In-vitro and in-vivo identification of novel positive allosteric modulators of the human dopamine D2 and D3 receptor

Authors:

Martyn Wood, Ali Ates, Veronique Marie Andre, Anne Michel, Robert Barnaby, Michel Gillard

UCB Biopharma SPRL, Chemin du Foriest, B-1420 Braine-l’Alleud, Belgium
Running Title page
Identification of dopamine D2/D3 receptor PAMs

Corresponding author: Martyn Wood;

e-mail: martyn.wood@ucb.com ; Tel: +32 (0)2 386 4019

GSM: +32 471 353987 Fax: +32 (0)2 386 6150

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Abbreviations.

CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; GPCR, G protein-coupled receptor ; GTPγS, guanosine 5'-O-(3-thio)triphosphate; HEK, human embryonic kidney;
HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); IBMX, 3-isobutyl-1-methylxanthine; NMDA, N-methyl-d-aspartate; PAM, positive allosteric modulator;
Abstract

Agonists at dopamine D2 and D3 receptors are important therapeutic agents in the treatment of Parkinson's disease. Compared to the use of agonists, allosteric potentiators offer potential advantages such as temporal, regional and phasic potentiation of natural signaling, and that of receptor subtype selectivity. We report the identification of a stereoselective interaction of a benzothiazol racemic compound which acts as a positive allosteric modulator (PAM) of the rat and human dopamine D2 and D3 receptors. The R-isomer did not directly stimulate the dopamine D2 receptor but potentiated the effects of dopamine. In contrast the S-isomer attenuated the effects of the PAM and the effects of dopamine. In radioligand binding studies, these compounds do not compete for binding of orthosteric ligands, but indeed the R-isomer increased the number of high affinity sites for \[^3H\]-dopamine without affecting Kd. We went on to identify a more potent PAM for use in native receptor systems. This compound potentiated the effects of D2/D3 signaling in-vitro in electrophysiological studies on dissociated striatal neurons and in-vivo on the effects of L-Dopa in the 6-OHDA contralateral turning model. These PAMs lacked activity at a wide variety of receptors, lacked PAM activity at related Gi–coupled G protein-coupled receptors and lacked activity at D1 receptors. However, the PAMs did potentiate \[^3H\]-dopamine binding at both D2 and D3 receptors. Together, these studies show that we have identified PAMs of the D2 and D3 receptors both in-vitro and in-vivo. Such compounds may have utility in the treatment of hypodopaminergic function.
Introduction.

There has been much interest in the identification of allosteric modulators of G protein-coupled receptors (GPCRs), both as tools to understand receptor mechanisms and as potential therapeutic agents (see Keov et al 2011; Conn et al, 2012). GPCRs represent the largest family of cell-surface receptors and a large number of marketed drugs directly activate or block signaling mediated via these receptors. However, for some GPCRs (e.g. peptide receptors) it has proven difficult to develop small molecules and for others achieving sufficient selectivity has been challenging due to the high degree of homology in the ligand binding site between GPCR subtypes (e.g. dopamine D2 and dopamine D3). Accordingly, much drug research has shifted to the identification of small molecules which target sites distinct from the orthosteric natural agonist and which induce a conformational change in the GPCR thereby allosterically modulating the receptor function. Allosteric ligands have a diverse range of activities including the ability to potentiate (positive allosteric modulator, PAM) or attenuate (negative allosteric modulator, NAM) the effects of the endogenous ligand, by affecting affinity and/or efficacy (Wootten et al, 2013). As well as subtype selectivity, allosteric modulators can present other potential advantages from a drug discovery perspective such as: lack of direct effect or intrinsic efficacy; only potentiating the effect of the native transmitter where and when it is released; and reduced propensity for inducing densensitisation arising from constant exposure to an agonist.

The monoamine dopamine acts via two families of GPCRs to modulate motor function, reward mechanisms, central processing and other physiological functions. The two
families are: D1-like, comprising dopamine D1 and D5 receptors which couple to the Gs and Golf G-protein and stimulating cAMP production; and D2-like which comprise the D2, D3 and D4 receptors which predominantly couple to Gi/o G-proteins and which attenuate cAMP production (see Neve et al, 2004; Beaulieu & Gainetdinov, 2011). D2 receptors exhibit a high degree of sequence homology with D3 receptors and they share a predicted binding site for dopamine and other ligands (Shi & Javitch, 2002).

Many therapeutic agents target the dopamine D2 and D3 receptors notably agonists such as ropinirole, pramipexole and rotigotine used in the treatment of motors disorders such as Parkinson’s (Perez-Lloret & Rascol, 2010) and antagonists such as atypical and typical antipsychotic drugs (Seeman et al 1976; Meltzer, 1999).

We therefore performed a high throughput screen on 80,000 compounds to identify novel allosteric modulators of the human dopamine D2 receptor. By determining changes in intracellular cAMP (HTRF, CisBio) we identified 3 hits which exhibited PAM properties at the dopamine D2 receptor. However, one compound was a racemic mixture (1,3-benzothiazol-2-yl(2-methyl-2,3-dihydro-indol-1-yl)methanone; Fig 1). On synthesis and chiral resolution of the two stereoisomers, one (the R stereoisomer) was shown to act as a PAM whilst the other displayed NAM properties. We report here the properties and characterisation of these compounds in recombinant systems on both function and radioligand binding, in native tissue and in-vivo in hemiparkinsonian rats.
Materials and Methods

Materials

$[^{35}]$-GTPγS and $[^{3}]$H]-dopamine were obtained from Perkin Elmer (Zaventem, Belgium). Complete protease inhibitor was obtained from Roche Diagnostics (Vilvoorde, Belgium); DNAse, 3-Isobutyl-1-methylxanthine (IBMX), forskolin, apomorphine from Sigma-Aldrich (Diegem, Belgium); cAMP dynamic range kit from Cisbio Bioassay (Codolet, France). Quinpirole, L741,626 were from Tocris (Abingdon, UK).

Compounds were synthesized and prepared in house, dissolved in DMSO and diluted in assay buffer such that the final concentration of DMSO in the assay was 1%.

Animals and ethics statement

All animal experiments were performed according to the Helsinki declaration and were conducted in accordance with the guidelines of the European Community Council directive 86/609/EEC and approved by the ethical committee from UCB Biopharma SPRL (LA1220040 and LA2220363). Male Sprague-Dawley rats (Janvier, France) were housed in cages (4 rats per cage) for one week before experimentation. They were kept on a 12:12 light / dark cycle with light on at 06:00 and at a temperature maintained at 20–21 °C and at humidity of approximately 40%. All animals had free access to standard pellet food and water before assignment to experimental groups. The animals weighed 250–275 g at the time of surgery and 400–450 g at the time of drug testing. Additional enrichment and welfare were provided (Enviro-dri, PharmaServ) before and
after the surgery. Animal health was monitored daily by the animal care staff. Surgeries were performed under ketamine and xylazine or under isoflurane anesthesia, and all efforts were made to minimize suffering. Sacrifice were done with CO₂ or when necessary by exsanguination.

**Cell culture and membrane preparation**

Chinese hamster ovary cells (CHO-K1) stably expressing the human dopamine D2 long receptor or the human D3 receptor were generated in house. Human embryonic kidney cells (HEK 293) transiently expressing the rat dopamine D2 long receptor were also generated in house using 293fectin (Life Technologies). CHO cells expressing human alpha₂C adrenergic receptors were obtained in collaboration with Dr. N. Moguilevsky, Free University of Brussels, Department of Applied Genetics (Brussels, Belgium). CHO cells expressing the human histamine H3 receptor were purchased from Euroscreen S.A. (Gosselies, Belgium). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. CHO cells were grown in DMEM-F12+GlutaMAX™-I medium (GIBCO®, Invitrogen Life Technology, Gent, Belgium) containing 10% fetal bovine serum (FBS) (BioWhittaker®, Lonza, Vervier, Belgium), 400 µg/mL Geneticin (GIBCO®, Invitrogen), 100 IU/mL Penicillin and 100 IU/mL Streptomycin (Pen-Strep solution, BioWhittaker®, Lonza).

Membranes were prepared from confluent cells grown in 500 cm² culture dishes. Cells were rinsed with 30 ml of phosphate-buffered saline (PBS, pH 7.4) and detached by a 5-10 min incubation in 30 ml of EDTA 1mM in PBS at 37°C and washed with 20 ml PBS
at 4°C. The cell suspension was centrifuged at 1,500 x g for 10 min at 4°C. The pellet was homogenized in 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 0.3 mM EDTA, 2 mM MgCl$_2$ buffer with complete protease inhibitor, using a glass/teflon homogenizer (10 strokes on ice at 1100 rpm). The cell pellet was then subjected to 2 freeze/thaw cycles and the membranes incubated for 10 min at 25°C with DNase (1 µl/ml). The membranes were then centrifuged at 40,000 x g for 25 min at 4°C and the final pellet suspended in a 20 mM Tris-HCl (pH 7.4), 250 mM sucrose buffer and stored in liquid nitrogen at a protein concentration of 2-8 mg/ml.

**Radioligand binding assays**

CHO D2 membranes (50-100 µg Pr per well) were incubated with $[^{3}H]$dopamine (0.05 nM to 100 nM in saturation studies; 0.6 nM in compound concentration-effect studies) for 120 min in 50 mM Tris buffer (pH 7.4 at 25°C, final volume 1 ml) containing (final) MgCl$_2$ (1 mM), pargyline (10 µM), ascorbic acid (1 mM) and test compound or DMSO (1% to define total binding) or apomorphine (10 µM to define non-specific binding, Burt et al, 1975). The reaction was terminated by rapid filtration (Glass fibre filters GF/B), the filters washed 4 times with ice-cold 50 mM Tris buffer and the retained radioactivity determined by liquid scintillation spectroscopy. All incubations were performed in duplicate or triplicate.

The same method was used for CHO D3 membranes except that the protein concentration was 3 µg Pr per well due to higher receptor expression.
[\textsuperscript{3}H]-raclopride binding to CHO D2 membranes was performed according to Wood et al (2014).

**Dopamine D2 stimulated \textsuperscript{35}S-GTP\textgamma S binding**

Membranes (5 µg Pr) expressing human D2 dopamine receptors were incubated for 15 min at 25°C in buffer (50 mM Tris-HCl (pH 7.4) containing 3 mM MgCl\textsubscript{2}, 50 mM NaCl, 1 µM GDP, 10 µg/ml saponin) containing test compound in a final volume of 0.2ml. \textsuperscript{35}S-GTP\gamma S (0.15-0.2 nM) was then added and the reaction continued for 60 min. Membrane-bound radioligand was separated by rapid filtration through glass fiber filters (GFB), washed 4 times with filtration buffer (ice-cold 50 mM Tris-HCl (pH7.4) and the retained radioactivity counted by liquid scintillation spectroscopy. All incubations were performed in duplicate or triplicate.

**cAMP cellular assays**

The effect of compounds on the levels of cAMP in the D2 CHO cells was assessed using cAMP dynamic kit 2 from Cisbio (Codolet, France) following the manufacturer’s instructions. Using homogenous time-resolved fluorescence (HTRF) technology, the assay is based on competition between native cAMP produced by cells and cAMP labelled with the dye d2. The tracer binding is visualized by an anti-cAMP antibody labeled with cryptate. Assays were performed in 384 wells with 5000 cells per well in a final volume of 80 µl. Cells were incubated with IBMX (500 µM final), compound (varying
concentrations) and forskolin (10 μM final) in the presence and absence of varying concentrations of dopamine with DMSO (1% final) for 60 min at ambient temperature. The reaction was terminated and the cells lysed, cAMP-d2 reagent and anti-cAMP antibody added and then incubated for 60 min at ambient temperature. The level of cAMP was then determined by measuring the fluorescence ratio (665nm/620nm). All incubations were performed in duplicate.

The same procedure was used for Lmtk-1 cells expressing the human dopamine D1 receptor except that forskolin was not included, the incubation was for 1 hour at room temperature with 20,000 cells per well.

Selectivity

To investigate the selectivity of any allosteric effect, compounds were evaluated at the Gi-coupled alpha-adrenergic2C receptor and histamine H3 receptors and the related D1, D3 and D4 receptors.

Histamine-stimulated [³⁵S]GTPγS binding was performed as described in Célanire et al (2009). Membranes (5-10 μg proteins) expressing human H₃ receptor were incubated for 15 minutes at 25°C in 0.2 ml of a 50 mM Tris-HCl buffer (pH 7.4) containing 3 mM MgCl₂, 50 mM NaCl, 1 μM GDP, 2 μg saponin, 1% DMSO and increasing concentrations of compounds for agonism/inverse agonism determination. Histamine and test compound were co-incubated for 60 min and 0.2 nM of [³⁵S]GTPγS was added to the samples and the incubation continued for another 30 min. Assays were
terminated by addition of ice-cold 50 mM Tris-HCl buffer (pH 7.4) followed by rapid filtration and radioactivity determined as above.

Binding of [3H]dopamine to the D3 receptor was performed as for the D2 receptor except that 3-5 μg protein was used per well and the concentration of [3H]dopamine was 2-3 nM.

Dopamine-stimulated [35S]GTPγS binding at the D4 receptor was performed as for the D2 receptor except that membranes were prepared from CHO cells expressing the human dopamine D4.4 receptor and that 10 μg protein of the membranes was used per well (Wood et al, 2014).

Dopamine-stimulated increases in cAMP at the dopamine D1 receptor in Ltk- cells (Wood et al, 2014) was performed as for the dopamine D2 receptor except that 20,000 cells per well were used and that the assay did not include forskolin.

Noradrenaline-stimulated [35S]GTPγS binding was performed as above using 10 μg protein from CHO cells expressing the human alpha2C adrenergic receptor in Tris buffer (pH 7.4) containing 1 mM MgCl2, 2 μg saponin, 1 μM GDP.

**Acutely dissociated striatal neurons**

Detailed procedures have been published (Cepeda et al. 1998, Flores-Hernandez et al. 2002, Cepeda et al. 2008). Adult rats were anesthetized with a mix of isoflurane and oxygen and sacrificed. Brains were dissected and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) NaCl, 130; NaH2PO4, 1.25; NaHCO3, 26; MgCl2, 5; CaCl2, 1; and glucose, 10. Coronal slices (350 μm) were...
transferred to a chamber containing ACSF (with 2 mM CaCl₂ and 2 mM MgCl₂) oxygenated with 95% O₂-5% CO₂ (pH 7.2–7.4, 290–310 mOsm, 25 ± 2°C). Dorsal striatum was dissected and treated for 15 min with papain (0.5 mg/ml, Calbiochem) at 35°C in a N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid] (HEPES)-buffered Hank’s balanced salts solution (Sigma) supplemented with (in mM) 1 pyruvic acid, 0.005 glutathione, 0.1 NG-nitro-L-arginine, and 1 kynurenic acid (pH 7.4, 300-310 mOsm). The tissue was rinsed with (mM) Na isethionate (140), KCl (2), MgCl₂ (2), CaCl₂ (0.1), glucose (23), and HEPES (15). Striatal slices were mechanically dissociated and plated onto a stage of an inverted microscope.

Standard whole-cell patch clamp techniques were used to obtain voltage clamp recordings. The internal solution consisted of (in mM) 175 N-methyl-D-glucamine, 40 HEPES, 2 MgCl₂, 10 EGTA, 12 phosphocreatine, 2 Na₂ ATP, 0.2 Na₂ GTP, and 0.1 leupeptin (pH 7.25, 265-270 mOsm). The external solution consisted of (in mM) 135 NaCl, 20 CsCl, 3 BaCl₂, 2 CaCl₂, 10 glucose, 10 HEPES, and 0.0003 tetrodotoxin (pH 7.4, 300-310 mOsm). Negative pressure was used to obtain tight seals (>1 GΩ) and data collected from neurons that had access resistances below 20 MΩ.

NMDA and quinpirole were dissolved in H₂O whereas the D2 antagonist L741,626 and the test compound were dissolved in DMSO. All experiments contained the same final DMSO concentration (1%). Drugs were applied through a pressure-driven fast perfusion system (Warner Instruments, Hamden, CT) using an array of application capillaries synchronized by pClamp. NMDA currents (3 sec duration every
15 sec) were evoked while holding the cell at -70 mV. The D2 agonist and antagonist were both pre-and co-applied. In separate cells, one concentration of the D2 agonist was tested before and after application of the tested compound or vehicle. Baseline for NMDA currents (100 μM) was established and modulation of NMDA currents by the D2 agonist, quinpirole was tested in neurons at one concentration. Quinpirole was then washed out, the test compound (10 μM) applied for 2 min before establishing a new baseline for NMDA currents, and re-testing quinpirole.

6-OHDA lesion

To protect norepinephrinergic neurons, animals were administered imipramine HCl (Sigma) 15 minutes before surgery. They were subsequently anesthetized with ketamine (Ceva, 75 mg/kg) and xylazine (Bayer, 10 mg/kg) and placed in stereotaxic frame (David Kopf Instrument). 6-OHDA was injected into the right ascending medial forebrain bundle at the following coordinates (in mm) relative to bregma and surface of the dura, AP = -3.5, ML = -1.5, DV = -8.7. Each rat received one injection of 6-OHDA HBr (4 μg/μl) over a period of 5 minutes (0.5 μl/min) for a total of 10 μg per rat. Animals were monitored for 3 weeks to ensure full recovery and habituation to the environment and experimenters.

On day 21 post surgery, all rats were challenged with a small dose of subcutaneously administered apomorphine (Sigma, 0.05 mg/kg). Rats showing more than 90 contralateral rotations (360°) over a 45-minute recording period were included in the study.
L-Dopa methyl ester (Sigma) was dissolved in physiological saline solution at a volume of 5 ml/kg. UCB compound (30 mg/kg, UCB) was intraperitoneally administered as a suspension at a dose volume of 5 ml/kg in vehicle (0.1% (w/v) Polysorbate 80 (Merck); 0.1% (w/v) 1510 silicone antifoam (VWR, UK) in 1.0% (w/v) methylcellulose (Sigma-Aldrich, UK)). The compound was prepared extemporaneously and homogenised by using a ultrasonic homogeniser (Covaris) and magnetic stirring.

**Behavioural recording**

Vehicle or UCB compound were administered as “add-on treatment” to a sub-threshold dose of L-Dopa (15 mg/kg) without any dopa decarboxylase inhibitor to avoid additional potential pharmacokinetic interaction. UCB compound or vehicle were administered intraperitoneally (ip) 15 minutes before the L-Dopa dose (15 mg/kg, ip).

Rotational behaviour was recorded using a computerized system. Rats were fixed in a harness and linked to mechanical sensors connected directly to a computer. Each 360° clock-wise or counter clock-wise turn was automatically recorded for up to 120 minutes at the maximum. Throughout the experiments, rats were allocated to individual test cages.

**Data analysis**

In all studies values are presented as means ± SEM from n separate experiments.

In electrophysiological studies data analyses were performed with Clampfit 10.3 (Molecular Devices, Wokingham UK). Group means for all measures were compared
using Student's t-tests (for two-group comparisons) and ANOVAs followed by Bonferroni t-tests (multiple-group comparisons) using SigmaStat software (SPSS, Chicago, IL, USA). Differences were considered to be statistically significant when $P<0.05$. 

In all other in-vitro assays, data analysis was performed in PRISM (GraphPad Software, La Jolla, CA, USA) using the sigmoidal dose-response equation for pharmacological studies and one-site specific equation for saturation studies.

The efficacy of the compound to modify the level of L-Dopa-induced contralateral rotations was assessed with two-way mixed ANOVA, combining the drug effect (2 levels) as between group factor with the time (12 levels) as within subjects factor. Statistical analyses were performed using the Statistica software (StatSoft Inc., OK, USA). For each test, statistical significance was assumed if $P<0.05$ and data were log-transformed prior to analysis.
Results

Effects on $[^{35}\text{S}]$-GTPγS binding

In membranes from CHO cells expressing the human dopamine D2 receptor, dopamine stimulated $[^{35}\text{S}]$-GTPγS binding with a $pEC_{50}$ of $6.46 \pm 0.05$ (mean ± s.e.m, n=8; 350 nM) and a stimulation of $91\% \pm 8$ over basal. The initial compound identified, at concentrations up to $10 \mu M$ lacked any significant effect on $[^{35}\text{S}]$-GTPγS binding in the same cells on its own (6% stimulation over basal ± 3 at $10 \mu M$, n=6; Fig 2A). However, in the presence of a low concentration of dopamine (30 nM) close to the EC20, the compound now stimulated $[^{35}\text{S}]$-GTPγS binding by $27\% \pm 6$ (fitted $Emax$) over basal (basal defined in the presence of dopamine) with a $pEC_{50}$ of $5.99\pm0.32$ (n=3; Fig 2B; 1000nM). Upon synthesis of the stereoisomers, the R isomer stimulated $[^{35}\text{S}]$-GTPγS binding in the presence of a low concentration of dopamine by $45\% \pm 3$ (n=3) with the same $pEC_{50}$ of $6.00\pm0.13$ whereas the S isomer lacked significant effects (Fig 2B).

The effects of the stereoisomers on the concentration-response curve to dopamine were then investigated (Fig 2C). At $10 \mu M$, the R isomer significantly increased the potency ($pEC_{50}$) of DA from $6.60\pm0.1$ to $7.19\pm0.1$ (250 nM to 65nM; n=3, $p<0.001$, F-test, GraphPad Prism) and increased the $Emax$ by 14 % ($p<0.01$, F-test). In contrast, the S isomer had no effect on the potency of DA to 6.44 (370 nM, not significant) but reduced the $Emax$ of dopamine by 20% ($p<0.01$, F-test).
We then examined whether the R isomer would potentiate the effects of other dopamine D2 receptor agonists. At 10 μM, the R isomer increased the potency of quinpirole (pEC50) from 6.21±0.08 to 6.69±0.11 (620 nM; mean ± s.e.m., n=4, p<0.01 Student’s paired t-test; Fig 2D). Quinpirole was a full agonist compared to dopamine (Emax 99 % ± 2 compared to dopamine fitted Emax=100%, mean ± s.e.m., n=4) and the presence of the R-isomer at 10 μM increased this Emax by 35% ± 2 (p<0.01, F-test, GraphPad Prism).

We next investigated the effects of the compounds in membranes expressing the rat dopamine D2 receptor. At the rat D2 receptor, dopamine stimulated [35S]-GTPγS binding with a pEC50 of 6.83 ± 0.16 (n=5; 150 nM). At the rat D2 receptor, the R isomer potentiated [35S]-GTPγS binding in the presence of a low concentration of dopamine (10nM) by 57% ± 6 over basal (fitted Emax and basal with basal defined as binding in the absence of compound but presence of 10nM dopamine)) with a pEC50 of 5.84±0.06 (n=5; 140nM). In the absence of dopamine, there was no significant effect.

**Effects on cAMP at the hD2L receptor**

In the presence of forskolin, dopamine produced a concentration-dependent decrease in cAMP levels with a pEC50 of 9.0 ± 0.1 (n=4; 1 nM). In the presence of 10 μM of the racemic initial compound, the potency (pEC50) of dopamine was increased to 9.6 ± 0.1 (Fig 3A; 0.25 nM). There was no effect of the compound on basal levels of cAMP and there was no change in the maximum response to dopamine. We then tested the effects of the stereoisomers on the DA concentration-response curve (changes in cAMP
assessed using HTRF ratio, Fig 3B). The R-isomer (10 μM) produced a significant shift in the potency of dopamine pEC$_{50}$ (EC$_{50}$) from 8.3 (4.6 nM) to 9.03 (0.9 nM) and the S-isomer had a small but non-significant reduction in dopamine potency to 8.1 (8 nM). This change in apparent dopamine potency was due to the correction of raw counts to nM cAMP using the standard curve.

Effects on D2 receptor binding

At concentrations up to 10 μM both the R- and S-isomers had no significant effect (<10%) on the binding of [³H]-raclopride to the human D2 receptor (data not shown). The effects of the potential allosteric modulators on the binding of the endogenous agonist, [³H]-dopamine, to the hD2 receptor in CHO cell membranes was then investigated. Previous literature has shown the importance of including Mg$^{++}$ ions both in the membrane preparation and in the assay buffer (Hamblin & Creese, 1982) and in the present studies the best window was obtained using 1 mM MgCl$_2$. The concentration of [³H]-dopamine used in competition studies was selected based on signal size and also on window size (specific: non-specific). The specific binding window was increased in the presence of pargyline and ascorbic acid compared to that in the absence and no specific filter binding was seen. No specific binding was seen in membranes prepared from cells lacking the D2 receptor. Control compounds such as apomorphine, chlorpromazine and (+)butaclamol produced a concentration-dependent full inhibition of [³H]-dopamine binding to the D2 receptor with a pEC$_{50}$ of 9.16±0.04 (n=3; 0.25 nM), 9.00±0.50 (n=4, 1 nM) and 8.58±0.10 (n=3; 2.6 nM) respectively.
contrast, the R isomer produced a concentration-dependent increase in $[^3]$H-dopamine binding (Fig 4A). This potentiation was difficult to quantify as the response did not saturate at the concentrations tested (we could not go to higher concentrations of the compound due to solubility) but a marked 100% increase in radioligand binding was seen at 10 μM of the compound and a significant effect was seen at 1 μM. The S isomer produced an inhibition of radioligand binding. Again the effect was difficult to quantify, but a significant inhibition of 16%±3 (n=3) was seen at 3 μM compound.

The effect of the isomers on the saturation binding of $[^3]$H-dopamine to the dopamine D2 receptor was then studied. Analysis of the saturation binding revealed the presence of 1 binding site (F-test, GraphPad Prism). Saturation studies were carried out in the presence and absence of 10 μM of the R and S isomers (Table 1). There was no significant effect on the apparent affinity of dopamine (Kd 15nM) but the maximum number of binding sites (Bmax; 3.2 ± 0.2 pmol/mgPr) was significantly increased in the presence of the R-isomer (P<0.5, Student’s paired t-test) whereas there was a small but non-significant reduction in the Bmax with the S-isomer (supplementary data). The Bmax for the antagonist radioligand $[^3]$H-raclopride in the same membranes was 17 pmol/mg Pr (data not shown).

In order to further explore the activities of these PAM effects at the dopamine receptors, we went on to identify [5-fluoro-4-(hydroxymethyl)-2-methoxyphenyl]-(4-fluoro-1H-indol-1-yl)methanone (see supplemental data for synthesis and structure) as a more potent and efficacious PAM suitable for in-vivo experimentation. On human D2 GTP$^{[35]}$S-
GTPγS binding, this improved PAM lacked significant effects on its own (<10% activation at 10 μM compared to maximal effect of dopamine) but potentiated the effects of a low concentration of dopamine with a pEC₅₀ of 6.2 ± 0.24 (n=5; 630 nM) and by an Erel of 89% over basal ± 12 (compared to 57% for the R isomer). This higher level of potentiation was evidenced by a greater shift or increase in the potency of dopamine of 15 fold (from 6.4nM to 0.4nM, Fig 4C) compared to 4 fold for the R-isomer. We therefore studied the effect of the improved PAM on the saturation binding of [³H]-dopamine as above. This PAM (10 μM) markedly increased the number of high affinity binding sites, giving a 3 fold increase markedly greater than that seen with the R-isomer and consistent with its higher efficacy (Table 2; Fig 5). The non-hydrolysable GTP analogue, Gpp(NH)p (32μM) reduced the affinity of [³H]dopamine binding and increased the apparent number of sites. The effect of the PAM was not affected by the presence of Gpp(NH)p.

**Selectivity assays**

To ensure that potentiation in dopamine mediated receptor signaling was not due to an effect at the G-protein level, experiments were conducted using the human alpha2C noradrenergic receptor and the histamine H3 receptor. No significant effects were seen at concentrations up to 10 μM on basal or agonist-stimulated [³⁵S]-GTPγS binding at either receptor for any of the compounds (data not shown but examples shown in supplementary data).
To examine selectivity further, the effects of the compounds were tested as allosteric modulators at human dopamine D1 and D3 receptors. At the D1 receptor, dopamine stimulated cAMP production with a pEC$_{50}$ of 8.6 ± 0.1 (n=4; 2.5 nM). All three compounds had no effect on the D1 response (data not shown), either on their own or in the presence of dopamine (1.5 nM) or on the dopamine concentration-response curve (supplementary data). In contrast, similar to their effects at the human dopamine D2 receptor, the R-isomer potentiated the binding of [3H]-dopamine to the D3 receptor and the S-isomer attenuated binding (Fig 4B). The compounds also lacked activity either on their own or in the presence of dopamine at the human dopamine D4 receptor (data not shown).

**Effects in electrophysiological studies in rat striatal neurons**

At concentrations above 3 μM, quinpirole dose-dependently decreased NMDA currents (ANOVA, F6,35=70.29, P<0.0001, Fig 6A). The D2 antagonist L-741,626 (30 μM; Hattori et al, 2006)) blocked 10 μM quinpirole effect (not shown) demonstrating that this effect is mediated by D2 receptor activation. In the presence of the D2 antagonist, we still observed a slight decrease of NMDA currents (-13.4±3.4%, n = 4). However it was similar to the decrease observed with the lower concentrations of quinpirole, suggesting that the remaining decrease is induced in part by current rundown. For all further studies, the effects of compounds were therefore compared to time-matched controls.

The R isomer tested alone at 10 μM did not have a significant effect on NMDA currents (-5.3±1.3%, n=29). In the presence of the R isomer (10 μM), the effect of quinpirole at 3,
10, 30, 100 and 300 μM (n=4-11) was increased (paired t-test P<0.05) and the potency of quinpirole pEC$_{50}$ was increased from 4.3 (52.5 μM) to 4.8 (15.9 μM) (Fig 6B). As a control, incubation in 1% DMSO failed to potentiate 3 μM quinpirole effect (n=5, Fig 6C). The PAM (R isomer) had no effect on the lowest concentration of quinpirole tested (1 μM) suggesting that any effect was not due to current rundown which would be present at all quinpirole concentrations.

**In-vivo effects on L-Dopa turning behaviour**

UCB compound significantly increased [F(1,14)=10.45, p<0.01] and prolonged [F(11,154)=50.55, p<0.0001] the L-Dopa-induced contralateral rotations (Fig 7). An effect of the treatment with time was also observed [F(11,154)=7.60, p<0.0001]. Additional statistical analysis showed that the group treated with the UCB compound had significant higher level of contralateral rotations than the vehicle-treated group for the first 60 minutes of the test.
Discussion

We report the identification of novel allosteric modulators of the dopamine D2 receptor and D3 receptors. We identified an initial hit from an HTS which lacked agonist effects on its own (at concentrations up to 10 μM), but which potentiated the effect of dopamine on the human D2 receptor in cAMP and on[^35S]-GTPγS binding. However, this initial hit was a mix of two isomers. We therefore separated the isomers and confirmed their conformation by X-ray— the R isomer with the methyl group up and the S isomer with the methyl group down. Compared to the racemic mixture, the R isomer produced a greater enhancement of D2-stimulated[^35S]-GTPγS binding whereas the S isomer appeared inactive, suggesting that the presence of the S isomer in the racemate attenuated the effects of the R isomer. We then compared the ability of the isomers to affect the concentration-response curve to the endogenous ligand, dopamine. The R-isomer increased both the potency and, to a lesser extent, the efficacy of dopamine, whereas the S-isomer produced a small but significant reduction in the maximal response to dopamine with no effect on dopamine potency. Similar results were seen on the ability of dopamine to inhibit cAMP levels, although a reduction in maximal response to dopamine in the presence of the S-isomer was not observed. This may reflect the greater receptor reserve seen in the cAMP pathway as evidenced by the markedly higher potency of dopamine in the cAMP assay (pEC$_{50}$ 9.0) than in the[^35S]-GTPγS binding assay (pEC$_{50}$ 6.6).
To check that this allosteric effect was mediated at the receptor level (as opposed to an effect on the G-protein), we then sought to determine if the compounds affect other Gi-coupled receptors. Using [35S]-GTPγS binding, we showed that neither compound had any significant affect as either an agonist or as a modulator at the Gi-coupled alpha-adrenergic 2C and histaminergic H3 receptors. Using the antagonist [3H]-raclopride no significant effect was seen at concentrations up to 10 μM indicating that the compounds did not compete at the orthosteric site. Using [3H]dopamine, we showed that the R isomer did not act as an orthosteric ligand to inhibit binding to the dopamine D2 receptor, but in fact increased the binding whereas the S isomer decreased binding. The increase in [3H]dopamine binding was consistent with its potency on [35S]GTPγS binding, although this was difficult to quantitate as solubility of the compounded precluded testing at concentrations greater than 10 μM. Similar effects on the binding of [3H]dopamine to the human dopamine D3 receptor were observed with both the R- and S-isomers. Novel allosteric binding sites on GPCRs have been suggested to offer selectivity benefits compared to the orthosteric site which is often highly conserved across receptor subtypes (Conn et al., 2009; Leach et al., 2010). In this study, we have shown selectivity for the D2 receptor over the D1 receptor, but not over the D3 receptor. There is a high degree of sequence similarity between the D2 and D3 receptors – over 78% within the transmembrane domains and a near identity of the residues inferred to form the dopamine binding site. For the antagonist eticlopride, 17 of the 18 residues which form its binding site are identical across the D2 and D3 receptors (Shi & Javitch, 2002; Chien et al., 2010). Interestingly SB269652 was identified as an allosteric
antagonist at both dopamine D3 and D2 receptors (Silvano et al, 2010). Recent studies have suggested that this compound acts as a bitopic ligand with the indole-2-carboxamide moiety interacting with an allosteric pocket (Lane et al, 2014) and which has a close structural similarity to the compounds identified here. This raises the possibility that the allosteric pocket is conserved between dopamine D2 and D3 receptors, but not across dopamine D4 or D1 receptors.

We had good evidence that the compounds acted as allosteric modulators at the dopamine D2 and D3 receptor, so we investigated the mechanism of this interaction by investigating their effects on the saturation binding of the orthosteric agonist $[^3]H$-dopamine. Since the description of this assay there have been few reports on its application, probably due to the low level of specific binding (Burt et al, 1975) compared to newer antagonist radioligands and the observation that the inclusion of Mg$^{++}$ in the membrane preparation is important (Hamblin & Creese, 1982). The affinity of $[^3]H$-dopamine determined in the present study is in good agreement with that obtained in rat tissue (5-10 nM, Burt et al, 1975) and in CHO cells (Torvinen et al, 2004). Our data showed that the PAM did not affect the affinity of $[^3]H$dopamine for the D2 receptor, but instead increased the proportion of receptors in the high affinity agonist state. This suggests that the PAM acts to enhance signaling or efficacy at the receptor. The change in potency of dopamine observed in the functional assays is probably due to a high receptor reserve leading to a ceiling in efficacy (see Kenakin, 2013). In support of
this (-)3-PPP has been reported as a partial agonist at the dopamine D2 receptor (Burris et al, 2002), but appeared as a full agonist in the present study (data not shown). GppNHp reduced the apparent affinity for [3H]-dopamine and increased the apparent number of sites, suggesting a conversion to a low affinity-inactive state. In support of this, the number of high-affinity sites labeled by [3H]-dopamine was lower than that seen with [3H]-raclopride. The lack of effect of Gpp(NH)p on [3H]-dopamine binding in the presence of the PAM suggests that this conformational change is independent of the G-protein.

Having demonstrated robust allosteric effects at the dopamine D2 receptor, it is important to show that these effects are maintained under physiological expression and signal transduction conditions. We show that the R isomer potentiates the effect of a dopamine D2/D3 agonist on a D2/D3 receptor mediated response in a native tissue system, rat striatal neurons. Activation of the D2/D3 receptor induced decreases of NMDA currents in adult rat striatal neurons and this was blocked by a dopamine D2/D3 receptor antagonist. In this assay, there was no effect of the compound alone, demonstrating a lack of agonist-like properties under physiological expression, but it increased the potency (EC_{50}) of quinpirole from 52 to 16 μM. The potency of quinpirole (pEC_{50} 4.28) was in agreement with other findings (Andre et al, 2010; Jocoy et al, 2011) and was consistent with a lower D2 receptor expression in the native tissue compared
to the recombinant systems expressing rat D2 receptors where the pEC$_{50}$ for quinpirole was 6.2.

In order to test the hypothesis that a D2 PAM should potentiate the effects of released dopamine in-vivo, we went on to identify [5-fluoro-4-(hydroxymethyl)-2-methoxyphenyl](4-fluoro-1H-indol-1-yl)methanone (supplementary data) as a more potent and efficacious PAM D2/D3 compound. This compound was slightly more potent, but was markedly more efficacious (almost 90% potentiation of GTP$\gamma$S assay compared to 40% for the initial PAM and 3 fold shift in Bmax of [$^3$H]-dopamine binding compared to 1.3), and it had improved physicochemical properties resulting in sufficient brain exposure for in-vivo testing. We have used the unilateral 6-OHDA lesion model in rats (Schwarting & Huston, 1996) to investigate the effects of the PAM using a threshold dose of L-Dopa which induced a low level of contralateral turns. This model was expected to be sensitive to the effects of a PAM. Indeed we saw that compound, at 30 mg/kg, potentiated the effects of a threshold dose of L-Dopa on contralateral turning in the 6-OHDA model. We confirmed that this was not due to an effect on L-Dopa metabolism as ether was no change in the plasma exposure of L-Dopa (R.Barnaby, personal communication). Unfortunately we did not have sufficient material to check the effect of the compound alone but no overt effect of the compound was seen in the pharmacokinetic study, supporting the hypothesis that the potentiation was due to an allosteric mechanism. These data indicate that a positive allosteric modulator of the dopamine D2/D3 receptor can potentiate dopamine effects on neuronal activity in rat brain.
In conclusion, we have shown a stereoselective interaction on the allosteric modulation of the dopamine D2 and D3 receptor. The R isomer acts as a PAM in that it lacks effects on its own, but potentiates the effects of dopamine at the D2 receptor on G-protein activation, cAMP signaling, and potentiates [³H]dopamine binding to the D2 receptor. The potentiation in Emax seen in [³⁵S]-GTPγS binding compared to the lack of effect on the downstream signaling, cAMP, together with the potentiation in Bmax and the lack of change in affinity for [³H]-dopamine, suggest that the R isomer acts as an efficacy modulator by stabilizing the active state of the receptor. In contrast the S isomer attenuates the effects of the R isomer and inhibits [³H]dopamine binding. Whilst the evidence for an allosteric action at the dopamine D3 receptor is not as robust as that at the dopamine D2 receptor, the potentiation of [³H]-dopamine binding in the absence of an effect on the orthosteric antagonist binding is consistent with the profile demonstrated at the D2 receptor. Unfortunately the R-isomer and S-isomers both produced a non-specific effect which interfered with our D3 receptor label-free functional assay (Wood et al, 2014) precluding further testing. Importantly we show that the PAM maintains its activity in native tissue and further that this potentiation was maintained in-vivo. In both these studies, an action at both dopamine D2 and D3 receptors could be involved and further studies with selective D3 receptor antagonists are needed. The identification of positive allosteric modulators at dopamine D2 and D3 receptors could have important applications in pathological conditions of hypodopaminergic function, such as in Parkinson’s disease and restless leg syndrome.
Acknowledgements: We would like to acknowledge the technical assistance of Mr David Urbain.
**Authorship contributions**

*Participated in research design:* Wood, Andre, Ates, Gillard, Michel, Barnaby.

*Conducted experiments:* Andre

*Performed data analysis:* Wood, Andre, Michel, Barnaby, Ates.

*Wrote or contributed to the writing of the manuscript:* Wood, Ates, Andre, Michel, Gillard
References.


Wood M, Dubois V, Scheller D, Gillard M (2014) Rotigotine is a potent agonist at dopamine D1 receptors as well as at dopamine D2 and D3 receptors. *Br J Pharmacol*, **172**: 1124–1135.

Legends for Figures

Figure 1: Chemical structure of the dopamine D2 receptor allosteric modulator showing the racemic centre.

Fig 2: Effect of compounds on D2 mediated $[^{35}\text{S}]$-GTP$\gamma$S binding. Results are means and s.e.m. from triplicate determinations in a single representative experiment which was repeated twice.

A. The R isomer lacks direct effects. Basal is the response in the absence of dopamine.
B. The R isomer causes a greater potentiation of D2 receptor signaling in the presence of a low concentration of dopamine than the racemate in a concentration-related manner. The S isomer lacked effects. Results are normalized to fitted max DA response and minimum (basal) response in presence of EC20 [DA].
C. In the presence of 10 $\mu$M of the R isomer, the potency and efficacy of dopamine was increased compared to dopamine alone. The presence of the S isomer (10 $\mu$M) had no effect.
D. In the presence of 10 $\mu$M of the R isomer, the potency and efficacy of quinpirole was increased compared to quinpirole alone.

Fig 3: Effect of compounds on D2 mediated cAMP levels. Results are means and s.e.m. from triplicate determinations in a single representative experiment which was repeated at least twice.

A. The presence of 10 $\mu$M of the racemate increases the potency of dopamine 4 fold to inhibit cAMP production at the human dopamine D2 receptor compared to dopamine alone. Shown is the data after conversion to [cAMP].
B. In the presence of 10 $\mu$M of the R isomer, the potency (5 fold) and efficacy of dopamine was increased compared to dopamine alone. There was a small non-significant reduction in the potency of dopamine in the presence of the S isomer (10 $\mu$M). Shown is the raw data in HTRF fluorescent ratio.
C. The improved compound at 10 $\mu$M increased the potency of dopamine by 15 fold.

Fig 4: Effect of compounds on $[^{3}\text{H}]$-dopamine binding to the human dopamine receptors. Results are means and s.e.m. from triplicate determinations in a single representative experiment which was repeated at least twice.
A. At the human D2L receptor, the R isomer caused a concentration-related increase in \(^{3}\text{H}\)-dopamine binding whereas the S isomer decreased binding.

B. At the human D3 receptor, the R isomer caused a concentration-related increase in \(^{3}\text{H}\)-dopamine binding whereas the S isomer decreased binding.

C. Fig 5: Effect of [5-fluoro-4-(hydroxymethyl)-2-methoxyphenyl](4-fluoro-1H-indol-1-yl)methanone (10 \(\mu\)M) on saturation binding of \(^{3}\text{H}\)-dopamine. Shown is the specific binding of \(^{3}\text{H}\)-dopamine in the presence and absence of the compound and are data from a single experiment with triplicate determination (A). In B we show the effect of GppNHp on the saturation binding of \(^{3}\text{H}\) dopamine and in C we show the lack of effect of GppNHp on the effect of the PAM.

Fig 6: Traces of 100 \(\mu\)M NMDA-mediated calcium and barium currents before and after co-application of 10 \(\mu\)M quinpirole, before and after incubation with the R isomer. B. Graph shows the dose-response effect of quinpirole or quinpirole+R isomer on NMDA currents. The dose-response curve was shifted to the left in the presence of PAMD2. C. While incubation in PAMD2 potentiated the effect of 3 \(\mu\)M quinpirole compared to vehicle control. Paired t-test *P<0.05; **P<0.01; ***P<0.001.

The blue line indicates D2 agonist+R isomer. the green line indicates D2 agonist alone.

Fig 7: Effect of improved PAM compound (30 mg/kg, ip) on the level of L-Dopa-induced contralateral rotations in unilateral 6-OHDA-lesioned rats (15 mg/kg, ip). *, p<0.05: mean significantly different from that of the vehicle-treated group (planned contrasts for every 10-min time interval).
### Tables

**Table 1:** Effects of the stereoisomers on the saturation binding of \[^{3}H\]dopamine to the human dopamine D2 receptor

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>+ R isomer (10 μM)</th>
<th>+ S isomer (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KD (nM)</td>
<td>15 ± 3</td>
<td>13 ± 3</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>Bmax (% control)</td>
<td>100 ± 6</td>
<td>137 ± 10*</td>
<td>82 ± 15</td>
</tr>
</tbody>
</table>

Data are means ± standard deviation from 3 separate experiments.

*p<0.05, Student’s paired t-test
Table 2: Effect of the improved PAM on the saturation binding of [³H]dopamine binding to the human dopamine D2 receptor

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>+ PAM (10 μM)</th>
<th>+ Gpp(NH)p (32 μM)</th>
<th>+ PAM + Gpp(NH)p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K_D (nM)</strong></td>
<td>18 ± 12</td>
<td>11 ± 4</td>
<td>87 ± 32*</td>
<td>20 ± 13</td>
</tr>
<tr>
<td><strong>Bmax (% control)</strong></td>
<td>100 ± 26</td>
<td>278 ± 51*</td>
<td>200 ± 120</td>
<td>289 ± 47*</td>
</tr>
</tbody>
</table>

Data are means ± standard deviation from 3-4 separate experiments.

*p<0.05, Student’s paired t-test compared to control
**A**

![Graph A](image1)

**B**

![Graph B](image2)

**C**

![Graph C](image3)

**D**

![Graph D](image4)
[\textsuperscript{35}S]GTP\gammaS Bound (c.p.m.)

\begin{align*}
\text{basal} & \quad 4000 \quad 8000 \quad 12000 \quad 16000 \quad 20000 \quad 24000 \\
\text{log[quinpirole].M} & \quad -10 \quad -9 \quad -8 \quad -7 \quad -6 \quad -5 \quad -4
\end{align*}

- R-isomer
- Control

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A

[D R U G ] log (M )

3H] Dopamine Bound (cpm)

-12 -11 -10 -9 -8 -7 -6 -5

0

500

1000

1500

2000

R -isomer

S -isomer

Apomorphine

[DRUG].log(M)

B

[D R U G ] log (M )

3H] Dopamine Bound (cpm)

-12 -11 -10 -9 -8 -7 -6 -5

0

500

1000

1500

R -isomer

S -isomer

Apomorphine

[DRUG].log(M)
Fig 5:

A

+ PAM at 10 μM Kd = 8 nM, Bmax=250 fmol

control Kd = 11 nM, Bmax=80 fmol

[^H]-dopamine]nM

B

control Kd = 11 nM, Bmax=80 fmol
active state

+ Gpp(NH)p Kd = 70 nM, Bmax=130 fmol
uncoupled, inactive state

[^H]-dopamine]nM

C

+ PAM 10 μM
+ PAM 10 μM
+ Gpp(NH)p

control

[^H]-dopamine]nM

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Fig 6:

A

B

C

-6 -5 -4 -3
0
2 0
4 0
6 0
8 0
1 0 0
C o n t r o l
+ R i s o m e r
-lo g [q u i n p i r o l e ] (M )

1 0 0 M N M D A c u r r e n t

-6 -5 -4 -3
0
2 0
4 0
6 0
8 0
1 0 0
C o n t r o l
+ R i s o m e r
-lo g [q u i n p i r o l e ] (M )

-6 -5 -4 -3
0
2 0
4 0
6 0
8 0
1 0 0
C o n t r o l
+ R i s o m e r
-lo g [q u i n p i r o l e ] (M )
Fig 7:
Molecular Pharmacology

In-vitro and in-vivo identification of novel positive allosteric modulators of the human dopamine D2 and D3 receptor

Authors:

Martyn Wood, Ali Ates, Veronique Marie Andre, Anne Michel, Robert Barnaby, Michel Gillard

UCB Biopharma SPRL, Chemin du Foriest, B-1420 Braine-l’Alleud, Belgium

Supplemental file

A. Selectivity data

1. Lack of effect on the R- and S-isomers on the histamine H3 –receptor mediated increase in $[^{35}\text{S}]$-GTP$\gamma$S binding. Shown is the stimulation by histamine and the effect of increasing concentrations of the test compounds in the presence of an EC20 concentration of histamine.
2. Lack of effect on the R-isomers on the noradrenaline-stimulated alpha2c adrenoceptor –receptor mediated increase in [\textsuperscript{35}S]-GTP\textsubscript{\gamma}S binding. Shown is the stimulation by histamine and the effect of increasing concentrations of the test compounds in the presence of an EC20 concentration of noradrenaline.

![Graph showing stimulation by histamine and effect of increasing concentrations of test compounds](image)

3. Lack of effect of the R-isomer on the response to dopamine at the human dopamine D1 receptor. Shown is the concentration-response curve to dopamine to stimulate cAMP levels (seen as reduction in HTRF response) in the absence (solid line – data points omitted for clarity) and presence (dashed line and data points) of 10 \textmu M of the R-isomer from triplicate determinations.

![Graph showing cAMP response to dopamine](image)

B. Effect of the R-isomer on [\textsuperscript{3}H]-dopamine saturation binding

4. The effect of the R-isomer on the saturation binding of [\textsuperscript{3}H]dopamine to the human dopamine D2 receptor. The presence of 10 \textmu M of the R-isomer had no significant effect on the Kd (26nM) but increased the apparent Bmax by 40%
5. Structure and synthesis of the improved compound [5-fluoro-4-(hydroxymethyl)-2-methoxyphenyl]-(4-fluoro-1H-indol-1-yl)methanone
Product (2).

A) 4-Bromo-2,5-difluorobenzoic acid.
Under an argon atmosphere, to a solution of 1,4-dibromo-2,5-difluorobenzene (1) (20.00 g, 73.6 mmol) in diethyl ether (160 mL) was added dropwise 1.6 M n-butyl lithium solution in hexane (1.05 eq, 49 mL) at -78 °C, and the mixture was stirred at same temperature for 2 min. The mixture was added quickly to the mixture of dry ice (about 100 g) and diethyl ether (200 mL), and the mixture was warmed up to room temperature. The precipitate was collected by filtration, and washed with diethyl ether. The obtained solid was treated with water (50 mL) and 1 M hydrochloric acid (150 mL), and extracted with diethyl ether. The organic layer was washed with brine and dried. The desiccant was removed by filtration and the filtrate was evaporated in vacuo. The resulting residue was washed with hexane, dried in vacuo to obtain 4-bromo-2,5-difluorobenzoic acid (14.8 g, 85 percent) as a pale yellow solid: 1H NMR (DMSO-d6) δ 7.78 (1H, dd, J = 6.4, 8.3 Hz), 7.89 (1H, dd, J = 5.9, 9.8 Hz).

B) Methyl 4-bromo-2,5-difluorobenzoate.
To a solution of 4-bromo-2,5-difluoro-benzoic acid (10.0 g, 42.36 mmol, 1 eq) in dry MeOH (140 mL) was added SOCl2 (6.2 mL, 84.72 mmol, 2 eq) at 0°C dropwise. The mixture was stirred at 70 °C for 3 h and the excess reagent was removed in vacuo. The residue was diluted with EA and washed with saturated NaHCO3 solution and brine. The organic phase was dried and concentrated to give 4-bromo-2,5-difluoro-benzoic acid methyl ester (9.4 g, 89percent) as a gray solid.
C) Methyl 4-bromo-5-fluoro-2-methoxybenzoate (2).
To a solution of methyl 4-bromo-2,5-difluorobenzoate (17.6 g, 70.4 mmol) in anhydrous DMF (200 ml) was added CH₃ONa (4.56g, 84.48 mmol). The mixture solution was stirred at RT for 16 h. The reaction mixture was diluted with 500 ml EA and washed with water (100mlx3). The organic layer was dried and concentrated to be purified by chromatography column (EA: PE=1:20) to afford 14.2 g product (77 percent).

Product (3).
A mixture of (2) (2.0 g, 7.6 mmol), HMPA (15 ml), Bu₃SnCH=CH₂ (3.4 g, 10.6 mmol) and trans-Dichlorobis(triphenylphosphine) palladium (II) (0.53 g, 0.76 mmol) was stirred under N₂ atmosphere at +80°C for 16 h and cooled. Water, EtOAc and n-hexane (100, 140 and 70 ml) were added, the organic layer was separated and the water one was extracted with EtOAc/ n-hexane. The organic layers were washed with water, dried over Na₂SO₄ and evaporated in vacuo. Compound (3) was purified by chromatography (silica gel 63-100 µm, ethyl acetate /hexane, 1:5). Fractions containing the target product were combined and evaporated in vacuo to give compound (3) methyl 5-fluoro-2-methoxy-4-vinylbenzoate (1.05 g, 66%).

Product (4).
A mixture of (3) (1.0 g, 4.75 mmol), THF (10 ml), NaIO₄ (1.3 g, 6 mmol) and 4%w/w solution of OsO₄ in CCl₄ (0.6 g, 2 mmol%) was stirred at RT for 6 h (TLC control). EtOAc (200 ml) was added, the organic layer was separated and the water one was extracted with EtOAc. The organic layer was dried over Na₂SO₄ and evaporated in vacuo. Compound (4) was purified by chromatography (silica gel 63-100 µm, ethyl acetate /hexane, 1:5). Fractions containing the target product were combined and evaporated in vacuo to give compound (4) methyl 5-fluoro-4-formyl-2-methoxybenzoate (0.72 g, 72%).

NMR of (4) (CDCl₃): 10.37 (s, 1H), 7.58(d, 1H), 7.39(d, 1H), 3.93(s, 3H), 3.92 (s, 3H).

Product (5).
NaBH₄ (powder, 0.25 g, 6.6 mmol) was added to a solution of (4) (0.7 g, 3.3 mmol) in THF (10 ml). The reaction mixture was stirred for 2 h (TLC control). Sat. NaHCO₃ and EtOAc (20 and 150 ml) were added, the organic layer was separated and the water one was extracted with EtOAc. The organic layer was dried over Na₂SO₄ and evaporated in vacuo. Compound (5) was purified by chromatography (silica gel 63-100 µm, ethyl acetate /hexane, 1:1). Fractions containing the target product were combined and evaporated in vacuo to give compound (5) methyl 5-fluoro-4-(hydroxymethyl)-2-methoxybenzoate (0.6 g, 85%).

Product (6).
A mixture of (5) (0.6 g, 2.8 mmol), THF (10 ml), water (3 ml) and LiOH (0.145 g, 6 mmol) was stirred at RT for 10 h (TLC control). 1M HCl (6.6 ml) was added and the reaction mixture was evaporated in vacuo and coevaporated in vacuo with MeCN (2*100 ml). DCM (50 ml), Et3N (1 g, 10 mmol) and TBS-Cl (1.5 g, 10 mmol) were added to the residue and the reaction mixture was stirred for 48 h. 1M citric acid and EtOAc (30 and 150 ml) were added, the organic layer was separated. The organic layer was dried over Na2SO4 and evaporated in vacuo. MeCN (80 ml) and water (20 ml) were added to the residue and the reaction mixture was stirred for 16 h (until compound (6) disappeared) (TLC control). The reaction mixture was evaporated in vacuo and coevaporated in vacuo with MeCN (100 ml). Compound (6) was purified by chromatography (silica gel 63-100 µm, ethyl acetate /DCM, 1:3). Fractions containing the target product were combined and evaporated in vacuo, to give compound (6) 4-([tert-butyl(dimethyl)silyl]oxy)methyl)-5-fluoro-2-methoxybenzoic acid (0.6 g, 67%).

**Product (7).**

DCC (0.5 g, 2.5 mmol) was added to a solution of (7) (0.6 g, 1.9 mmol), 4-fluoro-indole (0.34 g, 2.5 mmol) and DMAP (0.29 g, 2.4 mmol) in DCM (30 ml). The reaction mixture was stirred for 16 h. A solution of AcOH (0.3 g, 5 mmol) in DCM (10 ml) was added and the reaction mixture was filtered through SiO2 (50 ml) and washed with DCM, 100 ml. Filtrate was evaporated in vacuo. Compound (7) was purified by chromatography (silica gel 63-100 µm, DCM /hexane, 1:4 → 100% DCM). Fractions containing the target product were combined and evaporated in vacuo to give compound (7) 1-[4-([tert-butyl(dimethyl)silyl]oxy)methyl]-5-fluoro-2-methoxybenzoyl]-4-fluoro-1H-indole (0.57 g, 70%).

NMR (9) (CDCl3): 8.22(d, 1H), 7.3(m, 1H), 7.23(d, 1H), 7.13 (d, 1H), 7.04(d, 1H), 7.0(tr, 1H), 6.67(d, 1H), 4.87(s, 2H), 3.78(s, 3H), 1.0(s, 9H), 0.18(s, 6H).

**Product 8.**

1 M Bu4NF/THF solution (1.8 ml) was added to a solution of (9) (0.5 g, 1.16 mmol) and AcOH (0.55 g, 9 mmol) in THF (15 ml). The reaction mixture was stirred for 16 h and evaporated in vacuo. Compound 8 was purified by chromatography (silica gel 63-100 µm, DCM → DCM/EtOAc, 3:1. Fractions containing the target product were combined and evaporated in vacuo to give compound 8 {2-fluoro-4-[4-fluoro-1H-indol-1-yl]carbonyl]-5-methoxyphenyl}methanol (0.33 g, 90%).