Schmiedeberg Arch Pharmacol* **356**:328–334.
- Engel G, Göthert M, Hoyer D, Schlicker E, and Hillenbrand K (1986) Identity of inhibitory presynaptic 5-hydroxytryptamine (5-HT) autoreceptors in the rat brain cortex with 5-HT_{1B} binding sites. *Naunyn Schmiedeberg Arch Pharmacol* **332**:1–7.
- Horchler CL, McCauley JP Jr, Hall JE, Snyder DH, Moore WC, Hudzik TJ, and Chapdelaine MJ (2007) Synthesis of novel quinolone and quinoline-2-carboxylic acid (4-morpholin-4-yl-phenyl)amides: A late-stage diversification approach to potent 5HT_{1B} antagonists. *Bioorg Med Chem* **15**:939–950.
- Hoyer D, Hannon JP, and Martin GR (2002) Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol Biochem Behav* **71**:533–554.
- Huang Y, Bae SA, Roth BL, and Laruelle M (2005) Synthesis of potent and selective serotonin 5-HT_{1B} receptor ligands. *Bioorg Med Chem Lett* **15**:4786–4789.
- Hudzik T, Smolka J, Litwin L, Porrey T, and Pierson E (2003a) In vivo pharmacology of AZD1134, a novel 5-HT_{1B} antagonist. *Eur Neuropsychopharmacol* **13**:S181–S182.
- Hudzik TJ, Yanek M, Porrey T, Evenden J, Paronis C, Mastrangelo M, Ryan C, Ross S, and Stenfors C (2003b) Behavioral pharmacology of AR-A00002, a novel, selective 5-hydroxytryptamine_{1B} antagonist. *J Pharmacol Exp Ther* **304**:1072–1084.
- Marenco S, Carson RE, Berman KF, Herscovitch P, and Weinberger DR (2004) Nicotine-induced dopamine release in primates measured with [¹¹C]Raclopride. *Neuropsychopharmacology* **29**:259–268.
- Matzen L, van Amsterdam C, Rautenberg W, Greiner HE, Harting J, Seyfried CA, and Böttcher H (2000) 5-HT reuptake inhibitors with 5-HT_{1B/1D} antagonistic activity: a new approach toward efficient antidepressants. *J Med Chem* **43**:1149–1157.
- McCarthy TJ, Hogland U, Antoni G, Lindhe O, Sobolov S, Gray P, Schaeffer E, Poe R, Zhang L, Bergstrom M, et al. (2007) Discovery and qualification of the first 5HT_{1B} selective PET radiotracer using a novel PET radiotracer development paradigm. *Joint Molecular Imaging Conference*; 2007 Sept 8–11; Providence, RI. No. 0985, The Society for Molecular Imaging, Kerrville, TX.
- Moret C and Briley M (2000) The possible role of 5-HT_{1B/1D} receptors in psychiatric disorders and their potential as a target for therapy. *Eur J Pharmacol* **404**:1–12.
- Passchier J, Gee A, Willemsen A, Vaalburg W, and van Waarde A (2002) Measuring drug-related receptor occupancy with positron emission tomography. *Methods* **27**:278–286.
- Pierson ME, Andersson J, Nyberg S, McCarthy DJ, Varnas K, Finnema S, Gulyás B, Takano A, Karlsson P, Medd AM, et al. (2007) [¹¹C] AZ10419369: a selective 5-HT_{1B} receptor radioligand suitable for positron emission tomography (PET). Characterization in nonhuman primate and human brain. *American College of Neuropsychopharmacology Conference*; 2007 Dec 9–13; Boca Raton, FL. No. 101, American College of Neuropsychopharmacology, Nashville, TN.
- Pierson ME, Andersson J, Nyberg S, McCarthy DJ, Finnema SJ, Varnäs K, Takano A, Karlsson P, Gulyás B, Medd AM, et al. (2008) [¹¹C] AZ10419369: a selective 5-HT_{1B} receptor radioligand suitable for positron emission tomography (PET) characterization in the primate brain. *NeuroImage* **41**:1075–1085.
- Roberts C, Price GW, and Middlemiss DN (2001) Ligands for the investigation of 5-HT autoreceptor function. *Brain Res Bull* **56**:463–469.
- Sari Y (2004) Serotonin_{1B} receptors: from protein to physiological function and behavior. *Neurosci Biobehav Rev* **28**:565–582.
- Sari Y, Miquel MC, Brisorgueil MJ, Ruiz G, Doucet E, Hamon M, and Vergé D (1999) Cellular and subcellular localization of 5-hydroxytryptamine_{1B} receptors in the rat central nervous system: immunocytochemical, autoradiographic and lesion studies. *Neuroscience* **88**:899–915.
- Seneca N, Finnema SJ, Farde L, Gulyás B, Wikström HV, Halldin C, and Innis RB (2006) Effect of amphetamine on dopamine D₂ receptor binding in nonhuman primate brain: a comparison of the agonist radioligand [¹¹C]MNPDA and [¹¹C]raclopride. *Synapse* **59**:260–269.
- Smagin GN, Song D, Cross AJ, and Mrzljak L (2003) Activation of serotonin turnover and release in guinea pig brain by AZD1134, a novel high affinity 5-HT_{1B} receptor antagonist. *Annual Meeting of the Society for Neuroscience*; 2003 Nov 8–12; New Orleans, LA. No. 959.4, Society for Neuroscience, Washington, DC.
- Stenfors C, Hallerbäck T, Larsson LG, Wallsten C, and Ross SB (2004) Pharmacology of a novel selective 5-hydroxytryptamine_{1B} receptor antagonist, AR-A000002. *Naunyn Schmiedeberg Arch Pharmacol* **369**:330–337.
- Svenningsson P, Chergui K, Rachleff I, Flajolet M, Zhang X, El Yacoubi M, Vaugoeis JM, Nomikos GG, and Greengard P (2006) Alterations in 5-HT_{1B} receptor function by P11 in depression-like states. *Science* **311**:77–80.
- Udo de Haes JI, Cremers TI, Bosker FJ, Postema F, Tiemersma-Wegman TD, and den Boer JA (2005) Effect of increased serotonin levels on [18F] MPFF binding in the rat brain: fenfluramine vs the combination of citalopram and ketanserin. *Neuropsychopharmacology* **30**:1624–1631.
- Varnäs K, Hall H, Bonaventura P, and Sedvall G (2001) Autoradiographic mapping of 5-HT_{1B} and 5-HT_{1D} receptors in the post mortem human brain using [³H]GR 125743. *Brain Res* **915**:47–57.
- Varnäs K, Hurd YL, and Hall H (2005) Regional expression of 5-HT_{1B} receptor mRNA in the human brain. *Synapse* **56**:21–28.
- Westkaemper RB and Roth BL (2006) The serotonin receptors: from mol pharmacol to human therapeutics, in *The Serotonin Receptors: From Mol Pharmacol to Human Therapeutics* (Roth BL ed) pp 39–58, Humana Press, Totowa, NJ.
- Zgombick JM, Bard JA, Kucharewicz SA, Urquhart DA, Weinschenk RL, and Branchek TA (1997) Molecular cloning and pharmacological characterization of guinea pig 5-HT_{1B} and 5-HT_{1D} receptors. *Neuropharmacology* **36**:513–524.

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