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Discovery and characterization of a G protein-biased agonist that inhibits

β-arrestin recruitment to the D₂ dopamine receptor

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Abbreviations: D1R: D$_1$ dopamine receptor, D2R: D$_2$ dopamine receptor, D3R: D$_3$ dopamine receptor, D4R: D$_4$ dopamine receptor, D5R: D$_5$ dopamine receptor, EC$_{50}$: 50% excitatory concentration, IC$_{50}$: 50% inhibitory concentration

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ABSTRACT

A high-throughput screening campaign was conducted to interrogate a 380,000+ small molecule library for novel D_2 dopamine receptor modulators using a calcium mobilization assay. Active agonist compounds from the primary screen were examined for orthogonal D_2 dopamine receptor signaling activities including cAMP modulation and β-arrestin recruitment. While the majority of the subsequently confirmed hits activated all signaling pathways tested, several compounds showed a diminished ability to stimulate β-arrestin recruitment. One such compound (MLS1547) is a highly efficacious agonist at D_2 receptor-mediated G protein-linked signaling, but does not recruit β-arrestin, as demonstrated using two different assays. This compound does, however, antagonize dopamine-stimulated β-arrestin recruitment to the D_2 receptor. In an effort to investigate the chemical scaffold of MLS1547 further, we characterized a set of 24 analogs of MLS1547 with respect to their ability to inhibit cAMP accumulation or stimulate β-arrestin recruitment. A number of the analogs were similar to MLS1547 in that they displayed agonist activity for inhibiting cAMP accumulation, but did not stimulate β-arrestin recruitment (i.e., they were highly biased). In contrast, other analogs displayed various degrees of G protein signaling bias. These results provided the basis to use pharmacophore modeling and molecular docking analyses to build a preliminary structure-activity relationship of the functionally-selective properties of this series of compounds. In summary, we have identified and characterized a novel G protein-biased agonist of the D_2 dopamine receptor and identified structural features that may contribute to its biased signaling properties.

INTRODUCTION

Dopamine receptors mediate the many actions of dopamine in the brain and periphery. In mammals, five distinct dopamine receptors have been characterized, which are divided into two subfamilies based on their structure, pharmacology, and signaling properties (Sibley et al., 1992). D1-like receptors (D1R and D5R) are Gαs/olf coupled, while D2-like receptors (D2R, D3R and D4R) are Gα$_{i/o}$ coupled (Sibley et al., 1992). Dopamine receptors have also been shown to signal through recruitment and activation of the scaffolding protein β-arrestin (Beaulieu et al., 2007a). Among the dopamine receptors, the D2R is one of the most validated drug targets in neurology and psychiatry. However, most drugs targeting the D2R are problematic, either being less efficacious than desired or possessing adverse side effects due to the activation or blockade of multiple parallel signaling pathways. Despite recent advances, it remains unclear which signaling arms of the D2R are involved in the therapeutic effects of various agents used to treat neuropsychiatric disease states associated with the D2R.

One of the best characterized signaling pathways of the D2R is Gi/Go-mediated inhibition of adenylate cyclase, which reduces intracellular cAMP levels and thereby attenuates phosphorylation of the 32-kDa dopamine and cAMP-regulated phosphoprotein (DARPP-32)(Bateup et al., 2008) by PKA. DARPP-32 is a protein phosphatase that acts as an integrator of cell signaling of many neurotransmitters, including dopamine (Svenningsson et al., 2004). Reduction of DARPP-32 phosphorylation inhibits its activity and associated downstream signaling pathways (Bateup et al., 2008; Svenningsson et al., 2004). Notably, administration of antipsychotic drugs, such as haloperidol or clozapine, has been shown to increase the level of DARPP-32 phosphorylation (Pozzi et al., 2003). A more recently characterized signaling pathway for the D2R is activation of glycogen synthase kinase 3 β (GSK3β), which is G protein-independent and occurs through agonist recruitment of β-arrestin-2 to the receptor. This leads to the formation of a β-arrestin-dependent complex of protein kinase B (Akt), and protein phosphatase 2A, which results in dephosphorylation of Akt and subsequent activation of GSK3β (Beaulieu et al., 2008; Beaulieu et al., 2007b). Interestingly, Caron and colleagues have argued that *inhibition* of this pathway in D2R-expressing neurons is correlated with antipsychotic properties (Masri et al., 2008; Urs et al., 2012), whereas Roth and colleagues have suggested that *stimulation* of the D2R-β-arrestin pathway may actually enhance antipsychotic efficacy (Allen et al., 2011).

A promising approach to dissecting the importance of these signaling pathways, and resolving these associated controversies, is to study them using ligands that exhibit functionally-selective or biased signaling properties (Urban et al., 2007; Whalen et al., 2011). Many GPCRs are able to transduce signals through more than one intracellular pathway. Although in most cases the endogenous transmitter will activate all signaling pathways, synthetic agonists may preferentially activate one signaling pathway over another, or even activate one while inhibiting another (Kenakin, 2007a; Kenakin, 2007b; Kenakin, 2008; Mailman, 2007; Urban et al., 2007). While the mechanisms underlying functional-selectivity are not known, a leading hypothesis is that GPCRs can adopt multiple functionally "active" conformational states that are either stabilized or induced by these selective ligands (Kobilka and Deupi, 2007; Wess et al., 2008). Although relatively few biased ligands have been described for the D2R (Gay et al., 2004; Kilts et al., 2002; Lane et al., 2007; Lane et al., 2008; Mottola et al., 2002), existing examples strongly support the concept of the D2R being able to adopt multiple signaling-biased confirmations. Recently, a structural basis for functional-selectivity of several GPCRs has been proposed (Abdul-Ridha et al., 2014; Dror et al., 2013; Kruse et al., 2013; Liu et al., 2012; Wacker et al., 2013) suggesting that rational design of functionally-selective compounds may be possible.

Recently, Jin and colleagues developed and characterized a series of analogs of the atypical antipsychotic aripiprazole that are partial agonists of D2R-mediated β-arrestin recruitment yet fail to stimulate Gi-linked cAMP inhibition (Allen et al., 2011; Chen et al., 2012). In contrast, no biased ligands have been described with the opposing pharmacology for the D2R, that is, stimulation of G protein signaling pathways without activation of β-arrestin recruitment. We now report the discovery of a novel, highly efficacious G protein-biased agonist for the D2R that also antagonizes β-arrestin recruitment to the receptor. Identification of such functionally-selective ligands may provide the requisite pharmacological probes with which to dissect these two signaling pathways and elucidate their function *in vivo*. Functionally-selective agonists may also result in improved therapies for certain neuropsychiatric disorders, such as Parkinson's disease, in which D2R stimulation, is desired, and schizophrenia, where inhibition of D2R signaling is the goal.

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MATERIALS and METHODS

Calcium Mobilization Assay – Flp-In T-Rex 293 cells were stably transfected with human D2SR and Gqi5 protein using the Flp-In T-Rex expression system (Life Technologies, Grand Island, NY). The cell line was constructed by co-transfecting SFD2s/FRT/TO and pOG44, followed by hygromycin B selection. Gqi5/pIRESpuro3 (Clonetech) was then transfected into the D2R stable cell line, followed by selection with puromycin. D2R expression is controlled by tetracycline induction, while Gqi5 is continuously expressed.

D2R-stimulated calcium mobilization was measured using methods similar to those previously published by our laboratory (Chun et al., 2013). Cells were induced with 1 μM tetracycline added directly to the culture media and plated in 384- or 1536-well, optical, clear bottom, black-walled plates (Greiner Bio-one, Monroe, NC). 20 μL/well (20,000 cells/well) were added to 384-well plates, and 3 μL/well (4,000 cells/well) to a 1536-well plate. The next day, cells were incubated for 60 min at room temperature in the dark with Fluo-8 NW calcium dye in the presence of an extracellular signal quencher (Screen Quest™ Fluo-8 NW Calcium Assay Kit, AAT Bioquest, Inc., Sunnyvale, CA), as recommended by the manufacturer. The plates were then treated with various concentrations of agonist in the presence of 0.2 mM sodium metabisulfite. Plates were read kinetically in real time (every 0.6 sec) for 2 min after agonist addition. Compound additions were done in unison using a 384-tip onboard robotic pipet (384-well assays) or the onboard 1536-pintool (1536-well assays) while continuously reading at an excitation wavelength of 480 nm and an emission wavelength of 540 nm on an FDSS μCell (384-well assays) or an FDSS 7000 (1536-well assays) (Hamamatsu, Bridgewater, NJ). Data were recorded and quantified as maximum minus minimum (max-min) relative florescence units (RFU) within the assay window using FDSS software.

cAMP Inhibition Assay - D2R-mediated inhibition of forskolin-stimulated cAMP production was assayed using the DiscoveRx HitHunter assay kit (DiscoveRx Inc., Fremont, CA). CHO-K1 cells stably expressing the human D2R long isoform (DiscoveRx) were seeded in CP2 media at a density of 5,000 cells/well in 384-well black, clear-bottom plates. After 16-24 h of incubation at 37°C, 5% CO_2, and 90% humidity, the media was removed and replaced with 5 μl / well PBS. Cells were treated with 2.5 μl of various concentrations of compound diluted in PBS in the presence of an ~EC_{80} concentration of forskolin (100 μM) and 0.2 mM sodium metabisulfite and incubated for 60 min at 37°C, 5% CO_2, and 90% humidity. DiscoveRx HitHunter reagents were then added,

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and cells incubated in the dark at room temperature, according to manufacturer recommendations.

Luminescence was measured on a Hamamatsu FDSS µCELL (Hamamatsu Photonics K. K., Bridgewater, NJ) for 8.5 sec. Data were collected as RLUs and values were normalized to a percentage of the maximum forskolin-stimulated cAMP signal. Data fit to a single site model and the Hill coefficients of the concentration response curves did not significantly differ from unity.

β-Arrestin Recruitment Assay - The ability of the agonist-activated receptor to recruit β-arrestin-2 was determined using the DiscoveRx PathHunter (DiscoveRx Inc, Fremont, CA) technology that involves enzyme complementation of fusion-tagged receptor along with an arrestin recruitment modulating sequence and β-arrestin-2 proteins. Control experiments determined that this PathHunter receptor construct will couple to G protein mediated signaling with similar efficacy as an unmodified construct (data not shown). Assays were conducted, with minor modifications, as previously published by our laboratory (Banala et al., 2011; Bergman et al., 2013). Briefly, CHO-K1 cells expressing D2R long isoform (DiscoveRx) were seeded in CP media (DiscoveRx) at a density of 2,625 cells/well in 384-well black, clear-bottom plates. Following 24 h of incubation, the cells were treated with multiple concentrations of compound in PBS containing 1% DMSO and incubated at 37°C for 90 min. DiscoveRx reagent was then added to cells according to the manufacturers recommendations followed by a 30-60 min incubation at room temperature. Luminescence was measured on a Hamamatsu FDSS µCELL reader (Hamamatsu, Bridgewater, NJ). Data were collected as relative luminescence units (RLU) and subsequently normalized to a percentage of the control luminescence seen with a maximum concentration of dopamine, with zero percent being RLU seen in the absence of any compound. The Hill coefficients of the concentration response curves did not significantly differ from unity.

β-Arrestin BRET Assay – To directly assess induction of D2R-β-arrestin-2 interaction we employed a bioluminescence resonance energy transfer (BRET) assay that utilizes a cell line stably transfected with an Rluc-8 fusion-tagged D2R (short isoform) under a tetracycline-inducible promoter, as well as mVenus fusion-tagged β-arrestin-2 (Hamdan et al., 2005; Klewe et al., 2008). The cell line was constructed using Flp-In T-REX 293 cells (Invitrogen, Life Technologies) transfected with pIRESpuro3/mVenus/β-arrestin-2, where the mVenus tag is on the N-terminus of the human β-arrestin-2. Clones were then analyzed for expression of the construct following selection with 2 µg/ml puromycin. The cell line with the highest level of expression was

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then transfected with pcDNA5/FRT/TO-SFD2LRluc8 and POG44 followed by hygromycin selection and subsequent functional screening to select the final stable line. Addition of the Rluc-8 substrate coelenterazine *h* results in an emission at 485 nm. However, when in close proximity with mVenus, resonance energy transfer leads to a shift in the emission spectrum from 485 nm to 510-540 nm, thereby quantifying the interaction between the receptor and the β-arrestin-2 protein. Cells were induced for 24 h by addition of 1 µM tetracycline directly to the culture media resulting in membrane receptor expression of approximately 5.8 pmol/mg protein. Cells were then removed from the plates using Earle's balanced salt solution without calcium (EBSS-), pelleted by centrifugation, re-suspended (200,000 cells/ml) in Dulbecco's Phosphate Buffered Saline (DPBS) (Mediatech, Manassas, VA) plus 0.05 g/500 ml sucrose and seeded into 96-well solid bottom white assay plates (20,000 cells/well) (Greiner Bio-one, Monroe, NC). Cells were allowed to sit for 45 min at room temperature and were then treated with 5 µM coelenterazine *h* (Nanolight Technology, Pinetop, AZ), incubated for 5 min and then stimulated with agonist using an onboard robotics 8 channel pipet head in a Flexstation III (Molecular Devices, Sunnyvale, CA). Original data are collected 5 min after agonist addition as a ratio of 525nm/485nm emission. Data are expressed as normalized to the percentage of the maximum dopamine-induced ratio.

Radioligand Binding Assays – Radioligand competition binding assays were conducted with slight modifications as previously described by our laboratory (Chun et al., 2013). HEK293 cells stably transfected with human D1R, D2R, D3R, D4R, or D5R (Codex Biosolutions, Inc., Gaithersburg, MD) were dissociated from plates using EBSS-, and intact cells were collected by centrifugation at 900 $\times$ g for 10 min. Cells were re-suspended and lysed using 5 mM Tris-HCl and 5 mM $MgCl_2$ at pH 7.4 at 4°C. Cell lysate was pelleted by centrifugation at 30,000 $\times$ g for 30 min and re-suspended in EBSS with calcium at pH 7.4. Cell lysates (100 µl, containing ~8 µg protein for D2-like receptor assays or ~10 µg protein for D1-like receptor assays) were incubated for 90 min at room temperature with the indicated concentrations of MLS1547 and either 0.5 nM [^{3}H]-SCH23390 (D1R and D5R), or 0.5 nM [^{3}H]-methylspiperone (D2R, D3R, and D4R) in a final reaction volume of 250 µl. Non-specific binding was determined in the presence of 4 µM (+)-butaclamol. Bound ligand was separated from free by filtration through a PerkinElmer Unifilter-96 GF/C 96 well micro-plate using the PerkinElmer Unifilter-96 Harvester, washing 3 times, 1 ml per well in ice-cold assay buffer. After drying, 50 µl

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of liquid scintillation cocktail (MicroScint PS, Perkin Elmer, Waltham, MA) was added to each well, plates were sealed, and analyzed on a PerkinElmer Topcount NXT™.

Pharmacophore Modeling – All modeling was performed using tools in the Schrödinger suite (Schrödinger Inc., New York, NY). While the main goal of pharmacophore modeling is to explain the molecular features that are associated with active compounds, such capability can benefit from the inclusion of "inactive" compounds in the model building procedure. In our case, to differentiate G protein-biased from non-biased agonists in such a model we defined a so called "biased activity" measure that is similar to the "bias" factor in (Kenakin et al., 2012), but without taking the exponential form:

$$biased\ activity = \ \log_{10}\left(\frac{E_{max}}{EC_{50}}\right)_{G\ protein} - \log_{10}\left(\frac{E_{max}}{EC_{50}}\right)_{arrestin}$$

For G protein-biased agonists that did not exhibit measurable β-arrestin stimulation, we used the $\log_{10}(E_{max}/EC_{50})$ values from the cAMP assay to quantitate the biased activity of these ligands.

Based on the biased activity calculations described above, ten compounds, displaying either the strongest or weakest G protein-bias were selected: NCGC9125, NCGC9126, NCGC6387, NCGC9141 and MLS1547 for the biased set, and NCGC9134, NCGC9132, NCGC5872, NCGC9131 and NCGC6388 for the non-biased set (see Results). For each compound, the 3D structure was built using the program Maestro (v9.5), and a single protonation state was chosen, with the 4-position of the piperazine bearing the positive charge in all cases. Multiple conformers were generated using the ConfGen program (v2.5). At least 10 conformers per ligand were generated, which required sampling with the "intermediate" search strategy of the program.

The goal of a pharmacophore search is to identify the largest set of pharmacophore features with specific 3-dimentional relationships, i.e., inter-feature distances that are common amongst all active compounds. The pharmacophore model was built using the program Phase (v3.6). The default pharmacophore features include positive (P), negative (N), hydrogen-bond acceptor (A), hydrogen-bond donor (D), aromatic (R) and hydrophobic (H) types. For the compounds studied here, the following functional groups were assigned to the hydrophobic (H) feature using a procedure that has been described in (Greene, 1994):

isopropyl, aromatic halogens, aromatic CH_3, and methoxy-CH_3. The location of a given hydrophobic site is a weighted average of the positions of the non-hydrogen atoms in the associated fragment.

In addition, to allow ambiguous alignment of non-polar features, aromatic groups in the ligands were assigned to both the R and H feature types. When we assigned these features to the selected ligands, the largest number of features in a pharmacophore hypothesis that produced matches for all 5 biased compounds was found to be 4. These 4-point pharmacophores were of the HHPR, AHHP, HPRR and AHPR variants (the order of the features is arbitrary). For each of these variants, a number of 3D hypotheses were enumerated and clustered based on all occurring inter-feature distances. From this initial set of hypotheses, the one that best matched the actives was then determined by a more rigorous custom scoring function ("survival score"), which consisted of 1) the *alignment score* based on the RMSD of feature positions, 2) the *vector score* of aligned features with a directional character (aromatic, donor and acceptor), and 3) the pairwise *volume overlap* of all the ligands aligned to the pharmacophore. This default scoring of all possible variants revealed that the HHPR and HRPR hypotheses were best able to explain the data. To eliminate the hypotheses that also matched the non-biased compounds, the 5 most non-biased compounds were used to rescore the hypotheses ("inactive score"). The finally selected pharmacophore was the HRPR variant (see Results) with the best "survival"-"inactive" score.

Construction of a novel active D2R model – Our active D2R model was based on an active model of the D3R, for which an inactive crystal structure is available (Chien et al., 2010). The active D3R model was created by applying a set of spatial constraints obtained by comparing the inactive and active states of the β_2 adrenergic receptor (β2AR). In brief, the inactive and active structures of the β2AR (PDB: 2RH1(Cherezov et al., 2007) and PDB: 3SN6 (Rasmussen et al., 2011) respectively) were aligned and, by subtracting the coordinates of the Cα atoms of the inactive from the active form, a set of delta-coordinates were created. These deltas were added to the coordinates of the aligned D3R structure to generate a set of spatial constraints. Using these constraints, the inactive model of the D3R was transformed into an active form by using a hybrid minimization-Monte Carlo scheme, implemented in the program Prime (v3.3). The active D3R model was then used as a template in building the D2R homology model using Prime. Docking of compounds

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into the active-state model of the D2R was achieved using the program Glide (v6.0), using the SP scoring

function. To determine the binding mode of the congeneric series, a single reference compound (MLS1547)

was first docked in the orthosteric binding site (OBS) revealed by the bound eticlopride in the D3R structure,

which is formed by residues from TMs 3, 5, 6, and 7. We found the pyridine moiety of MLS1547 preferred to

point towards TM2. Interestingly, for two well-studied D2R antagonists, spiperone and azaperone that share a

common 4-fluophenyl-4-butanone moiety, as proposed/validated previously (Boeckler et al., 2005), if we

assume that such a moiety is bound in the OBS, then the pyridine moiety of azaperone similarly points towards

TM2 as MLS1547 (data not shown). We also docked MLS1547 in another D2R model in an active

conformation based on a D3R active model (Newman et al., 2012) and equilibrated using MD simulations.

Encouragingly results from both D2R models were consistent. All other compounds were then docked using

core-constraints on the core substructure shared by all compounds to ensure the core in the OBS adopted a

similar binding mode.

RESULTS AND DISCUSSION

In an effort to discover G protein-biased agonists of the D2R, we screened the ~380,000 compound

small molecule library in the Molecular Libraries Probe Production Center Network (MLPCN) at the National

Center for Advancing Translational Sciences (NCATS). The primary screen used a stably transfected cell line

expressing the human D2R and a chimeric Gqi5 protein enabling robust calcium mobilization upon activation of

the D2R. This screen identified 2,288 compounds with significant D_2-Gqi5 agonist activity, defined as

compounds that, when screened at 40 μM, elicited a response larger than 3-fold over the standard deviation of

mean signal from control wells with no compound. Agonists were also screened for calcium mobilization in

parental, non-transfected cell line and any compound showing activity was eliminated. The active compounds

were then subjected to verification by generating full concentration-response curves for each compound.

Subsequently, the hit compounds that exhibited full dose-response-activity relationships were evaluated in two

orthogonal assays - one was to evaluate their ability to inhibit cAMP accumulation, a primary G protein

signaling mechanism for the D2R (Bateup et al., 2008), and the other was to test their ability to recruit β-

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arrestin, an important signaling response independent of G protein activation (Beaulieu et al., 2007a; Beaulieu

et al., 2008). As would be expected, our results revealed that the vast majority of the hit compounds were

equally active in both the G protein- and β-arrestin-mediated signaling assays (e.g., see Supplemental Fig. 1).

However, a small subset of hits (about two dozen) showed either greatly diminished efficacy and/or potency in

the β-arrestin recruitment assay (see Supplemental section for screening details). One of these compounds,

MLS000051547 (MLS1547, Fig. 1A), was selected for further characterization as it appeared to have high

efficacy in the G protein-mediated signaling assays, but no measurable activity in the β-arrestin recruitment

assay.

Indeed, Fig. 1B shows that MLS1547 behaved as a highly efficacious agonist in the D_2-Gqi5 calcium

mobilization assay with an EC_{50} value of 0.37 µM and an Emax of ~90%. In order to insure that this activity is

physiological, and not restricted to the chimeric G protein nature of the Gqi5 assay, we examined the ability of

MLS1547 to inhibit forskolin-stimulated cAMP accumulation in cells stably expressing the D2R. Fig. 1C shows

that, similar to dopamine, MLS1547, completely inhibited the stimulation of cAMP by forskolin with an EC_{50} of

0.26 µM suggestive of it having high efficacy at D2R-mediated G protein-linked signaling. We further found

that the inhibition of cAMP accumulation by MLS1547 was completely blocked by co-treatment with the D2R

antagonist sulpiride, as shown in Fig. 1D. The IC_{50} of 22 nM for sulpiride's inhibition of MLS1547's action is

similar to sulpiride's potency for blocking dopamine's response in this cAMP assay (data not shown). Taken

together, these data indicate that MLS1547 is a highly efficacious agonist at the D2R for stimulating G protein-

mediated signaling.

The ability of MLS1547 to stimulate recruitment of β-arrestin to the D2R was evaluated using the

DiscoveRx β-arrestin PathHunter assay, which relies on the complementation and activation of β-galactosidase

when β-arrestin-2 is recruited to the receptor. Notably, β-arrestin-2 is the β-arrestin protein functionally

coupled to the D2R *in vivo* (Skinbjerg et al., 2009). While incubation with dopamine resulted in robust

recruitment of β-arrestin-2 in a dose-dependent manner, MLS1547 failed to exhibit activity in this assay (Fig.

2A) despite it being a highly efficacious agonist of G protein-mediated signaling. However, other possible

explanations for these discrepant results could include interference of the compound with the enzyme-linked

signaling resulting in a suppression of signal. To address these alternative explanations, we employed a bioluminescence resonance energy transfer (BRET) assay that directly measures the physical interactions between the β-arrestin-2 protein and the D2R. Furthermore this also serves as a control for a different cell background, as these assays were conducted in HEK293 cells. As seen in Fig. 2B, however, MLS1547 failed to stimulate any observable recruitment of β-arrestin-2 to the D2R in this assay, despite robust recruitment by dopamine. Taken together, these findings indicate that, while MLS1547 is a highly efficacious agonist at G protein-mediated signaling, it is unable to stimulate measurable β-arrestin recruitment, thus establishing it as an extremely G protein-biased agonist at the D2R.

Since MLS1547 activates G protein-linked pathways, yet does not stimulate β-arrestin recruitment, MLS1547 would be expected to antagonize the dopamine-induced β-arrestin response by blocking dopamine binding to the receptor. As seen in Fig. 2C, MLS1547 fully antagonized dopamine-mediated β-arrestin recruitment to the D2R in the DiscoveRx assay, with an IC_{50} of 9.9 μM. Similar results were obtained when MLS1547 was examined for antagonist activity in the D2R β-arrestin BRET assay, demonstrating an IC_{50} of 3.8 μM (Fig. 2D). In summary, while MLS1547 is an agonist of D2R-stimulated G protein-mediated signaling, it acts as an antagonist of the β-arrestin-mediated signaling pathway.

In order to determine the affinity of MLS1547 for the D2R, we used standard radioligand binding competition analyses. MLS1547 was found to completely displace [^{3}H]methylspiperone binding to the D2R with a calculated K_i value of 1.2 μM ± 0.2 (Fig. 3). Displacement studies were also conducted on the other dopamine receptor subtypes (Supplemental Fig. 2) resulting in K_i values of 2.3 ± 0.2 μM (D3R) and 0.32 ± 0.04 μM (D4R), mean ± SEM (n=3), suggesting a less than 10-fold difference in affinity between the members of the D2-like family. When the D1-like receptors were examined by measuring the ability of MLS1547 to displace [^{3}H]-SCH23390 binding to D_1 and D_5 receptors, the extrapolated K_i values for both receptors were >50 μM (Supplemental Fig. 2).

A series of MLS1547 analogs were either obtained from commercial sources, or, in a few cases, synthesized to investigate the structure-activity relationship (SAR) of pathway selectivity for this set of compounds (for purity and manufacturer see Supplemental Table 1). These analogs were assayed in the D2R

cAMP assay as well as in the DiscoveRx β-arrestin recruitment assay (Tables 1 and 2). Although none of the analogs were more potent than MLS1547, a subset of the analogs were also extremely G protein-biased such that they lacked β-arrestin recruitment activity (Table 1). However, another subset of compounds exhibited agonist activity in both the cAMP and β-arrestin assays with variable degrees of G protein signaling bias (Table 2). These findings were used to formulate a preliminary SAR of G protein bias in this series of compounds.

To do this, a pharmacophore model was constructed to distinguish between the completely G protein-biased (Table 1) and less/non-biased D2R agonists (Table 2). Specifically, we selected ten compounds, displaying either the strongest or weakest G protein-bias, and then assigned default pharmacophore features (positive (P), acceptor (A), donor (D), aromatic (R) and hydrophobic (H)) to these ligands (see Methods). We found that a 4-point HRPR hypothesis was best able to explain the experimental data (Fig. 4A/B). Specifically, the alignment of the "H" feature in these compounds is the key difference between the completely biased (Table 1) and less/non-biased (Table 2) agonists of this scaffold. As shown by two representative biased/non-biased pairs in Fig. 4, the completely biased compounds align optimally to the "H" feature, whereas the less/non-biased compounds do not (Fig. 4A/B).

In parallel, we docked these compounds into a novel active-state model of the D2R using MLS1547 as a reference compound. For the MLS1547 compound, we observed two plausible orientations with the pyridine moiety pointing towards either TM5 in the orthosteric binding site (OBS) or toward TMs 2 and 7, away from the OBS (data not shown). Based on the SAR derived from Table 1, it appears that the N in the pyridine ring likely makes an H-bond with a receptor residue, as there is a ~10 fold increase in the potency of MLS1547 compared to that of NCGC319125. When the pyridine points towards TM5, however, a corresponding H-bond donor cannot be identified in the OBS. Thus, it is more likely that MLS1547 adopts the alternate orientation with the pyridine pointing toward TMs 2 and 7, in which the N on the pyridine ring may interact with Thr369 in TM7 to form an H-bond. These docking results were confirmed using a model with the D2R in an active conformation based on another D3R active model (Newman et al., 2012) and equilibrated using molecular dynamics simulations (data not shown). Encouragingly, results from both models were similar.

Using the MLS1547 pose with the pyridine pointing toward TMs 2 and 7, we used a core-restrained protocol to dock the other compounds into the active D2R model in order to compare the binding modes of the

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fully G protein-biased vs. less/non-biased agonists. The resulting poses show that the completely biased

compounds from Table 1 have a significantly higher tendency to interact with the extracellular portion of TM5.

Specifically, the chloro (-Cl) group of MLS1547 interacts with a hydrophobic pocket formed by Ile184^{EL2},

Phe189$^{5.38}$, and Val190$^{5.41}$ (Fig. 4) (PDB ID D2, MLS1547, supplemental data). Interestingly, this is consistent

with our finding from the pharmacophore modeling that the fully G protein-biased, but not the less/non-biased

compounds, can be well-aligned to the "H" feature of the aforementioned HRPR pharmacophore (PDB ID

NCGC9141, NCGC5872, NCGC5873, supplemental data). While recent publications suggest a possible role

for aromatic -Cl groups in an H-bond formation, it is not atypical to consider -Cl as a hydrophobic group as well.

Indeed, there are multiple examples in the literature where a methyl/-Cl swap resulted in roughly the same

potency, and was better tolerated than the unsubstituted hydrogen (Baum et al., 2009).

Thus, it is tempting to speculate that the structural basis for the different efficacies of these two groups

of agonists critically depends on whether a compound can interact with the hydrophobic pocket near the

extracellular portion of TM5 mentioned above. Interestingly, interactions with the extracellular portion of TM5

have previously been proposed as the structural basis for the β-arrestin signaling bias of the serotonergic

agonist ergotamine at the 5HT$_{2B}$ receptor, whereas ergotamine is notably non-biased at the related 5HT$_{1B}$

receptor (Wacker et al., 2013). Thus, comparing crystal structures of ergotamine bound to both receptors, it

was found that the extracellular portion of TM5 is tilted significantly more towards the OBS in the 5HT$_{2B}$

receptor, compared to the 5HT$_{1B}$ receptor (Wacker et al., 2013). This suggests a mechanism whereby agonists

that prevent such a tilting would bias towards G protein activation, compared to the β-arrestin pathway. Future

experiments to test this SAR prediction for the D2R and other GPCRs will be required. It is interesting to note,

however, that in the D2R, mutations of four residues at the cytoplasmic end of TM5 disrupt β-arrestin

recruitment much more than they impact cAMP signaling (Lan et al., 2009), suggesting that differential

propagation of signals through TM5 may play an important role in determining signaling bias.

In summary, MLS1547 is the first example of a G protein-biased agonist of the D2R. While it robustly

activates G protein-mediated signaling, the compound does not promote β-arrestin recruitment to the receptor.

Rather, through occupancy of the receptor, MLS1547 functions as an antagonist of dopamine-induced β-

arrestin recruitment to the D2R. Administration of compounds with this pharmacological profile to animals

would be expected stimulate G protein-based signaling of the D2R while simultaneously inhibiting signaling

through the β-arrestin/Akt/GSK3β pathway. Such compounds may also engender less β-arrestin-mediated

receptor desensitization or internalization, thereby further amplifying the G protein signaling arm. Importantly,

MLS1547, despite displaying no measurable β-arrestin recruitment in our assays, appears to be a highly

efficacious agonist at G protein-mediated signaling for the D2R. Recently, β-arrestin-biased agonists have

been developed for the D2R that exhibit varying degrees of agonist efficacy (Allen et al., 2011; Chen et al.,

2012). Functionally selective probes for both of the major signaling arms of the D2R should now help to

dissect their roles in normal physiology and behavior as well as elucidate their involvement in the therapeutic

effects of various pharmaceutical agents.

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AUTHORSHIP CONTRIBUTIONS

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REFERENCES

Abdul-Ridha A, Lopez L, Keov P, Thal DM, Mistry SN, Sexton PM, Lane JR, Canals M and Christopoulos A (2014) Molecular determinants of allosteric modulation at the m1 muscarinic acetylcholine receptor. *The Journal of biological chemistry* **289**(9): 6067-6079.

Allen JA, Yost JM, Setola V, Chen X, Sassano MF, Chen M, Peterson S, Yadav PN, Huang XP, Feng B, Jensen NH, Che X, Bai X, Frye SV, Wetsel WC, Caron MG, Javitch JA, Roth BL and Jin J (2011) Discovery of beta-arrestin-biased dopamine D2 ligands for probing signal transduction pathways essential for antipsychotic efficacy. *Proceedings of the National Academy of Sciences of the United States of America* **108**(45): 18488-18493.

Banala AK, Levy BA, Khatri SS, Furman CA, Roof RA, Mishra Y, Griffin SA, Sibley DR, Luedtke RR and Newman AH (2011) N-(3-fluoro-4-(4-(2-methoxy or 2,3-dichlorophenyl)piperazine-1-yl)butyl)arylcarboxamides as selective dopamine D3 receptor ligands: critical role of the carboxamide linker for D3 receptor selectivity. *Journal of medicinal chemistry* **54**(10): 3581-3594.

Bateup HS, Svenningsson P, Kuroiwa M, Gong S, Nishi A, Heintz N and Greengard P (2008) Cell type-specific regulation of DARPP-32 phosphorylation by psychostimulant and antipsychotic drugs. *Nature neuroscience* **11**(8): 932-939.

Baum B, Mohamed M, Zayed M, Gerlach C, Heine A, Hangauer D and Klebe G (2009) More than a simple lipophilic contact: a detailed thermodynamic analysis of nonbasic residues in the s1 pocket of thrombin. *Journal of molecular biology* **390**(1): 56-69.

Beaulieu JM, Gainetdinov RR and Caron MG (2007a) The Akt-GSK-3 signaling cascade in the actions of dopamine. *Trends in pharmacological sciences* **28**(4): 166-172.

Beaulieu JM, Marion S, Rodriguiz RM, Medvedev IO, Sotnikova TD, Ghisi V, Wetsel WC, Lefkowitz RJ, Gainetdinov RR and Caron MG (2008) A beta-arrestin 2 signaling complex mediates lithium action on behavior. *Cell* **132**(1): 125-136.

Beaulieu JM, Tirotta E, Sotnikova TD, Masri B, Salahpour A, Gainetdinov RR, Borrelli E and Caron MG (2007b) Regulation of Akt signaling by D2 and D3 dopamine receptors in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**(4): 881-885.

Bergman J, Roof RA, Furman CA, Conroy JL, Mello NK, Sibley DR and Skolnick P (2013) Modification of cocaine self-administration by buspirone (buspar(R)): potential involvement of D3 and D4 dopamine receptors. *Int J Neuropsychopharmacol* **16**(2): 445-458.

Boeckler F, Lanig H and Gmeiner P (2005) Modeling the similarity and divergence of dopamine D2-like receptors and identification of validated ligand-receptor complexes. *Journal of medicinal chemistry* **48**(3): 694-709.

Chen X, Sassano MF, Zheng L, Setola V, Chen M, Bai X, Frye SV, Wetsel WC, Roth BL and Jin J (2012) Structure-functional selectivity relationship studies of beta-arrestin-biased dopamine D(2) receptor agonists. *Journal of medicinal chemistry* **55**(16): 7141-7153.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK and Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318**(5854): 1258-1265.

Chien EY, Liu W, Zhao Q, Katritch V, Han GW, Hanson MA, Shi L, Newman AH, Javitch JA, Cherezov V and Stevens RC (2010) Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science* **330**(6007): 1091-1095.

Chun LS, Free RB, Doyle TB, Huang XP, Rankin ML and Sibley DR (2013) D1-D2 dopamine receptor synergy promotes calcium signaling via multiple mechanisms. *Molecular pharmacology* **84**(2): 190-200.

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Dror RO, Green HF, Valant C, Borhani DW, Valcourt JR, Pan AC, Arlow DH, Canals M, Lane JR, Rahmani R, Baell JB, Sexton PM, Christopoulos A and Shaw DE (2013) Structural basis for modulation of a G-protein-coupled receptor by allosteric drugs. *Nature* **503**(7475): 295-299.

Gay EA, Urban JD, Nichols DE, Oxford GS and Mailman RB (2004) Functional selectivity of D2 receptor ligands in a Chinese hamster ovary hD2L cell line: evidence for induction of ligand-specific receptor states. *Molecular pharmacology* **66**(1): 97-105.

Greene JK, S. , Savoj, H., Sprague, P., Teig, S. (1994) Chemical Function Queries for 3D Database Search. *J Chem Inf Comput Sci* **34**: 1297-1308.

Hamdan FF, Audet M, Garneau P, Pelletier J and Bouvier M (2005) High-throughput screening of G protein-coupled receptor antagonists using a bioluminescence resonance energy transfer 1-based beta-arrestin2 recruitment assay. *Journal of biomolecular screening* **10**(5): 463-475.

Kenakin T (2007a) Collateral efficacy in drug discovery: taking advantage of the good (allosteric) nature of 7TM receptors. *Trends in pharmacological sciences* **28**(8): 407-415.

Kenakin T (2007b) Functional selectivity through protean and biased agonism: who steers the ship? *Molecular pharmacology* **72**(6): 1393-1401.

Kenakin T, Watson C, Muniz-Medina V, Christopoulos A and Novick S (2012) A simple method for quantifying functional selectivity and agonist bias. *ACS chemical neuroscience* **3**(3): 193-203.

Kenakin TP (2008) Pharmacological onomastics: what's in a name? *British journal of pharmacology* **153**(3): 432-438.

Kilts JD, Connery HS, Arrington EG, Lewis MM, Lawler CP, Oxford GS, O'Malley KL, Todd RD, Blake BL, Nichols DE and Mailman RB (2002) Functional selectivity of dopamine receptor agonists. II. Actions of dihydrexidine in D2L receptor-transfected MN9D cells and pituitary lactotrophs. *The Journal of pharmacology and experimental therapeutics* **301**(3): 1179-1189.

Klewe IV, Nielsen SM, Tarpo L, Urizar E, Dipace C, Javitch JA, Gether U, Egebjerg J and Christensen KV (2008) Recruitment of beta-arrestin2 to the dopamine D2 receptor: insights into anti-psychotic and anti-parkinsonian drug receptor signaling. *Neuropharmacology* **54**(8): 1215-1222.

Kobilka BK and Deupi X (2007) Conformational complexity of G-protein-coupled receptors. *Trends in pharmacological sciences* **28**(8): 397-406.

Kruse AC, Ring AM, Manglik A, Hu J, Hu K, Eitel K, Hubner H, Pardon E, Valant C, Sexton PM, Christopoulos A, Felder CC, Gmeiner P, Steyaert J, Weis WI, Garcia KC, Wess J and Kobilka BK (2013) Activation and allosteric modulation of a muscarinic acetylcholine receptor. *Nature* **504**(7478): 101-106.

Lan H, Liu Y, Bell MI, Gurevich VV and Neve KA (2009) A dopamine D2 receptor mutant capable of G protein-mediated signaling but deficient in arrestin binding. *Molecular pharmacology* **75**(1): 113-123.

Lane JR, Powney B, Wise A, Rees S and Milligan G (2007) Protean agonism at the dopamine D2 receptor: (S)-3-(3-hydroxyphenyl)-N-propylpiperidine is an agonist for activation of Go1 but an antagonist/inverse agonist for Gi1,Gi2, and Gi3. *Molecular pharmacology* **71**(5): 1349-1359.

Lane JR, Powney B, Wise A, Rees S and Milligan G (2008) G protein coupling and ligand selectivity of the D2L and D3 dopamine receptors. *The Journal of pharmacology and experimental therapeutics* **325**(1): 319-330.

Liu JJ, Horst R, Katritch V, Stevens RC and Wuthrich K (2012) Biased signaling pathways in beta2-adrenergic receptor characterized by 19F-NMR. *Science* **335**(6072): 1106-1110.

Mailman RB (2007) GPCR functional selectivity has therapeutic impact. *Trends in pharmacological sciences* **28**(8): 390-396.

Masri B, Salahpour A, Didriksen M, Ghisi V, Beaulieu JM, Gainetdinov RR and Caron MG (2008) Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proceedings of the National Academy of Sciences of the United States of America* **105**(36): 13656-13661.

MOL #90563

Mottola DM, Kilts JD, Lewis MM, Connery HS, Walker QD, Jones SR, Booth RG, Hyslop DK, Piercey M, Wightman RM, Lawler CP, Nichols DE and Mailman RB (2002) Functional selectivity of dopamine receptor agonists. I. Selective activation of postsynaptic dopamine D2 receptors linked to adenylate cyclase. *The Journal of pharmacology and experimental therapeutics* **301**(3): 1166-1178.

Newman AH, Beuming T, Banala AK, Donthamsetti P, Pongetti K, LaBounty A, Levy B, Cao J, Michino M, Luedtke RR, Javitch JA and Shi L (2012) Molecular determinants of selectivity and efficacy at the dopamine D3 receptor. *Journal of medicinal chemistry* **55**(15): 6689-6699.

Pozzi L, Hakansson K, Usiello A, Borgkvist A, Lindskog M, Greengard P and Fisone G (2003) Opposite regulation by typical and atypical anti-psychotics of ERK1/2, CREB and Elk-1 phosphorylation in mouse dorsal striatum. *Journal of neurochemistry* **86**(2): 451-459.

Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah ST, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skiniotis G, Weis WI, Sunahara RK and Kobilka BK (2011) Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* **477**(7366): 549-555.

Sibley DR, Monsma FJ, Jr., McVittie LD, Gerfen CR, Burch RM and Mahan LC (1992) Molecular neurobiology of dopamine receptor subtypes. *Neurochemistry international* **20 Suppl**: 17S-22S.

Skinbjerg M, Ariano MA, Thorsell A, Heilig M, Halldin C, Innis RB and Sibley DR (2009) Arrestin3 mediates D(2) dopamine receptor internalization. *Synapse* **63**(7): 621-624.

Svenningsson P, Nishi A, Fisone G, Girault JA, Nairn AC and Greengard P (2004) DARPP-32: an integrator of neurotransmission. *Annual review of pharmacology and toxicology* **44**: 269-296.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M and Mailman RB (2007) Functional selectivity and classical concepts of quantitative pharmacology. *The Journal of pharmacology and experimental therapeutics* **320**(1): 1-13.

Urs NM, Snyder JC, Jacobsen JP, Peterson SM and Caron MG (2012) Deletion of GSK3beta in D2R-expressing neurons reveals distinct roles for beta-arrestin signaling in antipsychotic and lithium action. *Proceedings of the National Academy of Sciences of the United States of America* **109**(50): 20732-20737.

Wacker D, Wang C, Katritch V, Han GW, Huang XP, Vardy E, McCorvy JD, Jiang Y, Chu M, Siu FY, Liu W, Xu HE, Cherezov V, Roth BL and Stevens RC (2013) Structural features for functional selectivity at serotonin receptors. *Science* **340**(6132): 615-619.

Wess J, Han SJ, Kim SK, Jacobson KA and Li JH (2008) Conformational changes involved in G-protein-coupled-receptor activation. *Trends in pharmacological sciences* **29**(12): 616-625.

Whalen EJ, Rajagopal S and Lefkowitz RJ (2011) Therapeutic potential of beta-arrestin- and G protein-biased agonists. *Trends in molecular medicine* **17**(3): 126-139.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. MLS1547 stimulates D2R G protein-mediated signaling. A, Structure of MLS000051547

(MLS1547). **B**, HEK293 cells stably expressing D2R and Gqi5 were assayed for MLS1547 stimulation of

calcium accumulation, as described in the Materials and Methods. Cells were stimulated with the indicated

concentrations of dopamine or MLS1547 (1547). EC_{50} and E_{max} values were obtained for dopamine and

MLS1547 and were 2.5 ± 0.3 nM, $101.7 \pm 0.1\%$, and 0.37 ± 0.2 μM, $89.3 \pm 4.3\%$ (mean $\pm$ SEM, n=3),

respectively. **C**, CHO cells stably expressing the D2R were assayed for MLS1547 inhibition of forskolin-

stimulated cAMP, as described in the Materials and Methods. Cells were stimulated with the indicated

concentration of dopamine or MLS1547 (1547). EC_{50} and E_{max} values were obtained for dopamine and

MLS1547 and were 0.06 ± 0.02 μM, $100.4 \pm 1.6\%$, and 0.26 ± 0.07 μM, $97.1 \pm 3.7\%$ (mean $\pm$ SEM, n=5),

respectively. **D**, CHO cells stably expressing D2R were assayed for sulpiride reversal of MLS1547 inhibition of

forskolin-stimulated cAMP accumulation. Cells were stimulated with an EC_{80} concentration of MLS1547 (1547)

in the presence of increasing concentrations of the D2R antagonist sulpiride. The IC_{50} value obtained for

sulpiride was 22.0 ± 2.8 nM (mean $\pm$ SEM, n= 4). Data are representative of 3-5 independent experiments run

in triplicate and plotted as a percentage of the maximum response observed with dopamine (**B** and **C**), or as a

percentage of the response seen with an EC_{80} concentration of MLS1547 (**D**), as indicated.

Figure 2. MLS1547 acts as an antagonist for dopamine-stimulated β-arrestin recruitment to the D2R. A,

DiscoveRx PathHunter cells were assayed for agonist-induced recruitment of β arrestin-2 to the D2R as

described in the Materials and Methods. Cells were stimulated with the indicated concentrations of dopamine

or MLS1547 (1547), and EC_{50} and E_{max} values were obtained for dopamine, 0.09 ± 0.03 μM and $99.0 \pm 0.6\%$,

respectively (mean $\pm$ SEM, n=3). MLS1547 failed to stimulate measurable β arrestin-2 recruitment. **B**,

HEK293 cells were stably transfected with Rluc-8-fused D2R and mVENUS-fused β-arrestin-2, stimulated with

various concentrations of dopamine or MLS1547 (1547) as indicated, and examined for BRET as described in

the Materials and Methods. EC_{50} values obtained for dopamine were 0.05 ± 0.01 μM and $99.8 \pm 1.9\%$,

respectively (mean $\pm$ SEM, n=3). MLS1547 failed to stimulate any measurable D2R-β arrestin-2 interactions.

Data are representative of 3-5 independent experiments run in triplicate and plotted as a percentage of maximum response observed with dopamine as indicated. **C,** DiscoveRx PathHunter cells were stimulated with an EC_{80} concentration of dopamine (1 μM), then assayed for the ability of MLS1547 to antagonize this response. Data are expressed as the percentage of the maximum response observed with 1 μM dopamine and represent the mean $\pm$ SEM values of three individual experiments performed in triplicate. The IC_{50} value for MLS1547 was calculated to be 9.9 $\pm$ 0.9 μM. **D,** The same cells described for the BRET assay in **B** above were stimulated with an EC_{80} of dopamine (1 μM) and assayed for the ability of MLS1547 to antagonize this response. Data are expressed as mean values of six independent experiments run in quadruplicate. The IC_{50} value for MLS1547 was 3.8 $\pm$ 1.8 μM (mean $\pm$ SEM, n=6).

Figure 3. Radioligand binding competition assay using MLS1547 and the D2R. Membranes from HEK293 cells stably transfected with the human D2R were harvested for radioligand competition binding assays as described in the Materials and Methods. Membranes were incubated with the indicated concentrations of MLS1547 and 0.5 nM [^{3}H]methylspiperone. The data are representative of four independent experiments and expressed as a percentage of the binding seen in the absence of any competing ligand. The K_i for MLS1547 was calculated to be 1.2 $\pm$ 0.2 μM (mean $\pm$ SEM, n=4).

Figure 4. Pharmacophore model for G protein-biased and non-biased agonist interactions with the D2R. Panels **A** and **B** HRPR pharmacophore with aligned biased compounds MLS1547 and NCGC9141. Biased compounds align well to all four features: two aromatic (beige), one hydrophobic (green) and a positive (blue) feature. Middle: Compared to the biased compound MLS1547, the non-biased compound NCGC5872 (left) lacks the -Cl group and cannot align both the hydrophobic and aromatic features, while the different attachment points of the naphthalene ring in compounds NCGC9141 and NCGC5873 cause the former, but not the latter to align well to the 4-point pharmacophore. In this case, one of the aromatic rings in NCGC9141 aligns the hydrophobic feature. Panels **C** and **D**, docked poses of biased (cyan) and unbiased (grey) compounds in an active model of the D2R. Note the more extensive interaction of non-polar features in the biased compounds with a hydrophobic pocket formed by residues in EL2 and TM5.

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D2.pdb. The D$_2$ Dopamine Receptor Model

MLS1547.pbd. Binding mode of MLS000051547 in the D2R Model

NCGC9141.pbd. Binding mode of compound NCGC00319141 in the D2R Model

NCGC5872.pbd. Binding mode of compound NCGC00345872 in the D2R Model

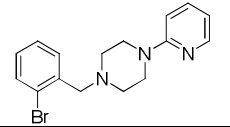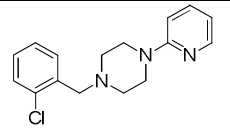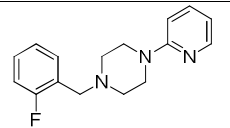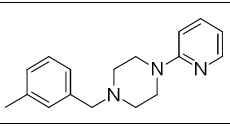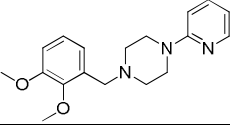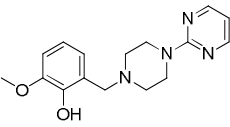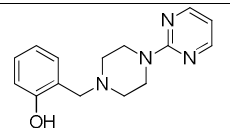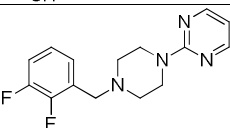**NCGC5873.pbd. Binding mode of the compound NCGC00345873 in the D2R Model**

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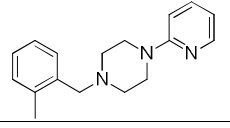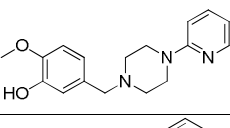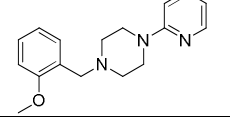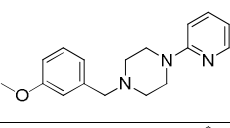
Table 1. Analogs of MLS1547 active in cAMP assays yet lacking β-arrestin recruitment activity. The compounds were assayed in agonist mode using the DiscoveRx cAMP and β-arrestin assays as described in the Materials and Methods.

Structure	Compound ID	D2R cAMP Response			D2R β-arrestin Recruitment	
		Emax (% Control ± SEM)	EC_{50} (μM ± SEM)	Log ($Emax/EC_{50}$)	Emax (% Control ± SEM)	EC_{50} (μM ± SEM)
	MLS000051547	97.1 ± 3.7	0.26 ± 0.07	2.57	Inactive	NA
	NCGC00319124	87.3 ± 14.7	5.5 ± 0.9	1.20	Inactive	NA
	NCGC00319127	93.9 ± 4.6	4.7 ± 1.7	1.30	Inactive	NA
	NCGC00319125	66.8 ± 3.9	2.8 ± 1.2	1.38	Inactive	NA
	NCGC00346387	94.8 ± 2.1	1.4 ± 0.8	1.83	Inactive	NA
	NCGC00319141	89.5 ± 5.2	0.7 ± 0.7	2.11	Inactive	NA
	MLS000860449	84.9 ± 2.7	4.5 ± 3.0	1.28	Inactive	NA
	NCGC0319129	74.8 ± 23.8	9.5 ± 5.6	0.90	Inactive	NA
	NCGC00319126	62.8 ± 7.7	1.1 ± 0.6	1.76	Inactive	NA
	Dopamine	100	0.06 ± 0.02	3.22	100	0.09 ± 0.03

Table 2. Analogs of MLS1547 active in both cAMP and β-arrestin assays. The compounds were assayed in agonist mode using the DiscoveRx cAMP and β-arrestin assays as described in the Materials and Methods.

Structure	Compound ID	D2R cAMP Response			D2R β-arrestin Recruitment			Bias activity
		Emax (% Control ± SEM)	EC$_{50}$ (μM ± SEM)	Log (Emax/EC$_{50}$)	Emax (% Control ± SEM)	EC$_{50}$ (μM ± SEM)	Log (Emax/EC$_{50}$)	
	NCGC00319139	95.5 ± 2.0	0.03 ± 0.01	3.50	89.3 ± 9.4	2.0 ± 0.5	1.65	1.85
	NCGC00319137	84.1 ± 4.5	0.02 ± 0.001	3.62	84.7 ± 2.5	1.6 ± 0.3	1.72	1.90
	NCGC00319136	93.8 ± 1.4	0.1 ± 0.04	2.97	76.4 ± 2.9	0.7 ± 0.07	2.04	0.93
	NCGC00092785	77.0 ± 10.9	0.04 ± 0.01	3.28	73.8 ± 1.2	0.4 ± 0.02	2.27	1.02
	NCGC00319134	86.1 ± 14.4	0.1 ± 0.04	2.94	74.7 ± 1.8	0.3 ± 0.03	2.40	0.54
	NCGC00319131	89.6 ± 0.5	0.2 ± 0.1	2.65	66 ± 0.2	0.8 ± 0.01	1.92	0.73
	NCGC00319132	78.9 ± 10	0.1 ± 0.01	2.90	59.2 ± 4.2	0.3 ± 0.04	2.30	0.60
	NCGC00319133	86.5 ± 14.7	0.01 ± 0.001	3.94	87.6 ± 2.0	0.2 ± 0.03	2.64	1.30
	NCGC00319130	71.0 ± 17.1	0.1 ± 0.02	2.85	66.0 ± 0.2	2.1 ± 0.4	1.50	1.35
	NCGC00319135	83.3 ± 14.4	1.4 ± 0.1	1.77	68.9 ± 4.9	13.2 ± 6.6	0.72	1.06
	NCGC00319140	92.5 ± 5.5	2.0 ± 0.2	1.67	66.2 ± 18.6	20.5 ± 9.6	0.51	1.16
	NCGC00319128	97.2 ± 0.7	0.4 ± 0.1	2.39	70.0 ± 5.9	3.4 ± 0.5	1.31	1.07
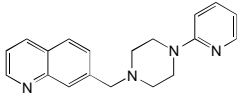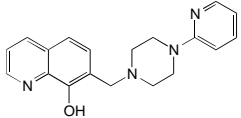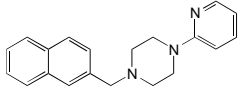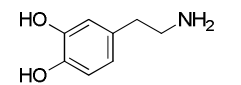
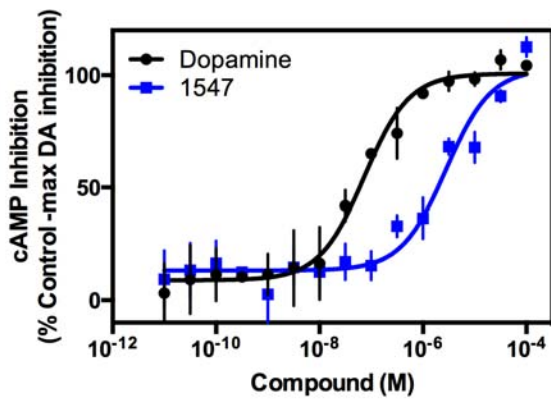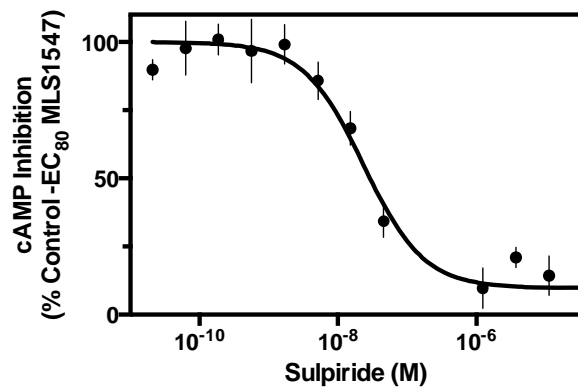| Structure	Compound							
	NCGC00346388	90.9 ± 1.9	0.2 ± 0.1	2.66	56.8 ± 7.1	1.0 ± 0.6	1.75	0.90
	NCGC00345872	97.1 ± 0.9	0.3 ± 0.1	2.51	58.0 ± 8.4	0.9 ± 0.3	1.81	0.70
	NCGC00345873	93.1 ± 1.6	0.1 ± 0.07	2.97	63.3 ± 8.9	0.9 ± 0.6	1.85	1.12
	Dopamine	100	0.06 ± 0.02	3.22	100	0.09 ± 0.03	3.05	0.18

Figure 1
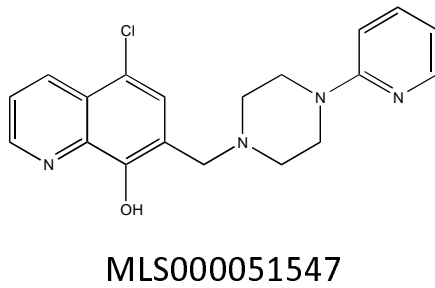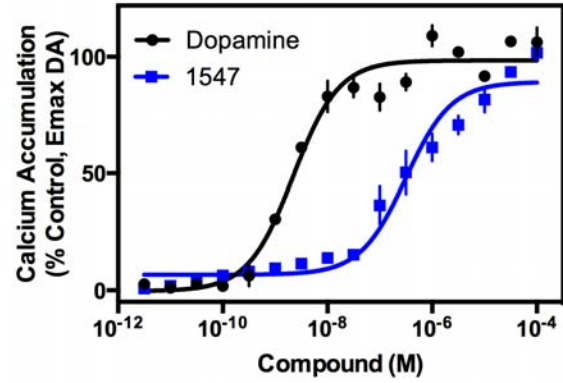
Figure 2

Figure 3

Figure 4