SUPPLEMENTAL MATERIALS – Molecular Pharmacology

Discovery and characterization of a G protein-biased agonist that inhibits β-arrestin recruitment to the D_2 dopamine receptor

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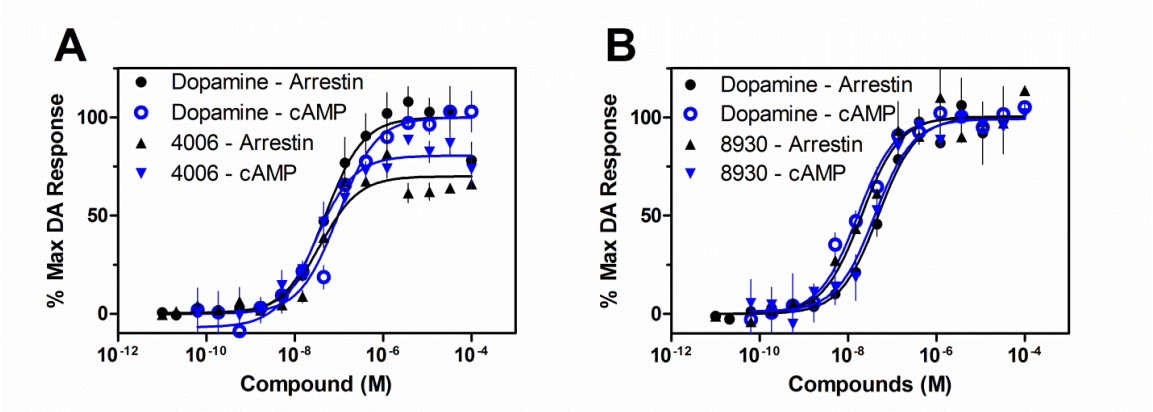
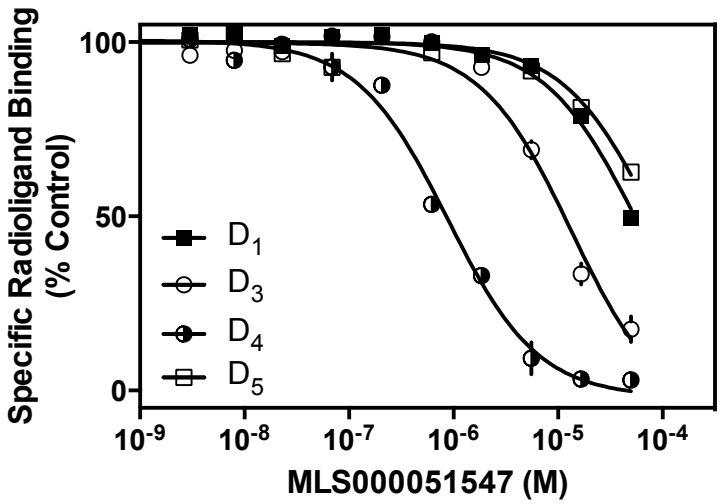
Screening Details:

Cells stably expressing the human D_2R and a chimeric Gqi5 protein enabling robust calcium mobilization upon activation of the D_2R were screened for calcium mobilization using a Hamamatsu FDSS 7000. Agonist screening was conducted by addition of the library compound followed by measurement of calcium mobilization as described in the Methods. The initial primary screen was conducted on ~380,000 unique compounds plated throughout 487 individual 1536-well plates at two concentrations of test compound, 2 μM and 10 μM. S/B for the assay was ~ 94 for agonists and Z' was 0.87 ± 0.12 over the course of the entire primary screen. The cherry-pick threshold was 25% agonist response compared to the full response seen with dopamine. Hit compounds were defined as all agonists with >40% agonism at either concentration. We then conducted a diversity structural pick of compounds back to 25% activity, which allowed us to select a total of 2400 compounds for confirmation, 2288 which were available and received. 1638 of these compounds replicated in a confirmation screen that was run using full concentration response curves in the D_2R-Gqi5 cells up to 40 μM (72% confirmation rate). DiscoveRx β-arrestin cells expressing the prostaglandin E2 receptor was run as a negative control for all agonists to ensure any signaling was D_2R mediated. The remaining compounds were then subjected to counter-screening in the β-arrestin recruitment assay. The majority of compounds did not demonstrate bias between the two signaling assays; however, 70 compounds were selected for further study, as they appeared to be selective agonists in D_2-Gqi5 calcium versus β-arrestin assays. These compounds were subjected to reanalysis and D_2R-mediated cAMP inhibition assays, and it was determined that many (61) of these compounds were weak partial agonists in both cell lines with slightly greater efficacy in D_2R-Gqi5 calcium assays, resulting in a weak or non existent bias. 9 remaining compounds showed varying degrees of Gi signaling bias with high efficacy including MLS1547, which was found to be completely inactive in β-arrestin recruitment and highly efficacious at cAMP inhibition. It was therefore chosen for further study.



Supplemental Figure 1. Examples of compounds with little to no bias between G protein and β-arrestin-mediated signaling. Compounds were assayed for agonist activity in both D_2R-mediated inhibition of cAMP and D_2R-mediated β-arrestin recruitment assays, as described in the Methods section. Results were normalized to 100% maximum dopamine response for each group. (**A)** NCGC00124006 was screened as an agonist in both assays giving Emax values of 80.5% in the cAMP inhibition assay and 69.9% in the β-arrestin recruitment assay. Corresponding EC_{50} values were 0.29 and 0.39 μM in the cAMP inhibition and β-arrestin recruitment assays, respectively. (**B**) NCGC00138930 was screened as an agonist in both assays giving Emax values of 99.3% in the cAMP inhibition assay and 100.4% in the β-arrestin recruitment assay. Corresponding EC_{50} values were 0.04 and 0.02 μM in the cAMP inhibition and β-arrestin recruitment assays, respectively. Data are representative graphs from screening assays. Dopamine was run as a non-biased control and mean values for dopamine are reported in Tables 1 and 2.



Supplemental Figure 2. Competition binding of MLS1547 for dopamine receptor subtypes. Membranes from HEK293 cells stably transfected with human D_1R, D_3R, D_4R, or D_5R (Codex Biosolutions, Inc., Gaithersburg, MD) were harvested for radioligand competition binding. Briefly, membranes were incubated with the indicated concentrations of MLS1547 and either 0.5nM [^{3}H]-SCH23390 (D_1R and D_5R), or 0.5nM [^{3}H]-methylspiperone (D_3R and D_4R). The curves shown are representative of four independent experiments done on different days. Data are expressed as a percentage of the binding seen for each individual receptor subtype in the absence of any competing ligand. K_i values were calculated using the Cheng-Prusoff equation and radioligand K_d values determined via saturation binding isotherms on each individual receptor. Average Ki values are reported in the Results section.

Supplemental Table 1. MLS1547 analog sources and purity.

Compound ID	Vendor[1]	Vendor ID	Purity[2]
NCGC00319124	Enamine	Z150743510	>99%
NCGC00319127	Enamine	Z150744366	>99%
NCGC00319125	Enamine	Z126931514	>99%
NCGC00346387	Synthesized	AED01-071	>99%
NCGC00319141	Chembridge	5266462	>99%
MLS000860449	Chembridge	5269631	>99%
NCGC00319129	Enamine	Z198142988	>99%
NCGC00319126	Enamine	Z220353854	>99%
NCGC00319139	Chembridge	5267052	>99%
NCGC00319137	Chembridge	5268791	>99%
NCGC00319136	Chembridge	5270283	>99%
NCGC00092785	Chembridge	5268596	>99%
NCGC00319134	Chembridge	5258813	>99%
NCGC00319131	Chembridge	5268509	>81%
NCGC00319132	Chembridge	5270410	>99%
NCGC00319133	Chembridge	5264064	>99%
NCGC00319130	Chembridge	5256816	>99%
NCGC00319135	Chembridge	5431822	>99%
NCGC00319140	Chembridge	5427011	>99%
NCGC00319128	Enamine	Z124005168	>99%
NCGC00346388	Synthesized	AED01-072	>99%
NCGC00345872	Synthesized	AED01-055	>99%
NCGC00345873	Synthesized	AED01-057	>99%

[1] "Enamine" is Enamine Ltd, Kiev, Ukraine. "Chembridge" is Chembridge Corporation, San Diego, CA. "Synthesized" indicates the compound was synthesized in house at NCATS, Rockville, MD.

[2] Analytical analyses were performed on a Waters Acquity LC/MS. A 6.8-minute gradient of 4 to 100% acetonitrile (containing 0.025% trifluoroacetic acid) in water (containing 0.05% trifluoroacetic acid) is used with an 8.6-minute run time at a flow rate of 0.8 mL/min. The column was an Agilent Poroshell, 3.0x 100 mm, with a 2.7 µm particle size. Purity determination was performed using a Diode Array Detector and an Evaporative Light Scattering Detector. Mass Determination was performed using a Waters Micromass ZQ mass spectrometer with electrospray. Data were analyzed using the Waters OpenLynx software.