Published in final edited form as:

ChemMedChem. 2016 August 19; 11(16): 1819–1828. doi:10.1002/cmdc.201600022.

A Novel Class of Dopamine D₄ Receptor Ligands Bearing the Imidazoline Nucleus[†]

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Abstract

Over the year, the 2-substituted imidazoline nucleus has been demonstrated to be a bioversatile structural motif. In this study, novel imidazoline derivatives, bearing a 3- and/or 4-hydroxy- or methoxy-substituted phenyl ring linked by an ethylene bridge to the position 2 of an *N*-benzyl- or *N*-phenethyl-substituted imidazoline nucleus, were prepared and studied at the D₂-like receptor subtypes. Binding studies highlighted that the *N*-phenethylimidazolines **10–12** and **15** were selective for D₄ over D₂ and D₃ receptors. In the functional assays the 3-methoxy-substituted derivative **12**, endowed with the highest D₄ affinity value, and its 3-hydroxy analogue **15** behaved as partial agonists with low intrinsic efficacy and as competitive D₄ antagonists when tested in the presence of the D₂-like receptor agonist quinpirole. The molecular docking analysis, performed at a homology model of human D₄ receptor developed by using the X-ray crystal structure of the antagonist-bound human D₃ receptor as template, was in accordance to the binding results and provided useful information for the design of novel imidazoline D₄ receptor ligands based on the scaffold **I**.

Graphical Abstract

Focusing on dopamine D₂-like receptors, we identified novel imidazoline ligands based on the bioversatile scaffold **I**. The most interesting compounds were selective for D₄ over D₂ and D₃

[†]This article is dedicated to Prof. Maria Pigni, who died on February 8, 2016.

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receptors and behaved as competitive D₄ antagonists. Useful information for a further optimization of this novel class of compounds emerged.

Keywords

imidazoline nucleus; privileged structure; selective D₄ receptor antagonists

Manuscript Classifications

drug design; receptors

Introduction

Privileged structures, including arylpiperidines, 1,4-benzodiazepin-2-ones, chromone, and benzimidazoles,¹ are frequently used in the design of biologically active molecules. Indeed, the appropriate decorations of these structures allow the development of compounds selective for different biological targets.² The 2-substituted imidazoline nucleus has also been demonstrated to be a bioversatile structural motif.³ Using this molecular core, over many years we have developed several ligands directed to different biological systems including α_2 -adrenergic and 5-HT_{1A}-serotonergic receptors and imidazoline binding sites (I-IBS). Some of these compounds showed interesting *in vivo* pharmacological properties such as antidepressant⁴ and antinociceptive⁵ activities, enhancement of morphine-induced analgesia,⁶ decrease of both acquisition and expression of morphine tolerance and dependence,⁷ as well as hyperanxiety-like behavior after alcohol intoxication,⁸ hypotensive and bradycardic effects.⁹

These ligands share the common scaffold **I** characterized by an aromatic area (Ar) linked to the position 2 of the imidazoline nucleus through a biatomic bridge (X) (Fig. 1).

Such a scaffold, conformationally restricted in a benzofuran ring, is also present in 2-BFI, currently considered a ligand of choice for the I₂-IBS subtype.¹⁰ We recently demonstrated

that 2-BFI can be transformed into dopaminergic agents through the insertion of suitable substituents at the aromatic area and imidazoline nitrogen. Indeed, its structurally related 5- or 6-hydroxyphenyl-*N*-benzyl derivatives **1** and **2** (Fig. 1) showed full D₂-like agonism with potencies comparable to that of dopamine.¹¹ These results opened the possibility of designing novel dopaminergic compounds using the 2-substituted imidazoline as a core template.

Dopamine receptors belong to the family of G-protein-coupled receptors (GPCRs) and are divided into D₁-like and D₂-like subgroups on the basis of sequence similarity and signal transduction properties. The first group includes the D₁ and D₅ subtypes, which transduce their signals via increasing intracellular cAMP levels. The D₂-like subfamily includes D₂, D₃ and D₄ subtypes, each of which reduce cAMP production as well as regulate the activity of various ion channels.¹² The varied expression of D₂-like receptors in the central nervous system and their modulation of several neurological processes, including reward, cognition, learning, and memory make them popular drug targets.¹³ The D₂ receptor is highly expressed in the corpus striatum, pituitary, and olfactory tubercle, with lower levels in thalamus, anterior cingulate, and retrosplenial cortex.¹⁴ D₂ full or partial agonists, in addition to L-DOPA, are widely used in the treatment of Parkinson's disease, whereas D₂ receptor antagonists can diminish the reinforcing effects of several drugs of abuse, including morphine and alcohol.¹³ First cloned and characterized in 1990,¹⁵ the D₃ receptor is principally localized in the ventral striatum, pallidum, nucleus accumbens, islands of Calleja, olfactory tubercle, and lateral septum.¹⁶ Different anatomical, pharmacological and genetic findings suggest that D₃ receptors might be associated with Parkinson's disease, schizophrenia, and drug addiction.¹⁷ In particular, the altered expression of D₃ receptors after exposure to drugs of abuse in rodents and in humans suggests that D₃ antagonists might be suitable candidates for the management of drug addiction.¹⁸ Finally, the D₄ receptor is primarily localized in the hippocampus and frontal cerebral cortex, with lesser expression in the corpus striatum and midbrain.¹⁹ The D₄ receptor is polymorphic in human populations, notably containing a 48-bp Variable Number Tandem Repeat (VNTR) polymorphism in the third exon, in a region that codes for the third intracellular loop of the receptor.²⁰ The length of the polymorphism varies from two (2 × 16 amino acids) to eleven (11 × 16 amino acids) repeats, with the most common alleles encoding 2 (2R), 4 (4R) or 7 (7R) repeats.²¹ The 7R allele has been reproducibly associated with attention deficit-hyperactivity disorder, indicating the important role that D₄ plays in regulating attentional processes. D₄ receptor agonists may be useful in reversing cognitive deficits in schizophrenia²² and they have been demonstrated to induce penile erection in rats.²³ Early reports indicated that D₄ receptor antagonists might be potential therapeutics in the treatment of drug dependence, including nicotine, morphine, cocaine and methamphetamine.²⁴

Considering the therapeutic potential of compounds targeting D₂-like receptors and encouraged by the observation that imidazoline derivatives might interact with them, in this study we designed novel ligands based on the scaffold **I** (Fig. 1) with the aim to target D₂-like receptors. In these molecules, the –CH₂CH₂– chain, also present in the endogenous ligand dopamine, was selected as the bridge between the phenyl ring, hydroxy- or methoxy-substituted in position 3 and/or 4, and the *N*-benzyl- or *N*-phenethyl-substituted imidazoline

nucleus (compounds **3–16**) (Fig. 2). The decorations on the phenyl and imidazoline moieties were suggested by evidences emerged from one of our previous studies.¹¹

The novel compounds were evaluated for their binding profiles at human D₂, D₃ or D₄ receptors. The 3-methoxy and 3-hydroxyphenyl-*N*-phenethyl derivatives **12** and **15**, respectively, were selected for functional studies at D₄ receptor. Finally, to rationalize the biological results, molecular modelling studies were performed.

Results and Discussion

Chemistry

The imidazolines **3–16** were prepared according to the synthetic procedure reported in Scheme 1. The condensation of the methyl esters **17**, **18**, **19**,²⁵ and **20**²⁶ with either *N*-benzylethylenediamine or *N*-phenethylethylenediamine in the presence of trimethylaluminum afforded the imidazolines **3–6** and **10–13**, respectively. The treatment of **4–6** and **11–13** with boron tribromide at –78 °C gave the imidazolines **7–9** and **14–16**, respectively.

In vitro biological studies

Binding studies—The compounds were preliminarily screened in binding studies on human cloned D₂, D₃ or D₄ receptors stably expressed in HEK 293 cells using [³H]-methylspiperone as a radioligand (Fig. 3). Detailed dose-response curves were determined only for ligands producing at least 70% displacement of the radioligand at 10 μM, as reported in Table 1.

The analysis of the results indicated that only the *N*-phenethyl derivative **14**, bearing a 4-hydroxy substituent on the phenyl ring, displayed D₂ receptor recognition ($K_i = 0.794 \mu\text{M}$). Moreover, **14** was also able to interact with D₃ receptor with a similar affinity ($K_i = 0.832 \mu\text{M}$). Slightly weaker D₃ receptor affinity ($K_i = 1.259 \mu\text{M}$) was provided by the 3-hydroxyphenyl-*N*-benzyl derivative **8**. Nevertheless, in this case a significant selectivity over D₂ and D₄ receptors was observed. The comparable affinity of **8** and **14** at D₃ receptors suggested that the recognition of this subtype was scarcely affected both by the position of the hydroxy substituent on the phenyl ring (3 or 4, respectively) and by the *N*-substituent (benzyl or phenethyl, respectively). In contrast, the *N*-substituent appeared to affect the binding affinity at D₄ receptor. Indeed, while all the *N*-benzyl derivatives (compounds **3–9**) produced less than 70% displacement of [³H]-methylspiperone (Fig. 3) at D₄ receptor, the *N*-phenethyl derivatives **10–12** and **15** displayed significant affinity for D₄ receptor as well as significant selectivity over D₂ and D₃ receptors. The D₄ binding affinity of the non-substituted derivative **10** increased by the introduction of one hydroxy or methoxy group in position 3 or 4 of the phenyl ring, with the exception of the 4-hydroxy substituent. In particular, the affinity values of **11**, **12**, **14**, and **15** suggested that the peculiar nature of the substituent and its position in the aromatic area modulated D₄ binding. Indeed, the insertion of a methoxy group at position 3 of the aromatic ring (compound **12**) proved to be particularly advantageous ($K_i = 0.095 \mu\text{M}$), whereas the same substitution at position 4 was less fruitful (**11**; $K_i = 0.501 \mu\text{M}$). Comparison of **11** and **12** with **14** and **15**, respectively,

suggested that the replacement of the methoxy with a hydroxy function was not advantageous for binding, producing instead a significant reduction of D₄ affinity. Finally, the results from Fig. 3 indicated that an aromatic area consisting of the 3,4-dimethoxy-phenyl (**6** and **13**) or catechol (**9** and **16**) moieties was not suitable for D₂-like receptor binding.

Functional studies—Compound **12**, endowed with the highest D₄ receptor affinity, and its hydroxy analogue **15** were selected to be evaluated in D_{4.4} receptor-expressing cells using a functional assay that relies on ligand-induced changes in the biomass proximal to the sensor surface of the microplate. After a 10 min baseline, cells were treated with the compound **12** or **15** alone and a partial agonist effect was observed with both compounds showing low intrinsic efficacy. After 20 min, the D₂-like receptor agonist quinpirole (QP) was added at a final concentration of 10⁻⁸ M and, as shown in Fig. 4A, **12** and **15** behaved as competitive antagonists. In order to obtain IC₅₀ values, peak effect of each concentration of QP alone or QP plus one of the compounds is shown as percentage of the effect of QP at 10⁻⁸ M. Values were fitted to a non-linear regression and IC₅₀ values were obtained (IC₅₀ = 0.950 μM and 3 μM for compounds **12** and **15**, respectively) (Fig. 4B).

Molecular modelling studies—To define the structural features underlying differences in binding affinities for this library of novel imidazoline derivatives, a molecular docking analysis was performed at a homology model of the human D₄ receptor developed by using the X-ray crystal structure of the antagonist-bound human D₃ receptor as template (pdb code: 3PBL; 2.89-Å resolution).²⁷ The availability of the crystal structure of the human D₃ receptor allows development of reliable homology models of other D₂-like receptors due to the high degree of amino acid sequence conservation, particularly in the transmembrane (TM) segments.²⁸ Since the D₃ receptor crystal structure was solved in complex with the high-affinity D₂/D₃ antagonist eticlopride, which binds the D₄ receptor with nanomolar affinity (48 nM),²⁹ this allows greater potential identification of key receptor-ligand interactions because the ligand-binding cavity is arranged in a ligand-binding state and thus more suitable for docking analysis. The developed D₄ receptor homology model was evaluated using the Protein Geometry Monitor application within MOE,³⁰ which provides a variety of stereochemical measurements for inspection of the structural quality in a given protein, such as backbone bond lengths, angles and dihedrals, Ramachandran φ-ψ dihedral plots, and sidechain rotamer and non-bonded contact quality. The developed model was then used for docking analyses performed with Autodock software through the Pyrx interface.³¹ A preliminary analysis was performed to test the ability of the docking tool to reproduce the experimental ligand arrangement within the ligand-binding pocket by simulating the binding mode of eticlopride at the D₃ receptor using the crystal structure. The docking protocol successfully reproduced the experimental eticlopride binding mode, with a RMS deviation of 0.77 Å (Fig. 5A). Subsequently, molecular docking analysis was performed using eticlopride at the developed D₄ homology model (Fig. 5B) as well as a set of known potent D₄ ligands (RO-10-5824, A-412997, PD-168077, and ABT724)³² to compare the simulated binding modes of these compounds and to depict possible shared interactions with the receptor residues.

Finally, the developed D₄ receptor model was employed as target for docking analysis of the new derivatives **8**, **10–12**, **14**, and **15**. The protonation state of these molecules at physiological pH was calculated with the MarvinSketch software online tool (available at the <https://www.chemaxon.com> web page).

The results of the docking studies present two possible binding modes of the analyzed imidazoline derivatives (Fig. 6). This may not be surprising considering that two similar phenethyl substituents are bound to the imidazoline core. While the heterocycle occupies a similar position in the cavity in both arrangements, the phenethyl arms are differently oriented in the two binding modes.

More in detail, binding mode 1 (Fig. 6A) presents the imidazoline scaffold at about the center of the binding cavity, with the *N*-phenethyl substituent pointing towards the TM5-TM6 residues and the 2-phenethyl group located between TM2-TM3-TM6-TM7 amino acids. The NH group of the protonated imidazoline gives a strong polar interaction with the carboxyl group of Asp115 (TM3) that results in the principal interaction with the cavity. The additional substituents on the 2-phenethyl group have a modulating effect on the D₄ affinity of the compounds. These groups are located in proximity of non-polar hydrophobic residues, like Phe91 (TM2), Leu111 (TM3), and Met112 (TM3). This may explain the higher binding affinity of the 3-OCH₃ substitution (**12**) at D₄ compared to the corresponding 3-OH substitution (**15**). Finally, the cavity occupancy of the synthesised compounds appears similar to the one of eticlopride, with analogue positioning of the substituents within the cavity sectors. Binding mode 2 (Fig. 6B) presents an opposite arrangement in respect to binding mode 1, with the imidazoline scaffold located almost in correspondence to the pyrrolidine group of eticlopride. The NH group of **12** matches the position of the positively charged group of the comparison compound, indicating a polar interaction with the Asp115 carboxyl function. The *N*-phenethyl substituent is located between Phe91 (TM2) and Arg186 (EL2) and points towards the extracellular environment, while the 2-phenethyl group is in close proximity to TM3-TM5-TM6 residues similar to the substituted phenyl ring of eticlopride. The additional substituents on the 2-phenethyl group are located in proximity to non-polar hydrophobic residues like Val116 (TM3), Leu187 (EL2), Val193 (TM5), Phe410 (TM6), and Phe411 (TM6). Thus, despite the dramatically different ligand orientation, the greater binding affinity of the 3-OCH₃ substitution (**12**) at D₄ compared to the corresponding 3-OH substitution (**15**) is also compatible with this binding mode. However, the presence of polar amino acids Thr120 (TM3), Ser196 (TM5), Ser197 (TM5), and Ser200 (TM5) would be expected to have increased interactions with -OH substituents rather than methoxy groups (therefore leading to an increased affinity for compounds **14** and **15** compared to **11** and **12**). Furthermore, binding mode 2 makes the ligand occupy the cavity not completely due to the externally oriented arrangement of the *N*-phenethyl group; this appears detrimental for a stable ligand-target interaction. Therefore, comparing the two possible arrangements of the synthesized compounds and considering also that the binding mode 1 conformations have a significantly higher docking scores than the binding mode 2 ones, we may conclude that the binding mode 1 arrangements are the more probable at the D₄ receptor and that they may represent starting points for a further optimization of the derivatives.

Considering the *N*-benzyl derivative **8**, both binding modes were observed from docking results even if the compound could not occupy the same position of the *N*-phenethyl substituted analogues but resulted in slightly displaced binding modes. In our opinion, the reduced flexibility of the *N*-benzyl derivative does not allow the compound to be well inserted in the binding cavity and to properly interact with the receptor residues, leading to a loss of D₄ affinity with respect to the *N*-phenethyl analogues.

Conclusion

The present study confirms the versatility of the imidazoline nucleus to support ligands directed to D₂-like receptors. In particular, the novel *N*-phenethylimidazolines **10–12** and **15** prove to be selective D₄ ligands. Among them, compound **12**, characterized by a 3-methoxyphenyl linked by an ethylene chain to the position 2 of the *N*-phenethylimidazoline ring, shows the highest affinity. In the functional assays, **12** and its hydroxy analogue **15** behave as partial agonists with low intrinsic efficacy and as competitive D₄ antagonists when tested in the presence of the D₂-like receptor agonist QP. The molecular docking study appears in accordance to the binding results and suggests the preferred binding mode of the novel compounds at the human D₄ receptor cavity. Such a study provides useful information for a further optimization of this novel class of derivatives based on the scaffold **I** and for the development of novel pharmacological tools targeting the D₄ receptor.

Experimental section

Chemistry

Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR and NMR spectra were recorded on Perkin-Elmer 297 and Varian Mercury AS400 instruments, respectively. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), or m (multiplet). IR spectral data (not shown because of the lack of unusual features) were obtained for all compounds reported and are consistent with the assigned structures. The microanalyses were recorded on FLASH 2000 instrument (ThermoFisher Scientific). The elemental composition of the compounds agreed to within $\pm 0.4\%$ of the calculated value. Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040–0.063 mm, Merck) by flash chromatography. Compounds were named following IUPAC rules as applied by ChemBioDraw Ultra (version 11.0) software for systematically naming organic chemicals. The purity of the novel compounds was determined by combustion analysis and was 95%.

1-Benzyl-2-phenethyl-4,5-dihydro-1H-imidazole (3)—A solution of *N*-benzylethylenediamine (0.98 g, 6.5 mmol) in dry toluene (10 mL) was added dropwise to a stirred solution of 2M trimethylaluminum (3.5 mL, 6.5 mmol) in dry toluene (5 mL) at 0 °C in nitrogen atmosphere and stirred at room temperature for 1 h. The solution was cooled to 0 °C and a solution of **17** (Aldrich) (0.57 g, 3.5 mmol) in dry toluene (10 mL) was added dropwise. The reaction mixture was heated to reflux for 20 h, cooled to 0 °C, and quenched cautiously with MeOH (0.8 mL) followed by H₂O (0.25 mL). After the addition of CHCl₃ (11.0 mL), the mixture was stirred for 30 min at 55 °C to ensure the precipitation of the

aluminum salts, then it was filtered and the organic layer was extracted with 2 N HCl. The aqueous layer was made basic with 10% NaOH and extracted with CHCl₃. The organic layer was dried over Na₂SO₄, filtered, and evaporated to give a residue, which was purified by flash chromatography eluting with CHCl₃/MeOH 95:5 to obtain a colorless oil: (0.53 g, 57% yield); ¹H NMR (CDCl₃) δ (ppm) = 2.56–2.62 (m, 2H, PhCH₂CH₂C), 3.03–3.10 (m, 2H, PhCH₂CH₂C), 3.24 (t, *J*=9.8 Hz, 2H, NCH₂CH₂N), 3.65 (t, *J*=9.8 Hz, 2H, NCH₂CH₂N), 4.22 (s, 2H, NCH₂Ph), 7.16–7.37 (m, 10H, ArH). The free base was transformed into the oxalate salt, which was crystallized from EtOH; mp: 148–149 °C; Anal. calcd for C₁₈H₂₀N₂·C₂H₂O₄: C 67.78, H 6.26, N 7.90, found: C 67.99, H 6.13, N 7.75.

1-Benzyl-2-(4-methoxyphenethyl)-4,5-dihydro-1H-imidazole (4)—This compound was prepared starting from **18** (Aldrich) and *N*-benzylethylenediamine following the procedure described for **3**: an oil was obtained (48% yield). ¹H NMR (CDCl₃) δ (ppm) = 2.55 (m, 2H, PhCH₂CH₂C), 2.96 (m, 2H, PhCH₂CH₂C), 3.23 (t, *J*=9.6 Hz, 2H, NCH₂CH₂N), 3.74 (t, *J*=9.6 Hz, 2H, NCH₂CH₂N), 3.77 (s, 3H, OCH₃), 4.24 (s, 2H, NCH₂Ar), 6.81 (d, *J*=8.4 Hz, 2H, ArH), 7.11–7.40 (m, 7H, ArH). The free base was transformed into the O,O'-dibenzoyl tartrate salt, which was crystallized from 2-PrOH; mp: 147–148 °C; Anal. calcd for C₁₉H₂₂N₂O·C₁₈H₁₄O₈: C 68.09, H 5.56, N 4.29, found: C 67.88, H 5.67, N 4.14.

1-Benzyl-2-(3-methoxyphenethyl)-4,5-dihydro-1H-imidazole (5)—This compound was prepared starting from **19**²⁵ and *N*-benzylethylenediamine following the procedure described for **3**: an oil was obtained (55% yield). ¹H NMR (CDCl₃) δ (ppm) = 2.58 (t, *J*=8.2 Hz, 2H, PhCH₂CH₂C), 3.02 (t, *J*=8.2 Hz, 2H, 2H, PhCH₂CH₂C), 3.25 (t, *J*=9.5 Hz, 2H, NCH₂CH₂N), 3.72 (t, *J*=9.5 Hz, 2H, NCH₂CH₂N), 3.78 (s, 3H, OCH₃), 4.25 (s, 2H, NCH₂Ph), 6.78 (m, 2H, ArH), 7.15–7.39 (m, 7H, ArH). The free base was transformed into the oxalate salt, which was crystallized from EtOH; mp: 131–132 °C; Anal. calcd for C₁₉H₂₂N₂O·C₂H₂O₄: C 65.61, H, 6.29, N, 7.29, found: C 65.86, H, 6.13, N, 7.39.

1-Benzyl-2-(3,4-dimethoxyphenethyl)-4,5-dihydro-1H-imidazole (6)—This compound was prepared starting from **20**²⁶ and *N*-benzylethylenediamine following the procedure described for **3**: an oil was obtained (46% yield). ¹H NMR (CDCl₃) δ (ppm) = 2.58 (m, 2H, PhCH₂CH₂C), 2.92 (m, 2H, PhCH₂CH₂C), 3.14 (t, *J*=9.6 Hz, 2H, NCH₂CH₂N), 3.78 (t, *J*=9.6 Hz, 2H, NCH₂CH₂N), 3.82 (s, 6H, OCH₃), 4.23 (s, 2H, NCH₂Ph), 6.76 (m, 2H, ArH), 7.17–7.36 (m, 6H, ArH). The free base was transformed into the oxalate salt, which was crystallized from EtOH; mp: 160–162 °C. Anal. calcd for C₂₀H₂₄N₂O₂·C₂H₂O₄: C 63.76, H 6.32, N 6.76, found: C 63.91, H 6.36, N 6.69.

4-(2-(1-Benzyl-4,5-dihydro-1H-imidazol-2-yl)ethyl)phenol (7)—To a solution of **4** (1 g, 3.57 mmol) in CH₂Cl₂ (5 mL) at –78 °C, a 2 M solution of BBr₃ (32.16 mmol) in CH₂Cl₂ was added dropwise. The mixture was left to warm to rt and stirred overnight. It was cooled again to –78 °C, and MeOH (6 mL) was carefully added. After stirring at rt for 15 min, the solvent was removed and the residue was recrystallized from methanol (67% yield); mp: 207–208 °C; ¹H NMR (DMSO) δ (ppm) = 2.82 (t, *J*=7.3 Hz, 2H, PhCH₂CH₂C), 2.98 (t, *J*=7.3 Hz, 2H, PhCH₂CH₂C), 3.41 (t, *J*=10.2 Hz, 2H, NCH₂CH₂N), 3.77 (t, *J*=10.2 Hz, 2H,

NCH₂CH₂N), 4.29 (s, 2H, NCH₂Ar), 5.62 (br s, 1H, OH, exchangeable with D₂O), 6.73 (d, *J*=8.3 Hz, 2H, ArH), 6.98 (d, *J*=8.3 Hz, 2H, ArH), 7.13–7.37 (m, 5H, ArH), 7.54 (br s, 1H, OH, exchangeable with D₂O); Anal. calcd for C₁₈H₂₀N₂O.HBr: C 59.84, H 5.86, N 7.75, found: C 59.59, H 5.98, N 7.63.

3-(2-(1-Benzyl-4,5-dihydro-1H-imidazol-2-yl)ethyl)phenol (8)—This compound was prepared starting from **5** following the procedure described for **7**. The obtained residue was recrystallized from MeOH (62% yield); mp: 168–169 °C; ¹H NMR (DMSO) δ (ppm) = 2.83 (t, *J*=7.5 Hz, 2H, PhCH₂CH₂C), 2.98 (t, *J*=7.5 Hz, 2H, PhCH₂CH₂C), 3.39 (t, *J*=9.9 Hz, 2H, NCH₂CH₂N), 3.81 (t, *J*=9.9 Hz, 2H, NCH₂CH₂N), 4.28 (s, 2H, NCH₂Ar), 5.46 (br s, 1H, OH, exchangeable with D₂O), 6.60–6.71 (m, 2H, ArH), 6.88 (s, 1H, ArH), 7.12–7.39 (m, 6H, ArH); Anal. calcd for C₁₈H₂₀N₂O.HBr: C 59.84, H 5.86, N 7.75, found: C 60.01, H 6.02, N 7.67.

4-(2-(1-Benzyl-4,5-dihydro-1H-imidazol-2-yl)ethyl)benzene-1,2-diol (9)—This compound was prepared starting from **6** following the procedure described for **7**. The obtained residue was recrystallized from MeOH (44% yield); mp: 213–214 °C; ¹H NMR (DMSO) δ (ppm) = 2.48 (m, 2H, PhCH₂CH₂C), 2.91 (m, 2H, PhCH₂CH₂C), 3.21 (t, *J*=9.8 Hz, 2H, NCH₂CH₂N), 3.73 (t, *J*=9.8 Hz, 2H, NCH₂CH₂N), 4.39 (s, 2H, NCH₂Ph), 5.38 (br s, 1H, OH, exchangeable with D₂O), 5.81 (br s, 1H, OH, exchangeable with D₂O), 6.70 (s, 1H, ArH), 7.15–7.41 (m, 7H, ArH); Anal. calcd for C₁₈H₂₀N₂O₂.HBr: C 57.30, H 5.61, N 7.43, found: C 57.53, H 5.80, N 7.25.

1,2-Diphenethyl-4,5-dihydro-1H-imidazole (10)—This compound was prepared starting from **17** (Aldrich) and *N*-phenethylethylenediamine following the procedure described for **3**: an oil was obtained (62% yield); ¹H NMR (CDCl₃) δ (ppm) = 2.41 (m, 2H, PhCH₂CH₂C), 2.76–2.81 (m, 4H, PhCH₂CH₂C and NCH₂CH₂Ph), 3.18 (m, 2H, NCH₂CH₂Ph), 3.37 (t, *J*=9.7 Hz, 2H, NCH₂CH₂N), 3.74 (t, *J*=9.7 Hz, 2H, NCH₂CH₂N), 7.15–7.42 (m, 10H, ArH). The free base was transformed into the O,O'-dibenzoyl tartrate salt, which was crystallized from EtOH; mp: 132–133 °C; Anal. calcd for C₁₉H₂₂N₂.C₁₈H₁₄O₈: C 69.80, H 5.70, N 4.40, found: C 69.59, H 5.56, N 4.57.

2-(4-Methoxyphenethyl)-1-phenethyl-4,5-dihydro-1H-imidazole (11)—This compound was prepared starting from **18** (Aldrich) and *N*-phenethylethylenediamine following the procedure described for **3**: an oil was obtained (55% yield); ¹H NMR (CDCl₃) δ (ppm) = 2.29 (t, *J*=8.2 Hz, 2H, PhCH₂CH₂C), 2.80–2.86 (m, 4H, PhCH₂CH₂C and NCH₂CH₂Ph), 3.22 (t, *J*=7.2 Hz, 2H, NCH₂CH₂Ph), 3.37 (t, *J*=9.7 Hz, 2H, NCH₂CH₂N), 3.72 (t, *J*=9.7 Hz, 2H, NCH₂CH₂N), 3.78 (s, 3H, OCH₃), 6.82 (d, *J*=7.9 Hz, 2H, ArH), 7.07 (m, 2H, ArH), 7.15 (d, *J*=7.9 Hz, 2H, ArH), 7.27–7.42 (m, 3H, ArH). The free base was transformed into the O,O'-dibenzoyl tartrate salt, which was crystallized from 2-PrOH; mp: 84–85 °C; Anal. calcd for C₂₀H₂₄N₂O.C₁₈H₁₄O₈: C 68.46, H 5.74, N 4.20, found: C 68.58, H 5.85, N 4.04.

2-(3-Methoxyphenethyl)-1-phenethyl-4,5-dihydro-1H-imidazole (12)—This compound was prepared starting from **19**²⁵ and *N*-phenethylethylenediamine following the

procedure described for **3**: an oil was obtained (49% yield); ^1H NMR (CDCl_3) δ (ppm) = 2.41 (m, 2H, $\text{PhCH}_2\text{CH}_2\text{C}$), 2.75–2.81 (m, 4H, $\text{PhCH}_2\text{CH}_2\text{C}$ and $\text{NCH}_2\text{CH}_2\text{Ph}$), 2.98 (t, $J=7.3$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{Ph}$), 3.27 (t, $J=9.6$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 3.81 (t, $J=9.6$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 3.83 (s, 3H, OCH_3), 6.81–6.85 (m, 4H, ArH), 7.15–7.53 (m, 5H, ArH). The free base was transformed into the O,O'-dibenzoyl tartrate salt, which was crystallized from 2-PrOH; mp: 95–97 °C; Anal. calcd for $\text{C}_{20}\text{H}_{24}\text{N}_2\text{O}\cdot\text{C}_{18}\text{H}_{14}\text{O}_8$: C 68.46, H 5.74, N 4.20, found: C 68.59, H 5.88, N 4.06.

2-(3,4-Dimethoxyphenethyl)-1-phenethyl-4,5-dihydro-1H-imidazole (13)—This compound was prepared starting from **20**²⁶ and *N*-phenethylethylenediamine following the procedure described for **3**: an oil was obtained (50% yield); ^1H NMR (CDCl_3) δ (ppm) = 2.38 (m, 2H, $\text{PhCH}_2\text{CH}_2\text{C}$), 2.56–2.67 (m, 4H, $\text{PhCH}_2\text{CH}_2\text{C}$ and $\text{NCH}_2\text{CH}_2\text{Ph}$), 3.01 (m, 2H, $\text{NCH}_2\text{CH}_2\text{Ph}$), 3.25 (t, $J=9.9$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 3.61 (t, $J=9.9$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 3.80 (s, 6H, OCH_3), 6.74–6.83 (m, 2H, ArH), 6.89 (s, 1H, ArH), 7.27–7.38 (m, 5H, ArH). The free base was transformed into the oxalate salt, which was crystallized from EtOH; mp: 155–157 °C; Anal. calcd for $\text{C}_{21}\text{H}_{26}\text{N}_2\text{O}_2\cdot\text{C}_2\text{H}_2\text{O}_4$: C 64.47, H 6.59, N 6.54, found: C 64.36, H 6.71, N 6.68.

4-(2-(1-Phenethyl-4,5-dihydro-1H-imidazol-2-yl)ethyl)phenol (14)—This compound was prepared starting from **11** following the procedure described for **7**. The obtained residue was treated with saturated NaHCO_3 and extracted with CHCl_3 . The organic layer was dried over Na_2SO_4 , filtered, and evaporated to give an oil. The free base was transformed into the O,O'-dibenzoyl tartrate salt which was recrystallized from 2-PrOH (71% yield); mp: 152–153 °C; ^1H NMR (DMSO) δ (ppm) = 2.46 (m, 2H, $\text{PhCH}_2\text{CH}_2\text{C}$), 2.69–2.89 (m, 4H, $\text{PhCH}_2\text{CH}_2\text{C}$ and $\text{NCH}_2\text{CH}_2\text{Ph}$), 3.38 (t, $J=6.9$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{Ph}$), 3.61 (t, $J=10.3$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 3.84 (t, $J=10.3$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 5.56 (br s, 1H, OH, exchangeable with D_2O), 6.67 (d, $J=8.1$ Hz, 2H, ArH), 6.91 (d, $J=8.1$ Hz, 2H, ArH), 7.24–7.38 (m, 5H, ArH); Anal. calcd for $\text{C}_{19}\text{H}_{22}\text{N}_2\text{O}\cdot\text{C}_{18}\text{H}_{14}\text{O}_8$: C 68.09, H 5.56, N 4.29, found: C 68.29, H 5.41, N 4.44.

3-(2-(1-Phenethyl-4,5-dihydro-1H-imidazol-2-yl)ethyl)phenol (15)—This compound was prepared starting from **12** following the procedure described for **7**. The obtained residue was treated with saturated NaHCO_3 and extracted with CHCl_3 . The organic layer was dried over Na_2SO_4 , filtered, and evaporated to give an oil. The free base was transformed into the oxalate salt, which was recrystallized from EtOH (68% yield); mp: 117–118 °C; ^1H NMR (DMSO) δ (ppm) = 2.33 (m, 2H, $\text{PhCH}_2\text{CH}_2\text{C}$), 2.68–2.75 (m, 4H, $\text{PhCH}_2\text{CH}_2\text{C}$ and $\text{NCH}_2\text{CH}_2\text{Ph}$), 2.98 (t, $J=7.1$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{Ph}$), 3.31 (t, $J=10.0$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 3.73 (t, $J=10.0$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 5.28 (br s, 1H, OH, exchangeable with D_2O), 6.73–6.81 (m, 2H, ArH), 6.86 (s, 1H, ArH), 7.26–7.43 (m, 6H, ArH); Anal. calcd for $\text{C}_{19}\text{H}_{22}\text{N}_2\text{O}\cdot\text{C}_2\text{H}_2\text{O}_4$: C 65.61, H 6.29, N 7.29, found: C 65.90, H 6.13, N 7.17.

4-(2-(1-Phenethyl-4,5-dihydro-1H-imidazol-2-yl)ethyl)benzene-1,2-diol (16)—This compound was prepared starting from **13** following the procedure described for **7**. The organic layer was dried over Na_2SO_4 , filtered, and evaporated to give an oil. The free base was transformed into the oxalate salt, which was recrystallized from EtOH (41% yield); mp:

143–145 °C; ^1H NMR (DMSO) δ (ppm) = 2.10 (m, 2H, $\text{PhCH}_2\text{CH}_2\text{C}$), 2.65–2.71 (m, 4H, $\text{PhCH}_2\text{CH}_2\text{C}$ and $\text{NCH}_2\text{CH}_2\text{Ph}$), 3.06 (t, $J=7.2$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{Ph}$), 3.21 (t, $J=10.1$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 3.73 (t $J=10.1$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 5.38 (br s, 1H, OH, exchangeable with D_2O), 5.77 (br s, 1H, OH, exchangeable with D_2O), 6.72 (s, 1H, ArH), 7.16–7.40 (m, 7H, ArH); Anal. calcd for $\text{C}_{19}\text{H}_{22}\text{N}_2\text{O}_2\cdot\text{C}_2\text{H}_2\text{O}_4$: C 62.99, H 6.04, N 7.00, found: 63.12, H 6.16, N 7.13.

Biology

[^3H]*N*-methyispiperone binding assay—HEK293 cells stably expressing human D_2 , D_3 , or D_4 receptors were grown in a 50:50 mix of DMEM and Ham's F12 culture media, supplemented with 20 mM HEPES, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1X antibiotic/antimycotic, 10% heat-inactivated fetal bovine serum, and 200 $\mu\text{g}/\text{ml}$ hygromycin (Life Technologies, Grand Island, NY) and kept in an incubator at 37 °C and 5% CO_2 . Upon reaching 80–90% confluence, cells were harvested using pre-mixed Earle's Balanced Salt Solution (EBSS) with 5 μM EDTA (Life Technologies) and centrifuged at 3000 rpm for 10 min at 21 °C. The supernatant was removed and the pellet was resuspended in 10 ml hypotonic lysis buffer (5 mM MgCl_2 5 mM Tris, pH 7.4 at 4 °C) and centrifuged at 20,000 rpm for 30 min at 4 °C. The pellet was then resuspended in fresh EBSS buffer made from 8.7 g/L Earle's Balanced Salts without phenol red (US Biological, Salem, MA), 2.2 g/L sodium bicarbonate, pH to 7.4. A Bradford protein assay (Bio-Rad, Hercules, CA) was used to determine the protein concentration and membranes were diluted to 500 $\mu\text{g}/\text{mL}$ and stored in a –80 °C freezer for later use.

On test day, all test compounds were freshly dissolved in 30% DMSO and 70% H_2O , typically to a stock concentration of 1 mM or 100 μM . If free-base compounds did not readily go into solution, 10 μl of glacial acetic acid was added. Each test compound was then diluted into 13 half-log serial dilutions using 30% DMSO vehicle; final test concentrations ranged from 100 μM to 10 pM. Frozen membranes were diluted in fresh EBSS to a 100 $\mu\text{g}/\text{mL}$ stock for D_2 or D_3 binding, 200 $\mu\text{g}/\text{mL}$ for D_4 binding. Radioligand competition experiments were conducted in glass tubes containing 300 μL fresh EBSS buffer with 0.2 mM sodium metabisulfite, 50 μl of diluted test compound, 100 μL of membranes (10 μg total protein for D_2 and D_3 binding, 20 μg for D_4 binding), and 50 μL of [^3H]*N*-methyispiperone (0.4 nM final concentration; Perkin Elmer). Nonspecific binding was determined using 10 μM butaclamol (Sigma-Aldrich, St. Louis, MO) and total binding was determined with 30% DMSO vehicle. All compound dilutions were tested in triplicate and the reaction incubated for one hour at room temperature. The reaction was terminated by filtration through Whatman GF/B filters, presoaked for one hour in 0.5% polyethylenimine, using a Brandel R48 filtering manifold (Brandel Instruments, Gaithersburg, MD). The filters were washed 3 times with 3 ml of ice cold EBSS+ buffer and transferred to scintillation vials. 3 mL CytoScint liquid scintillation cocktail (MP Biomedicals, Solon, OH) was added and vials were counted using a Perkin Elmer Tri-Carb 2910 TR liquid scintillation counter (Waltham, MA). IC_{50} values for each compound were determined from dose-response curves and K_i values were calculated using the Cheng-Prusoff equation.³³ Reported K_i values were determined from at least three independent experiments.

Functional assay—Analysis of the signaling properties of the D₄ receptor compounds was assessed using DMR technique. This technology enables real-time detection of integrated cellular responses in living cells. It relies on the detection of refractive index alterations on biosensor-coated microplates that originate from stimulus-induced changes in the total biomass proximal to the sensor surface. DMR refers to cellular processes that occur when biomolecules change their localization within a cell. A number of cellular behaviors are associated with mass relocation, such as protein trafficking, morphological changes, cytoskeletal rearrangement, receptor internalization or adhesion. DMR is detected by passing polarized broadband light through the bottom portion of cells grown on microtiter plates in which grating surfaces are embedded and by recording a change on the wavelength of outgoing light. HEK 293 cells stably expressing D_{4.4} receptors were grown overnight in a fibronectin-coated 96-well biosensor microplate at a density sufficient to achieve confluence after 18–24 hours and kept in an incubator at 37°C and 5% CO₂. The day of the experiment cells were washed five times with HBSS and were kept for two hours inside the reader to allow temperature equilibration (routinely the DMR measurements will be performed at 28 °C). After equilibration a baseline was measured during 10 minutes to define a zero level and to ensure that the signal is stable. After that, cells were treated with either buffer or increasing concentrations of compounds **12** and **15** and the signal was recorded for 20 minutes. Finally, QP at a final concentration of 10⁻⁸ M was added to the cells and the signal was recorded for 60 minutes. A ligand-induced pm shift was measured over time and baseline (buffer treated cells) was subtracted. The peak effect (minute 4 after QP addition) of each concentration of compounds 12 and 15 was used to obtain dose-response curves and IC₅₀ values were calculated from non-linear regression. Response is shown as percentage versus QP 10⁻⁸ M. Reported IC₅₀ values were determined from three independent experiments. DMR response was measured using Enspire Multimode plate reader Label-free system.

Molecular modelling

All molecular modelling studies were performed on a 2 CPU (PIV 2.0–3.0 GHZ) Linux PC. Homology modelling, energy minimization, and docking studies were carried out using MOE (version 2010.10) suite.³⁰ All ligand structures were optimized using RHF/AM1 semiempirical calculations and the software package MOPAC implemented in MOE was utilized for these calculations.³⁴

Building of the human D₄ receptor model—The crystal structure of the human D₃ receptor in complex with eticlopride was retrieved from the Protein Data Bank (<http://www.rcsb.org>; pdb code: 3PBL; 2.89-Å resolution²⁷). A multiple alignment of the dopamine receptor primary sequences was built within MOE as preliminary step. The boundaries identified from the used X-ray crystal structure of the human D₃ receptor were then applied for the corresponding sequences of the TM helices of the D₄ receptor. The missing loop domains were built by the loop search method implemented in MOE. Once the heavy atoms were modeled, all hydrogen atoms were added, and the protein coordinates were then minimized with MOE using the AMBER99 force field.³⁵ The minimizations were performed by steepest descent steps followed by conjugate gradient minimization until the RMS gradient of the potential energy was less than 0.05 kJ mol⁻¹ Å⁻¹. Reliability and

quality of the model was checked using the Protein Geometry Monitor application within MOE, which provides a variety of stereochemical measurements for inspection of the structural quality in a given protein, like backbone bond lengths, angles and dihedrals, Ramachandran ϕ - ψ dihedral plots, and sidechain rotamer and nonbonded contact quality.

Molecular docking analysis—All compound structures were docked into the binding site of the A_{2A}AR using Autodock 4.2.6 software and PyRx interface.³¹ Lamarckian genetic algorithm was employed for this analysis with the following settings: 50 runs for each ligand; 2,500,000 as maximum number of energy evaluations; 27,000 as maximum number of generations; 0.02 as rate of gene mutation and 0.8 as rate of crossover. The grid box was set with 50, 50, and 50 points in the *x*, *y*, and *z* directions, respectively, with the default grid spacing of 0.375 Å.

Acknowledgments

This work was supported by grants from the University of Camerino (Fondo di Ateneo per la Ricerca 2014–2015) and by the NIDA Intramural Research Program.

Abbreviations

I-IBS	imidazoline binding sites
GPCRs	G-protein-coupled receptors
QP	quinpirole
pm	picometers
DMR	dynamic mass redistribution

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Fig. 1.
Molecular structures of scaffold **I**, 2-BFI, and compounds **1** and **2**.

Fig. 2.
Chemical structures of compounds **3–16**.

Fig. 3.

Preliminary binding screen of compounds **3–16** at human cloned D₂, D₃, and D₄ receptors stably expressed in HEK 293 cells. Each compound was tested in triplicate at a concentration of 10 μ M for its displacement of the [³H]*N*-methylspiperone radiotracer. 70% displacement (dotted line) was considered a successful “hit” justifying full dose-response evaluation at all three receptors.

Fig. 4.

(A) Dynamic mass redistribution (DMR) shown as ligand-induced response in picometers (pm) over time in HEK 293 cells stably expressing $D_{4.4}$ receptors. After 10 minutes of baseline, compounds **12** (left) and **15** (right) were added to the cells at increasing concentrations and the DMR response was measured for 20 minutes. Next, QP at a final concentration of 10^{-8} M was added and the DMR response was measured for 60 minutes. Values are means $\pm$ SEM from a representative experiment performed in triplicate. (B) Dose-response curves using peak effects of each concentration (minute 4) expressed as percentage versus of QP 10^{-8} M. IC_{50} values were obtained from non-linear regression curves (IC_{50} = 0.950 μ M and 3 μ M for compounds **12** and **15**, respectively). Values are means $\pm$ SEM of 3 experiments performed in triplicate.

Fig. 5.
(A) The experimental (green, sticks representation) binding mode of eticlopride within the D₃ receptor binding cavity is successfully reproduced (pink, balls & sticks representation) by the docking tool. (B) Simulated binding mode of eticlopride within the cavity of the D₄ receptor model. The cavity is represented as molecular surface.

Fig. 6. Binding mode 1 (A) and binding mode 2 (B) of compound **12** at the D₄ receptor cavity. Key residues for ligand-receptor interaction are indicated (see text for details).

Scheme 1.

Reagents and conditions: (a) *N*-benzylethylenediamine for **3–6**, *N*-phenylethylenediamine for **10–13**, $Al(CH_3)_3$, toluene, reflux; (b) BBr_3 , dichloromethane, $-78^\circ C$.

Table 1

Binding affinity values, expressed as K_i (μM), of **8**, **10–12**, **14**, and **15** at human D_2 , D_3 and D_4 receptors.^a

Compd	R_1	R_2	R_3	D_2 $K_i \pm \text{SEM}, \mu\text{M}$	D_3 $K_i \pm \text{SEM}, \mu\text{M}$	D_4 $K_i \pm \text{SEM}, \mu\text{M}$
8	H	OH	$\text{CH}_2\text{C}_6\text{H}_5$	>10	1.259 ± 0.11	>10
10	H	H	$\text{CH}_2\text{CH}_2\text{C}_6\text{H}_5$	>10	>10	0.758 ± 0.06
11	OCH_3	H	$\text{CH}_2\text{CH}_2\text{C}_6\text{H}_5$	>10	>10	0.501 ± 0.04
12	H	OCH_3	$\text{CH}_2\text{CH}_2\text{C}_6\text{H}_5$	>10	>10	0.095 ± 0.03
14	OH	H	$\text{CH}_2\text{CH}_2\text{C}_6\text{H}_5$	0.794 ± 0.27	0.832 ± 0.24	>10
15	H	OH	$\text{CH}_2\text{CH}_2\text{C}_6\text{H}_5$	>10	>10	0.589 ± 0.05

^a Each K_i value represents data from at least three independent experiments, each performed in triplicate. Binding assays are described in detail in the Experimental Methods.