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Integrated Strategy for Use of Positron Emission Tomography in Nonhuman Primates to Confirm Multitarget Occupancy of Novel Psychotropic Drugs: An Example with AZD3676

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ABSTRACT

Positron emission tomography (PET) is widely applied in central nervous system (CNS) drug development for assessment of target engagement in vivo. As the majority of PET investigations have addressed drug interaction at a single binding site, findings of multitarget engagement have been less frequently reported and have often been inconsistent with results obtained in vitro. AZD3676 [*N,N*-dimethyl-7-(4-(2-(pyridin-2-yl)ethyl)piperazin-1-yl) benzofuran-2-carboxamide] is a novel combined serotonin (5-hydroxytryptamine) 5-HT_{1A} and 5-HT_{1B} receptor antagonist that was developed for the treatment of cognitive impairment in Alzheimer's disease. Here, we evaluated the properties of AZD3676 as a CNS drug by combining in vitro and ex vivo radioligand binding techniques, behavioral pharmacology in rodents, and PET imaging in nonhuman primates. Target engagement in the nonhuman primate brain was assessed in PET studies by determination of drug-induced occupancy using receptor-selective radioligands. AZD3676 showed preclinical properties consistent with CNS drug potential, including nanomolar receptor affinity and efficacy in rodent models of learning and memory. In PET studies of the monkey brain, AZD3676 inhibited radioligand binding in a dose-dependent manner with similar affinity at both receptors. The equally high affinity at 5-HT_{1A} and 5-HT_{1B} receptors as determined in vivo was not predicted from corresponding estimates obtained in vitro, suggesting more than 10-fold selectivity for 5-HT_{1A} versus 5-HT_{1B} receptors. These findings support the further integrated use of PET for confirmation of multitarget occupancy of CNS drugs. Importantly, earlier introduction of PET studies in nonhuman primates may reduce future development costs and the requirement for animal experiments in preclinical CNS drug development programs.

Introduction

Discovery and development of drugs for diseases of the central nervous system (CNS) represent a major challenge to the pharmaceutic industry, with a higher rate of attrition than for other therapeutic areas (Kola and Landis, 2004; Miller, 2010). Given the complexity of the brain and cerebral vasculature, one of the main challenges in the development of efficacious CNS drugs is to predict brain exposure and target engagement based primarily on drug affinity in vitro, animal behavior data, and drug concentrations in plasma (Liu et al., 2005; Summerfield and Jeffrey, 2006; Reichel, 2009).

Additional information can be obtained by positron emission tomography (PET), an imaging methodology that allows the concentration and time course of a drug molecule as labeled with a radionuclide to be traced within the body. This approach, commonly referred to as PET microdosing (Bergström et al., 2003), has over recent years been applied to study the brain exposure of large sets of established CNS

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ABBREVIATIONS: AZD3676, *N,N*-dimethyl-7-(4-(2-(pyridin-2-yl)ethyl)piperazin-1-yl) benzofuran-2-carboxamide; BP_{ND}, binding potential; AZ10419369, 5-methyl-8-(4-methylpiperazin-1-yl)-*N*-(4-morpholin-4-ylphenyl)-4-oxochromene-2-carboxamide; WAY-100635, *N*-[2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]-*N*-pyridin-2-ylcyclohexanecarboxamide; CFC, contextual fear conditioning; CNS, central nervous system; $f_{u,\ plasma}$, unbound fraction in plasma; 5-HT, 5-hydroxytryptamine (serotonin); $K_{i,\ plasma}$, plasma concentration required for 50% receptor occupancy; MRI, magnetic resonance imaging; NAD-299, (*R*)-3-*N*,*N*-dicyclobutylamino-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide hydrogen (2*R*,3*R*)-tartrate monohydrate; PET, positron emission tomography; R-8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; ROI, region of interest; WAY-100634, *N*-[2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]pyridin-2-amine.

drugs and experimental compounds using systematic methods of analysis (Gunn et al., 2012; Schou et al., 2015).

For the use of PET to demonstrate drug binding to the intended target a different approach has to be applied. Here, a target-specific radioligand is used, and an indirect measure of target engagement is obtained by assessing the extent of competitive binding, or occupancy, induced by the drug. This approach has been widely applied, and there are numerous reports on the application of PET to assess drug binding at a single binding site (reviewed by Grimwood and Hartig, 2009).

A common observation for early developed antipsychotic and antidepressant drugs is that they have affinity for more than one receptor or transporter. This has led to several hypotheses on the potential therapeutic benefit of drug action at several targets. Indeed, a few PET investigations have examined established drugs and reference compounds and confirmed high occupancy at multiple binding sites (Rabiner et al., 2002; DeLorenzo et al., 2011; Comley et al., 2013; Stenkrona et al., 2013; Takano et al., 2013; Ding et al., 2014).

The finding that efficacious drugs on the market may act at multiple targets has been taken as a rationale for a multitude of industrial projects aiming at the development of novel drugs having multiple actions. This poses a particular challenge for medicinal chemistry. First, the chemists have to synthesize molecules having multiple affinities at various CNS targets. Second, and maybe even more challenging, the relative affinity at the different targets has to be of the same order to make it likely that more than one of the intended targets are engaged at clinical treatment.

However, a concern raised by several of the initial investigations is that relative affinities of multitarget drugs predicted based on in vitro affinity estimates often differ from relative affinities obtained in vivo using PET (Rabiner et al., 2002; DeLorenzo et al., 2011; Takano et al., 2013). In vitro radioligand binding experiments are often conducted under different experimental conditions, using buffer systems, pH and ionic strengths, and incubation protocols that have been optimized for the radioligand and target examined. Such optimizations for single targets may yield biased estimates of relative target affinity at physiologic conditions, so there is need for more effective strategies for preclinical development of novel molecules expected to have dual or multiple actions in humans.

AZD3676 [*N*,*N*-dimethyl-7-(4-(2-(pyridin-2-yl)ethyl)piperazin-1-yl)benzofuran-2-carboxamide (Fig. 1)] is a high-affinity antagonist at both the serotonin (5-HT) 5-HT_{1A} and 5-HT_{1B} receptors that was developed for the treatment of cognitive impairment in Alzheimer's disease. As antagonists at the 5-HT_{1A} and 5-HT_{1B} receptors have been found to enhance learning and memory through independent mechanisms in animal models (Åhlander-Lüttgen et al., 2003; Ögren et al., 2008), our project was initiated based on the hypothesis that antagonism at both these receptors may provide more efficacious pharmacologic treatment than a compound selective for any of the receptors.

In our drug discovery project on AZD3676 we applied a partly novel cross-species approach to build confidence in an expected dual drug action in humans in vivo. The preclinical pharmacology of AZD3676 was characterized by an integrated strategy using in vitro methods for assessment of drug binding to target receptors, behavioral pharmacology, and ex vivo occupancy in rodents followed by PET imaging in nonhuman primates. In the PET studies, the relationship between AZD3676 plasma exposure and drug-induced occupancy was assessed using the 5-HT_{1A} and 5-HT_{1B} receptor radioligands [^{11}C]WAY100635 [*N*-[2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]-*N*-pyridin-2-ylcyclohexanecarboxamide] and [^{11}C]AZ10419369 [5-methyl-8-(4-methylpiperazin-1-yl)-*N*-(4-morpholin-4-ylphenyl)-4-oxochromene-2-carboxamide], respectively. The final objective was to predict the exposure levels and doses required in clinical trials to ensure dual action at both targets.

Materials and Methods

In Vitro Pharmacologic Characterization

After preliminary routine screening of affinity in recombinant cells, we performed extended pharmacologic characterization of AZD3676 using competitive radioligand binding studies in monkey brain homogenates. Monkey brain tissue was homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM EDTA, incubated for 15 minutes at 37°C in the presence of 1 mM GTP and centrifuged for 10 minutes. The pellet was resuspended in membrane preparation buffer and centrifuged. The final pellet was resuspended in 0.32 M sucrose, 50 mM Tris-HCL, and 10 mM EDTA and stored at −70°C.

The concentrations of AZD3676 were selected based on affinity estimates obtained in the initial screening using recombinant human 5-HT_{1A} and 5-HT_{1B} receptors (0.16 and 2.3 nM, respectively). The 5-HT_{1A} receptor binding studies were conducted using the radioligand [^{3}H]WAY-100635 (Hall et al., 1997) at concentration 80 pM and AZD3676 concentrations ranging from 1 pM to 100 nM in 50 mM Tris-HCl buffer, pH 7.4, containing 4 mM $MgCl_2$ and 1 mM EDTA. Nonspecific binding was determined in the presence of 1 μM NAD-299 [(*R*)-3-*N*,*N*-dicyclobutylamino-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide hydrogen (2*R*,3*R*)-tartrate monohydrate] (Johansson et al., 1997).

For studies of 5-HT_{1B} receptor binding, we performed incubations with the radioligand [^{3}H]AZ10419369 (Maier et al., 2009) at concentration 0.3 nM and increasing concentrations (0.01 nM–1 μM) of AZD3676 in 50 mM Tris-HCl buffer, pH 7.4, containing 4 mM $CaCl_2$, 4 mM $MgCl_2$, and 1 mM EDTA. Nonspecific binding was determined in the presence of 1 μM methiothepin.

In both homogenate studies the incubations were performed for 16 hours at room temperature and in duplicate on separate days using 11 concentrations of AZD3676. Bound and free radioligand were separated by filtration through Whatman GF/B filters (GE Healthcare Life Science, Maidstone, United Kingdom) pretreated with polyethylenimine and subsequently washed with buffer (20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl for 5-HT_{1A} and 5 mM Tris-HCl, pH 7.4, for 5-HT_{1B} receptor studies) using a Brandel cell harvester (Brandel, Gaithersburg, MD).

In Vitro Binding of AZD3676 to Plasma Proteins

The plasma protein binding of AZD3676 (0.4–20 μM) was determined, in accordance with ultrafiltration procedures previously described by Eriksson et al. (2005) in mouse, rat, guinea pig, cynomolgus monkey, and human plasma. Plasma was prepared by thawing, mixing, and centrifugation. The pH of the plasma was adjusted to 7.4 by CO_2 incubation before the ultrafiltration.

Receptor Occupancy in Mouse, Rat, and Guinea Pig Brains

The studies were approved by the Animal Ethics Committee of the Swedish Board of Agriculture (Dnr. S115/07; S8/09; S74/09). Animals were allowed at least 1 hour of room habituation before they were injected with AZD3676. Studies of 5-HT_{1A} receptor occupancy were conducted in C57BL/6 mice (n = 4–8 animals per dose), Sprague Dawley rats (n = 4–8 per dose), and Dunkin Hartley guinea pigs (n = 3 per dose), and the studies of 5-HT_{1B} receptor occupancy were conducted in Dunkin Hartley guinea pigs (n = 3 per dose). Studies

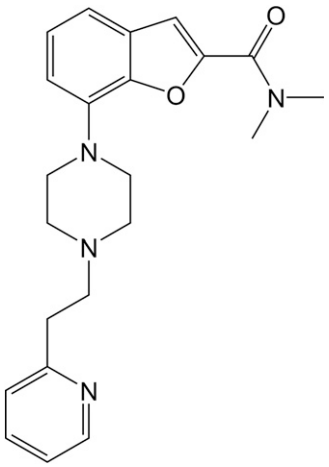

Fig. 1. Molecular structure of AZD3676.

of 5-HT_{1A} receptor occupancy were conducted in mice and rats for prediction of occupancy at efficacious doses based on in vivo pharmacology studies in mice.

Similar to reports for other 5-HT_{1B} receptor compounds (Oksenberg et al., 1992) AZD3676 showed species differences in binding to the 5-HT_{1B} receptor, with about 100-fold lower affinity for mouse than for primate receptors. The guinea pig was selected as a model species in studies of 5-HT_{1B} receptor occupancy because AZD3676 has been found to have similar affinity at the guinea pig and primate 5-HT_{1B} receptors (0.5 nM versus 2.3 nM for humans).

Occupancy of AZD3676 at the 5-HT_{1A} and 5-HT_{1B} receptors was assessed using the radioligands [^{3}H]WAY-100635 (specific radioactivity: 57–76 Ci/mmol) and [^{3}H]AZ10419369 (specific radioactivity: 54 Ci/mmol), respectively, according to procedures described by Maier et al. (2009). For [^{3}H]WAY-100635 the total radioactivity injected was 1 μCi/animal for mice, 7.5 μCi/animal for rats, and 10 μCi/animal for guinea pigs. The corresponding radioactivity was 15 μCi/guinea pig for [^{3}H]AZ10419369.

Studies of 5-HT_{1A} receptor occupancy were performed after administration of AZD3676 at s.c. doses of 0.03, 0.1, 0.3, 1.0, and 3.0 μmol/kg for mice, 0.1, 1.0, 3.0, and 10.0 μmol/kg for rats, and 0.3, 1.0, and 10.0 μmol/kg for guinea pigs, and studies of 5-HT_{1B} receptor occupancy in guinea pigs were performed with AZD3676 doses of 0.3, 1.0, 3.0, and 10.0 μmol/kg s.c.

In studies of 5-HT_{1A} and 5-HT_{1B} receptor occupancy, the animals received vehicle or AZD3676 followed by i.v. injections of [^{3}H]WAY-100635 or [^{3}H]AZ10419369, respectively, 5 minutes later for studies in mice and guinea pigs and 30 minutes later in rats.

Animals were sacrificed 30 minutes after radioligand injection. This time interval was defined in accordance with previous ex vivo studies using [^{3}H]AZ10419369 (Maier et al., 2009) and [^{3}H]WAY-100635 (Hirst et al., 2008). The time relative to AZD3676 administration (35 minutes for mice and guinea pigs and 60 minutes for rats) was selected based on the time profile for CNS exposure for the three species and guided by the physiologic response (reversal of agonist-induced hypothermia) recorded at different time points after AZD3676 administration as well as receptor-binding kinetics determined in vitro (unpublished observations). The brain was removed and dissected into selected regions (striatum, frontal cortex, hippocampus, and cerebellum). Tissue regions were weighed and solubilized overnight, and the radioactivity was measured using a TriCarb2900 scintillation counter (PerkinElmer, Waltham, MA).

Binding to the 5-HT_{1A} receptor was determined in hippocampus and frontal cortex and binding to the 5-HT_{1B} receptor was determined in striatum and frontal cortex, regions known to have a high density of the respective receptor subtype. The binding potential was calculated as the difference in binding (dpm/mg) between the target region and cerebellum divided by the binding in the cerebellum. Occupancy was determined as the percentage difference in binding potential after AZD3676 administration relative to vehicle treatment.

In Vivo Pharmacology

Efficacy in Mouse Contextual Fear Conditioning Model with Induced Memory Deficit. The studies were approved by the Animal Ethics Committee of the Swedish Board of Agriculture (Dnr S6/09). Contextual fear conditioning (CFC) evaluates the learned aversion of an animal for an environment that has previously been associated with a mild aversive stimulus. This test has been shown to depend on hippocampus and amygdala functions. Two different approaches were used to address AZD3676 effects on serotonergic-induced deficit and cholinergic-induced deficit. Studies were conducted in C57BL/6 mice (n = 10–12 per treatment group).

1. *R-8-OH-DPAT-induced memory deficit.* The 5-HT_{1A} receptor agonist R-8-OH-DPAT [8-hydroxy-2-(di-n-propylamino)tetralin] (Arvidsson et al., 1981) has been reported to induce memory deficits, including aversive learning impairments, in rodents (Sanger and Joly, 1989; Carli et al., 1992; Misane and Ögren, 2000; Lüttgen et al., 2005). The learning impairments induced by R-8-OH-DPAT in different memory tests are likely an effect of postsynaptic 5-HT_{1A} receptor stimulation (Carli et al., 1993; Misane et al., 1998; Misane and Ögren, 2000). In the mouse CFC model, R-8-OH-DPAT has been shown to induce memory deficit at a dose high enough to affect the postsynaptic 5-HT_{1A} receptors (0.6 μmol/kg s.c.). During the training session the animal was placed in a box and allowed to explore the environment. After a defined time, the animal was exposed to a short-lasting (2-second) electric shock current (0.7 mA) and allowed to stay in the same environment for 30 seconds to make the association with the aversive experience and the environment. Twenty-four hours later the animal was placed in the same box. During the test session the animals were videotaped, and the freezing response was scored manually afterward based on the video recordings.
2. *Scopolamine-induced memory deficit.* Scopolamine was used to mimic the cholinergic deficits found in Alzheimer disease patients (Lenz et al., 2012). During the training session, the animal was placed in a box and allowed to explore the environment. After a defined time, the animal was exposed to a short-lasting (2-second) electric shock current (0.7 mA) and allowed to stay in the same environment for 30 seconds to make the association. After this 30-second period, the animal was once again exposed to an electric shock current (again 0.7 mA) for 2 seconds to facilitate the aversive experience. Again, the animal was left in the box for 30 seconds to create an association with the aversive experience and the environment. Twenty-four hours later the animal was placed in the same box. During the test session the animals were videotaped, and the freezing response was scored manually afterward.

PET Studies in Monkeys

Carbon-11 Labeling. [^{11}C]WAY-100635 was prepared by ^{11}C-acylation of WAY-100634 with cyclohexane[carbonyl-^{11}C]chloride (Pike et al., 1995), as previously described in detail by Hall et al. (1997). [^{11}C]AZ10419369 was prepared by *N*-methylation of the desmethyl precursor 8-(1-piperazinyl)-5-methylchrom-2-en-4-one-2-(4-morpholinophenyl) carboxamide (AstraZeneca R&D, Wilmington, DE), using carbon-11 methyl triflate, as has been described in the literature (Pierson et al., 2008; Andersson et al., 2011).

PET and Magnetic Resonance Imaging Procedures. The study was approved by the Animal Ethics Committee of the Swedish Animal Welfare Agency (Dnr 145/08, 399/08, and 386/09) and was performed according to the "Guidelines for Planning, Conducting and

Documenting Experimental Research" (Dnr 4820/06-600) of the Karolinska Institutet as well as the "Guide for the Care and Use of Laboratory Animals" (Clark et al., 1997). Four cynomolgus monkeys (weighing 3.8–6.2 kg) were supplied by Astrid Fagraeus Laboratory, Karolinska Institutet, Solna, Sweden. Anesthesia was induced by intramuscular injection of ketamine hydrochloride (approximately 10 mg/kg) and maintained by the administration of a mixture of sevoflurane, oxygen, and medical air after endotracheal intubation. The monkey was observed continuously during the PET experimental days. Body temperature was maintained by Bair Hugger Model 505 (Arizant Healthcare, Eden Prairie, MN) and monitored by an esophageal thermometer. Heart and respiration rates were continuously monitored during the experiment. We noted no anesthesia or drug-related effects on the vital parameters or other functions that we measured (oxygen saturation, rectal temperature). The monkey head was immobilized with a head-fixation system (Karlsson et al., 1993).

PET measurements were conducted using the High Resolution Research Tomograph (Siemens Molecular Imaging, Knoxville, TN). In each PET measurement, a sterile physiologic phosphate buffer (pH 7.4) solution of the radiotracer was injected as a bolus into a sural vein during 5 seconds with a simultaneous start of PET data acquisition. List-mode data were acquired continuously for 123 minutes starting at the time of radioligand injection and were subsequently reconstructed as previously described elsewhere (Varrone et al., 2009) with a series of 34 time frames.

For examination of 5-HT_{1A} receptor occupancy, two PET measurements with [^{11}C]WAY-100635 were conducted 3 hours apart at baseline and during AZD3676 administration, respectively, in each of three monkeys. The radioactivity injected ranged from 101 to 109 MBq. For examination of 5-HT_{1B} receptor occupancy, three PET measurements with [^{11}C]AZ10419369 (baseline and high and low dose) were planned in each of three monkeys, but due to technical problems during one of the experimental sessions only two measurements were conducted in one of the monkeys. A fourth session was therefore undertaken at baseline and after additional doses of AZD3676 in this monkey. The radioactivity injected ranged from 157 to 167 MBq.

AZD3676 is predicted to have a small volume of distribution and a short biologic half-life, requiring an extended release formulation in a clinical setting. Thus, to achieve therapeutic exposure conditions AZD3676 was administered as a constant intravenous infusion starting approximately 30 minutes before the radioligand injection using a bolus and infusion paradigm. At the start of infusion, a low dose of AZD3676 was administered over 5 minutes. Subsequently a higher maintenance dose was administered over 130 minutes. Maintenance doses of AZD3676 administered in the measurements with [^{11}C]WAY-100635 were 0.16, 0.32, and 0.82 μmol/kg, respectively. Corresponding doses in the measurements with [^{11}C]AZ10419369 studies were 0.1, 0.124, 0.16, 0.3, 0.38, 0.85, and 1.8 μmol/kg, respectively.

PET Data Analysis. For anatomic designation, magnetic resonance imaging (MRI) scans of the individual monkey brains were obtained using a 1.5 T General Electric Signa (GE Healthcare, Milwaukee, WI) system (for details, see Schou et al., 2013). Regions of interest (ROIs) for the prefrontal cortex, occipital cortex, and cerebellum were manually delineated on the T1-weighted MRI scans using an in-house image analysis software (Roland et al., 1994). Brain MRIs were coregistered to the averaged brain PET images using SPM5 (Wellcome Trust Centre for Neuroimaging, London, United Kingdom). Time-activity curves were generated by pooling ROIs for each anatomic region and applying the pooled ROIs to PET images using the affine transformation matrix acquired from coregistration of the MRI.

For all measurements with [^{11}C]WAY-100635 and [^{11}C]AZ10419369 regional binding potential (BP_{ND}) values for the prefrontal cortex and occipital cortex, respectively, were estimated with the simplified reference tissue model (Lammertsma and Hume, 1996) using the cerebellum as reference region. Estimation of BP_{ND} values was performed using PMOD version 3.2. Receptor occupancy was calculated as the percent reduction in BP_{ND} values after AZD3676 treatment relative to that obtained at baseline. The calculated receptor occupancy was then correlated to AZD3676 plasma exposure according to a hyperbolic equation (Karlsson et al., 1995) with maximum occupancy set at 100%. The inhibition constant corresponding to the AZD3676 plasma concentration required for half-maximum receptor occupancy ($K_{i,\ plasma}$) was estimated by nonlinear fitting of the relationship between AZD3676 plasma concentration and receptor occupancy using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

$$\text{Occupancy}\ (\%) = \frac{Occ_{max}\ C_{avg,PET}}{K_{i,plasma} + C_{avg,PET}}$$

Statistical Analysis

Differences in freezing behavior between treatment groups in the CFC model were assessed using one way analysis of variance followed by Dunnett's multiple comparison test.

Results

In Vitro Pharmacologic Characterization. Mean K_i values of AZD3676 for the monkey 5-HT_{1A} and 5-HT_{1B} receptor, as determined using competitive binding studies in brain homogenates at room temperature, were found to be 0.13 nM (range: 0.12–0.14 nM) and 2.4 nM (range: 1.6–3.6 nM), respectively.

In Vitro Binding of AZD3676 to Plasma Proteins. For all species investigated, the protein binding of AZD3676 was concentration dependent, with unbound fraction in plasma ($f_{u,\ plasma}$) ranging from 0.052 to 0.20 for cynomolgus monkeys and 0.013–0.077 for humans at the concentrations studied (0.4–20 μM). At the predicted C_{max} (~1 μM) the $f_{u,\ plasma}$ value for AZD3676 in mice, rats, guinea pigs, cynomolgus monkeys, and humans was 0.092, 0.081, 0.37, 0.060, and 0.014, respectively. Studies conducted using human serum albumin and alpha-1-acid glycoprotein supported that the major component of the binding and the concentration dependence of $f_{u,\ plasma}$ was associated with binding to the alpha-1-acid glycoprotein.

Receptor Occupancy in Mouse, Rat, and Guinea Pig Brains. Administration of AZD3676 dose-dependently reduced the binding of the radioligands [^{11}C]WAY-100635 and [^{3}H]AZ10419369 to 5-HT_{1A} and 5-HT_{1B} receptors in target regions, whereas the binding in the cerebellum did not differ from that in vehicle-treated animals (results not shown). In studies of 5-HT_{1A} receptor binding in the frontal cortex of mice and rats after s.c. administration of AZD3676, the dose required for half-maximum receptor occupancy was 0.05 and 0.17 μmol/kg, respectively. For guinea pigs the doses required for half-maximum occupancy were 0.3 μmol/kg s.c. for 5-HT_{1A} receptors and 1.2 μmol/kg s.c. for 5-HT_{1B} receptors (Fig. 2).

In Vivo Pharmacology. For both memory-deficit models there was a statistically significant effect of treatment condition (one-way analysis of variance, $P < 0.01$). Both the positive control compound NAD299 (3 μmol/kg s.c.; $P < 0.01$) and AZD3676 (10 μmol/kg s.c.; $P < 0.05$) reverted the memory deficits induced by 0.6 μmol/kg s.c. R-8-OH-DPAT (memory-deficit model 1; Fig. 3A). Also, AZD3676 both at 3 ($P < 0.01$) and 10 μmol/kg s.c. ($P < 0.05$) reverted the memory deficit induced by 1 μmol/kg s.c. scopolamine (memory-deficit model

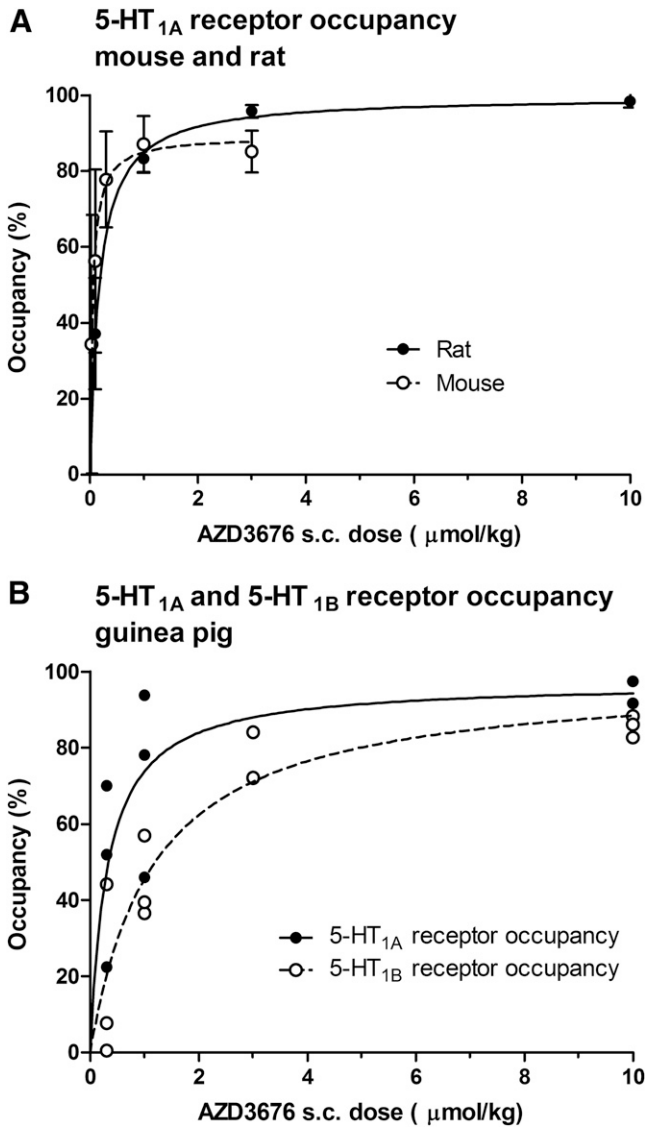

Fig. 2. Receptor occupancy determined ex vivo in mouse, rat, and guinea pig frontal cortex after administration of different doses of AZD3676. (A) 5-HT_{1A} receptor occupancy in mouse and rat brain (n = 4–8). (B) 5-HT_{1A} and 5-HT_{1B} receptor occupancy in guinea pig brain (n = 3). Occupancy at the 5-HT_{1A} and 5-HT_{1B} receptors was assessed using the radioligands [^{3}H]WAY-100635 and [^{3}H]AZ10419369, respectively.

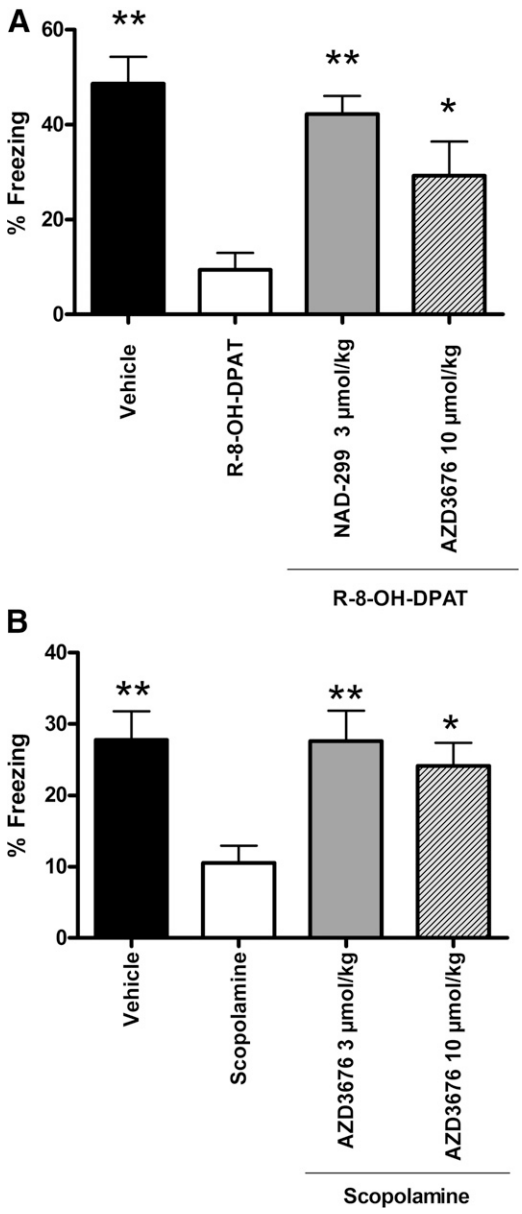

Fig. 3. Effects of AZD3676 on contextual fear conditioning in mice treated with (A) the 5-HT_{1A} receptor agonist R-8-OH-DPAT (0.6 μmol/kg s.c.) and (B) the muscarinic receptor antagonist scopolamine (1 μmol/kg s.c.). In *A*, the 5-HT_{1A} receptor antagonist NAD-299 was used as a reference compound. Values are expressed as mean and S.E.M. (n = 10 in *A* and 12 in *B*). *P< 0.05; **P< 0.01 versus R-8-OH-DPAT or scopolamine treatment.

2; Fig. 3B). Also, after oral administration of 1 μmol/kg, AZD3676 significantly reverted the scopolamine-induced memory deficit ($P < 0.05$; results not shown).

PET Receptor Occupancy in Monkeys. At baseline conditions the regional distribution of radioactivity in the cynomolgus monkey brain was consistent with the binding patterns previously reported in PET studies using the radioligands [^{11}C]WAY-100635 (Farde et al., 1997) and [^{11}C]AZ10419369 (Pierson et al., 2008), respectively (Fig. 4). Pretreatment with AZD3676 reduced radioactivity in target regions in a dose-dependent manner. The relationship between receptor occupancy and average AZD3676 plasma concentration at time of PET measurement could be described by a hyperbolic function with maximum occupancy constrained to 100% (Fig. 5). The total AZD3676 plasma concentration required for 50% receptor occupancy ($K_{i,\ plasma}$) was estimated to be 84 nM for 5-HT_{1A} and 72 nM for 5-HT_{1B} receptors.

Discussion

The preclinical pharmacology of AZD3676, a novel antagonist at 5-HT_{1A} and 5-HT_{1B} receptors, was characterized by an integrated approach, combining in vitro and ex vivo radioligand binding methods, behavioral pharmacology in rodents, and PET imaging in nonhuman primates. AZD3676 was found to have nanomolar receptor affinity, pharmacologic properties consistent with memory enhancement in rodents, and dual target engagement in the nonhuman primate brain.

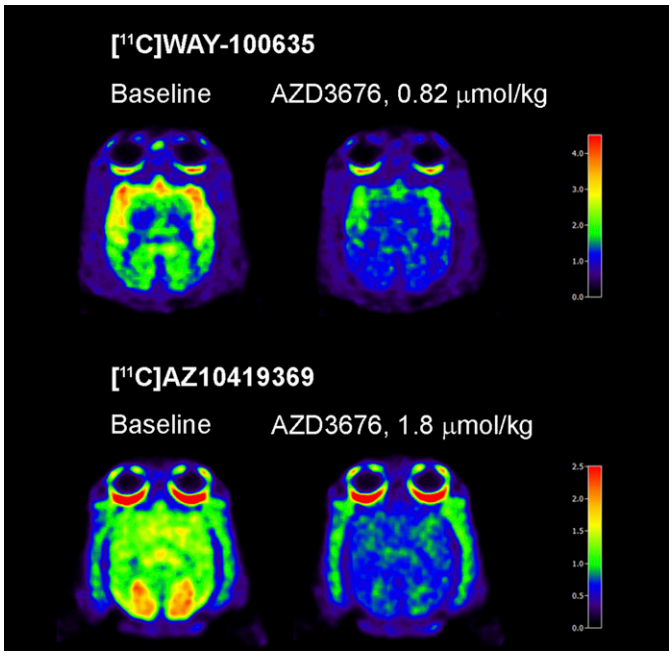

Fig. 4. AZD3676-induced receptor occupancy in the monkey brain: PET images of averaged radioactivity at baseline and during AZD3676 administration. 5-HT_{1A} receptor occupancy was determined using [^{11}C]WAY-100635 (upper panel), and 5-HT_{1B} receptor occupancy was determined using [^{11}C]AZ10419369 as a radioligand (lower panel). Image intensity is presented in standardized uptake value units.

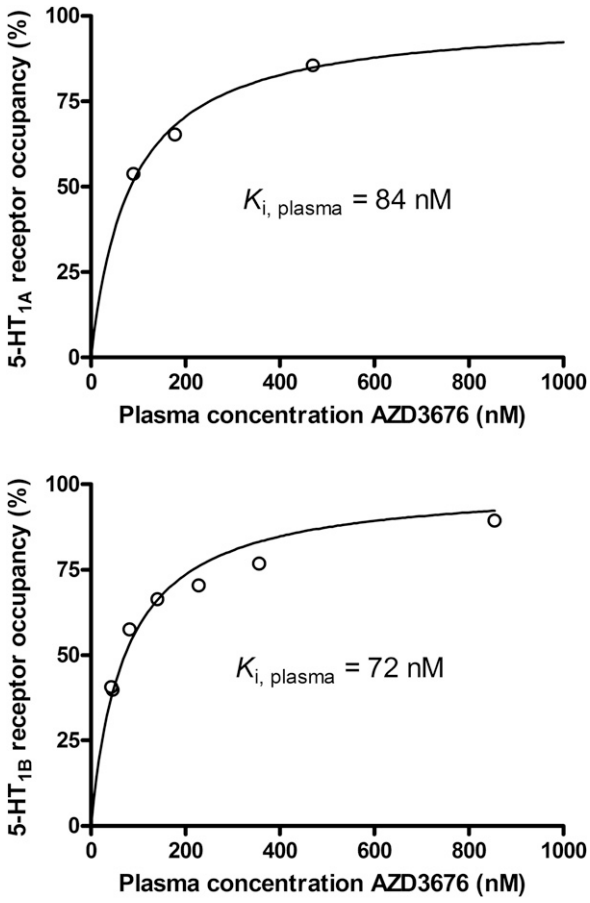

Fig. 5. AZD3676-induced receptor occupancy in the monkey brain: receptor occupancy as a function of the total AZD3676 plasma concentration. 5-HT_{1A} receptor occupancy was determined using [^{11}C]WAY-100635 (upper panel), and 5-HT_{1B} receptor occupancy was determined using [^{11}C]AZ10419369 as a radioligand (lower panel).

In PET measurements with selective radioligands for 5-HT_{1A} and 5-HT_{1B} receptors, AZD3676 inhibited the binding at both receptors in a dose- and concentration-dependent manner, thus confirming target engagement in the primate brain. The affinity was similar at both receptor subtypes with $K_{i,\ plasma}$ estimates of 84 nM and 72 nM for 5-HT_{1A} and 5-HT_{1B} receptors, respectively, as calculated from the total plasma concentration of AZD3676.

The similar affinity values were not predicted from in vitro measurements, which rather indicate a more than 10-fold higher affinity at the 5-HT_{1A} versus the 5-HT_{1B} receptor. As plasma protein binding of AZD3676 was not measured in association with PET studies, K_i values determined in vitro and in vivo cannot be directly compared. However, the in vivo K_i (based on unbound plasma concentrations) could be estimated based on $f_{u,\ plasma}$ values determined in independent samples of cynomolgus monkey plasma (0.06). This approach yielded consistent estimates of K_i for binding at the 5-HT_{1B} receptor (4 nM, in vivo versus 2.4 nM, in vitro), whereas the affinity was more than 10-fold lower in vivo for the 5-HT_{1A} receptor (5 nM, in vivo versus 0.13 nM, in vitro).

Several previous PET investigations of multitarget occupancy have found discrepancies in relative affinities compared with that predicted from radioligand binding assays in vitro (Rabiner et al., 2002; DeLorenzo et al., 2011; Takano et al., 2013). Although reasons for these discrepancies are not understood, it is likely that the artificial environments of in vitro binding assays do not fully mimic physiologic conditions and may cause aberrations that differ between targets.

Although ex vivo radioligand binding studies are, to a higher extent, expected to be representative for the conditions in vivo, translation of findings to human conditions are limited by interspecies variability in receptor binding kinetics (Erreboe et al., 1995) and brain disposition (Syvänen et al., 2009). These binding parameters commonly differ between primates and rodents but have often been found to be consistent when comparing nonhuman primates and human subjects (Varnäs et al., 2011). The present findings indicating species variation in relative target affinity further confirm the value of PET in nonhuman primates as a translational tool for assessment of multitarget engagement in vivo.

Behavioral pharmacology studies were undertaken to establish the receptor occupancy induced at doses required for memory enhancement in rodents. As assessed using behavioral pharmacology studies and ex vivo receptor occupancy in mouse brain, AZD3676 reverted R-8-OH-DPAT-induced and scopolamine-induced memory deficits at doses corresponding to 5-HT_{1A} receptor occupancy of 80%. Based on this information, in combination with the exposure-occupancy relationship for nonhuman primates, the initial doses in clinical trials of AZD3676 should target total plasma exposures of 340 nM (or 20 nM based on the unbound drug concentrations). At the predicted efficacious doses of AZD3676, the 5-HT_{1A} receptor occupancy was 80%. The corresponding occupancy at the 5-HT_{1B} receptor was approximately 70% when extrapolated from our studies in guinea pigs. This extent of target engagement is consistent with that previously reported for antagonists at other binding sites (Grimwood and Hartig, 2009). Occupancy

in the range of 60%–90% has been reported at efficacious doses of antagonists at other G protein-coupled receptors, transporters, and ligand-gated ion channels, an exception being the group of atypical antipsychotics, which require lower dopamine D2/D3 receptor occupancy (30%–70%) than those of classic antipsychotics. The lower extent of D2/D3 receptor occupancy required for these compounds could partly be explained by binding to additional targets (Grimwood and Hartig, 2009).

The 5-HT_{1A} receptor antagonist lecozotan was developed by the former company Wyeth as a cognitive-enhancing ligand for treatment of patients with Alzheimer's disease. The compound was reported to be well tolerated in healthy young and elderly subjects (Patat et al., 2009); however, some adverse effects of lecozotan were described, and the drug later failed in phase II clinical trials in part as consequence of these side effects (Ramirez et al., 2014). AZD3676 represents a novel strategy for the treatment of cognitive impairment by combining the enhancing effect of 5-HT_{1A} receptor antagonism and simultaneous inhibition of presynaptic 5-HT_{1B} receptors on nerve terminals. Thereby, an additive effect on transmitter release in the brain is expected. It is possible that the combined pharmacology involving both 5-HT_{1A} and 5-HT_{1B} receptors will allow for a lower occupancy at 5-HT_{1A} receptors to generate the necessary therapeutic effect and thereby avoid 5-HT_{1A} receptor-driven side effects. However, the benefit of combined antagonism at 5-HT_{1A} and 5-HT_{1B} receptors remains to be proven in clinical trials.

In conclusion, AZD3676 shows preclinical characteristics consistent with CNS drug properties, including dual nanomolar receptor affinity, efficacy in animal models of memory impairment, and target engagement in the primate brain. The findings further support the integrated use of PET in nonhuman primates as a tool to confirm multitarget engagement of psychotropic drugs. Importantly, the outcome of the present multidisciplinary approach suggests that earlier introduction of PET studies in nonhuman primates may reduce the requirement for initial rodent experiments and associated costs in preclinical stages.

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Wrote or contributed to the writing of the manuscript: Varnäs, Farde, Juréus, Johnström, Schött, Schou, Ahlgren, Malmborg, Halldin.

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