AMG 580: A Novel Small Molecule Phosphodiesterase 10A (PDE10A) Positron Emission Tomography Tracer

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ABSTRACT
Phosphodiesterase 10A (PDE10A) inhibitors have therapeutic potential for the treatment of psychiatric and neurologic disorders, such as schizophrenia and Huntington’s disease. One of the key requirements for successful central nervous system drug development is to demonstrate target coverage of therapeutic candidates in brain for lead optimization in the drug discovery phase and for assisting dose selection in clinical development. Therefore, we identified AMG 580 [1-(4-(3-(4-(1H-benzo[d]imidazole-2-carbonyl)phenoxy)pyrazin-2-yl)piperidin-1-yl)-2-fluoropropan-1-one], a novel, selective small-molecule antagonist with subnanomolar affinity for rat, primate, and human PDE10A. We showed that AMG 580 is suitable as a tracer for lead optimization to determine target coverage by novel PDE10A inhibitors using triple-stage quadrupole liquid chromatography–tandem mass spectrometry technology. [1H]AMG 580 bound with high affinity in a specific and saturable manner to both striatal homogenates and brain slices from rats, baboons, and human in vitro. Moreover, [18F]AMG 580 demonstrated prominent uptake by positron emission tomography in rats, suggesting that radiolabeled AMG 580 may be suitable for further development as a noninvasive radiotracer for target coverage measurements in clinical studies. These results indicate that AMG 580 is a potential imaging biomarker for mapping PDE10A distribution and ensuring target coverage by therapeutic PDE10A inhibitors in clinical studies.

INTRODUCTION
Phosphodiesterase (PDE) 10A (PDE10A) is a dual substrate phosphodiesterase, which hydrolyzes cAMP and cGMP (Sodertling et al., 1999). cAMP and cGMP are intracellular second messages playing pivotal functions in regulation of broad spectrum cellular activities, including modulation of synaptic transmission, neurotransmitter synthesis, storage and release, and neuronal growth and differentiation (Conti and Beavo, 2007; Nishi et al., 2008). PDE10A is highly expressed in the central nervous system and, with the exception of testis, expressed only at low levels in peripheral tissues (Seeger et al., 2003; Coskran et al., 2006; Lakics et al., 2010). In the central nervous system, PDE10A protein is

ABBR eviations: AMG 579, 1-(4-[3-(4-(1H-benzo[d]imidazole-2-carbonyl)phenoxy)pyrazin-2-yl)piperidin-1-yl]ethane; AMG 580, 1-(4-(3-(4-(1H-benzo[d]imidazole-2-carbonyl)phenoxy)pyrazin-2-yl)piperidin-1-yl)-2-fluoropropan-1-one; AMG 3807, (R)-1-(4-(3-(4-(1H-benzo[d]imidazole-2-carbonyl)phenoxy)pyrazin-2-yl)piperidin-1-yl)-2-fluoropropan-1-one; AMG 3808, (S)-1-(4-(3-(4-(1H-benzo[d]imidazole-2-carbonyl)phenoxy)pyrazin-2-yl)piperidin-1-yl)-2-fluoropropan-1-one; AMG7980, 5-[6,7-dimethoxycinnolin-4-yl-N-isopropyl-3-methylpyridin-2-amine; AUC, area under curve; BAY 73-6691, 1-2-chlorophenyl-6-(2R)-3,3,3-trifluoro-2-methylpropyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidine-4-one; BRL-50481, 3-(4-N,N-dimethylsulfonamido)-4-methyl-nitrobenzene; DMSO, dimethylsulfoxide; EHNA, erythro-9-2-hydroxy-3-nonyl]adenine; HPLC, high-performance liquid chromatography; HPMC, hydroxypropyl methylcellulose; ID, injected dose; IMA107, (R)-5-(3-fluropropyl-1-yl)-N-methyl-2-(3-methyliquinaxalin-yl)-N-(tetrahydro-2H-pyran-4-yl)pyrazol[1,5-a]pyrimidine-7-amine; [18F]JNJ42259152, 2-[(4-[2-(18F-fluorooethyl)-4-(4-pyridinyl)-1H-pyrazol-3-yl]phenoxy)methyl]-3,5-dimethyl-pyridine; LC-MS/MS, triple-stage quadrupole liquid chromatography–tandem mass spectrometry; Lu AE2886, 6-methyl-2-[2-(1'-13C-methyl)-4-phenyl-1H-imidazol-2-yl]-ethyl-1,2,4-triazole[1,5-a]pyridine; MNI 654, 2-[2-(2-fluorooethyl)-1H-imidazol-6-yl]-7-methyl-4-oxo-3,4-dihydroquinazolin-2-yl]ethyl-4-isopropoxysindoline-1,3-dione; MNI 659, 2-[(4-(3-(4-fluorooxyloxy)phenyl)-7-methyl-4-oxo-3,4-dihydroquinazolin-2-yl]ethyl-4-isopropoxysindoline-1,3-dione; MP10, 2-[(4-[1-methyl-4-pyridin-4-yl-1H-pyrazol-3-yl)phenoxy)methylquinoline; MSA, methanesulfonic acid; PDE, phosphodiesterase; PDE10A, phosphodiesterase 10A; PET, positron emission tomography; RO-201724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; ROI, regions of interest; SD, Sprague-Dawley; SPR, surface plasmon resonance; STR, striatum; SUV, standardized uptake value; THA, thalamus; TP-10, 2-[(4-[4-pyridin-4-yl-1(2,2,2-trifluoroethyl)pyrazol-3-yl]phenoxy)methylquinoline.

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predominantly localized in the medium spiny neurons of the striatum (STR), which is the main relay for glutamatergic cortical and dopaminergic input within the basal ganglia. PDE10A is expressed in both direct (D1) and indirect (D2) pathway neurons, but not in striatal interneurons (Xie et al., 2006, 2008). The unique expression patterns of PDE10A in the STR suggest that interference of PDE10A enzyme activity may result in alterations of behaviors regulated by the neuronal circuits (Siuciak et al., 2006, 2008). Inhibition of PDE10A in the direct pathway neurons may lead to the potentiation of D1 receptor signaling, while inhibition of PDE10A in the indirect pathway neurons causes the potentiation of the A2A receptor and inhibition of D2 receptor signaling (Nishi et al., 2008, 2011). Therefore, inhibition of PDE10A has been hypothesized to be beneficial in psychiatric and neurologic diseases involving the basal ganglia, such as schizophrenia (Menniti et al., 2007; Schmidt et al., 2008) and Huntington’s disease (Kleiman et al., 2011). Selective inhibitors of PDE10A have been reported to be efficacious in preclinical rodent behavioral models of schizophrenia, such as phencyclidine-stimulated hyperlocomotor activity, prepulse inhibition model, and conditioned avoidance response (Siuciak et al., 2006, 2008; Grauer et al., 2009). Moreover, the PDE10A inhibitor TP-10 (2-[[4-[4-pyridin-4-yl]-2-fluoropropan-1-one], a novel PDE10A antagonist tracer has been demonstrated to ameliorate pathology and locomotor behavior in rat and R6/2 mouse models of Huntington’s disease (Giampà et al., 2009, 2010).

One of the key questions for central nervous system drug development is to understand if compounds can reach sufficient target coverage in the brain to achieve desirable efficacy. Positron emission tomography (PET) imaging is widely used to assess target occupancy and to establish a correlation between target coverage in the brain to achieve desirable efficacy. Positron emission tomography (PET) imaging is widely used to assess target coverage in the brain to achieve desirable efficacy. PET. Additional studies are underway to further investigate its potential usage as a PDE10A PET ligand for human brain imaging.

**Materials and Methods**

**Animals**

All experiments were conducted under approved research protocols by Amgen’s Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals guidelines in facilities accredited by the Association for the

**TABLE 1**

Summary of AMG 580 properties

<table>
<thead>
<tr>
<th>PDEs</th>
<th>Reference Cpd IC₅₀</th>
<th>AMG580 IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1B</td>
<td>Nimodipine 578 nM &gt;30 µM</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>PDE2A1</td>
<td>EHNA 840 nM &gt;30 µM</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>PDE3A</td>
<td>Enoximone 7.6 nM &gt;30 µM</td>
<td>0.13 nM</td>
</tr>
<tr>
<td>PDE4D2</td>
<td>Rolipram 3.7 nM &gt;30 µM</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>PDE5A</td>
<td>Slidenafil 8.6 nM &gt;30 µM</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>PDE6</td>
<td>Zaprinast 23300 nM &gt;30 µM</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>PDE7A1</td>
<td>BRL50481 389 nM &gt;30 µM</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>PDE8A1</td>
<td>Diprydamole 14900 nM &gt;30 µM</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>PDE9A2</td>
<td>Bay37-659 439 nM &gt;30 µM</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>PDE10</td>
<td>0.13 nM</td>
<td>1.2 nM</td>
</tr>
<tr>
<td>PDE11A4</td>
<td>Diprydamole 245 nM &gt;30 µM</td>
<td>1.2 nM</td>
</tr>
</tbody>
</table>

AUC, area under the curve; ER, efflux ratio; F_app, unbound free fraction; h, human; ID, injected dose; P_app, apparent permeability coefficient; P_glycoph, P-glycoprotein; PSA, polar surface area; r, rat; STR, striatum; THA, thalamus.
Assessment and Accreditation of Laboratory Animal Care. Adult male Sprague-Dawley (SD) rats (250–280 g) were purchased from Harlan (Livermore, CA). Rats were group housed on a filtered, forced air isolation rack, and maintained on sterile wood chip bedding in a quiet room on a 12-hour light/dark cycle, with food and water available ad libitum. Animals were allowed for minimum 3 days of adaptation to the laboratory conditions prior to being used in the experiments. Baboon striatal samples were purchased from the Southwest Foundation for Biomedical Research (San Antonio, TX). Left hemisphere, rostral striatal samples were acquired for homogenate binding assessments. For autoradiography, a 1-cm-thick slab of the left hemisphere containing the STR were obtained. Brain tissues from rat and baboon were frozen immediately upon dissection on dry ice. All samples were shipped on dry ice and stored at −80°C until use.

**Human Samples**

Human brain tissue samples were acquired under agreement with hospitals in the local Los Angeles area and Amgen Tissue Acquisition Center. Brain tissue samples with an area of 1 cm² and a thickness of 0.5 cm (final of 0.5 cm³ volume) were dissected from the donors, obtained 3–12 hours postmortem and then embedded in optimal cutting temperature compound. For autoradiography, a 1-cm-thick slab of the left hemisphere containing the STR were obtained from a 40-year-old male. The male sample obtained for homogenate binding assessments was from a 47-year-old Caucasian. The female sample obtained was from a 38-year-old Caucasian. The causes of death of all human sample donors were due to accidents, not associated with diseases, and therefore considered to be normal tissue samples. All samples were shipped on dry ice and stored at −80°C until use.

**Compound Synthesis and Radiolabeling**

All selective PDE10 inhibitors described here were synthesized by the Medicinal Chemistry Department, Amgen, Inc. (Thousand Oaks, CA). AMG 580 was radiolabeled with [3H] with a specific activity of 84.9 Ci/mmol and a radiochemical purity of 99.4%. The synthesis of [18F]AMG 580 was performed using an ELIXYS automated radiochemical synthesizer (Sofie Biosciences, Culver City, CA). Briefly, [18F]fluoride was eluted from an ion exchange column using a Kryptofix 222 (Sigma-Aldrich, St. Louis, MO) potassium carbonate solution into a reaction vessel and evaporated under helium flow for 5 minutes at 70°C. The reaction vessel was then washed with acetonitrile and dried at 100°C under vacuum. A solution of AMG 580 precursor in dimethylsulfoxide (DMSO) was added to the reaction vessel, heated to 70°C for 20 minutes, and followed by cooling to 40°C and dilution with high-performance liquid chromatography (HPLC)–grade water. The diluted solution was passed through a solid-phase extraction cartridge (Sep-Pak C18 Light; Waters Corp., Milford, MA), which was then rinsed with HPLC-grade water before elution using ethanol. The solution was loaded onto a HPLC column (Luna C18, 10 µm, 250 × 10 mm; Phenomenex, Inc., Torrance, CA) and eluted using 45:55 v/v of acetonitrile and 10 mM NaH2PO4 at 4 ml/min. The [18F]AMG 580 fraction was collected, diluted with sterile water, passed through a second solid-phase extraction cartridge, rinsed with water, and eluted using ethanol. The final product was passed through a 0.22 µm filter and into a vial containing sterile saline for injection. The specificity for [18F]fluoride labeling was 110 mCi/µmol.

**PDE10A Potency and Selectivity Assays**

Functional inhibition of PDE activity was measured essentially as described in the immobilized metal ion affinity–based fluorescence polarization time-resolved fluorescence resonance energy transfer assay kit protocol (Molecular Devices, Sunnyvale, CA). AMG 580 or control compound was serially diluted in 100% DMSO from a 3 mM stock. Recombinant PDE enzymes (PDE1–5, -7, -9, and -11) or bovine retinal lysate (PDE6) and cyclic adenosine monophosphate or cyclic guanidine monophosphate substrates were diluted in complete reaction buffer (10 mM Tris-HCl, pH 7.2, 10 mM MgCl2, 0.05% NaN3, 0.01% Tween 20, and 1 mM freshly added dithiothreitol). The ability of AMG 580 to inhibit the activity of PDE1–9 and -11 was measured using a time-resolved fluorescence resonance energy transfer–based functional assay. The IC50 value was calculated from a 22-point dose-response curve run in duplicate. Reference compounds for PDE1–9 and PDE11 were used as positive controls.

**In Vivo Kinetics and Distribution of AMG 580 and Enantiomer (AMG 3807 and AMG 3808) in Rat Brain Using Triple-Stage Quadrupole Liquid Chromatography Mass Spectrometry**

Tracers formulated in 20% Captisol (pH 4) with methanesulfonic acid (MSA) were administered to male SD rats at 10 µg/kg of body weight via bolus intravenous tail vein injection. Animals were euthanized at varying times postinjection and the tracer concentrations (ng/g) were measured using LC-MS/MS. N = 4 per data point.

**TABLE 2**

<table>
<thead>
<tr>
<th>Enantiomer</th>
<th>IC50 (nM)</th>
<th>Ki (nM)</th>
<th>T1/2 (min)</th>
<th>kin (1/min)</th>
<th>koff (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMG3807</td>
<td>0.04</td>
<td>0.6</td>
<td>3.1</td>
<td>6.4 × 10⁶</td>
<td>3.8 × 10⁻³</td>
</tr>
<tr>
<td>AMG3808</td>
<td>0.14</td>
<td>0.7</td>
<td>0.8</td>
<td>2.2 × 10⁷</td>
<td>1.5 × 10⁻²</td>
</tr>
</tbody>
</table>

FIG. 2. AMG 580 in vivo kinetics and distribution. (A) Time course and tissue distribution of AMG 580 in vivo in rats using LC-MS/MS. AMG 580 (10 µg/kg) was administered by intravenous injection and the samples (STR, blue circle; THA, purple triangle, plasma, red square) were collected at each indicated time point postinjection and the tracer concentrations (ng/g) were measured using LC-MS/MS. N = 4 per data point. (B) Comparison of striatal uptake of AMG 580 and its enantiomers AMG 3807 and AMG 3808. The structures of AMG 3807 and AMG 3808 only differ from AMG 580 in that they are single enantiomers. The uptake values were represented as percentage of ID per gram of tissue (% ID/g). Blue circle, more active enantiomer AMG 3807; green circle, AMG 580; purple circle, less active enantiomer AMG 3808. N = 4 per data point.
10, 20, 30, 60, 120, and 240 minutes postdosing. Blood and brain tissue samples were collected. Brain samples were weighed and HPLC-grade water was added (20% w/v), followed by homogenization using a Covaris E110 Acoustic Homogenizer (Model E210; Covaris, Inc., Woburn, MA). Samples were analyzed for content of compounds by high-performance liquid chromatography in conjunction with triple-stage quadrupole–tandem mass spectrometry (LC-MS/MS) (API 4000 LC-MS/MS; Applied Biosystems, Carlsbad, CA), as described previously (Hu et al., 2012).

In Vivo PDE10A Inhibitor AMG 579 Target Occupancy Measurement Using Tracer AMG 580 with LC-MS/MS. PDE10A inhibitor AMG 579 [1-(4-(3-(4-(1H-benzo[d]imidazole-2-carbonyl)phenoxy)
pyrazin-2-yl)piperidin-1-yl)ethanone (Hu et al., 2014) was formulated in 2% hydroxypropyl methylcellulose (HPMC)/1% Tween 80 (pH 2.2) with MSA. Vehicle (N = 6) or AMG 579 (at dosages of 0.1, 0.3, 1, 3, 10, and 30 mg/kg, with a dose volume of 5 ml/kg, N = 4) was administered orally in male SD rats. 90 minutes post oral dosing, 5 mg/kg of AMG 580 was injected to rats via lateral tail vein (with a dose volume of 0.5 ml/kg). 30 minutes after injecting AMG 580, animals under isoflurane were euthanized by decapitation; samples of blood and brain tissues were then collected for analysis as described previously. Later, it was found that an alternative formulation [30% hydroxypropyl β-cyclodextrin, 1% HPMC (pH 2) with MSA] yielded better solubility of AMG 580. Therefore, occupancy of AMG 580 at 10 and 30 mg/kg in the alternative formulation was performed and data were combined with the original formulation.

STR, with the highest PDE10A expression, was used as the target tissue and the thalamus (THA) was treated as the reference tissue (Hu et al., 2012). Occupancy was calculated based on the reference tissue model determined using the following equations:

\[
BP = \frac{STR}{THA} - 1, \quad \% \text{Occupancy} = 100 \times \left(1 - \frac{BP_{\text{Drug}}}{BP_{\text{Vehicle}}}\right),
\]

where BP is the binding potential.

**Surface Plasmon Resonance Spectroscopy Binding Assay**

Surface plasmon resonance (SPR) measurements were performed on a BiaCore T100 (GE Healthcare, Piscataway, NJ). Recombinant protein, human His-PDE10A (amino acid 442-779, consisting of catalytic domain), was generated at Amgen; all other reagents were purchased from GE Healthcare or Sigma-Aldrich. PDE10A (approximately 3000 response units) was coupled to the CMS chip using standard amine coupling. The immobilization running buffer consisted of 10 mM HEPES (pH 7.4) with 150 mM NaCl; immobilization steps consisted of a 7-minute 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/N-hydroxysuccinimide activation step [200 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 50 mM N-hydroxysuccinimide], 0.2 minute 20 mg/ml PDE10A in 10 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.0), and 7-minute 1 M ethanolamine hydrochloride (pH 8.5). Binding kinetics was measured with a 20 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 5 mM MgCl₂, and 2% (v/v) DMSO. AMG 580 stock solution was prepared at 10 mM concentration in DMSO and diluted with running buffer to concentrations of 8, 40, 200, and 1000 nM. Kinetic titration experiments were performed by injecting compound solutions of ascending concentrations in 2-minute intervals onto the PDE10A surface. All experiments

**Fig. 5.** \(K_D\) and \(B_{\text{max}}\) measurement of \(^{3}H\)AMG 580 binding to striatal homogenates of rats, baboon, and human. Saturation binding was determined by incubating rat STR homogenates with concentrations of \(^{3}H\)AMG 580 ranging from 0.01 to 50 nM in a final volume of 200 µl. Nonspecific binding was determined using excess of unlabeled AMG 580 at 1 µM. Red circles represent total binding, blue circles represent nonspecific binding, and green circles represent specific binding. GraphPad Prism 5 software was used for the nonlinear regression of the curve fit. Data presented as mean ± S.E.M. Error bars in the graph represent N = 4 data points. (A) Rat STR; (B) baboon rostral STR; (C) human male putamen; and (D) human female putamen.

**TABLE 3**

\(K_D/B_{\text{max}}\) values from saturation binding studies of \(^{3}H\)AMG 580 with rat, baboon, and human striatal samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>(K_D) (pM)</th>
<th>(B_{\text{max}}) (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat STR</td>
<td>51.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Baboon R-STR</td>
<td>71.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Human male putamen</td>
<td>83.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Human female putamen</td>
<td>84.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>
were performed at 25°C. The data were processed and analyzed using the T100 evaluation software and CLAMP analysis software package (Center for Biomolecular Interactions, University of Utah, Salt Lake City, UT). The sample response was baseline corrected by subtracting reference flow cell data to correct for systematic noise and baseline drift. The response from blank injections was used to double reference the binding data. The kinetic parameters were established by fitting the data to a 1:1 binding model that included a mass transfer limitation term.

**Kd/Bmax Measurements and Binding Displacement**

Brain homogenates were prepared from rat, baboon, and human STR using a Covaris E110 Acoustic Homogenizer, as described previously (Hu et al., 2012). For saturation binding experiments, rat STR homogenate (10 μg/well) or baboon and human STR homogenate (25 μg/well) were incubated with [3H]AMG 580 in a 96-well plate with or without nonradiolabeled AMG 580 in assay buffer (50 mM Tris, pH 7.5, 5 mM MgCl₂, 1/2 Roche complete protease inhibitor tablet (Indianapolis, IN), EDTA free) for 1 hour at room temperature. The binding reactions were terminated by rapid filtration through a GF/C filter, presoaked in 0.3% polyethylenimine for 30 minutes, and immediately rinsed 6 times with 4°C washing buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂). The filter plates were dried in a vacuum oven at 56°C for approximately 40 minutes, and 50 μl of Microscint-20 (PerkinElmer, Waltham, MA) was added per well. Plates were sealed and read on a Packard (Meriden, CT) TopCount NXT plate reader for 2 min/well.

**In Vitro Binding Data Analysis**

In vitro radioligand binding data were analyzed by nonlinear regression analysis using GraphPad Prism software, version 5 (GraphPad, Inc., San Diego, CA), which fit binding curves to the following equations. For the saturation binding experiments:

\[
B = B_{max} \times L / (K_d + L),
\]

where \(B\) is the concentration of bound ligand, \(B_{max}\) is maximal number of binding sites, \(K_d\) is the ligand dissociation constant, and \(L\) is ligand concentration. For the competition binding experiments:

\[
B = B_{low} \left( B_{high} - B_{low} \right) / \left( 1 + 10^{\left( \log IC_{50} - L \right)/n} \right),
\]

where \(B\) is the concentration of bound ligand, \(B_{low}\) is the bottom plateau of the y-axis, \(B_{high}\) is the top plateau of the y-axis, \(IC_{50}\) is the inhibitor concentration for half-maximal binding inhibition, \(L\) is ligand concentration, and \(n\) is the Hill coefficient. For the binding displacement experiments: the y-axis in the competition binding experiment was defined as the percent of control, which was calculated from the control wells. The high control was an average of eight wells containing [3H]AMG 580 plus brain homogenates (without inhibitor compound), and the low control was an average of eight wells containing [3H]AMG 580 in the presence of increasing concentrations of selected nonradiolabeled compounds (PDE10A-specific inhibitors or other non-PDE10A phosphodiesterase inhibitors) ranging from 5 pM to 100 nM. The concentration of nonradiolabeled compounds that inhibited 50% of the specific binding of [3H]AMG 580 was determined (IC₅₀); using the \(K_d\) values obtained, the \(K_i\) values were calculated according to the Cheng and Prusoff (1973) equation.

**Fig. 6.** Binding displacement of [3H]AMG 580 to rat, baboon, and human STR with selective PDE10 inhibitors: AMG 580, AMG 579, AMG 0074, and AMG 7980. Dose-response curves of various PDE10 inhibitors were determined in competition binding studies using rat STR (solid circle), baboon rostral STR (open circle), human male putamen (solid square), and human female putamen (open square) homogenates with approximately 130 pM of [3H]AMG 580 in the presence of increasing concentrations of compound from 0.005 to 100 nM. Curves were analyzed using nonlinear regression in GraphPad Prism. Results expressed as mean ± S.E.M., error bars in graph represent n = 2 per data point. \(K_i\) values are listed in Table 4.
Percent of control = \( \frac{cpm_{\text{test well}} - ave_{\text{low}}}{ave_{\text{high}} - ave_{\text{low}}} \) \( K_i = \frac{IC_{50}}{1 + (L/K_D)} \)

The \( K_i \) values were calculated according to Cheng and Prusoff (1973), where IC\(_{50}\) is the inhibitor concentration for half-maximal binding inhibition, \( L \) is the input ligand concentration, and \( K_D \) is the ligand dissociation constant.

**Autoradiography**

SD rats were sacrificed by CO\(_2\) inhalation and then decapitated. Brains were quickly removed from the skull, frozen in 2-methylbutane and stored at \(-80^\circ\text{C}\) until use. Baboon and human samples consisted of frozen slabs (approximately 1-cm-thick) of the left hemisphere. Twenty-micrometer sections containing the STR were cut from frozen brain tissue at \(-20^\circ\text{C}\) using a cryostat and placed onto microscope slides. Slides were stored at \(-80^\circ\text{C}\) until use. For autoradiography experiments, slides containing adjacent brain sections were incubated in the same solution with addition of 10 \( \mu \text{M} \) nonradiolabeled, structurally unrelated PDE10 antagonist AMG 0074 (Verhoest et al., 2006). After incubation, slides were washed 3 times in ice cold binding buffer, dipped into distilled water to remove buffer salts, and dried under a stream of cold air. Autoradiographic images were acquired for 24 hours in a Beta Imager 2000 (Biospace, Paris, France) and digitized using M3 Vision software (Biospace). Images were assembled into one photograph using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA).

**Micro-PET Imaging**

Four male SD rats (200 g; Harlan, Indianapolis, IN) were imaged using an Inveon PET/CT scanner (Siemens Medical Solutions USA, Inc., Malvern, PA). Anesthesia was induced using 2–4% isoflurane in 100% oxygen followed by 1–2% isoflurane during image acquisition. Animals were placed (2 per acquisition) into a custom three-dimensional printed animal holder and supplied with supplemental heating (37°C) and physiologic monitoring using a BioVet monitoring system (m2m Imaging Corp., Cleveland, OH). A bolus intravenous injection of [\( ^{18}\text{F} \)]AMG 580 (40–60 \( \mu \text{Ci} \), injected mass of 0.17–0.26 \( \mu \text{g} \) per animal, with labeling specificity of 110 mCi/\( \mu \text{mol} \) in 300 \( \mu \text{l} \) saline, was administered via a tail vein catheter immediately after starting 120-minute dynamic PET image acquisition. List mode data were histogrammed into 1/2, 2/2, 17/3, 6/5, 2/15, 6/30, 17/300, and 3/600 second frames and images were reconstructed using a three-dimensional ordered subset-expectation maximization algorithm (two iterations) followed by a maximum a posteriori algorithm (16 subsets, 18 iterations, \( \beta = 0.03 \)) provided by Inveon Acquisition Workplace 1.5 (Siemens Medical Solutions USA Molecular Imaging, Knoxville, TN).

**Micro-PET Image Analysis**

Analysis of PET images was performed using Inveon Research Workplace (Siemens Medical Solutions USA Molecular Imaging). Manually generated regions of interest (ROI) containing the STR and cerebellum were traced on multiple slices in the coronal plane. Uptake was quantified by calculating the standardized uptake value (SUV) as:

\[
\text{SUV} = \frac{[C_{\text{ROI}}(t)]}{(\text{ID}/\text{BW})},
\]

where \( C_{\text{ROI}}(t) \) is the radioactivity concentration contained in the ROI at time \( t \), \( \text{ID} \) is the injected dose of radioactivity, and \( \text{BW} \) is the body weight.
weight (Zasadny and Wahl, 1993). All data were decay corrected to the time of tracer injection and the ROI radioactivity concentration was calculated using the calibration factor measured from a phantom of known volume and radioactivity concentration.

Results

In Vitro Characteristics and In Vivo Kinetic Properties of AMG 580. AMG 580 (Fig. 1A), exhibiting in vitro properties suitable for a tracer, was identified from a focused medicinal chemistry campaign. Details of medicinal chemistry effort that led to the identification of AMG 580 will be described in a separate communication. AMG 580 is a potent PDE10A inhibitor with IC\textsubscript{50} of 0.13 nM and is highly selective over other PDEs (without significant inhibition up to 30 \textmu M) (Fig. 1B; Table 1). AMG 580 showed good membrane permeability and was not a P-glycoprotein substrate as determined by an efflux ratio of 1 (Table 1). LC-MS/MS technology was used to assess the kinetics and tissue distribution of AMG 580 by microdosing (10 \textmu g/kg) in rats in vivo. AMG 580 exhibited good tracer properties; it showed high specific uptake in the STR with a value of 4.6% ID/g, target-to-reference ratio at 16, while those values of racemic AMG 580 were in between (Fig. 2B). Both molecules (AMG 3807 and AMG 3808) and the racemic mixture (AMG 580) were highly potent and selective. We choose the racemic molecule AMG 580 for further characterization because it reached our tracer selection criteria and had the advantage of rapid purification post \[^{18}\text{F}\] fluoride labeling.

In Vivo Saturable Striatal Uptake of AMG 580 in Rats. High saturable specific binding in the target tissue in vivo is a prerequisite for a tracer to be able to measure target-ligand interactions. AMG 580 was administered at a range of dosages from 0.1 to 300 \mu g/kg in rats by bolus intravenous injection. Striatal tissues, thalamic tissues, and blood samples were collected at 30 minutes postdosing, at which the STR uptake of AMG 580 peaked (Fig. 2A). AMG 580 concentrations in the tissues were determined with LC-MS/MS. Specific STR uptake was determined by subtracting the tracer levels in the THA (as the reference tissue for nonspecific binding due to its low PDE10A expression level) from the levels in the STR (as total binding due to its high PDE10A expression level) (Fig. 3A). The resulting curve indicated that specific striatal binding of AMG 580 was able to reach saturation in vivo. The ED\textsubscript{50} (dosage to reach 50% of plateau) was determined by nonlinear regression to arrive at a value of 200 \pm 12 \mu g/kg. The dosage of tracer used for studying target occupancy of testing a compound should not exceed 5% of occupancy by the tracer itself (Chernet et al., 2005). Therefore, 5 \mu g/kg of AMG 580 (with approximately 2.4% occupancy) was chosen for all of the subsequent studies. Plotting the percent of target occupancy against the tracer concentration in the plasma (Fig. 3B) revealed plasma EC\textsubscript{50} of 140.7 \pm 11.3 nM (with unbound free fraction IC\textsubscript{50,Fx} = 1.0 \pm 0.1 nM). This study also enabled measurement of PDE10A target density in the STR that is accessible to the tracer in vivo, with a value of 1.1 pmol/mg of wet tissue (Fig. 3B).

In Vivo PDE10 Target Occupancy of AMG 579 Measurement Using AMG 580 as a Tracer. The usability of AMG 580 as a tracer for occupancy measures was determined

Fig. 7. No effect on \[^{3}H\]AMG 580 binding to rat STR by other non-PDE10A inhibitors. Binding of approximately 130 pM of \[^{3}H\]AMG 580 to rat STR in the presence of each individual non-PDE10A inhibitors, as indicated by the legends, with concentrations from 0.005 to 100 nM. Nonradiolabeled AMG 580 was used as the positive control. Curves were analyzed using nonlinear regression in GraphPad Prism. Results expressed as mean \pm S.E.M., error bars in the graph represent n = 2 per data point.

Fig. 8. In vitro autoradiography of \[^{3}H\]AMG 580 of rat, baboon, and human STR. Binding of 1 nM \[^{3}H\]AMG 580 to horizontal (A and B) and coronal (C and D) rat brain and coronal baboon (E and F) and human (G and H) brain slices. Nonspecific binding was assessed in the presence of 10 \mu M AMG 074, a selective PDE10A inhibitor (B, D, F, and H). Scale bars represent 1 cm.
by a target occupancy dose-response study. Concentrations of tracer and blocker were determined by LC-MS/MS in the STR (target tissue), THA (reference tissue), and plasma. Target occupancy values were derived based on tracer concentrations detected in the STR and THA using the reference tissue model equation. Target occupancy of a highly potent and highly selective PDE10A inhibitor, AMG 579 (Hu et al., 2014), was observed by displacement of tracer AMG 580 (at 5 μg/kg i.v.) in a dose-dependent manner, ranging from 4.4% occupancy at the 0.1 mg/kg dose up to 63% at the 30 mg/kg dose (Fig. 4B). An additional maximum exposure study was conducted using a different formulation (30% hydroxypropyl β-cyclodextrin, 1% HPMC pH 2 w/MSA) vehicle designed to reach higher AMG 579 exposure levels (Fig. 4A). This formulation yielded higher plasma exposure levels and target occupancy values, with a mean occupancy of 74.9% at 10 mg/kg and 86.7% at 30 mg/kg (compared with 50 and 63%, respectively, using the previous formulation) (Fig. 4B). Combining the results from the two studies indicated that AMG 579 target occupancy correlated with AMG 579 plasma exposure levels, with an $R^2$ value of 0.89 (Fig. 4C). The calculated ED$_{50}$ was 4.5 mg/kg (Fig. 4B). The EC$_{50}$ value was determined to be 2.0 μM by drug exposure levels in the total plasma (Fig. 4C), while the unbound free EC$_{50,fu}$ value was about 7.9 nM (unbound free fraction in rat was 0.004 for AMG 579). Drug exposure in the STR area (target tissue) showed good correlation with target occupancy, with EC$_{50}$ of 0.7 μM (striatal EC$_{50,fu}$ = 2.9 nM) (Fig. 4D). This study demonstrates that AMG 580 can be used as a PDE10 tracer to accurately measure in vivo target occupancy of the PDE10 inhibitor AMG 579 in rats, and the target occupancy correlates with drug plasma exposure levels.

**Measurements of $[^{3}H]$AMG 580 $K_i/B_{max}$ and $K_i$ Cross-Species and Specificity Assessment of AMG 580.** AMG 580 was labeled with tritium to assess $K_i/B_{max}$ in homogenates prepared from rat, baboon, and human striatal tissues. The tracer $[^{3}H]$AMG 580 bound to striatal homogenates in a saturable manner (Fig. 5) with nonspecific binding defined in the presence of excess amount of nonradiolabeled compound. The binding affinity was calculated using nonlinear regression to an equilibrium dissociation constant ($K_D$) of 51.7 pM for rat STR, 71.9 pM for baboon STR, 83.1 pM from human male putamen, and 84.7 pM for human female putamen (Table 3). The value was lower compared with that measured by the SPR assay ($K_D = 0.6–0.7$ nM), in which only the catalytic domain of human PDE10A was employed due to technical feasibility. The discrepancy of $K_D$ values may result from: (1) different assay formats between radioligand binding and SPR; (2) different forms or proteins used, native full length protein in tissue homogenate versus purified protein containing catalytic region; and (3) a combination of different forms of proteins used in different assay formats. $B_{max}$ of $[^{3}H]$AMG 580 for PDE10A binding on rat was calculated to be 2.2 pmol/mg of protein, whereas baboon and human gave similar values calculated between 1.0 and 1.4 pmol/mg of protein (Table 3).

A series of binding displacement experiments were conducted to determine the $K_i$ values of selective PDE10 inhibitors using $[^{3}H]$AMG 580 as the tracer. In the first set of experiments, chemically distinct, nonlabeled, and selective PDE10 inhibitors, AMG 580 (IC$_{50} = 0.13$ nM), AMG 579 (IC$_{50} = 0.19$ nM) (ketobenazidazole series) (Hu et al., 2014), AMG 0074 (IC$_{50} = 1.0$ nM) (quinoline series) (Verhoest et al., 2009), and AMG 7980 (IC$_{50} = 1.9$ nM) (cininoline series) (Hu et al., 2012) were used to compete for $[^{3}H]$AMG 580 binding to rat, baboon, and human striatal homogenates (Fig. 6; Table 4). All compounds were able to displace $[^{3}H]$AMG580 binding in a competitive manner (Fig. 6), with the $K_i$ values for rat, baboon, and human striatal tissues listed in Table 4. Selectivity was demonstrated by showing that inhibitors selective for other PDEs, but not PDE10A did not displace $[^{3}H]$AMG 580 binding to rat STR, e.g., Nimodipine (PDE1), EHNA (erythro-9-(2-hydroxy-3-nonyl)adenine; PDE2), Enoximone (PDE3), Rolipram (PDE4), RO-201724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PDE4), Sildenafil (PDE5), BRL-50481 (3-(N,N-dimethylsulfonamido)-4-methyl-nitrobenzene; PDE7), Dipyridamole (PDE8), and Bay 73-6691 (1-(2-chlorophenyl)-6-(2R)-3,3-trifluoro-2-methylpropyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidine-4-one; PDE9) (Fig. 7). Specific binding of $[^{3}H]$AMG 580 to the STR was also confirmed visually by autoradiography in rat, baboon, and human brain slices (Fig. 8). Binding to rat (Fig. 8, A and C), baboon (Fig. 8E), and human (Fig. 8G) STR was blocked by
an excess of nonradiolabeled PDE10A inhibitor AMG 7980 (Fig. 8, B, D, F, and H).

AMG 580 was also characterized using an in vitro safety pharmacology panel to assess off-target binding and function. Briefly, the potential effects of AMG 580 on 116 receptors/ channels using radioligand binding assay (i.e., binding displacement of each target’s respective radioligand by unlabeled AMG 580) (Supplemental Table 1) and 32 enzymes (Supplemental Table 2) were investigated. The assays were carried out at a concentration of 1 μM AMG 580 in duplicate. Results showing an inhibition higher than 50% is considered to represent significant effects, therefore AMG 580 possessed no significant inhibition on any of the targets tested. In addition, the in vitro effects of AMG 580 on the hERG channel current were examined. The half-maximal inhibitory concentration (IC50) for the inhibitory effect of AMG 580 on hERG potassium current was estimated to be greater than 3 μM (data not shown).

Micro-PET Imaging with [18F]AMG 580. [18F]AMG 580 showed rapid penetration into the brain and retention in distinct regions. In vivo micro-PET images illustrated abundant radioactivity in the PDE10A enriched areas of the brain (Fig. 9). The highest accumulation of activity was observed in the STR, reaching a SUV of 2.77 ± 0.25 at 52.5 minutes after injection, with moderate accumulation observed in the nucleus accumbens (SUV = 0.94 ± 0.24). Distribution to the cerebellum, a region largely devoid of PDE10A functional protein expression, was measured at a SUV of 0.58 ± 0.01. Similarly, uptake in the frontal cortex and hippocampus was low and comparable to background. Kinetics of [18F]AMG 580 distributions to the STR and cerebellum are plotted in the tissue-time activity curves shown in Fig. 10. In agreement with the observations made using LC-MS/MS technology, micro-PET imaging measured the peak of striatal tracer uptake occurring approximately 30 minutes after injection. Uptake plateaued between 30 and 60 minutes, followed by a gradual washout. Of note, radioactivity concentration in the cerebellum decreased from 0 to 40 minutes, and remained relatively constant from 40 to 120 minutes after injection.

Discussion

Previously, we reported a high-affinity, selective PDE10A tracer AMG 7980 (Hu et al., 2012; Hwang et al., 2014). AMG 7980 has a fast off-rate, limiting its utility for PET imaging in higher species. To identify PDE10A tracer candidates with suitable retention time on the target, SPR spectroscopy was used for the off- and on-rate assessment of the compounds using the purified human PDE10A catalytic domain described previously (Hu et al., 2012). This allowed us to eliminate compounds with fast off-rate (cutoff criterion: approximately 10-fold slower than AMG 7980). With the combination of more stringent selection criterion and a LC-MS/MS in vivo screen of unlabeled candidate molecules, we identified a promising tracer candidate. AMG 580, a novel small molecule PDE10A tracer, belongs to a keto-benzamidazole scaffold with high affinity to (approximately 50–80 pM by [3H]AMG 580 binding), high potency to (IC50 = 0.13 nM in vitro biochemical assay), and high selectivity over other PDEs (IC50 > 10 μM). AMG 580 reached peak uptake in the STR between 30 and 60 minutes post bolus intravenous injection of microdosage and slowly declined in the target tissues (Fig. 2A), while AMG 7980 showed a rapid kinetics in the target tissues (Hu et al., 2012; Hwang et al., 2014). Longer retention on target and fast washout in the reference tissues contributed to the higher target-to-reference ratio (AUC STR/THA = 23) of AMG 580 compared with AMG 7980 (AUC STR/THA = 9) (Hu et al., 2012).

AMG 580 was characterized and evaluated rigorously both in vitro and in vivo for its tracer candidacy. Using [3H]AMG 580, we performed K/D/Bmax measurement on striatal homogenates from rats, baboons, and humans. The rat striatal Bmax values, which represent the target densities determined by two different PDE10A tracers, AMG 580 and AMG7980 (Hu et al., 2012), were similar; however, AMG 580 exhibited much higher affinity in rat STR (Kd = 52 pM) than AMG 7980 (Kd = 940 pM; Hu et al., 2012). The specific binding of [18F]AMG 580 to rat striatal homogenate can be displaced by PDE10A inhibitors but not by well characterized inhibitors to other PDEs. The autoradiography results with [3H]AMG 580 on brain slice sections showed that the signals are highly localized in the striatal area across three different species—rat, baboon and human—which is consistent with previously published PDE10A expression patterns (Seeger et al., 2003; Coskran et al., 2006; Lakics et al., 2010). The distributions of PDE10A in brains of different species from previous studies were mainly at mRNA levels and protein levels by various techniques; however, the levels of PDE10A target accessible to a selective small molecule PDE10A inhibitor have not been addressed in higher species. We used [3H]AMG 580 to determine PDE10A target densities (1.2–1.4 pmol/mg) in human brain tissues (putamen) from both male and female donors (Fig. 5, C and D; Table 3).

A target-specific radiotracer can be a very powerful translational tool in preclinical research to correlate target occupancy with preclinical efficacy of drug candidates and support compound selection. Using LC-MS/MS technology, we demonstrated that AMG 580 can be employed as a tracer to measure target occupancy of a PDE10A inhibitor (AMG 579) in rodents in vivo. AMG 580 is a racemic molecule. Both enantiomers were potent and showed high affinity to PDE10A (Fig. 2B; Table 2). Although AMG 3807 showed slightly better properties as a tracer than its enantiomer AMG 3808, we selected racemic AMG 580 as a tracer candidate mainly for two reasons: (1) the in vitro and in vivo properties of AMG 580 met or exceeded the selection criteria; and (2) a rapid purification procedure could be developed after [18F] fluoride labeling. We showed that [18F]AMG 580 can be used successfully for micro-PET imaging in rats (Figs. 9 and 10), and that close agreement was observed between LC-MS/MS and micro-PET measurements.

A significant amount of effort has recently been devoted to generating PDE10A PET tracers in research and drug development in relation to treating schizophrenia and Huntington’s disease. To this end, several PDE10A PET tracers have been developed by us and others, including [11C]MP10 (Andrés et al., 2011), [18F]JNJ42259152 (Celen et al., 2010), [13C]AMG 7980 (Hwang et al., 2014), [13C]Lu AE92686 (Kehler et al., 2014), [18F]MNI 659 and 654 (Barret et al., 2014), and [13C]IMA107 (Plisson et al., 2014). After a survey of the two available tracers at the time of this work, together with our previous work (Hwang et al., 2014), we sought a tracer that could be radiolabeled with fluorine-18 because of its longer half-life and was distinct in structure from MP10 and the MP10 analog JNJ42259152. In the current study, we identified and characterized AMG 580, a novel PDE10A antagonist tracer with a distinct chemical structure compared with previously published tracers, which exhibits the
desirable tracer profiles, including subnanomolar PDE10A potency and binding affinity, high specificity, optimal in vitro and in vivo kinetic properties, and selective tissue uptake. Future studies using 18F-PAMG 580 in human subjects will provide insight into how it compares with other tracers for clinical studies assessing PDE10A distribution in patient populations, such as schizophrenia and Huntington's disease, and target occupancy by therapeutic agents.

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Authorship Contributions


Contributed new reagents or analytic tools: Hu, Thiel, Davis.


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References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzyme reaction. Biochem Pharmacol 22:3099–3108.


D1 receptor signaling in striatal neurons.


