Dopamine D_4 Ligands and Models of Receptor Activation: 2-(4-Pyridin-2-ylpiperazin-1-ylmethyl)-1*H*-benzimidazole and Related Heteroarylmethylarylpiperazines Exhibit a Substituent Effect Responsible for Additional Efficacy Tuning

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Received November 7, 2003

A series of subtype selective dopamine D_4 receptor ligands from the hetroarylmethylphenylpiperazine class have been discovered that exhibit a remarkable structure−activity relationship (SAR), revealing a substituent effect in which regiosubstitution on the terminal arylpiperazine ring can modulate functional or intrinsic activity. Other structure-dependent efficacy studies in the dopamine D_4 field have suggested a critical interaction of the heteroarylmethyl moiety with specific protein microdomains in controlling intrinsic activity. Our studies indicate that for some binding orientations, the phenylpiperazine moiety also plays a key role in determining efficacy. These data also implicate a kinetic or efficiency term, contained within measured functional affinities for agonists, which support a sequential binding and conformational stabilization model for receptor activation. The structural similarity between partial agonist and antagonist, within this subset of ligands, and lack of bioisosterism for this substituent effect are key phenomena for these hypotheses.

Introduction

G-protein-coupled receptors (GPCRs) involved in second messenger signaling are one of the most studied classes of targets for drug discovery.[1] They comprise greater than 1% of the human genome,[2] represent the molecular targets for over 45% of all currently marketed drugs, and may account for one-third of current pharmaceutical research.[3] Investigations of these proteins and corresponding high-affinity ligands have probably contributed more to the conceptualization of pharmacological agonism or antagonism than any other class. Competitive agonists and antagonists for these receptors, utilizing the same recognition site, often reveal a continuum of ligand efficacy or intrinsic activity. Current models of activation mechanisms propose conformational isomers or states of the protein, thereby affecting the morphology of the recognition site surface but while still employing common site residues.[4] The realization that receptors can have constitutive intrinsic activity[5] in their native environment, presumably in the absence of ligand, has complicated the understanding of protein conformational control of intrinsic activity and the role of ligand stabilization in this process. Driven by selective ligand binding or stabilization, conformational selection models and model variants, incorporating sequential binding and conformational stabilization, form the basis for most models of activation involving ligand mediation.[6]

The complex structures and associated cellular machinery of membrane-embedded GPCRs have hindered purification and limited detailed characterization of protein structural morphology or ligand binding orientation that can be obtained through traditional X-ray or NMR techniques. Ligand recognition site description and relative binding orientation of given dopamine receptor ligands are proposed on the basis of eloquent mutagenesis studies[7] and the comparative binding data using mutants from these studies. Site-directed mutagenesis data can indicate key residues involved in translation of conformational information through certain helices to the G protein but do not give detailed information with regard to ligand binding orientation. Other general descriptions of ligand binding can be derived from structure−activity relationship (SAR) data. The ubiquitous incorporation of piperazine derivatives within ligands having high affinity for the A-family or aminergic class of GPCR receptors supports a consistent binding interaction.[8] This interaction would correlate to the protonated basic amine of endogenous biogenic amine agonist with a highly conserved aspartic acid (ASP) in transmembrane-spanning domain 3 (TM3) ($ASP^{3.32}$)[9] and is one definitive feature of the competitive recognition site and ligand pharmacophore. In addition to TM3 for binding, another general structural determinate is TM6, implicated in activation mechanisms.[10] This large class or supergenus of arylpiperazines derivatives can therefore be considered as privileged structures[11] for this family of GPCR receptors. The compounds in this study are from this class.

The dopamine receptor has been the target for many therapeutic drug strategies.[12] Dopamine D_4 antagonists were pursued as a possible intervention for schizophre-

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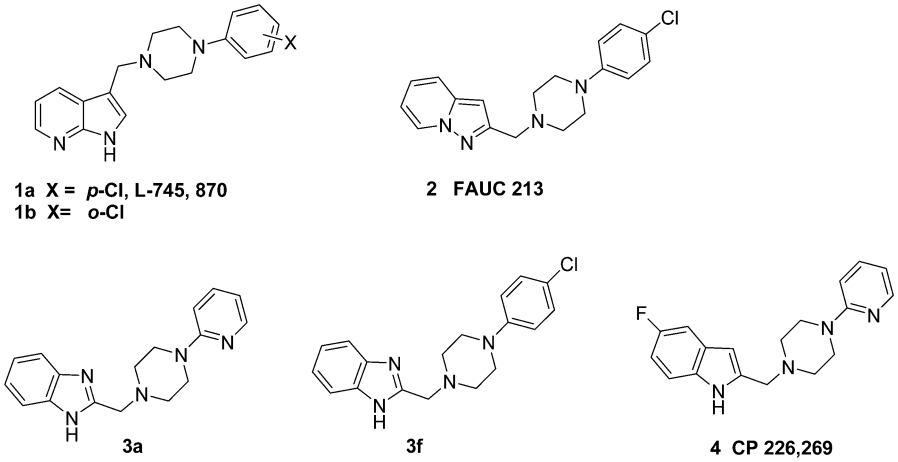
10.1021/jm0305669 CCC: $27.50
Published on Web 03/23/2004

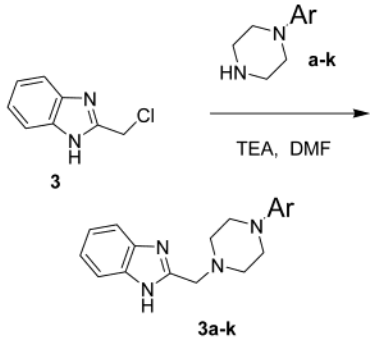
Chart 1. Structurally Similar Heteroarylmethylarylpiperazines: Selective D_4 Ligands



nia (Chart 1).[13] The D_4 selective antagonist to advance the farthest in development for this indication, L-745,-870 (**1a**),[14] failed to demonstrate clinical efficacy. Much debate has centered on the true intrinsic activity of **1a** and whether it is indeed a partial agonist,[15] thus highlighting the difficulties in understanding the range of ligand intrinsic activity or how this measure or aspect of a ligand's biochemical profile relates to ligand affinity or a ligand's dissociation constant for a given low-energy conformation of the receptor. Compound **2** has been reported[16] as a "complete antagonist" with regard to studies on **1a**. In the Gmeiner et al. communication, comparison of efficacy data from mitogenesis functional assays, D_4/D_2 selectivities, and mutagenesis data from D_2 and D_4 receptors has provided a better understanding of ligand binding orientations and transmembrane domains involved in receptor activation for ligands **1a** and **2**.[16]

Recently, a therapeutic target for selective D_4 agonists has been disclosed.[17] We have identified compound **3a**, a selective D_4 agonist, for treatment of male erectile dysfunction (MED).[18] From the intense search for selective D_4 antagonists, a few selective partial D_4 agonists, such as **4**, were reported.[19] The structural similarity of **1a** and **4** stimulated our investigation of this class of compounds to determine SAR for intrinsic activity at the D_4 subtype and led to the discovery of **3a**. As part of that SAR study, we reported that within this narrow class of benzimidazoylmethyl-4-arylpiperazines, substitution on the terminal piperazine aryl ring dramatically influenced intrinsic activity. In this report we examine this substituent effect in more detail using sets of monosubstituted congeners related to **3a** and contrast data using binding competition, previous studies on this class of compounds, and models of GPCR receptor activation. Both the structural similarity between antagonists and agonist and the wide range of substituents that produce the observed substituent effect support this effect as not being related to enthalpy contributions[20] to the ΔG of binding or ligand dissociation constant for a given conformation of the protein. Some conclusions with regard to ligand binding orientation are suggested; however, more significantly, a kinetic component or efficiency terms, reflected in both the observed EC_{50} and intrinsic activity of functionally active ligands, which correlate to stabilization of a transition state between two conformations are indicated.

Scheme 1



Results

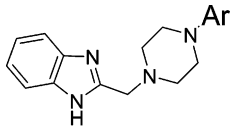
Synthesis of compounds **3a**−**k** were accomplished by reacting commercially available 2-chloromethylbenzimidazole (**3**) with the appropriate arylpiperazine (**a**−**k**) in DMF at room temperature in the presence of triethylamamine (Scheme 1). Arylpiperazines **a** and **c**−**k** were commercially available. Piperazine derivatve **b**, the 3-pyridyl isomer, was prepared by Buchwald coupling of 3-bromopyridine and piperazine.

Compound **1b** was prepared using the procedure described for the synthesis of **1a**, using azaindole, formaldehyde, and the arylpiperazine derivative in acetic acid and water, by substituting 2-chlorophenylpiperazine for the 4-chloro derivative.[21] Compounds **2** and **4** were prepared as previously described.[16,18]

D_4 efficacy or intrinsic activities of ligands were measured using recombinant human D_4 receptor expressed in HEK-293 cells also expressing a cotransfected $G\alpha_{qo5}$ protein.[22] D_4 ligand binding affinity was determined by radioligand competition against [^{3}H]spiperone,[23] using membranes from the engineered HEK-293 cells. D_2 binding affinity was determined by [^{3}H]spiperone competition using membranes from human D_2 transfected HEK-293 cells.

Our original SAR study began with two sets of ligands: (a) variation of the benzofused azaheterocyle of both **1a** and **4**; (b) analogues with different selected substituents on the terminal piperazine aryl ring. We discovered that the novel benzimidazole analogues of both azaindole **1a** or indole **4** produced high-affinity D_4 ligands **3f** and **3a**, respectively. The calcium flux assay revealed that, like **4**, **3a** was a potent partial agonist.

Table 1. Human D_4 Binding Affinity and Functional Data for Benzimidazole Analogues **3a**−**k**



3a-k

compd	aryl substituent (AR)	binding affinity K_i [a]	EC_{50} [b]	% efficacy[c] (intrinsic activity)
3a	2-pyridyl	167 ± 2.4	12.4 ± 0.5	61.3 ± 3.4
3b	3-pyridyl	376 ± 38	ND	24.9 ± 4.9
3c	4-pyridyl	>10000	NA	4.8 ± 0.8
3d	2-ClC_6H_4	8.5 ± 0.6	1.0 ± 0.1	43.0 ± 5.9
3e	3-ClC_6H_4	9.6 ± 0.6	NA	3.8 ± 0.6
3f	4-ClC_6H_4	14.8 ± 3.0	NA	4.7 ± 0.7
3g	C_6H_5	52 ± 5.8	10.1 ± 0.8	37.5 ± 4.0
3h	2-$OCH_3C_6H_4$	4.3 ± 0.3	5.6 ± 0.5	47.5 ± 4.7
3i	3-$OCH_3C_6H_4$	26.5 ± 1.6	NA	5.0 ± 0.4
3j	4-$OCH_3C_6H_4$	20.4 ± 0.15	NA	4.8 ± 0.5
3k	4-FC_6H_4	79.6 ± 6.3	NA	9.9 ± 2.6

[a] Competition against [^{3}H]spiperone, expressed as nM (±SEM, $n \geq 3$). [b] Concentration giving 50% signal, expressed as nM (±SEM, $n \geq 3$). NA: not applicable. ND: not determined. [c] % activation relative to 10 μM dopamine (±SEM, $n \geq 3$).

Shown in Table 1 are EC_{50} values, percent efficacy, and corresponding binding affinities for sets of monosubstituted arylpiperazine congeners related to **3a** and **3f**.

Of the three pyridyl isomers, only the 2-pyridyl analogue (**3a**) is a potent partial agonist with an EC_{50} of 12 nM and achieving 61% activation relative to 10 μM dopamine. The 3-pyridyl isomer (**3b**) has weaker affinity on the basis of binding data and an appreciable lower intrinsic activity or the ability to activate the receptor. The 4-pyridyl isomer (**3c**) has weak affinity for the receptor. This data set indicates that a nitrogen atom in the ortho position of the terminal aryl ring could play a key role in molecular interaction between the ligand and receptor. The binding affinities were consistent within the three monosubstituted chlorophenyl analogues (**3d**−**f**), revealing a higher affinity than the corresponding pyridyl analogues; however, the same SAR phenomenon for receptor activation is observed. The *o*-chloro analogue (**3d**) produced a partial 43% response with a potent EC_{50} of 1 nM. The EC_{50} is also indicative of high affinity but may only appear to correlate well with the competition binding data for this ligand. Although the *m*- and *p*-chloro substituted analogues (**3e**, **3f**) have similar apparent dissociation constants, on the basis of radioligand competition, they lack the ability to induce calcium mobilization. The three monomethoxy substituted analogues also exhibit an identical trend. Only the 2-methoxy analogue (**3h**) is a potent partial agonist producing approximately 50% of the response of 10 μM dopamine. The other two methoxy analogues (**3i**, **3j**) have potent affinity but cannot function as agonists. The unsubstituted phenyl analogue (**3g**) indicates that a hydrogen substituent in this ortho position is sufficient in favoring an active form of the receptor. In contrast, a *p*-fluoro substituent precludes substantial agonist function (compare **3g** to **3k**), consistent with the trend that substituents in the meta or para position other than hydrogen do not lead to ligands having agonist function.

Ligand **1b**, the *o*-chloro analogue of **1a**, was prepared to test the generality of this ortho effect toward the azaindole series. Human D_4 functional data for this analogue, the reference partial agonist **4**, and ligand **2** in our chimeric system are shown in Table 2. The change of the *p*-chloro substituent (**1a**) to the ortho position (**1b**) produces little effect on intrinsic activity. Ligand **1b** is a weak partial agonist similar to **1a** exhibiting only approximately 20% activation at micromolar concentrations. Null cell experiments and blockade of the calcium flux assay by D_4 antagonists confirm these signals for **1a, 1b** and **2** are from interactions with dopamine D_4 receptors (data not shown). The potent partial agonism observed for **4** agrees well with the reported profile of this compound.[19a] Ligand **2** produces little activation of the D_4 receptor, similar to **1a**, but activation occurs at much lower concentrations. This comparison provides an example of the dual nature and independent measurement of affinity and efficacy.

Table 2. Intrinsic Activities of Terminal Monochlorosubstituted Phenylpiperazine Analogues in the hD_4 Calcium Flux Assay with Cotransfected $G\alpha_{qo5}$ Protein

compd	EC_{50} [a]	intrinsic activity[b]
1a	2230 ± 1010	20 ± 2.9
1b	10000[c]	19 ± 2.8
2	154 ± 56	19 ± 3.6
4	32 ± 7.4	46 ± 4.1

[a] Concentration giving 50% signal, expressed as nM (±SEM, $n \geq 3$). [b] % activation relative to 10 μM dopamine (±SEM, $n \geq 3$). [c] Highest dose tested.

Table 3. Human D_2/D_4 Receptor Subtype Selectivity of Monochloro-Substituted Phenylpiperazine Analogues Determined Using [^{3}H]spiperone Binding Competition

compd	D_4[a]	D_2[a]	D_2/D_4
1a	0.4 ± 0.05	448 ± 54	1120
1b	719 ± 109	>10000	>14
2	2.7 ± 0.5	1310 ± 583	489
3d	8.5 ± 0.6	>10000	>1176
3e	9.6 ± 0.6	>10000	>1041
3f	14.8 ± 3.0	>10000	>756

[a] K_i value, from binding competition against [^{3}H]spiperone, expressed as nM (±SEM, $n \geq 3$).

D_2/D_4 selectivity data were used as an additional probe of ligand-binding orientation. Subtype selectivity was measured for monochlorophenyl analogues **3d**, **3e**, **3f**, **1a**, **1b**, and **2** and are shown in Table 3. Ligands **3d**−**f** are D_4/D_2 selective at the highest concentrations tested and support similar binding modes for these three analogues. By comparison of **1a** and **1b**, compound **1b** has a much weaker affinity at D_4, presumably as a result of changes in ligand interaction of the chlorophenyl moiety. Compound **2** is similar in D_4 affinity and selectivity to **1a**, as proposed by the previous overlay models.[16]

Discussion

Details of ligand binding orientation obtained through docking models are one fundamental aspect of understanding mechanisms of receptor activation. Compound **2**, described as a "complete" antagonist,[16] was designed as part of a study on antagonists related to **1a**. The reported efficacy tuning for ligands **1a** and **2** is proposed by using docking models that overlay the common *p*-chlorophenylpiperazine moiety for the two ligands. D_2/D_4 selectivity was used as one factor to establish relative ligand orientation within the recognition site and at-

tributed to interaction of the phenylpiperazine moiety with the TM2,3,7 microdomain. Following this model, the heteroarene subunits of the two ligands then engage with different vectors into a cluster of residues in the TM5,6 microdomain affecting different degrees of receptor activation. Compound **1b** displays similar weak agonism or efficacy as **1a** (Table 2) and supports a similar interaction of the azaindole with the TM5,6 microdomain. The generality of the ortho effect seen for the benzimidazole analogues does not translate to the chlorophenylazaindole analogues and suggests subtle differences in binding orientation for the monochlorophenyl derivatives between the azaindole and benzimidazole series. The change in chlorosubstitution for **1b** as reflected by interaction with the TM2,3,7 microdomain produces the observed differences for **1a** and **1b** in D_2 and D_4 affinities. However, the D_2/D_4 selectivity data and the efficacy data still support similar general ligand binding orientation and receptor activation mechanism for **1b** compared to that proposed for **1a**.

The complexity introduced by our study of the benzimidazole analogues is centered on an apparent key role of the terminal arylpiperazine moiety. Substituents in the ortho position of this ring, possessing a diverse range of substituent parameters, produce a similar efficacy effect, while the heteroarene moiety is kept constant. Much of our data could fit the rationale of Gmeiner et al., since all ortho-substituted analogues could be argued to adopt a similar binding orientation, one different from the meta and para analogues. These two types of terminal ring orientations would then produce agonism or antagonism by altering the position of the pendent benzimidazole engaging with the TM5,6 domain. It is hard to rationalize why the minimal size of an ortho lone pair (2-pyridyl, **3a**) or ortho hydrogen (**3g**) has the same effect or influence on binding orientation as the larger ortho substituents in our study, such as the *o*-chloro analogue (**3d**) or the even larger *o*-SMe and *o*-nitro analogues reported in our previous SAR study.[18] Neither polarity nor the electron-withdrawing nature of the ortho substituent produces modulation of the generality of this ortho effect. This lack of obvious Hansch−Mitscher bioisosterism[24] for this positional substituent implies that this substituent is not in close contact with the protein in the putative "agonist" binding orientation for these ortho-substituted congeners. This lack of bioisosterism also precludes associating this effect with typical SAR parameters, such as electronic effects on the π systems of the aryl rings, effects on the pK_a of the important protonated piperazine nitrogen, or ligand conformational or shape analysis that normally relates enthalpy contributions of protein−ligand interaction to binding energy. Our three monochloro analogues have remarkably similar D_4 binding affinities and D_2/D_4 selectivites (Table 3). These data support similar binding orientations for these three benzimidazole analogues but are distinct from analogues derived from **1** and **2**. The similar affinities also suggest a lack of close contact with the protein for the chloro group of meta and para analogues **3e** and **3f**.

Bound ligand orientation and associated helical domain changes provide a basic mechanistic model of receptor activation. Difficulty remains in understanding how measured biochemical parameters for a given

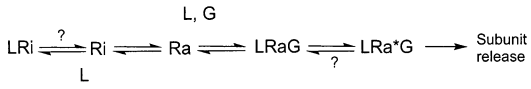


Figure 1. Two receptor state or conformational control model of receptor activation: **Ri**, antagonist or inactive receptor conformation; **Ra**, high agonist affinity or active receptor conformation; **L**, ligand; **G**, G-protein; **LRaG**, initial ligand bound, active receptor−G protein complex (specific order or sequence is not implied with regard to ligand, receptor, and G protein association in formation of bound active receptor complex); **LRa*G**, bound transitional complex leading to receptor activation and G protein subunit release.

ligand correlate to general models of ligand interaction and receptor activation. Two fundamental concepts or models[6] of receptor activation are conformational selection and sequential binding−conformational stabilization. A general receptor activation model is shown in Figure 1.

Two-state models of conformational selection are formed on the basis of equilibrium ligand interaction with multiple conformational states.[25] There are convincing data for an initial conformational selection process by a ligand between **Ri** and **Ra**. This concept is supported by GTP shift experiments,[23,26] the use of cells that do not contain G proteins that can react with expressed receptors,[27] and data using pertussis toxin,[28] which prevent association of specific G proteins and receptors. It is known that antagonists often recognize both affinity states (**Ri** and **Ra**) of the receptor as manifested in biphasic binding curves of agonists versus radioligand antagonists.[23,27a,29] By use of antagonist radioligands, agonist affinities for both states in D_2 and D_4 receptors have been reported in these studies. Full agonists, having catecholamine structures, display a significant difference in affinities for the high- and low-affinity states. Restated, these full agonists have a high selectivity for the agonist state, as predicted by the simplest two-state model. By use of binding data from competition using radioligands of different functional profiles or conformational selectivity, intrinsic activities of competing ligands have been successfully predicted.[30] This general conformational selection model is also useful in explaining constitutive activity in the absence of ligand based on the basal position of the ratio **Ra**/**Ri**. Biphasic binding curves suggest that antagonists can identify both states. This implies that ligand affinity for the active state (**LRaG**) is by itself insufficient for receptor activation. Introduction of additional conformational selection states has been introduced to explain these phenomena and expand this model.[25c]

Sequential binding−conformational stabilization models more thoroughly encompass concepts of induced fit[31] and easily embrace ligand-mediated helical or transmembrane domain changes identified using mutagenesis data and docking models. Additional ligand-mediated receptor conformational changes after formation of **LRaG** leading to **LRa*G** and G protein subunit dissociation mechanistically distinguish agonist binding. Our three monochlorophenyl analogues (**3d**−**f**) have similar potent single nanomolar binding affinities competing against the antagonist spiperone. These data together with the structural similarity of these analogues strongly suggest that both the partial agonist **3d** and the antagonist type ligands **3e** and **3f** are nonselective with regard to the two affinity states of the D_4

receptor in our membrane assays. It should be noted that data are not presented in this study to support the assumption that **3e** and **3f** as well as **3i** and **3j** are D_4 antagonists. In our previous SAR study,[18] antagonist data were given for compounds that are close analogues to **3a**, and recently, **3f** and **3j** have been reported as D_4 receptor antagonists.[32]

Theoretically, extensive dose−response competition curves for antagonist radioligand with a reference full agonist, having sufficient selectivity between the two receptor conformations, can reveal a biphasic curve that may be manipulated by GTP shift experiments. However, we do not observe a biphasic curve when competing our partial agonists against the antagonist spiperone, and for these partial agonists, it seems more plausible that a lack of ligand selectivity between the conformations exists. If ligand affinity selectivity is less than 10-fold between the two conformations, the presence of two states would not be detected by a Hill analysis of binding data.

Although not indicated to be in close contact with the protein in bound ground states by the lack of influence on the binding affinities, the *m*- and *p*-chloro substituents for **3e** and **3f** could create steric interactions with residues moving to accommodate subsequent changes in protein conformation. This unfavorable interaction would thereby raise the activation energy for required protein movement to gain access to **LRa*G**. **LRa*G** can represent a higher energy species or transition state to the penultimate conformation leading to subunit dissociation. Whether it is a ground-state conformation or not, the rationale of protein−ligand interaction contributing to a ΔG of binding is a unifying thermodynamic principle. The ortho substituent could favor these subsequent conformational changes after binding. The nature of such a positive effect could be in the lowering of the activation energy to the key transition state as governed by the Arrhenius equation. This proposal complicates the understanding of ligand stabilization correlating to **LRa*G**, since it is not supported by known bioisosterism for this substituent. Therefore, the ortho effect may represent a lower energy pathway relative to a negative effect or higher activation energies exhibited by the meta and para analogues. Both possibilities define kinetic effects for these substituents.

Conceptually, incorporation of kinetic parameters or velocities of formation rates into these models are easily accommodated. With some analogy to enzyme kinetics, the velocity of formation for a sequence of bound conformations can be partially decoupled from the dissociation constant in that the highest dissociation constant may not produce the most efficient formation rate.[33] This kinetic term can then be reflected in both ligand EC_{50} and intrinsic activity. Agonist or partial agonist affinity for **Ri** modulates the concentration of **LRaG** ([**LRaG**] = $[R_o]$ − [LRi]) and can have a dramatic effect on the velocity or rate of formation of **LRa*G** by reducing the concentration term in the rate equation.

Ligand-driven partitioning of the receptor pool may also be largely a kinetic process and not a thermodynamic process, analogous to the Curtin−Hammet principle.[34] This process can be driven by energy manipulation (phosphorylation) within the cell and by the large entropy term of the G-protein dissociation. This type of driving force may be difficult to replicate in our equilibrium binding assays with truncated cellular machinery resulting from membrane preparation. Equilibrium affinities as measured by binding assays therefore are constrained by assay conditions and time frames. Protein−ligand interactions as described by induced fit models for receptor activation, measuring several changes or movements of the bound ligand−receptor−G protein, are more complex. The difficulty in assigning dissociation constants to ligand interaction in multiconformational state processes has been documented.[26,35]

The use of the $G\alpha_{qo5}$ chimeric protein might be questioned in our study as providing a tool for rapid drug discovery but one having little relevance to the true pharmacology of the D_4 receptor. Chimeric systems have been validated for the closely related D_2 receptor.[36] The similarity of our D_4 binding data for the antagonist spiperone[22] and agonist dopamine (data not shown) to literature values with the native signaling Gio suggests the desired morphology of the recognition site in our chimeric system relative to the native D_4 receptor; however, a caveat regarding the stoichiometry of the remaining G protein in our membrane binding system is valid. The correlation of our D_4 receptor activation data for **2**[16] and the reference compound **4**[19a] also supports the validity of our system in relation to the native system. Our value for the EC_{50} of **1a** is considerably less potent than some reports,[15,16] but it is consistent with the predominant D_4 antagonist profile of this compound. Even considering potential limitations,[37] the engineered system provides a valuable tool for direct observation of G-protein-linked receptor activation and understanding the ligand's role in unified activation mechanisms.

Conclusions

Structural similarity and consistent binding affinities within a set of benzimidazoylmethylarylpiperazine ligands, which display a range of intrinsic activity, indicate these ligands have poor selectivity for high- and low-affinity states of the dopamine D_4 receptor. This is consistent with conformational selection models and the partial agonism observed for the ortho-substituted analogues. It is equally consistent with the known recognition of both states by antagonists, which are represented by the meta- and para-substituted analogues. The lack of substituent bioisosterism and conformational selectivity indicates that kinetic terms relating activation energies between conformational changes are significant components of observed affinity. These changes of the receptor−ligand−G protein complex occur after initial agonist binding. Kinetic parameters are prominent in both the observed EC_{50} and the intrinsic activity for functional agonist and partial agonist ligands. A subtle balance in ligand stabilization of an active ground state(s), yet not introducing activation barriers between subsequent conformations along the reaction coordinate of the receptor−ligand complex, appears to describe receptor activation for this set of partial agonists. These data support a sequential binding and conformational stabilization model under kinetic control within the cell. Agonists are a class of ligands for small-molecule drug development that possess complex combinations of dissociation constants and

kinetic parameters as part of their biochemical profile, both of which can be manipulated, somewhat independently, through SAR strategies.

Experimental Section

All solvents were of anhydrous reagent grade from commercial sources. Unless otherwise noted, all chemicals and reagents were obtained commercially and used without purification. The ^{1}H NMR spectra were obtained at 300 MHz on a Nicolet/GE QE300 spectrometer. Chemical shifts are reported in parts per million (ppm) relative to TMS or TSP as an internal standard. Mass spectra were obtained on a Kratos MS-50 instrument in DCI/NH_3 mode. Elemental analyses were performed by Robertson Microlit Laboratories, Inc., Madison, NJ. Flash chromatography was carried out using silica gel 60 (E. Merck, 230−400 mesh) or prepacked 40 mm silica gel columns from BioTage. Thin-layer chromatography was performed on 250 μM silica-coated glass plates from EM Science. Samples were analyzed by HPLC−MS-ELSD on an Open Access Finnigan Navigator/Agilent 1100/Sedere Sedex 75 system using a Phenomenex Luna C_8 column (5 μm, 2.1 mm × 50 mm). The elution system used was a gradient of 10−100% over 4.5 min at 1.5 mL/min, and the solvent was either acetonitrile/0.1% aqueous TFA or acetonitrile/10 mM ammonium acetate. The MS was operated in the +APCI mode. Melting points were determined on a Buchi 510 melting point apparatus and are uncorrected.

3-{[4-(2-Chlorophenyl)piperizin-1-yl]methyl}-1*H*-pyrrolo[2,3-*b*]pyridine (1b) (35%) (Glass). Compound **1b** was prepared by the literature procedure described for **1a** by substituting 2-chlorophenylpiperazine for the 4-chloro derivative.[21] ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.88 (m, 2H), 3.10 (m, 2H), 5.25 (s, 2H), 6.52 (d, J = 3.4 Hz, 2H), 6.96 (m, 1H), 7.03 (m, 1H), 7.09 (dd, J = 7.8, 4.8 Hz, 1H), 7.20 (m, 1H), 7.32 (dd, J = 7.8, 1.7 Hz, 1H), 7.93 (dd, J = 7.8, 1.2 Hz, 1H), 8.33 (dd, J = 4.8, 1.4 Hz, 1H); MS (DCI/NH_3) m/z 327 (M + H)$^+$. Anal. ($C_{18}H_{19}ClN_4$) C, H, N.

Compounds 3b, 3c, 3e, 3f, 3i, 3j, 3k. These compounds were prepared by the general procedure given for **3a** (see below). Compounds **3d**, **3g**, and **3h** were previously prepared by a similar method.[18]

2-(4-Pyridin-2-yl-piperazin-1-ylmethyl)-1*H*-benzimidazole, Dihydrochloride Salt (3a). To a rapidly stirred solution of 2.5 g (15.4 mmol) of 2-pyridylpiperazine in 10 mL of 1:1 CH_3CN/DMF in a large round-bottom flask in a water bath at 20 °C was added 2.55 g (15.4 mmol) of 2-chloromethylbenzimidazole as a powder over 2 min. Triethylamine (3.2 mL, 1.5 equiv) was added, and the reaction mixture was stirred for 24 h. The reaction was processed by adding 250 mL of 0.4 M aqueous Na_2CO_3, then extracting the product with 220 mL of 10:1 CH_2Cl_2/butanol. The organic phase was washed with 300 mL of H_2O, dried over Na_2SO_4, then concentrated in vacuo to yield a tan powder. Recrystallization from 330 mL of 1:10 ethanol/hexane gave 1.66 g (37%) of the free base as a white powder. Mp 220−221 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.09 (dd, J = 4.5, 1.8 Hz, 1 H), 7.41−7.58 (m, 3 H), 7.14 (m, 2 H), 6.81 (d, J = 8.7 Hz, 1 H), 6.62 (dd, J = 6.6, 4.5 Hz, 1 H), 3.77 (s, 2 H), 3.52 (t, J = 4.5 Hz, 4 H), 2.55 (t, J = 4.5 Hz, 4 H); MS m/z 294 (M + H)$^+$.

The crystalline dihydrochloride monohydrate salt was found to have high aqueous solubility (>65 mg/mL) and was prepared by addition of 2.4 equiv of 12 M hydrochloric acid to a stirred suspension of 350 mg of the free base in methanol. The clear solution obtained deposited large, well-formed crystals, which were collected by filtration and washed with ethyl acetate and ethanol. Mp 235−237 °C. Anal. ($C_{17}H_{19}N_5$·2.0HCl·1.0H_2O), C, H, N.

2-(4-Pyridin-3-ylpiperazin-1-ylmethyl)-1*H*-benzimidazole (3b). A mixture of piperazine (22.6 g, 260 mmol), 3-bromopyridine (6.7 g, 42 mmol), NaO-t-Bu (5.6 g, 58 mmol), Pd_2dba_3 (20 mg, 0.022 mmol), and P(t-Bu)$_3$ (2 mg, 0.012 mmol) in 25 mL of *o*-xylene was stirred at ambient temperature for 1 h and then heated at reflux for 4 h. After the mixture cooled, solids were removed by filtration and the filtrate was concentrated in vacuo. The residue was purified by chromatography to give 3.6 g (100%) of 1-(3-pyridyl)piperazine as a colorless oil. By use of this material without further purification, **3b** was prepared by the general procedure (39%). Mp 192−193 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.64 (t, J = 6 Hz, 4 H), 3.24 (t, J = 6 Hz, 4 H), 3.79 (s, 2 H), 7.15 (m, 2 H), 7.21 (dd, J = 7, 6 Hz, 1 H), 7.30 (m, 1 H), 7.43 (d, J = 7.5 Hz, 1 H), 7.57 (d, J = 7.5 Hz, 1 H), 7.98 (dd, J = 1, 5 Hz, 1 H), 8.28 (d, J = 4.5 Hz, 1 H), 12.31 (brs, 1 H); MS (DCI/NH_3) m/z 294 (M + H)$^+$. Anal. ($C_{17}H_{19}N_5$) C, H, N.

2-(4-Pyridin-4-ylpiperazin-1-ylmethyl)-1*H*-benzimidazole (3c). 3c was purified by reversed-phase HPLC and isolated as the CF_3CO_2H salt (28%) (glass). ^{1}H NMR (CD_3OD, 300 MHz) δ 2.83 (t, J = 5 Hz, 4 H), 3.83 (t, J = 5 Hz, 4 H), 4.2 (s, 2 H), 7.29 (d, J = 7.8 Hz, 1 H), 7.60 (dd, J = 6, 3 Hz, 2 H), 7.78 (dd, J = 6, 3 Hz, 2 H), 8.14 (d, J = 7.8 Hz, 1 H); MS (DCI/NH_3) m/z 294 (M + H)$^+$. Anal. ($C_{17}H_{19}N_5$·2.1CF_3CO_2H·0.9H_2O) C, H, N. F: calcd, 21.80; found, 21.31.

2-{[4-(3-Chlorophenyl)piperizin-1yl]methyl}-1*H*-benzimidazole (3e). 3e was purified by reversed-phase HPLC and isolated as the CF_3CO_2H salt (64%) (glass). ^{1}H NMR (CD_3OD, 300 MHz) δ 2.92 (t, J = 5 Hz, 4H), 3.38 (t, J = 5 Hz, 4H), 4.23 (s, 2H), 6.82 (dd, J = 7.5, 1.8 Hz, 1H), 6.92 (dd, J = 8.7, 1.8 Hz, 1H), 6.95 (t, J = 1.8, 1H), 7.20 (t, J = 8.7 Hz, 1H), 7.55 (dd, J = 6, 3 Hz, 2H), 7.76 (dd, J = 6, 3 Hz, 2H); MS (DCI/NH_3) m/z 327 (M + H)$^+$. Anal. ($C_{18}H_{19}ClN_4$·1.2CF_3CO_2H·0.3H_2O) C, H, F, N.

2-{[4-(4-Chlorophenyl)piperizin-1yl]methyl}-1*H*-benzimidazole (3f). 3f was purified by reversed-phase HPLC and isolated as the CF_3CO_2H salt (64%) (glass). ^{1}H NMR (CD_3OD, 300 MHz) δ 2.93 (t, J = 5 Hz, 4H), 3.33 (t, J = 5 Hz, 4H), 4.23 (s, 2H), 6.95 (d, J = 7.8 Hz, 1H), 7.21 (d, J = 7.8 Hz, 1H), 7.55 (dd, J = 6, 3 Hz, 2H), 7.77 (dd, J = 6, 3 Hz, 2H); MS (DCI/NH_3) m/z 327 (M + H)$^+$. Anal. ($C_{18}H_{19}ClN_4$·1.3CF_3CO_2H·0.7H_2O): C, F, N. H: calcd, 4.49; found, 3.98.

2-{[4-(3-Methoxyphenyl)piperizin-1yl]methyl}-1*H*-benzimidazole (3i). 3i was purified by reversed-phase HPLC and isolated as the CF_3CO_2H salt (69%) (glass). ^{1}H NMR (CD_3OD, 300 MHz) δ 3.00 (t, J = 5 Hz, 4H), 3.48 (t, J = 5 Hz, 4H), 3.78 (s, 3H), 4.25 (s, 2H), 6.52 (dd, J = 7.5, 1.8 Hz, 1H), 6.59 (t, J = 1.8, 1H), 6.65 (dd, J = 9, 1.8 Hz, 1H), 7.20 (t, J = 9.0 Hz, 1H), 7.55 (dd, J = 6, 3 Hz, 2H), 7.76 (dd, J = 6, 3 Hz, 2H); MS (DCI/NH_3) m/z 323 (M + H)$^+$. Anal. ($C_{19}H_{22}N_4O$·1.7CF_3CO_2H·0.4H_2O) C, H, F, N.

2-{[4-(4-Methoxyphenyl)piperizin-1yl]methyl}-1*H*-benzimidazole (3j) (41%) (Glass). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.76 (m, 4 H), 3.14 (m, 4 H), 3.77 (s, 3 H), 3.92 (s, 2 H), 6.88 (m, 4 H) 7.26 (dd, J = 6.10, 3.05 Hz, 2 H), 7.59 (dd, J = 5.76, 3.05 Hz, 2 H); MS (DCI/NH_3) m/z 339 (M + H)$^+$. Anal. ($C_{19}H_{22}N_4O$) C, H, N.

2-{[4-(4-Fluorophenyl)piperizin-1yl]methyl}-1*H*-benzimidazole (3k) (12%) (Glass). ^{1}H NMR (300 MHz, CD_3OD) δ 2.71 (m, 4 H), 3.16 (m, 4 H), 3.86 (s, 2 H), 6.95 (d, J = 6.44 Hz, 4 H), 7.22 (dd, J = 6.10, 3.05 Hz, 2 H), 7.54 (dd, J = 5.93, 3.22 Hz, 2 H); MS (DCI/NH_3) m/z 311 (M + H)$^+$. Anal. ($C_{18}H_{19}FN_4$) C, H, N.

Human $D_{4.4}$ HEK FLIPR Assay. Human $D_{4.4}$ was coexpressed with $G\alpha_{qo5}$ in HEK293 cells as described.[22] Cells were plated into 96-well, black-wall/clear-bottom microplates (Biocoat, Becton Dickinson, Boston, MA) at 20 000 cells per well. After 2 days of culture, the culture medium was removed by aspiration and replaced by 0.1 mL of DPBS (Dulbecco's phosphate-buffered saline with D-glucose and sodium pyruvate) containing 0.04% Pluronic F-127 and 4 μM Fluo-4, fluorescent calcium indicator dye. After incubation for 1 h at room temperature, the cells were washed four times with DPBS in a plate washer (Molecular Devices). After the final wash, 150 μL of DPBS was added to each well. Fluorometric imaging plate reader (FLIPR 384, Molecular Devices) transferred 50 μL from the compound plate to the cells and recorded fluorescence reading for 3 min (every second for the first minute and every 5 s for the next 2 min). The instrument software normalizes the fluorescent reading to give equivalent

initial readings at time zero, and all the data were normalized to the response of 10 μM dopamine.

^{3}[H]spiperone Human $D_{4.4}$ Binding Assay. Human dopamine $D_{4.4}$ receptor-transfected HEK-293 cells[22] were cultured in DMEM supplemented with 10% fetal calf serum, 1 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Rockville, MD). For membrane preparation, the cells were seeded into a Cell Factory (VWR, Plainfield, NJ) and the confluent cells were rinsed with PBS and detached with cell dissociation buffer (Invitrogen). The resulting cell suspension was centrifuged, and the pellet was homogenized by a Polytron for 10 s in 50 mM Tris-HCl, pH 7.4. Membrane aliquots were stored at −80 °C until use.

Binding assays[22] were initiated by addition 250 μL of membrane to 200 μL of [^{3}H]spiperone (125 Ci/mmol) and were incubated at room temperature for 2 h. Nonspecific binding was determined in the presence of 10 μM haloperidol (RBI-Sigma). The incubation buffer consisted of 50 mM Tris-HCl, pH 7.4, 5 mM KCl, 120 mM NaCl, 5 mM $MgCl_2$, and 1mM EDTA. In competition binding studies, agonists or antagonists were prepared with 0.1% ascorbic acid in the buffer. The final concentration for [^{3}H]spiperone was 0.1 nM. The reaction was terminated by rapid filtration through UniFilter-96 GF/B filers, using a Filtermate harvester (Packard, Meriden, CT). Filters were washed three times with 1 mL of ice-cold 50 mM Tris-HCl, pH 7.4. Radioactivity was measured by a TopCount microplate scintillation counter (Packard, Meriden, CT). Proteins were determined by the BCA protein assay kit (Pierce, Rockford, IL) using BSA as a standard.

^{3}[H]spiperone Human D_{2L} Binding Assay. Cloned human dopamine D_{2L} receptor cells (hD_{2L}-HEK293)[38] were cultured in RPMI medium supplemented with 10% fetal calf serum, 1 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. For membrane preparation, the cells were seeded into a Cell Factory (VWR, Plainfield, NJ) and the confluent cells were rinsed with PBS and detached with cell dissociation buffer (Invitrogen/Life Sciences, Rockville, MD). The resulting cell suspension was centrifuged, and the pellet was homogenized by Polytron for 10 s in 50 mM Tris-HCl, pH 7.4. Membrane aliquots were stored at −80 °C until use. Saturation binding assays were conducted with 20 μg of hD_{2L} membrane using 0.01−2 nM [^{3}H]spiperone (Amersham, Arlington Heights, IL) in 50 mM Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM Mg Cl_2, and 2 mM $CaCl_2$. Nonspecific binding was determined in the presence of 10 μM haloperidol. K_d values determined in this way were 0.09 nM. In competition assays, concentrations of compounds (0.1−10000 nM) were competed with 0.1 nM [^{3}H]spiperone. Nonspecific binding was also determined in the presence of 10 μM haloperidol. After incubation for 1 h at room temperature, samples were filtered and counted. Nonlinear regression analysis derived IC_{50} values were converted to K_i values using GraphPad Prism software.

Acknowledgment. The authors thank and acknowledge discussions and manuscript review with Professor Peter Beak of the University of Illinois−Urbana-Champaign, Professor Les Mitscher of the University of Kansas, and both Dr. Michael Jarvis and Dr. Yvonne Martin of Abbott Laboratories.

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JM0305669