Discovery of D1 Dopamine Receptor Positive Allosteric Modulators: Characterization of Pharmacology and Identification of Residues that Regulate Species Selectivity

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ABSTRACT

The present studies represent the first published report of a dopamine D1 positive allosteric modulator (PAM). D1 receptors have been proposed as a therapeutic target for the treatment of cognitive deficits associated with schizophrenia. However, the clinical utility of orthosteric agonist compounds is limited by cardiovascular side effects, poor pharmacokinetics, lack of D1 selectivity, and an inverted dose response. A number of these challenges may be overcome by utilization of a selective D1 PAM. The current studies describe two chemically distinct D1 PAMs: Compound A 1-(rel-1S,3R,6R)-6-(benzo[d][1,3]dioxol-5-yl)bicyclo[4.1.0]heptan-3-yl)-4-((2-bromo-5-chlorobenzyl) piperazine] and Compound B [rel-(9R,10R,12S)-N-(2,6-dichloro-3-methyl[phenyl]-12-methyl-9,10-dihydro-9,10-ethanoanthracene-12-carboxamide]. Compound A shows pure PAM activity, with an EC50 of 230 nM and agonist activity at the D2 receptor in D2-expressing human embryonic kidney cells. Compound B shows superior potency (EC50 of 43 nM) and selectivity for D1 versus D2 dopamine receptors. Unlike Compound A, Compound B is selective for human and nonhuman primate D1 receptors, but lacks activity at the rodent (rat and mouse) D1 receptors. Using molecular biology techniques, a single amino acid was identified at position 130, which mediates the species selectivity of Compound B. These data represent the first described D1-selective PAMs and define critical amino acids that regulate species selectivity.

Introduction

Schizophrenia is a neuropsychiatric disorder characterized by positive symptoms, negative symptoms, and cognitive deficits. Working memory, a critical component of cognition, has been associated with functional outcomes in schizophrenic patients (Green et al., 2000). Because current antipsychotic medications do not address cognitive deficits in schizophrenia, a large unmet clinical need for treating these symptoms remains.

Dopamine is a catecholamine neurotransmitter found in both the brain and the periphery. Dopamine receptors can be categorized into two groups: D1-like (D1 and D5 receptors) and D2-like (D2–D4 receptors) (Sibley and Monsma, 1992). Within the brain, particularly in the prefrontal cortex, dopamine plays an important role in regulating cognition (Puig et al., 2014). Brozoski et al. (1979) first demonstrated the critical role of dopamine in regulating working memory in nonhuman primates by showing that depletion of dopamine in the cortex impaired performance on a delayed alternation performance task. Moreover, this effect was reversed with levodopa treatment (Brozoski et al., 1979). Subsequent studies have demonstrated that administration of a full or partial agonist of D1-like receptors reversed working memory deficits induced by ketamine administration or in aged monkeys (Cai and Arnsten, 1997; Castner and Goldman-Rakic, 2004; Nakako et al., 2013). Data from these studies support the hypothesis that the development of a subtype-selective D1 agonist or partial agonist may be beneficial for the treatment of cognitive deficits associated with schizophrenia.
To date, a number of D1-like agonists have been developed and tested preclinically, but clinical success of these agonists has been hindered by a number of issues. These include lack of D1 selectivity (against both D2-like and D5 receptors), poor pharmacokinetics, and adverse cardiovascular effects. Specifically, dihydrexidine is a D1-like full agonist with similar affinity for D1 and D5 and roughly 10-fold selectivity over D2-like receptors (Mottola et al., 2002). Dihydrexidine showed improvement in a working memory task in patients with schizotypal personality disorder (Rosell et al., 2015). The D1 agonist produg ABT-431 also caused hypotension at doses that demonstrated clinical efficacy versus negative symptoms (Rascov et al., 1999), D1 and D5 receptors are expressed in vascular and renal tissues and both modulate blood pressure, although their individual roles are still being investigated (Zeng et al., 2007). Indeed, the only clinically approved D1 agonist, fenoldopam, is approved for the treatment of hypertension and hypertensive crisis.

Positive allosteric modulators (PAMs) represent an alternative approach to orthosteric agonists (compounds that interact with the native ligand-binding site). PAMs can increase the affinity and/or efficacy of the orthosteric agonist for its target receptor by acting at a site other that the native ligand-binding site (allosteric) and represent a novel approach for the development of antipsychotic agents. The PAM approach to central nervous system pharmacology has already had clinical validation. In GABAergic systems, PAMs (such as the prototype diazepam) are equally effective to orthosteric agonists, with significantly better safety and tolerability profiles (Costa, 1991). Importantly, so-called pure G protein–coupled receptor (GPCR) PAMs, which lack intrinsic agonist activity within a specific signaling pathway, have been described. These compounds modulate the basal tone of the endogenous ligand in a manner that conserves spatial and temporal elements of native neurotransmission (Christopoulos and Kenakin, 2002; Wootten et al., 2013). Indeed, multiple PAMs have been identified for GPCRs, which may circumvent the challenges of orthosteric agonists (Conn et al., 2009; Koov et al., 2011). The present studies herein describe two novel D1 PAM chemotypes represented by Compound A [1-((rel-1S,3R,6R)-6-(benzo[d][1,3]dioxol-5-yl) bicyclo[4.1.0]heptan-3-yl)-4-(2-bromo-5-chlorobenzyl)piperazine] (piperazine series and Compound B [rel-(9R,10R,12S)-N-(2,6-dichloro-3-methylphenyl)-12-methyl-9,10-dihydro-9,10-ethanoanthracene-12-carboxamide] (ethanoanthracene series), which show selectivity for D1 and lack intrinsic agonist activity. Of these two PAMs, Compound B was selective for the human D1 versus rat D1 receptor, and this selectivity was mediated by a single amino acid residue within the human sequence. These studies are the first to describe a D1 PAM and provide structural information regarding key amino acids that regulate the PAM activity, thus providing a path forward for the development of D1 PAMs as potential therapeutics.

Materials and Methods

Cell culture and transfection reagents were purchased from Invitrogen (Carlsbad, CA). cAMP homogeneous time-resolved fluorescence kits were purchased from Cisbio (Bedford, MA). Dopamine, dopamine agonists, and antagonists were purchased from Sigma-Aldrich (St. Louis, MO).

Heterologous Expression. Human embryonic kidney (HEK) and Chinese hamster ovary cells were transfected with cDNA containing the human, rat, or mouse dopamine D1 receptor, and stable cell lines were developed using the following sequence information: human DRD-1 NM_000794.3, human DRD-2 NM_000795.3, human DRD-4 NM_000797.9, human DRD-5 NM_000798.9, rat DRD-1 NM_012546, and mouse DRD-1 NM_010076. Transient transfections were performed using Lipofectamine and Lipofectamine Plus reagent (Invitrogen) following the manufacturer’s instructions.

Primary Neuronal Cultures. Cortical and striatal tissue was removed from embryonic day 19 rat embryos. Tissue was dissociated using the Papain dissociation system (Worthington, Lakewood, NJ). Following dissociation, cells were cultured in neurobasal medium plus B27 supplement. Primary neurons were used in functional assays between days 12 and 14 in culture.

cAMP Accumulation Assay. The cAMP accumulation assay was performed according to the manufacturer’s instructions. Briefly, cells were plated into polylysine-coated 384-well plates. The medium was removed and replaced with 20 μl of Hanks’ balanced salt solution plus 0.3 μl of serial diluted PAM compounds or control diluent for 15 minutes. An EC20 of dopamine (∼EC20 for the PAM mode) or buffer (for the agonist mode) was added to the cells and incubated at room temperature for 30 minutes. The dopamine EC20 was determined for each experiment and ranged from 2 to 10 nM for transfected HEK cells and 300 to 600 nM for primary neuronal cultures. Anti-cAMP cryptate and D2-labeled cAMP were added to the wells, and the plates were incubated for 1 hour. The plates were read on an Envision plate reader (PerkinElmer, Waltham, MA).

D1 Receptor Mutagenesis. D1 alanine mutants in a PCXP-nFlag-DRD1/PC DNA3 expression vector were created by site-directed mutagenesis. Two hundred nanograms per 200 nl wild-type (WT) or mutant DNA was added per well of a 384-well amine plate (BD Biosciences, San Jose, CA) using the Labcyte ECHO550 (Sunnyvale, CA). A 1:20 dilution of TransFast transfection reagent (Promega, Madison, WI) in serum-free Dulbecco’s modified Eagle’s medium high glucose medium without additive (Life Technologies, Grand Island, NY) was added to the DNA plate following the manufacturer’s instructions and mixed by shaking for 15 minutes at room temperature. Ten thousand HEK293T cells in assay medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum; Gibco, Madison, WI) were then dispensed per well in the assay plate and incubated for 18–24 hours at 37°C and 5% CO. The cell medium was aspirated, and cells were washed once with phosphate-buffered serum (Life Technologies). For the PAM mode cAMP assay, 8 μl IBMX (3-isobutyl-1-methylxanthine) buffer (Sigma-Aldrich) was added to each well, followed by the addition of 100 nl in an 11-point, 5-fold serial dilution (final concentrations ranged from 80 μM to 1.3 nM). Two microliters of dopamine (Sigma-Aldrich) at a final concentration of 2.5 nM (∼EC20) was then added. Cells were incubated for 30 minutes at room temperature, and the homogeneous time-resolved fluorescence cAMP assay was conducted according to the manufacturer’s specifications. Briefly, 5 μl anti-cAMP cryptate and 5 μl D2-labeled cAMP, both in lys buffer, were added to the cells of the well plate, 20 μl were transferred to a proxi plate (PerkinElmer), and the plates were incubated for 1 hour at room temperature. Fluorescence was quantitated (fluorescence ratio of 665:620 nm) by PerkinElmer Envision. Activation data for the test compound over a range of concentrations were plotted as the percentage activation of the test compound (100% = maximum response). After correcting for background, the EC50 values were determined. The EC50 is defined as the concentration of the test compound that produces 50% of the maximal response and was quantified using the four-parameter logistic equation to fit the data.

Generation of D1 Chimera. All human/rat chimera constructs and the human R to Q 130-point mutant were provided by the Life Technologies Gene Art custom de novo synthesis service. Start codons were preceded by kozak consensus sequences CCACC. All sequences included 5’ and 3’ Gateway adapters, and final sequences were
expressed in a Gateway-modified pIRES neo2 vector (Clontech, Mountain View, CA). The rat Q130 R mutant and rat/human + Q130R [rat/human chimera (depicted in Fig. 5), with a single amino acid Q130 converted back to the corresponding amino acid R] point mutants were generated using the Stratagene QuickChange Lightning site-directed mutagenesis kit (La Jolla, CA) and expressed in pIRES neo2.

Molecular Modeling. The Structure Prediction Wizard within Prime (version 3.1; Schrödinger, LLC, New York, NY) was used to generate a homology model starting from a hand-edited alignment of a human D1R sequence, with the β2-adrenergic receptor (β2AR) protein sequence from the X-ray crystal structure of the nanobody-stabilized agonist-bound β2AR (PDB ID 3P0G) (Rasmussen et al., 2011). D1R residues 23–232 and 267–346 were included, and intracellular loop 3 (residues 233–266) was omitted. The initial model was generated using default model-building parameters, and loops were refined with Prime. Following energy minimization of the model using the OPLS2-2005 force field (Jorgensen et al., 1996; Jorgensen and Tirado-Rives, 1998; Shivakumar et al., 2010), the D1 agonist dinapsoline (Ghosh et al., 1996) was modeled into the orthosteric site. Using the OPLS2-2005 force field (Jorgensen et al., 1996; Jorgensen and Tirado-Rives, 1998; Shivakumar et al., 2010), the D1 agonist dinapsoline (Ghosh et al., 1996) was modeled into the orthosteric site. In the modeled binding pose, the protonated amine of dinapsoline forms a salt bridge with conserved D1033.32, and each hydroxyl group was hydrogen bonded to either S1985.42 or S2025.46 [for residues predicted to lie within transmembrane helices, Ballesteros-Weinstein indices (Ballesteros and Weinstein, 1995) are provided as superscripts]. Mutagenesis studies (Pollock et al., 1992; Tomic et al., 1993; Kong et al., 2006) have shown that these residues are important for dopamine binding to D1R, and in the crystal structure of the β2AR bound to carazolol (PDB ID 3P0G) (Rasmussen et al., 2011), the agonist forms similar interactions with the corresponding residues. D1R residues 11–22 were omitted during the initial homology model generation, and when it was determined that inclusion of these residues in the model was desirable, they were appended to the N terminus of the initial model (transmembrane [TM] 1) in a canonical α-helical conformation, as observed in the β2AR structural template, and the N terminus of this modified structural model was acetylated. Following energy minimization of the revised D1R/dinapsoline model using the OPLS2-2005 force field (Jorgensen et al., 1996; Jorgensen and Tirado-Rives, 1998; Shivakumar et al., 2010), a single 100-nanosecond molecular dynamics simulation (isothermal-isobaric ensemble) in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine lipid bilayer with full explicit solvation (transferable intermolecular potential 3 point water) (Jorgensen et al., 1983) at 300 K was performed on a Linux cluster using the NAMD version 2.8 program (Phillips et al., 2005) and the AMBER/CHARMM force field (Weiner et al., 1984; Cornell et al., 1995; Wang et al., 2004; Hornak et al., 2006; Lindorff-Larsen et al., 2010). The simulation time of 100 nanoseconds is adequate for alleviating high-energy unfavorable interactions in the initial model and refolding modeled loops into low-energy conformations, but is unlikely to yield the native structure of the D1R, especially if the initial model was inaccurate. In-house scripts were used to monitor the protein and ligand root-mean-square deviation (RMSD) values, with respect to the initial structure as well as the total energy of the system and other parameters. After approximately 25 nanoseconds, large changes in protein and ligand RMSD were completed and the mean of all the heavy atom protein and ligand RMSD values averaged over the entire simulation was 3.2 and 1.7 Å, respectively (Supplemental Fig. 1). Key hydrogen bonding interactions between the ligand and D1033.32, S1985.42, and S2025.46 were maintained during the simulation (Supplemental Figs. 2 and 3). The mean structure from the 100th nanosecond of the simulation was subjected to limited energy minimization to yield a final model.

Data Analysis. Data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). All values are shown as the mean ± standard deviation unless otherwise indicated. Multiple comparisons were made using an analysis of variance, followed by Newman-Keuls post hoc analysis. In all cases, F < 0.05 was considered to be statistically significant.

Results

Following a high-throughput screen of the Bristol-Myers Squibb chemical library, two D1 PAM chemotype series were identified and designated as piperazines and ethanoanthracenes. Representative compounds from each chemical series are depicted in Fig. 1. The potency and selectivity of these compounds were evaluated in a dopamine receptor panel.
TABLE 1
Selection of D1 PAMs in heterologous expression systems

<p>| Potency of D1 PAMs in Chinese hamster ovary and HEK cells expressing the human isomer of D1. Activity of D1 PAMs at other dopamine receptors was evaluated in Chinese hamster ovary cells expressing D2, D4, or D5 receptors. Values represent the mean IC50 values (± 1 S.D.). To determine agonist and PAM activity, compounds were tested in the absence of dopamine (agonist mode) or in the presence of an EC50 of dopamine (PAM mode). |
|-----------------|-----------------|
|                  | D1 PAM EC50     |</p>
<table>
<thead>
<tr>
<th>Compound A</th>
<th>Compound B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese hamster ovary</td>
<td>210 ± 30 (n = 2)</td>
</tr>
<tr>
<td>HEK agonist</td>
<td>250 ± 60 (n = 2)</td>
</tr>
<tr>
<td>D1 PAM</td>
<td>&gt;30,000 (n = 2)</td>
</tr>
<tr>
<td>D2 PAM</td>
<td>330 ± 100 (agonist)</td>
</tr>
<tr>
<td>D4 PAM</td>
<td>&gt;30,000 (n = 2)</td>
</tr>
<tr>
<td>D5 PAM</td>
<td>&gt;30,000 (n = 2)</td>
</tr>
</tbody>
</table>

Using both Chinese hamster ovary and HEK cell backgrounds in the absence (agonist mode) or presence of an EC50 of dopamine (PAM mode). Data presented in Table 1 show that members of the ethanoanthracene series, represented by Compound B, were selective D1 PAMs with no agonist activity. Because we were unable to generate a D3-expressing cell line sufficient for evaluating compound activity, selectivity data at this receptor remain to be determined. Compounds from the piperazine series, represented by Compound A, showed D1 PAM activity but also agonist activity at the D2 receptor (Table 1). As agonism at D2 receptors would worsen positive symptoms of schizophrenia, we chose to focus efforts on the ethanoanthracene series. Compound B was tested in a fold-shift assay to determine the maximal shift in dopamine potency seen with this compound. Data presented in Fig. 2 show that Compound B is a high fold-shift compound (produces a maximal 18-fold shift in dopamine potency in a CAMP accumulation assay), with a potency (shift50) value of 0.4 μM (0.2–0.6 μM). This shift50 value corresponds to the affinity (Kp) of the PAM for D1 in the absence of the agonist according to the allosteric ternary complex model (Christopoulos and Kenakin, 2002). Based upon this model, the affinity of the PAM in the presence of the agonist can be calculated to be approximately 0.02 μM, although this could not be measured directly in the current study. To confirm the activity of this series in a native D1 cellular background, we generated rat primary neuronal cultures and characterized the activity of D1 receptors in this neuronal system. Table 2 summarizes the potency of dopamine and dopamine agonists in primary cortical neurons. Dopamine increased CAMP production in primary cortical cultures, with a potency of 354 nM (±167 nM; n = 8, Table 2). The effect of dopamine in CAMP production was completely blocked by the D1 antagonist SCH23390 [(R)(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine], but was unaffected by the D2 antagonist sulpiride (Fig. 3). The D1 partial agonists SKF-38393 [(±)-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol] and SKF-89359 [6-chloro-2,3,4,5-tetrahydro-3-methyl-1(3-methylphenyl)-1H-3-benzazepine-7,8-diol] showed nanomolar potency, with sub-maximal CAMP activation (relative to dopamine control), whereas the D1 full agonist SKF-81297 [(±)-6-chloro-2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine] increased CAMP levels to a level comparable to that of dopamine (Table 2). Expression profiling of cortical neurons also confirmed D1 receptors as the highest expressing dopamine receptors in these neuronal preparations (data not shown). Collectively, these data confirm that this rat primary neuronal culture system has endogenous D1 receptors and validate the use of this endogenous cellular system for evaluating D1 PAM activity.

Activity of Compound B was evaluated in rat primary neurons in both the agonist and PAM mode. Compound B showed minimal PAM activity (Ymax < 20% of dopamine control), with no agonist activity in rat primary cortical neurons (Fig. 4A). For comparison, Compound A was also tested in rat primary neurons. Consistent with data in transformed cell lines, Compound A showed no agonist activity, but showed increased CAMP production (Ymax = 50% of dopamine control) in the presence of an EC50 of dopamine (Fig. 4A). These data were unexpected as Compound B was five times more potent than Compound A, and both showed similar Ymax values (63.6 and 64.3, respectively) in D1-expressing HEK cells. To explore this apparent disconnect between cells transfected with human D1 receptors and rat primary neurons, Compound B and Compound A were evaluated in HEK cells expressing the rat D1 receptor. Consistent with data in rat primary neurons, this experiment showed that Compound A exhibited PAM activity at rat D1 receptors, whereas Compound B was inactive at rat D1 receptors (Fig. 4B). To determine if this reduced activity at rat D1 receptors was consistent for other compounds from the ethanoanthracene series and to evaluate activity at non-human primate D1 receptors, an additional set of ethanoanthracene compounds was evaluated in HEK cells expressing, rat, cynomolgus (cyno), and human D1 receptors (Supplemental Fig. 4; Table 3). Consistent with the decreased activity at rat D1 receptors for Compound B, all structurally similar compounds showed reduced activity (less potent and...
decreased \( Y_{\text{max}} \) at the rat D1 receptor, whereas activity was comparable between human and cyno D1 receptors.

To identify the amino acid(s) involved in this species selectivity, two parallel work streams were initiated. First, an alanine scan was conducted to replace key amino acids within the human D1 receptor. For these studies, select point mutants were generated based on their position within the receptor protein, their ability to retain dopamine signaling (potency within 10-fold of the wild-type receptor), and a level of overall receptor expression comparable to the wild-type receptor. For each of these mutants, a PAM concentration response curve was run in the presence of an EC\(_{20}\) of dopamine and PAM EC\(_{50}\) values were generated. Because we were interested in amino acids that contributed to loss of efficacy of Compound B at the rat D1 receptor, we focused on mutants that showed the largest difference in potency between Compound B and Compound A. One point mutant evaluated, five showed a shift in potency greater than 4-fold for Compound B versus Compound A (Table 4).

In parallel with mutagenesis efforts, the potency of each compound was evaluated at a series of human-rat D1 chimeric receptors in an effort to identify the amino acid(s) that contributed to the species selectivity of Compound B (Fig. 5). In all of these chimeras, the potency of dopamine was unchanged, suggesting that orthosteric binding and effector coupling was unchanged by the chimera (Table 5). Moreover, in the full-length rat receptor, conversion of the human amino acid R130 completely restored the activity of Compound B (Table 5). In the rat N terminus through extracellular loop two of the human D1 was exchanged with the analogous rat sequence. Collectively, these data suggest that the binding site for Compound B might lie between the start of TM1 and the end of TM4. Further analysis of the human and rat D1 sequences in this region identified only three amino acids that differ between the human and rat sequence: F92, L, S943.23 → P, and R130 → Q (human → rat changes). Of these, F92, which is located in extracellular loop 1, and S943.23 are conserved in the mouse and did not affect the potency of Compound B in the alanine mutagenesis studies. To further test the role of R130, located in intracellular loop 2 (ICL2), in contributing to the species selectivity of Compound B, two point mutants were generated by substituting the rat glutamine at position 130 with the human amino acid arginine. In the rat N terminus through TM5 chimera, substitution of Q130 with the human amino acid R130 completely restored the activity of Compound B (Table 5). Moreover, in the full-length rat receptor, conversion of this single amino acid to the human amino acid (R130) restored activity of Compound B. To confirm this observation, full fold-shift cAMP accumulation assays were performed in parallel utilizing HEK293 cells transiently expressing either wild-type human D1, wild-type rat D1, or the rat Q130R D1 mutant. In these experiments, Compound B produced a maximal 11-fold (6- to 19-fold) increase in dopamine potency, with a measured shift\(_{50}\) value of 0.4 \( \mu \text{M} \) (0.1–2 \( \mu \text{M} \)) (Fig. 6), in agreement with the results obtained from similar experiments using cells stably expressing human D1 (Fig. 2). Compound B produced no observable effect on dopamine responses in HEK293 cells transiently expressing wild-type rat D1. However, in cells transiently expressing the rat
Q130R mutant D1 receptor, both the cooperativity and potency of Compound B were fully restored, with shift_max = 11-fold (7- to 18-fold) and shift50 = 0.5 μM (0.2–2 μM), values that were indistinguishable from those observed at human D1 (Fig. 6). Collectively, these data suggested a critical role of arginine 130 in the human D1 receptor in the activity/species selectivity of Compound B.

In Fig. 7, the locations of R130 and the residues where alanine mutation more negatively impacts the EC50 of Compound B than Compound A (Table 4) are highlighted on a molecular dynamics-refined homology model of the D1R/dinapsoline complex. This model was generated using the X-ray crystal structure of the nanobody-stabilized agonist-bound 2AR as a template (Rasmussen et al., 2011). Based on the model (and as expected from sequence proximity), R130 in ICL2 is close to V1193.48 and W1233.52, which are located on the membrane-exposed face of α-helix transmembrane region TM3. The distances from the Cα atom of R130 to those of V1193.48 and W1233.52 are 16 and 10 Å, respectively. The EC50 values of Compound B against these mutants are more than 900-fold larger than the EC50 measured in WT HEK cells. In the model, residue V582.38 is at the intracellular end of the transmembrane region TM2 helix. The inter-Cα distance for R130 and V582.38 is 15 Å, and the V582.38A mutation increases the EC50 of Compound B by 220-fold relative to WT. M135 is located in ICL2 about 9 Å from R130, and the EC50 of Compound B against the mutant is about 42-fold larger than Compound A (Table 4) are highlighted in Fig. 7, the residues discussed above are also close to the D1R-conserved D1203.49, R1213.50, and Y1223.51 motif, which is important in modulating the constitutive activity of GPCRs (Scheer et al., 1996; Capra et al., 2004; Rovati et al., 2007). ICL2 is hypothesized to form part of the Compound B binding site, and this loop has been proposed to stabilize the inactive state of GPCRs. Binding of Compound B proximal to these two D1R structural elements could potentially impact their function, contributing to the observed PAM activity of the compound.

Compound B is primarily hydrophobic/aromatic in nature, and the amide is the only polar moiety in the molecule. With the exception of R130, all of the residues discussed above have hydrophobic side chains that could make favorable van der Waals contacts with this PAM. A π-cation interaction between the side chain of R130 and one of the aromatic rings in the compound is also plausible. In the rat D1R, this residue is a glutamine that cannot form an analogous interaction with the ligand. Absence of this putative π-cation interaction in the rat D1R could contribute to the observed species selectivity of Compound B.

As discussed above, the PAM-binding site may include parts of ICL2 and the N- and C-terminal ends of TM α-helices 2 and 3, respectively. However, it is also possible that the mutations that diminished the PAM activity of Compound B impact the binding of Compound B at some other site in D1R through an allosteric mechanism. Alternatively, the

### Table 3

Characterization of additional ethanoanthracene compounds in HEK cells expressing rat, human, or cyno D1 receptors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human D1</th>
<th>Cyto D1</th>
<th>Rat D1</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>EC50 (%)</td>
<td>Y_max (%)</td>
<td>nM</td>
</tr>
<tr>
<td>ETC-1</td>
<td>38 ± 8</td>
<td>82 ± 12.3</td>
<td>48 ± 16</td>
</tr>
<tr>
<td>ETC-2</td>
<td>77 ± 10</td>
<td>72 ± 19</td>
<td>65 ± 20</td>
</tr>
<tr>
<td>ETC-3</td>
<td>254 ± 122</td>
<td>57 ± 9.7</td>
<td>425 ± 53</td>
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### Table 4

Top amino acid mutants showing loss of activity for Compound B.

<table>
<thead>
<tr>
<th>D1 Variant</th>
<th>Compound A</th>
<th>Compound B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (%)</td>
<td>Y_max (%)</td>
</tr>
<tr>
<td>WT</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td>W123A</td>
<td>600</td>
<td>20</td>
</tr>
<tr>
<td>V119A</td>
<td>1800</td>
<td>35</td>
</tr>
<tr>
<td>V58A</td>
<td>730</td>
<td>22</td>
</tr>
<tr>
<td>F95A</td>
<td>380</td>
<td>37</td>
</tr>
<tr>
<td>M135A</td>
<td>650</td>
<td>21</td>
</tr>
</tbody>
</table>

Potency of Compounds B and A was determined in HEK cells transiently expressing various human D1 point mutants as described in Materials and Methods. For each mutant, the endogenous amino acid and the predicted position with the human D1 protein sequence are given. Values represent the calculated nanomolar potency (± S.D.). Y_max values represent the maximal increase in cAMP relative to dopamine control.
mutations could mitigate the positive allosteric modulation effects of Compound B by altering the interactions of the mutant D1R with the G protein and/or modifying the level of constitutive activity of the receptor while not directly impacting the binding of Compound B. Since two of the residues where mutations significantly diminished the PAM activity of Compound B (V119^3.48 and W123^3.52) immediately flank the D120^3.49, R121^3.50, and Y122^3.51

**TABLE 5**

<table>
<thead>
<tr>
<th>Chimera</th>
<th>Dopamine</th>
<th>Compound A</th>
<th>Compound B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50</td>
<td>Ymax</td>
<td>EC50</td>
</tr>
<tr>
<td></td>
<td>nM</td>
<td>%</td>
<td>nM</td>
</tr>
<tr>
<td>Human</td>
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<td>245 ± 115</td>
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<td>Rat</td>
<td>48 ± 17</td>
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<td>270 ± 168</td>
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<tr>
<td>Rat N terminus</td>
<td>16 ± 4</td>
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<td>662 ± 213</td>
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<tr>
<td>Rat ECL2</td>
<td>21 ± 3</td>
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<td>712 ± 168</td>
</tr>
<tr>
<td>Rat TM6/ECL3</td>
<td>34 ± 9</td>
<td>100</td>
<td>960 ± 197</td>
</tr>
<tr>
<td>Rat ICL3</td>
<td>49 ± 38</td>
<td>100</td>
<td>296 ± 175</td>
</tr>
<tr>
<td>Rat C terminus</td>
<td>22 ± 17</td>
<td>100</td>
<td>237 ± 243</td>
</tr>
<tr>
<td>Human/rat</td>
<td>37 ± 25</td>
<td>100</td>
<td>311 ± 165</td>
</tr>
<tr>
<td>Rat/human</td>
<td>54 ± 31</td>
<td>100</td>
<td>210 ± 110</td>
</tr>
<tr>
<td>Rat/Q130R</td>
<td>74 ± 47</td>
<td>100</td>
<td>273 ± 125</td>
</tr>
<tr>
<td>Rat/human + Q130R</td>
<td>80 ± 12</td>
<td>100</td>
<td>345 ± 50</td>
</tr>
</tbody>
</table>

ECL, extracellular loop.
motif in D1R, this latter possibility also seems likely. At present, the available data are insufficient to discriminate between these possibilities.

**Discussion**

Compounds A and B were identified as D1 PAMs and optimized following a high-throughput screen of the Bristol-Myers Squibb chemical library. These compounds represent two distinct chemotypes and are the first reported D1 receptor PAMs. Both Compounds A and B showed nanomolar PAM potency, with no agonist activity (Fig. 1; Table 1). Moreover, Compound B showed selectivity against other dopamine receptors, including D2, D4, and D5 receptors. Although D3 heterologous expression systems have been used as models to support D3 receptor-binding studies, we were unable to generate a D3-expressing cell line with a functional readout sufficient to support evaluation of PAM activity. Therefore, functional activity of these PAMs at the D3 receptor remains to be determined. However, these D1 PAMs do provide the initial steps toward the development of D1 PAMs for the potential treatment of cognitive dysfunction associated with psychiatric disorders, such as schizophrenia.

The development of D1 agonists for the treatment of cognitive dysfunction has been limited by two major factors. First, D1 agonists used in the clinic have been associated with hypotension, limiting the clinical dose (Blanchet et al., 1998; Rascol et al., 1999). To date, no D1 selective compounds have been identified to delineate the role of D1 versus D5 receptors in regulating blood pressure. However, preclinical data may support a greater role of the D5 receptor in regulating blood pressure, relative to the D1 receptor. For example, D5 receptors were shown to play an important role in regulating blood pressure.

**Table 6**

<table>
<thead>
<tr>
<th>Residue</th>
<th>D1R</th>
<th>D2R</th>
<th>D3R</th>
<th>D4R</th>
<th>D5R</th>
</tr>
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<tr>
<td>58</td>
<td>Y</td>
<td>T</td>
<td>T</td>
<td>P</td>
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<tr>
<td>130 ICL1</td>
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<td>R</td>
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<tr>
<td>135 ICL2</td>
<td>M</td>
<td>S</td>
<td>S</td>
<td>G</td>
<td>M</td>
</tr>
</tbody>
</table>

**Fig. 6.** Fold-shift analysis of Compound B at (A) wild-type human D1, (B) Q130R mutant rat D1, or (C) wild-type rat D1. HEK cells transiently expressing these various D1 receptors were treated with various concentrations of dopamine in the presence or absence of increasing concentrations of Compound B. The cooperativity (\(\alpha\) or shift\(_{max}\)) and shift\(_{50}\) values reported represent the mean and 95% confidence intervals from three experiments.

**Fig. 7.** Homology model of the D1R/dinapsoline complex, TMs, extracellular loops (ECL), and ICL are labeled. Key residues identified via alanine mutagenesis and human/rat chimera studies are depicted in a cyan ball and stick representation. The D1R D120\(^{5.49}\), R 130\(^{5.50}\), and Y122\(^{3.51}\) conserved motif and the agonist dinapsoline are shown in a magenta and orange ball and stick representation, respectively. The image was created with the PyMOL Molecular Graphics System (version 1.6.0.0; Schrödinger, LLC).
hypertension in D3 knockout mice (Wang et al., 2013). Genetic deletion of D5 receptors in mice results in elevated blood pressure (Hollon et al., 2002; Yang et al., 2004), whereas the role of D1 receptors in regulating blood pressure is still unclear (Albrecht et al., 1996; Wang et al., 1999). A selective D1 PAM may also have a reduced hypotensive liability compared with an agonist at both the D1 and D5 receptors simply because a D1 PAM would increase activity of fewer renal or vascular dopamine receptors. Although it was beyond the scope of this study, the lack of activity at D5 receptors may therefore reduce the hypotensive liability of the identified D1 PAMs relative to D1 agonists that lack D1 versus D5 receptor selectivity.

A second challenge with D1 receptor agonists is that preclinically, agonists have shown an inverted U dose response in that too much or too little D1 receptor activation can have adverse effects on cognition (for a review, see Williams and Castner, 2006). Because cognitive deficits associated with schizophrenia are hypothesized to result from a hypofunction of the prefrontal dopaminergic tone (Davis et al., 1991), a D1 PAM would be beneficial as it would enhance the effect of endogenous dopamine without direct activation of the D1 receptor.

One unexpected finding in the current study was the lack of activity of Compound B in rat primary neuronal cultures. This lack of activity in a rat endogenous expression system was confirmed in HEK cells overexpressing the D1 receptor (Fig. 4). The human and rat D1 sequence share a >90% sequence homology, with the majority of this sequence divergence occurring at the N and C termini. Based on these findings, we sought to determine the critical amino acid(s) that mediate species selectivity of Compound B. Alanine mutagenesis is a rapid screening method for identifying key amino acids important for binding of radioligands (Gregory et al., 2013). We adapted this approach to identify amino acids that contribute to the species selectivity of our D1 PAMs. In a parallel approach, a series of D1 human/rat chimeras were generated to further evaluate regions critical for PAM activity. Collectively, these data identified a critical amino acid, R130, as directly mediating the species selectivity of Compound B. Moreover, alanine mutagenesis studies suggest that other amino acids in ICL2 as well as proximal regions of TM helix 2 and TM helix 3 were critical in mediating the activity of Compound B (Table 4). These data suggest the binding region for the Compound B chymotype includes the second intracellular loop and possibly the second and third transmembrane helices of the D1 receptor. However, it is also possible that Compound B binds elsewhere in the D1R, with its activity negatively impacted by the mutations discussed above via an allosteric mechanism, modulation of receptor/G protein interactions, or mutation-induced changes in the level of constitutive activity of D1R. There are insufficient data to distinguish between these possibilities at present.

Little information has been published as to the contribution of this region in the function or expression of the D1 receptor. However, the amino acids identified from these studies are in close proximity to the glutamic acid/aspartic acid-arginine-tyrosine motif, which has been rigorously studied across many GPCRs (for a review, see Rovati et al., 2007). For example, changes in the glutamic acid or aspartic acid motif can increase the constitutive activity of the GPCR or increase agonist affinity and efficacy (Scheer et al., 1996; Capra et al., 2004). Chung et al. (2002) proposed that the ICL2 of some GPCRs takes on a helical structure and stabilizes the inactive state of the receptor. It is possible that our current D1 PAM (Compound B) modified the tertiary structure of the ICL2 in a manner that enhances efficacy of the orthosteric ligand without increasing the constitutive activity.

In summary, the current studies provide the first description of PAMs selective for the D1 receptor: Compound B and Compound A (Fig. 1). These compounds may be useful tools for understanding the contribution of increases in D1 versus D5 receptor activity across multiple physiologic systems, from the kidneys to the brain. Moreover, using molecular biology approaches and species differences, we were able to map the amino acids critical in the activity of our D1 PAM (Compound B), thus providing a structural scaffold for the development of future D1 PAMs. Collectively, these findings represent the initial steps toward the development of D1 PAMs for the potential treatment of neuropsychiatric diseases.

Authorship Contributions


Conducted experiments: Lewis, Hunihan, Watson, Beno, Ferrante, Molski, Kong, Cvijic, Rockwell, Alt, Brown.

Contributed new reagents or analytic tools: Lewis, Hunihan, Gentles, Hu, Huang, Bronson, Beno, Macor, Hendriksen, Molski, Kong, Cvijic, Alt, Brown.


Wrote or contributed to the writing of the manuscript: Lewis, Hunihan, Beno, Alt, Brown.

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