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Dopamine D3 Receptor Selective Ligands with Varying Intrinsic Efficacies at Adenylyl Cyclase Inhibition and Mitogenic Signaling Pathways

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Abstract

A panel of eight structurally related pairs of 2,3-dichloro and 2-methoxy substituted 4-phenylpiperazines with nanomolar affinity and selectivity at D3 dopamine receptors has been synthesized. Compounds in which a heterocyclic (2-phenyl pyridyl, 3-phenyl pyridyl, benzothiophen or benzofuran) moiety is adjacent to the amide was varied and/or a double bond (*trans*-butenyl) replaced the four carbon aliphatic chain linking the arylamide with the 4-phenylpiperazine moiety were compared for a) affinity at human D2 and D3 dopamine receptors using a competitive radioligand binding assay, b) intrinsic efficacy using a forskolin-dependent adenylyl cyclase inhibition assay and c) intrinsic efficacy using a mitogenic assay. The efficacy of these compounds for both the cyclase and the mitogenic assays were compared to quinpirole, which is a full agonist that is selective for D2-like dopamine receptors. In the initial studies four pairs of 2,3-dichlorophenyl analogues compounds, differing by the presence or absence of a *trans*-olefin in the four carbon linking chain, were evaluated. Generally, the compound with the *trans*-butenyl linker had a) 2-fold higher D3 receptor binding selectivity compared to the D2 receptor and b) reduced intrinsic efficacy at both D2 and D3 receptors. Subsequently, four pairs of the corresponding 2-methoxy substituted compounds were evaluated in a similar manner. In each pair of these compounds the reverse effects on D3 receptor binding selectivity and efficacy were observed, since the compounds with the *trans*-butenyl linker had a) lower D3 receptor binding selectivity and b) similar or slightly increased intrinsic efficacy at D3 receptors in the cyclase assay. Furthermore, we observed that all of the 16 novel compounds were a) more efficacious for the D3 receptor cyclase inhibition assay than for the D3 receptor mitogenic assay and b) exhibited the same or greater efficacy at D3 compared to D2 receptor (with the exception of one compound). Based upon the results of these studies we concluded that the heterocyclic amide moiety is the pivotal structural element determining the intrinsic efficacy of our substituted phenylpiperazine D3 receptor selective compounds. The magnitude of the efficacy of the compound is modulated by both a) the substituent(s) on the phenyl piperazine and b) the saturation of the four carbon chain (butyl vs, butenyl) that links the arylamide and the phenylpiperazine. In addition, these data provide evidence that our ligands can have differing efficacies for the cyclase inhibition and the mitogenic activation signaling pathways. This form of functional selectivity appears to apply to our D3 receptor selective compounds since a) compounds that are essentially full agonists at the cyclase assay appear to be only partial agonists in the mitogenic assay and b) compounds that are partial agonists in our cyclase assay are partial agonists or antagonists in the mitogenic assay. These findings are relevant for defining the *in vivo* mechanism of action of this class of

substituted phenylpiperazines when they exhibit behavioral modifying actions in animal models of pathological disorders.

Keywords

dopamine receptors; D2-like dopamine receptors; D3 receptors; D3 selective compounds; intrinsic efficacy; functional selectivity

INTRODUCTION

There are three dopaminergic pathways in the mammalian brain; the nigrostriatal pathway, the mesocorticolimbic pathway and the tuberoinfundibular pathway. These systems are involved in movement coordination, cognition, emotion, affect, memory and the regulation of prolactin secretion by the pituitary. Alterations in the dopaminergic pathways have been implicated in the pathogenesis of neurological, neuropsychiatric and hormonal disorders, including Parkinson's Disease, schizophrenia and hyperprolactinemia (Missale et al., 1998; Lee et al., 1978; Kapur and Mamo, 2003; Nieoullon, 2002; Jardemark et al., 2002; Cunnah and Besser, 1991; Korczyn, 2003; Luedtke and Mach, 2003). In addition, modulation of the dopaminergic pathways is thought to occur as a consequence of acute and chronic abuse of psychostimulants, including cocaine and amphetamines (Uhl et al., 1998; Nader et al., 1999; Volkow et al., 2002; Newman et al., 2005) or the chronic pharmacotherapeutic use of L-dopa in the treatment of Parkinsonian disorders (Cenci, 2007).

Molecular genetic studies of G protein coupled receptors have defined two major types or families of dopamine receptors, the D1-like (D1 and D5 receptor subtypes) and D2-like (D2, D3 and D4 receptor subtypes) receptors based upon structural and pharmacological similarities. For example, D1-like receptors are structurally similar and positively linked to the activation of adenylyl cyclase via coupling to the Gs/Golf class of G proteins (Herve et al., 2001). Conversely, stimulation of the D2-like receptors results in coupling with the Gi/Go class of G proteins, leading to the inhibition of adenylyl cyclase activity (Sibley et al., 1993; Sealfon and Olanow, 2000; Vallone et al., 2000). In addition to the inhibition of adenylyl cyclase activity, agonist activation of D2-like receptors can lead to mitogenic activation, phospholipase D (PLD) stimulation, increased activity of G protein-regulated inwardly rectifying potassium channels (GIRK), MAP kinase activation and the activation of the Na⁺/H⁺ exchanger (Huff et al., 1998; Kuzhikandathil et al., 1998; Senogles, 2003; Neve et al., 2004).

The D2 and D3 dopamine receptor subtypes have approximately 46% amino acid homology. However, the transmembrane spanning (TMS) regions of the D2 and D3 receptors, which are thought to construct the ligand binding site, share 78% homology (Sokoloff et al., 1990). Despite the similarities in the structure, the D2 and D3 receptors differ in their a) neuroanatomical localization, b) levels of receptor expression, c) coupling efficacy (response magnitude) in response to agonist stimulation and d) regulation and desensitization (Joyce 2001; Kuzhikandathil et al., 2004).

Because of the high degree of homology between D2 and D3 receptor binding sites, it has been difficult to obtain compounds that can bind selectively to either the D2 or the D3 dopamine receptor subtypes (Newman et al., 2005; Luedtke and Mach, 2003). However, recently D2 or D3 dopamine receptor selective agonists and antagonists have been developed (Grundt et al., 2005, 2007; Chu et al., 2005; Vangveravong et al., 2006; Newman et al., 2009). It is hoped that these subtype selective compounds will be useful pharmacologic tools to precisely dissect the role of these two D2-like receptor subtypes in a

variety of experimental physiological and behavioral models, including the reinforcing and toxic properties of cocaine, socialization, memory, fine motor skills, neuropsychiatric symptoms and the regulation of interneuronal activity in the basal ganglia (Canales and Iversen, 2000; Witkin et al., 2004; Gendreau et al., 2000). However, before the *in vivo* effect of these compounds can be interpreted mechanistically, molecular pharmacological evaluation of this class of compounds is required to define their binding selectivity and intrinsic efficacy at D2-like receptors.

In this report we examine some of the pharmacologic properties of four pairs of structurally related 2,3-dichloro substituted and four pairs of 2-methoxy substituted 4-phenylpiperazines. Each of these pairs of compounds differs by the presence or absence of a *trans* olefin in the butyl linking chain. Two of the pairs also differ by small changes in the structure of the arylamide moiety. We compared the intrinsic efficacy of each pair at both D2 and D3 receptors using an adenylyl cyclase inhibition assay and a mitogenic assay. We found they had varying a) selectivity for binding at the D3 dopamine receptor compared to the D2 receptor subtypes and b) intrinsic efficacy and functional selectivity at the D3 receptor. By defining the structural elements of our D3 receptor selective ligands that confer selectivity and intrinsic efficacy we hope to lay the foundation for determining which pharmacological property/properties might be predictive of therapeutic efficacy in animal models of psychostimulant reinforcement/addiction, neuropsychiatric disorders and/or neurological disorders.

METHODS AND MATERIALS

Radioligand binding filtration assays and data analysis

A filtration binding assay was used to characterize the binding properties of 8 pairs of novel D3 dopamine receptor selective compounds at human D2 and D3 dopamine receptors. Competition curves were performed using ^{125}I -IABN (Luedtke et al., 2000) and human D2 or D3 dopamine receptors stably expressed in HEK 293 cells using the pIRESneo2 bicistronic expression vector (Clontech). The binding buffer contained 50 mM Tris-HCl, 10 mM EDTA, and 150 mM NaCl at pH 7.4. Nonspecific binding was defined using 4 μM (+)-butaclamol. For competition experiments the radioligand concentration was approximately equal to the K_d value and the concentration of the competitive inhibitor ranges over 5 orders of magnitude. Binding was terminated by the addition of cold wash buffer (10 mM Tris-HCl/150 mM NaCl, pH 7.4) and filtration over a glass-fiber filter (Schleicher and Schuell No. 32). Filters were washed with 10 ml of cold buffer and the radioactivity was quantitated. A Packard Cobra gamma counter was used for ^{125}I -labeled radioligands (efficiency = 75%). The protein concentration was determined using a BCA reagent (Pierce) with bovine serum albumin as the protein standard.

Data from competitive inhibition experiments was modeled using nonlinear regression analysis (Magar, 1972) to determine the concentration of inhibitor that inhibits 50% of the specific binding of the radioligand (IC_{50} value). Competition curves were modeled for a single site using the following equation:

$$B = \frac{B_o}{1 + (L/\text{IC}_{50})} + B_{ns}$$

where **B** is the amount of ligand bound to tissue, **B_o** is the amount of ligand bound in the absence of competitive inhibitor, **L** is the concentration of the competitive inhibitor, **B_{ns}** is the nonspecific binding of the radioligand (defined using a high concentration of a structurally dissimilar competitive inhibitor) and **IC₅₀** is the concentration of competitive

inhibitor that inhibits 50% of the total specific binding. Data from competition dose response curves were analyzed using Tablecurve program (Jandel). IC₅₀ values were converted to equilibrium dissociation constants (**K_i values**) using the Cheng and Prusoff correction (1973).

Adenylyl cyclase assays and data analysis

A whole cell cyclic AMP accumulation assay was used with stably transfected HEK cells expressing human D2 or D3 dopamine receptors. This assay is an adaptation of the original method of Shimizu, Daly and Creverling (1969) (Su et al., 1976). Confluent (60–80%) HEK cells transfected with recombinant D2-like dopamine receptors are grown in DMEM/10% fetal calf serum containing geneticin in 75 cm³ flasks. The endogenous ATP pool is radioactively labeled by preincubating the cells in serum-free DMEM/25 mM Hepes medium containing 2 µCi/ml of [2,8-³H]-adenine (20 to 50 Ci/mmol) in a 5% CO₂ incubator at 37 °C for 75 min. The radioactive medium was removed by aspiration. Serum-free DMEM (5.6 ml) containing 25 mM HEPES, pH 7.4 and 0.1mM of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) was added to the flask. A 400 µl ml aliquot of cells was removed and added to 50 µL of forskolin (100 µM) and 50 µl of test drug or quinpirole in DMEM-HEPES. The cells were incubated for 20 minutes at 37 °C (vortexing every 5 minutes). The reaction was terminated by addition of 0.5 ml of 10% trichloroacetic acid (w/v) containing 1.0 mM unlabeled cyclic AMP as a recovery standard. Both the [³H]-cyclic AMP and the [³H]-ATP fractions were collected following separation using columns of Dowex AG-50W-X4 and alumina, as described by Saloman et al. (1974). The final yield of [³H]-cyclic AMP is corrected for column recovery of unlabeled cyclic AMP determined spectrophotometrically (OD₂₅₉). The results are reported as percent conversion of [³H]-ATP into [³H]-cyclic AMP.

Mitogenic Assays

The mitogenesis analysis was performed under contracts to the Addiction Treatment Discovery Program within the Division of Pharmacotherapies and Medical Consequences of Drug Abuse (**DPMC**) of NIDA. This assay is based upon a ³H-thymidine uptake by cells that are proliferating. For their mitogenic assay, DTRD uses human D2 or D3 receptors expressed in CHO cells that are plated in 96-well plates at a density of 5,000 cells/well. After a 24 hour culture, cells are washed twice with culture medium without fetal calf serum and incubated with test drugs. Then ³H-thymidine (1 pCi/ well) is added for 2 hours and cells are harvested by vacuum filtration through Whatman GF/C fiber filters and rinsed 15 times with 200 µl of 50 mM potassium phosphate buffer (pH = 7.4) containing 150 mM NaCl. Radioactivity retained on filters is determined by liquid scintillation counting (Pilon et al., 1994).

Compounds

PG01042; *N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)butyl)-4-(pyridin-3-yl)benzamide; PG01041; (*E*)-*N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)but-2-enyl)-4-(pyridin-3-yl)benzamide; CJB 090; *N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)butyl)-4-(pyridin-2-yl)benzamide; PG01037; (*E*)-*N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)but-2-enyl)-4-(pyridin-2-yl)benzamide; PG01059; *N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)butyl)benzofuran-2-carboxamide; PG01055; (*E*)-*N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)but-2-enyl)benzofuran-2-carboxamide; PG01032; *N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)butyl)benzo[*b*]thiophene-2-carboxamide; PG01030; (*E*)-*N*-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)but-2-enyl)benzo[*b*]thiophene-2-carboxamide; PG848; *N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)-4-(pyridin-3-yl)benzamide; PG845; (*E*)-*N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)but-2-enyl)-4-(pyridin-3-

yl)benzamide; PG849; *N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)benzofuran-2-carboxamide; PG844; (*E*)-*N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)but-2-enyl)benzofuran-2-carboxamide; PG838; *N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)benzo[*b*]thiophene-2-carboxamide; PG841; (*E*)-*N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)but-2-enyl)benzo[*b*]thiophene-2-carboxamide were all synthesized, purified and characterized in the Medicinal Chemistry Section, NIDA-IRP according to literature procedures. CJB 090 was first reported in Newman et al., 2003, PG01042, PG01041, PG01037, PG01059, PG01055, PG01032 and PG01030 were first described in Grundt et al., 2005. PG849, PG844, PG838 and PG841 were first described in Newman et al., 2009. PG848 and PG 845 were designed and prepared specifically for this study and the synthetic methods used were identical to those reported for the other ligands.

RESULTS

We previously reported that subtle changes in structure of substituted phenylpiperazines leads to varying binding selectivity at D2 and D3 dopamine receptors (Grundt et al., 2005; Grundt et al., 2007). A comparison of this group of compounds is now expanded to include four pairs of structurally related 2,3-dichloro and four pairs of 2-methoxy substituted derivatives.

We previously reported that full agonists at human D2 or D4 dopamine receptor subtypes in HEK 293 cells lead to a >80% decrease in forskolin-dependent stimulation of adenylyl cyclase activity (Luedtke et al., 2000). In our initial studies with human D3 receptors expressed in HEK cells we observed very low levels of cyclase inhibition (<10%), which is consistent with previous reports (Sokoloff et al., 1990; Robinson and Caron, 1997). Over the course of our studies we identified a population of stably transfected HEK cells that express the D3 receptor at high levels and in which agonist stimulation leads to a 35–40% inhibition of forskolin-dependent stimulation of adenylyl cyclase. Composite data of dose response curves for the effect of quinpirole, a D2-like receptor selective full agonist, on our D2 and D3 dopamine receptor-expressing HEK cells is shown in Figure 1. The EC₅₀ values for quinpirole at both D2 and D3 receptors is in a nanomolar range, whereas the dissociation constant is $>100 \times 10^9$ moles/liter. This observation is likely due to the high levels of receptor expression directed by the pIRES bicistronic expression vector (Clontech). The levels of expression of D2 and D3 receptors in these transfected cells lines was determined using direct radioligand binding protocols using the high affinity antagonist ¹²⁵I-IABN (Luedtke et al., 2000). Our estimates of the affinity (K_d value) and levels of receptor expression (B_{max} values) are a) 0.04 ± 0.01 (n=8) and $57,941 \pm 14,686$ fmoles/mg protein (n = 5) for D2 and b) 0.04 ± 0.01 (n=8) and $4,202 \pm 1,516$ fmoles/mg protein (n = 5) for the D3 dopamine receptor subtypes. Therefore, it appears that <10% occupancy of the binding sites is required to achieve maximum inhibition of adenylyl cyclase.

The experimental strategy for the cyclase studies in this manuscript was to administer the test compound at a final concentration $10\times$ the K_i value (approximately 90% receptor occupancy). Quinpirole was included in each experimental set as the reference D2-like receptor full agonist (D2, 1 μ M; D3 100 nM) and haloperidol was included as a prototypic antagonist (D2, 10 nM; D3 500 nM). Each test compound was rated as the percent efficacy relative to the full agonist. In our initial control studies we consistently found that quinpirole and dopamine gave us a similar magnitude of efficacy at both D2 and D3 receptors, indicating that quinpirole was a *bona fide* full agonist (data not shown).

Each of the compounds discussed in this report were also evaluated for activity in a mitogenic assay. It had been previously reported that agonist stimulation of D2 or D3 dopamine receptors can lead to an increase in mitogenic activity (Pilon et al., 1994).

Representative dose-response curves for the mitogenic assay of 2,3-dichlorophenyl piperazines using D2 or D3 dopamine receptors are shown in Figure 2. The strategy used by DTRD is to first test for intrinsic efficacy over a range of concentrations (generally from 1×10^{-10} to 1×10^{-6} Molar). If mitogenic activity is not observed ($n = 1$) then competition experiments ($n = 2$) are performed to verify that the compound exhibits the ability to inhibit quinpirole-dependent mitogenesis. If the compound exhibits activity in the mitogenic assay, then the assay is repeated ($n = 2$) to obtain an estimate of the efficacy relative to quinpirole.

For our initial studies we focused on the 2,3-dichloro-phenylpiperazine derivatives. We used our adenylyl cyclase inhibition assay to ask if our compounds had similar or different intrinsic efficacy at D2 and D3 dopamine receptor subtypes. Figure 3 shows the rank-order of intrinsic efficacy of the eight 2,3-dichloro phenylpiperazine compounds at D2 and D3 receptors. The order of the compounds is presented based upon decreasing intrinsic efficacy (left to right) at D3 dopamine receptors. The first observation is that there are no inverse agonists in this panel of compounds. More specifically, none of the compounds enhanced forskolin-dependent stimulation of adenylyl cyclase activity. The second observation is that although these compounds are structurally related, their intrinsic efficacy at D3 receptors varies from 14% to 86%, relative to quinpirole. The third observation is that the relative rank-order-of-efficacy is not the same for D2 and D3 dopamine receptor subtypes. For example, PG01042 was found to be a strong agonist at D3 receptors but it is a weak partial agonist at D2 receptors. CJB090 appears to have equal relative efficacy at both D2 and D3 receptors (partial agonist). For five of the compounds (PG01042, PG01041, PG01059, PG01032, and PG01055) the relative magnitude of the intrinsic efficacy (percent intrinsic activity relative to the full agonist) is consistently greater for D3 receptors than for D2 receptors. Therefore, despite the high degree of structural homology between the D2 and the D3 dopamine receptor subtypes, the 2,3-dichloro compounds generally exhibited preferential subtype intrinsic efficacy.

A comparison of the intrinsic efficacy of our 2,3-dichloro-phenylpiperazine compounds at D3 receptors for the adenylyl cyclase assay and the mitogenic assay is shown in Figure 4. We found that while each of the compounds exhibited some activity in the adenylyl cyclase assay only two compounds, PG01042 and CJB090, were active as partial agonists in the mitogenic assay. While some of our compounds were partial agonists for both assays, other compounds were partial agonists in the adenylyl cyclase inhibition assay and antagonists in the mitogenic assay. PG01059 is an example of a 2,3-dichloro-phenylpiperazine that is a partial agonist for cyclase inhibition (59.5 ± 6.9 % of maximal activity) while in the mitogenesis assay it a) exhibits no intrinsic efficacy (Figure 7) and b) inhibits the activity of the full agonists quinpirole (data not shown). The majority of our 2,3-dichloro compounds exhibited functional selectivity for the D3 receptor (partial agonists in one assay but antagonists in the second assay). A comparison of the effect of these compounds at D2 receptors indicated that none of the compounds exhibited mitogenic activity, with only weak activity ($< 40\%$ relative to quinpirole) in the adenylyl cyclase inhibition assay (*data not shown*). In addition, none of the compounds were intrinsic efficacy in the mitogenic assay for D2 or D3 receptors without also being active in the cyclase inhibition assay.

We then asked if we could identify the structural elements that were contributing to the D2-like receptor selectivity and intrinsic efficacy of these compounds. In Figures 5, 6, 7, and 8 we show structures of the four pairs of 2,3-dichloro phenylpiperazines in which each pair differ only by the saturation in the 4-carbon chain (butyl vs, butenyl). For each compound we also list the a) binding affinity (K_i values) at D2 and D3 receptors, b) selectivity at D2 receptors (D2:D3 ratio), c) relative intrinsic efficacy for adenylyl cyclase inhibition at D2 and D3 receptors and d) relative intrinsic efficacy for the mitogenic assay at D2 and D3 receptors. Replacing the saturated aliphatic chain linking the heterocyclic amide with the 4-

phenylpiperazine moiety with a *trans* double (*trans*-butenyl) bond generally a) increased D3 dopamine receptor binding selectivity (with the exception of PG01055) and b) decreased intrinsic efficacy using a forskolin-dependent adenylyl cyclase inhibition and c) decreased mitogenic activity. The magnitude of the change in binding selectivity was generally about two-fold, but for the PG01059 and PG01055 pair the selectivity decreased slightly. This change in binding selectivity appears to be primarily due to a decrease in affinity at D2 receptors, although the affinity at D3 receptors was, sometimes, also affected.

For each pair of 2,3-dichloro compounds, the substitution of the *trans* olefin bond resulted in a decrease in the intrinsic efficacy at D3 receptor for the cyclase assay of approximately 50%. This substitution also resulted in a decrease of intrinsic efficacy at D2 receptors for this assay. In addition, although we did observe some activity in the mitogenic assay at D2 and D3 receptors for PG01041 and CJB090, none of the compounds containing the double bond exhibited intrinsic efficacy at D2 or D3 receptors. In summary, it appears that incorporation of the *trans*-butenyl bond into the class of 2,3-dichloro substituted 4-phenylpiperazine compounds decreases the intrinsic efficacy of the compounds at both D2 and D3 receptors.

We then examined the composition of the heterocyclic amide group. First, changing the position of the nitrogen in the 4-pyridyl-phenyl group from the 2-position to the 3-position (CBJ090 vs. PG01042) resulted in a two-fold decrease in the binding selectivity at D3 receptors (Figures 5 and 6). This change in selectivity appears to be primarily due to a decrease in D2 receptor affinity. However, that increase in selectivity was accompanied by a 2-fold decrease in intrinsic efficacy of the D3 receptor in the adenylyl cyclase assay with a slight gain in activity at the D2 receptor. There appears to be little, if any, effect on a) D2 or D3 receptor-mediated mitogenesis or b) D2 receptor-mediated cyclase inhibition. A similar pattern of change was observed for compounds containing the *trans*-butenyl double bond (PG01037 and PG01041).

Second, substituting a sulfur in the benzothiophen moiety for an oxygen of the benzofuran (PG01032 vs. PG01059) led to increased selectivity, but the magnitude of that increase depended also upon the saturation of the 4 carbon chain linking the heterocyclic group to the piperazine moiety (Figures 7 and 8). These changes in selectivity appear to be due to changes in both D2 and D3 receptor binding affinity. This structural change also resulted in increased intrinsic efficacy at adenylyl cyclase inhibition for D2 and D3 receptors, however with little or no effect at D2 dopamine receptors for the compounds that contained the *trans* olefin bond (PG01030 vs. PG01055). Whereas compounds containing the 4-pyridyl-phenyl group exhibited full to partial agonist activity with the cyclase assays (Figures 5 and 6), compounds containing the benzothiophen or the benzofuran groups (Figures 7 and 8) generally exhibited a) less cyclase activity with b) no mitogenic activity. In summary, the composition of the heterocyclic group appears to play a pivotal role in the binding selectivity and the intrinsic efficacy of the class of 2,3-dichloro substituted 4-phenylpiperazine compounds but the saturation and relative structural rigidity of the four carbon linker modulates that selectivity.

Next we evaluated an analogous four pairs of compounds, except instead of a 2,3-dichlorophenyl piperazine, these compounds contained a 2-methoxyphenyl piperazine moiety (Figure 10, 11, 12 and 13). Figure 9 shows the rank-order of intrinsic efficacy of the eight 2-methoxyphenyl piperazine compounds at D2 and D3 receptors. The order of the compounds is again presented based upon decreasing intrinsic efficacy (left to right) at D3 dopamine receptors. First, as with the 2,3-dichloro derivatives, no inverse agonists were found. Second, as with the 2,3-dichloro derivatives, the intrinsic efficacy of the 2-methoxy analogues varied at D3 receptors but the variation in activity appears less dramatic (from

34% to 77%). The third observation is that again the relative rank-order-of-efficacy is not the same for D2 and D3 dopamine receptor subtypes. For example, PG 582 has the greatest intrinsic efficacy at the D2 receptor but the least activity at the D3 receptor (Figures 9 and 11). However, as with the 2,3-dichloro compounds, the intrinsic efficacy at D3 receptors of our 2-methoxyphenyl piperazine derivatives generally appeared to be equal to or exceed the activity at D2 receptors. Therefore, both the 2,3-dichloro and the 2-methoxy derivatives generally exhibited preferential subtype intrinsic efficacy. Our fourth observation was that, in contrast to the data obtained for the 2,3-dichloro compounds, we found that the 2-methoxy compounds containing the saturated four carbon chain had approximately the same or slightly higher (rather than lower) intrinsic efficacy than compounds containing the *trans* olefin bond at D3 receptors in the cyclase assay (Figure 10, 11, 12, 13 and 14).

It was observed that all of our 2-methoxy compounds lacked mitogenic activity at D3 receptors. Therefore, by substituting the 2,3-dichlorophenyl moiety in PG01042 (Figure 5) to a 2-methoxy group (PG 848; Figure 10) a D2 and D3 receptor partial mitogenic agonist was converted to an antagonist. A similar result was observed for compounds CJB090 (Figure 6) and PG 582 (Figure 11). The composition of the substitution on the phenylpiperazine group also influences intrinsic efficacy observed in the D3 receptor mediated cyclase inhibition assay, although the effect appeared to be more profound for the compounds containing the *trans*-butenyl linker (Figure 15).

The intrinsic efficacy of the heterocyclic amide group of the 2-methoxy derivative was compared to the results obtained for the 2,3-dichloro compounds. The 4-pyridyl-phenyl group with the nitrogen at the 3-position (PG 848 and PG 845 (Figure 10)) had the greatest efficacy at D3 receptors in this group of eight compounds. This result was consistent with what we had observed for the 2,3-dichloro compounds (PG01042 and PG01041) (Figures 5 and 16A). Compounds containing the benzothiophen or the benzofuran moiety exhibited moderate to weak partial agonist activity at D3 receptors (35–49% activity) with no mitogenic activity at D2 or D3 receptors (Figures 12 and 13), which again is similar to what we observed with the 2,3-dichloro containing compounds (16–60% cyclase activity) (Figures 7, 8 and Figure 16B).

Furthermore, the incorporation of a *trans* double bond into the four carbon linking chain consistently decreased D3 versus D2 receptor binding selectivity of the compounds containing the 2-methoxy-phenyl moiety. This observation is in contrast to our findings for the panel of 2,3-dichloro-phenyl compounds, in which the incorporation of the double bond was found to increase D3 receptor selectivity. In both groups of compounds the pattern was essentially consistent. With the exception of the PG01059/PG01055 pair, substitution of a *trans* double bond in the 2,3-dichlorophenyl piperazines increased the D2:D3 affinity ratio, whereas substitution of the *trans* double bond in the 2-methoxyphenyl piperazines decreased the D2:D3 affinity ratio. When the double bond is introduced into the 2,3-dichlorophenyl piperazines, the major effect was to decrease binding affinity at the D2 receptor subtype, with little or no effect on affinity at D3 receptors. Consequently D3 receptor selectivity was increased 2- to 6-fold. However, for the 2-methoxyphenyl piperazine compounds, substitution of a *trans* double bond decreased affinity at both D2 and D3 receptors such that D3 receptor binding selectivity decreased.

We then examined the composition and intrinsic efficacy of the heterocyclic amide group of the 2-methoxy-phenyl analogues and compared them to what we had observed for the 2,3-dichloro compounds. Again, the 4-pyridyl-phenyl amide with the pyridyl nitrogen at the 3-position (PG 848 and PG 845) had the greatest efficacy at D3 receptors in this group of eight compounds (Figure 9). This result was consistent with what we had observed for the 2,3-dichloro compounds. Compounds containing the benzothiophen or the benzofuran moiety

exhibited moderate to weak partial agonist activity at D3 receptors (35–49% activity) with no mitogenic activity at D2 or D3 receptors, which is similar to what we observed with the 2,3-dichloro-substituted containing compounds (16–60% cyclase activity). Substitution of the benzothiophen group for the benzofuran generally resulted in no change or decreased intrinsic efficacy in the cyclase assay for both D2 and D3 receptors (PG 849 vs. PG 838; PG 844 vs. PG 841) (Figures 12, 13 and 16A), which again is consistent with what we observed for the 2,3-dichloro derivatives (PG01059 vs. PG01032; PG01055 vs. PG01030) (Figures 7, 8 and 16B).

DISCUSSION

This report is a comparison of the binding and functional properties of a select panel of novel chloro or methoxy substituted 4-phenylpiperazine compounds linked by a four carbon chain to an aryl amide that exhibit high affinity and binding selectivity for D3 dopamine receptors, compared to the D2 receptor subtype. Initially our focus was on determining how small structural differences in these compound might affect D2 versus D3 receptor subtype binding selectivity. We have evaluated hundreds of compounds in this class for D2-like receptor binding selectivity and we have not found an example in this class that are D2 receptor selective. Therefore, this class of compounds appears to be exploiting variations in the topography of the binding sites of these two dopamine receptor subtypes which favors D3 receptor selectivity. We recognized that the length and composition of the 2,3-dichloro and 2-methoxy substituted 4-phenylpiperazines, as well as the composition of the 4-carbon chain, are important factors in the ability of these compounds to bind differentially to D2 and D3 dopamine receptors (Newman et al., 2005, 2009; Grundt et al., 2007). In addition, these factors also influence the intrinsic efficacy. Therefore, this panel of compounds has both varying selectivity for D3 versus D2 dopamine receptor subtypes, as well as varying efficacy at the D3 dopamine receptor.

The pharmacological term efficacy is classically used to quantitatively describe the magnitude of a response produced by the binding of a ligand to a receptor, generally in comparison to the endogenous (full) agonist. The magnitude and direction of the response relative to the endogenous agonist leads to characterizing a compound as a neutral antagonist, a partial agonist, a full agonist or an inverse agonist. Contemporary models of receptor activation recognize that the energy associated with the binding reaction can result in a multiplicity of possible changes in receptor configuration, leading to activation of an intracellular second messenger pathway with varying magnitude. Kobilka and co-workers very elegantly demonstrated this multiplicity of receptor conformational changes by monitoring the fluorescence lifetimes of an environmentally sensitive fluorescent reporter molecule covalently linked to the β_2 -adrenergic receptor following administration of receptor selective a) full agonist, b) partial agonist and c) antagonist (Ghanouni et al., 2001).

Although there are now several examples of three-dimensional crystal structures of rhodopsin and other GPCRs, there are no examples of a receptor in a fully activated state. However, computational and biophysical studies have been performed to predict the structural alterations that might be associated with GPCR activation. In a computational study of agonist-induced conformational changes associated with bovine rhodopsin activation, Bhattacharya and co-workers (2008b) predicted a series of sequential steps associated with receptor activation that might be common to GPCR receptor family. Studies by Grossfield and colleagues (2008) indicate that receptor activation is accompanied by a substantial change in the internal hydration of the rhodopsin molecule. In a study of the hypothetical ligand-stabilized conformational states of the human β_2 -adrenergic receptor, the receptor conformations for the binding of five different ligands of varying intrinsic efficacy were studied, including a full agonist, a partial agonist, a weak partial agonist, an

antagonist/weak partial agonist and an inverse agonist. Each ligand was found to stabilize a slightly different receptor conformation (Bhattacharya et al., 2008a). Therefore, it would seem likely that the varying intrinsic efficacy observed for our compounds at D3 receptors is the consequence of each ligand stabilizing a slightly different conformation of the D3 dopamine receptor.

The questions then become a) what information can be gained from this comparison of the binding and intrinsic efficacy of these pairs of compounds that can be applied to the future design of D3 receptor selective substituted 4-phenylpiperazine compounds and b) can these studies provide insight into the molecular basis for the signal mechanisms associated with D2-like receptor activation? The first conclusion is that the composition, position and orientation of the electron donor and acceptor groups within the heterocyclic ring adjacent to the amide are critical for establishing the optimal interactions with amino acid side chains required for receptor activation. The differences in efficacy between PG01042 and CJB090 or between PG 848 and PG 582, which differ only in the position of the nitrogen of a 4-pyridyl-phenyl group, clearly demonstrate the importance of the aryl amide ring structure. The second conclusion is that modification of the phenylpiperazine or the four carbon chain linker can modulate intrinsic efficacy.

However, it is now known that a single receptor subtype, including the D2-like receptors, can activate multiple signaling pathways (Neve, 2004). In addition, there is evidence that ligands can have differing efficacy and/or potency for different effector pathways. While the magnitude of the efficacy of a compound is a function of the ligand-dependent change in protein conformation, the magnitude (or even potency) of a response that is being measured may differ from one messenger pathway to another, even within the same cell (Clarke, 2005; Cohen et al., 2005). For members of the G protein coupled receptor (GPCR) family each receptor state may have a different efficiency of coupling to G proteins or for the activation of non G-protein mediated signaling pathways. Therefore, ligand-dependent receptor conformations may mediate selective signaling (Kenakin, 2003; Kenakin and Onaran, 2002; Clarke, 2005; Clarke and Bond, 1998). This revision of classic receptor theory has been referred to as functional selectivity (Gay et al., 2004; Kenakin, 2003; Clarke, 2005; Urban et al., 2007). Functional selectivity has been previously reported to apply to the activation of D2 dopamine receptors (Mailman and Gay, 2004; Gay et al., 2004).

The functional selectivity of our D3 receptor selective compounds is manifested by a marked difference in activity at the adenylyl cyclase inhibition and mitogenic assays. One example of this selectivity is exhibited by compound PG01059, which is a partial agonist at D3 receptors in the adenylyl cyclase assay ($59.5 \pm 6.9\%$ relative to the full agonist quinpirole) but is both a) devoid of efficacy in the mitogenic assay (Figures 4 and 7) and b) capable of inhibiting the activity of quinpirole in this assay (data not shown). A second example is the comparison of the activity of compound CJB090, which is a partial agonist for both the cyclase and mitogenic assays, and compounds PG01059, PG01041, PG01032 and PG01055. These latter four compounds have intrinsic efficacy in the cyclase assay of equal or greater magnitude than CJB090, yet they appear to lack efficacy in the mitogenic assay (Figures 4, 5, 6, 7 and 8) with the ability to inhibit quinpirole mitogenic activity (data not shown).

Based upon these observations we are drawn to two conclusions about sequential signaling for the D3 dopamine receptor with substituted phenylpiperazines. First, the mitogenic response and the cyclase inhibition response for D3 receptor signaling can be separable (Figure 4). PG01059 and PG01041 are examples of compounds that are partial agonists for cyclase inhibition which are devoid of intrinsic efficacy for mitogenesis. Therefore, the signal for activating cyclase inhibition does not necessarily require simultaneous signaling

for mitogenesis. Second, since we only observe mitogenic signaling in the context of cyclase inhibition, the signal for mitogenesis does require simultaneous coupling to cyclase inhibition. For the 16 compounds described in this report (and other compounds that were tested), we have not found any examples of a compound that has intrinsic efficacy in the mitogenic assay while exhibiting no (or even less) activity in the cyclase inhibition assay. This second conclusion is, of course, tempered by the possible exception we may find in future studies or by the discovery of a novel structural class of D3 receptor selective compounds whose SAR properties are inconsistent with the properties of our substituted phenylpiperazines.

Compounds containing the fused, structurally rigid benzothiophen and benzofuran rings are not intrinsically active in the D2 or D3 receptor mitogenic assays. Therefore, the composition of the heterocyclic amide group appears to be pivotal for signaling selectivity. It is worth noting that the lead compound for this family of compounds, NGB 2904 (which has a fluorenyl amide, linked to a 2,3-dichloro substituted 4-phenylpiperazine via a butyl chain), is an antagonist at D3 receptors for both the cyclase inhibition assay and the mitogenic assay. The importance of the heterocyclic group in signaling is further exhibited by the fact that changing the position of a nitrogen of a 4-pyridyl-phenyl group from the 2-position to the 3-position (CBJ090 vs. PG01042) results in increased coupling to cyclase inhibition at D3 receptors, with little to no effect on the mitogenic response.

Further comparison of the structures of our compounds indicates that none of our compounds with the *trans*-butenyl component show intrinsic efficacy in the D2 or D3 receptor mitogenic assay. Since structurally related compounds containing the single bonded 4-carbon spacer are partial agonists, it appears that the saturation and resulting structural rigidity of the 4 carbon chain can affect the intensity of the signaling pathways. This ability to modulate signaling intensity is further exemplified by the observation that, for the majority of compounds, the magnitude of the adenylyl cyclase inhibition is either attenuated or enhanced with the incorporation of the *trans*-butenyl bond (Figures 5, 6, 7 and 8).

In previous studies we have found that altering the length or composition of the 4-carbon spacer can dramatically alter affinity and/or binding selectivity. For example, derivatives of substituted 4-phenylpiperazines that contain two, three or five single bonded carbon spacers have decreased affinity and/or selectivity relative to the four carbon compounds (Robarge et al., 2000). Therefore, the four carbon spacer is the critical length for the proper juxtaposition of the heterocyclic amide and the 4-phenylpiperazine groups (Newman et al., 2005; Boekler and Gmeiner, 2006). We now report that not only the length but also the composition of the 4-carbon spacer plays an important role in both receptor subtype binding selectivity and ligand intrinsic efficacy. Interestingly, incorporation of an alkyne triple bond into the 4-carbon spacer dramatically decreases binding affinity at both D2 and D3 receptors in the 2,3-dichlorophenylpiperazine class (Newman et al., 2003).

One possible explanation for the difference in intrinsic efficacy of compounds containing the aliphatic butyl chain and the *trans*-butenyl chain may be the difference in the flexibility/rigidity of the ligand. In this scenario, increased molecular rigidity due to the *trans*-double bond constrains the possible receptor conformational changes associated with ligand-dependent activation. This constraint of protein movement results in converting a) an almost full agonist to a partial agonist (PG01042 vs. PG01041 for D3 receptor inhibition of adenylyl cyclase) or b) a partial agonist to an antagonist (PG01042 vs. PG01041 for D2 and D3 receptor mitogenic activity or PG01032 vs. PG01030 for the inhibition of adenylyl cyclase). This explanation is consistent with the data obtained using the 2,3-dichloro derivatives (Figures 5 through 8) but does not account for the data obtained for the 2-

methoxy analogues since the incorporation of the *trans*-butenyl bond resulted in increased cyclase efficacy at D3 receptors (Figures 10 through 13).

An alternative explanation is that the substituted phenylpiperazine moieties might be thought of as an allosteric modulator of the aryl amide group, which would bind to the orthosteric site (or possibly vice versa). This hypothetical allosteric site would, obviously, have to be juxtaposed in close proximity to the orthosteric site. The 4-carbon chain spacer would be important for a) maintaining the proper orientation of the phenylpiperazine and the aryl amide groups and b) achieving the high affinity ligand/receptor interaction, whereas the binding of the phenylpiperazine or the aryl amide alone would likely be a low affinity interaction. The interaction of the substituted phenylpiperazine with the allosteric site might constrain the types of receptor conformational changes that could be achieved by the binding of the orthosteric group. Precedence for this mechanism of action comes from the studies by Valant and co-workers (2008) in which they propose that the muscarinic partial agonist McN-A-343 acts as a bitopic orthosteric/allosteric ligand, in which intrinsic efficacy and functional selectivity of this compound is the consequence of the covalent coupling of an allosteric moiety with an orthosteric ligand.

A third (and not mutually exclusive) possibility is that the composition of both the substituted phenylpiperazine and the 4 carbon chain play an important role in determining the positioning of the aryl amide within the neurotransmitter binding site. A slight shift or rotation of the aryl amide group within the binding site could result in the observed changes in intrinsic efficacy at D3 receptors, receptor subtype binding selectivity and/or D3 receptor functional selectivity.

Based upon our studies, we propose the following criteria for the design of substituted 4-phenylpiperazine compounds. First, if a neutral antagonist is the desired compound, then an extended aryl amide group (such as the fluorenyl group found in NGB 2904 or the benzothiophen and benzofuran rings discussed here) with a *trans*-butenyl chain and 2,3-dichlorophenyl moiety would be optimal. An antagonist of this type is generally desirable as a radiotracer for *in vivo* or *in vitro* binding studies. Second, if a D3 receptor partial agonist at cyclase inhibition but devoid of mitogenic activity is desired, then a compound with a 2-methoxy phenyl substitution would be optimal because all of these compounds exhibit low to no intrinsic efficacy in the mitogenesis assay. Third, if a D3 receptor selective compound with efficacy for both cyclase inhibition and mitogenesis is desired, then a flexible aryl amide substituted at the *para* position would be required, with a flexible 4-carbon spacer. Finally, the extended length of the substituted 4-phenylpiperazine compounds may preclude the development of compounds that are full agonists at both the cyclase inhibition and mitogenesis by preventing the receptor from achieving the conformational alterations that might be required for full agonist activity, as found in the more compact compounds such as dopamine, quinpirole or PD 128,907.

In conclusion, we anticipate that a better understanding of the parameters of structure-activity relationships for D2 and D3 dopamine receptor selective compounds could lead to a rational approach for developing receptor subtype selective and functionally selective pharmacotherapeutic agents for the treatment of neurological diseases, neuropsychiatric disorders and behavioral disorders associated with the abuse of psychostimulants. Ligand specific conformational changes would likely be responsible for the different arrays of cyclase and mitogenic signaling that we have reported in this communication (Swaminath et al., 2005; Urban et al., 2007). The substituted 4-phenylpiperazine compounds represent a unique family of synthetic dopaminergic ligands and have provided a novel strategy to investigate the activation process of D3 dopamine receptors.

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Abbreviations

DPMC	Division of Pharmacotherapies and Medical Consequences of Drug Abuse
ECL	extracellular loop
IABN	5-iodo, 2,3-dimethoxy-azabicyclo[3.3.1]nonan-3 -yl benzamide
IBMX	isobutylmethylxanyhine
ICL	intracellular loop
TMS	transmembrane spanning

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Figure 1. Normalized Dose Response Curves of the Inhibition of Forskolin-Dependent Stimulation of Adenylyl Cyclase by Quinpirole

Inhibition curves for the D2-like selective full agonist quinpirole were analyzed using a one site model with the TABLECURVE program, where the a) values for no inhibition (100% maximum stimulation) were constrained and b) values for maximal inhibition were not constrained. Individual values for the inhibition of cyclase activity are the mean $\pm$ S.E.M. with $n = 3$. IC_{50} values were 1.3 nM and 0.9 nM for D2 (, solid line) and D3 (, dashed line) receptors, respectively. The maximal inhibition observed was approximately 90% and 33% for D2 and D3 receptors, respectively. The K_i values for the low affinity state of the two D2-like receptors are also shown in the figure. Levels of expression of human D2 and D3 dopamine receptors in HEK cells are $57,941 \pm 14,686$ fmol/mg protein and $4,202 \pm 1,516$ fmol/mg protein, respectively.

Figure 2. Representative Dose-Response Curves for the Mitogenic Assay

Representative dose-response curves for the mitogenic assay using D2 (*left top and bottom panels*) or D3 (*right top and bottom panels*) dopamine receptors are shown for compounds CJB090 (*top two panels*) and PG01041 (*bottom two panels*). The curves for the test compounds (from 1×10^{-11} to 1×10^{-6} Molar) are shown as solid triangles (lower curve: $\blacktriangle$). A reference curve for the full agonist quinpirole (higher curve: $\triangle$) is also performed (from 1×10^{-10} to 1×10^{-6} Molar).

Figure 3. Comparison of the Efficacy for Adenylyl Cyclase Inhibition at Human D2L and D3 Receptors for the 2,3-Dichlorophenyl Substituted Piperazines

A comparison of the mean $\pm$ S.E.M. for the maximal intrinsic efficacy for the inhibition of adenylyl cyclase using human D2 (hatched bars) or D3 (solid bars) dopamine receptors expressed in HEK 293 cells is shown. Mean values ($n = 3$) were normalized relative to the activity observed for the full agonist quinpirole (1000 nM for D2 and 100 nM for D3). The order of the compounds is based upon the rank order of efficacy at D3 receptors.

Figure 4. Comparison of the Efficacy for Adenylyl Cyclase Inhibition and Mitogenesis at Human D3 Receptors for the 2,3-Dichlorophenyl Substituted Piperazines

A comparison of the mean $\pm$ S.E.M. for the maximal intrinsic efficacy for the inhibition of adenylyl cyclase (solid bars) and mitogenic activity (hatched bars) using human D3 dopamine receptors expressed in HEK 293 cells is shown. For both assays the mean values ($n = 3$) were normalized relative to the activity observed for the full agonist quinpirole (1000 nM for mitogenesis and 100 nM for cyclase). The order of the compounds is based upon the rank order of efficacy at D3 receptors for the adenylyl cyclase inhibition assay.

	PG01042	PG01041
D2 affinity	18.2 ± 1.8 (3)	54.5 ± 3.9 (3)
D3 affinity	0.47 ± 0.09 (3)	0.87 ± 0.18 (4)
D2:D3 ratio	39	63
% Max D2 AC inhibition	27.1 ± 3.6 (4)	16.2 ± 5.8 (3)
% Max D3 AC inhibition	85.2 ± 10.9 (5)	41.8 ± 9.7 (5)
% Max D2 Mitogenesis	28.0 (2)	0 (1)
% Max D3 Mitogenesis	27.9 (2)	0 (1)

Figure 5. Structural and Functional Comparison of the 2,3-Dichlorophenyl Substituted Piperazines PG01042 and PG01041

The affinity (K_i value: nM) for the binding to the low affinity state of human D2 and D3 dopamine receptors was obtained using competitive radioligand binding experiments with ^{125}I -IABN as the radiotracer. Maximal inhibition of AC was achieved using the test ligands at concentrations approximately $10\times$ the K_i values (PG01041, 500 nM for D2 and 10 nM for D3; PG01042, 200 nM for D2 and 10 nM for D3; quinpirole, 1000 nM for D2 and 100 nM for D3). The maximal percent inhibition (% Max) was obtained by normalizing to the mean maximal inhibition achieved using the full agonist quinpirole (*mean values in parentheses $\pm$ S.E.M.*). For this set of experiments the maximal inhibition at D2 receptors was $89.3 \pm 1.1\%$ ($n = 5$) and at D3 receptors it was $35.0 \pm 3.6\%$ ($n = 5$). The percent maximum mitogenesis is based upon dose response curves (one concentration per decade) using a maximum of 10^{-5} M test drug. The numerical values are the mean $\pm$ the S.E.M. and the number in the parentheses is the number of independent experiments (n).

	CJB090	PG01037
D2 affinity	24.8 ± 3.9 (6)	93.3 ± 6.9 (4)
D3 affinity	0.4 ± 0.12 (3)	0.7 ± 0.09 (3)
D2:D3 ratio	62	133
% D2 Max AC inhibition	38.6 ± 1.7 (4)	20.2 ± 3.5 (3)
% D3 Max AC inhibition	39.9 ± 8.5 (3)	18.0 ± 6.8 (6)
% D2 Max mitogenesis	22.4 (2)	0 (1)
% D3 Max mitogenesis	29.7 (2)	0 (1)

Figure 6. Structural and Functional Comparison of the 2,3-Dichlorophenyl Substituted Piperazines CJB090 and PG01037

The affinity (K_i value: nM) for the binding to the low affinity state of human D2 and D3 dopamine receptors was obtained using competitive radioligand binding experiments with ^{125}I -IABN as the radiotracer. Maximal inhibition of AC was achieved using the test ligands at concentrations approximately 10 $\times$ the K_i values (CJB090, 250 nM for D2 and 5 nM for D3; PG01037, 1000 nM for D2 and 10 nM for D3; quinpirole, 1000 nM for D2 and 100 nM for D3). The maximal percent inhibition (% Max) was obtained by normalizing to the mean maximal inhibition achieved using the full agonist quinpirole (*mean values in parentheses $\pm$ S.E.M.*). For this set of experiments the maximal inhibition at D2 receptors was $87.2 \pm 2.7\%$ ($n = 4$) and at D3 receptors it was $36.6 \pm 2.1\%$ ($n = 5$). The percent maximum mitogenesis is based upon dose response curves (one concentration per decade) using a maximum of 10^{-5} M test drug. The numerical values are the mean $\pm$ the S.E.M. and the number in the parentheses is the number of independent experiments (n).

	PG01059	PG01055
D2 affinity	49.4 ± 6.3 (6)	114.5 ± 34.6 (6)
D3 affinity (Ki value nM)	0.7 ± 0.15(6)	1.9 ± 0.46 (6)
D2:D3 ratio	71	60
% Max D2 AC inhibition	32.1 ± 4.4 (3)	12.9 ± 2.4 (3)
% Max D3 AC inhibition	59.5 ± 6.9 (5)	35.5 ± 8.6 (5)
% Max D2 Mitogenesis	0 (1)	0 (1)
% Max D3 Mitogenesis	0 (1)	0 (1)

Figure 7. Structural and Functional Comparison of the 2,3-Dichlorophenyl Substituted Piperazines PG01059 and PG01055

The affinity (Ki value: nM) for the binding to the low affinity state of human D2 and D3 dopamine receptors was obtained using competitive radioligand binding experiments with ¹²⁵I-IABN as the radiotracer. Maximal inhibition of AC was achieved using the test ligands at concentrations approximately 10× the Ki values (PG01059, 500 nM for D2 and 20 nM for D3; PG01055, 1000 nM for D2 and 20 nM for D3; quinpirole, 1000 nM for D2 and 100 nM for D3). The maximal percent inhibition (% Max) was obtained by normalizing to the mean maximal inhibition achieved using the full agonist quinpirole (*mean values in parentheses ± S.E.M.*). For this set of experiments the maximal inhibition at D2 receptors was 89.3 ± 1.1% (n = 4) and at D3 receptors it was 35.0 ± 3.6% (n = 5). The percent maximum mitogenesis is based upon dose response curves (one concentration per decade) using a maximum of 10⁻⁵ M test drug. The numerical values are the mean ± the S.E.M. and the number in the parentheses is the number of independent experiments (n).

	PG01032	PG01030
D2 affinity (Ki value nM)	63.0 ± 6.8 (3)	148.7 ± 5.6 (3)
D3 affinity (Ki value nM)	0.8 ± 0.15 (3)	1.1 ± 0.03 (3)
D2:D3 ratio	79	135
% Max D2 AC inhibition	20.3 ± 1.6 (3)	15.6 ± 1.5 (4)
% Max D3 AC inhibition	36.9 ± 6.8 (5)	15.8 ± 5.2 (5)
% Max D2 Mitogenesis	0 (1)	0 (1)
% Max D3 Mitogenesis	0 (1)	0 (1)

Figure 8. Structural and Functional Comparison of the 2,3-Dichlorophenyl Substituted Piperazines PG01032 and PG01030

The affinity (Ki value: nM) for the binding to the low affinity state of human D2 and D3 dopamine receptors was obtained using competitive radioligand binding experiments with ¹²⁵I-IABN as the radiotracer. Maximal inhibition of AC was achieved using the test ligands at concentrations approximately 10× the Ki values (PG01032, 500 nM for D2 and 10 nM for D3; PG01030, 200 nM for D2 and 10 nM for D3; quinpirole, 1000 nM for D2 and 100 nM for D3). The maximal percent inhibition (% Max) was obtained by normalizing to the mean maximal inhibition achieved using the full agonist quinpirole (*mean values in parentheses ± S.E.M.*). For this set of experiments the maximal inhibition at D2 receptors was 87.2 ± 2.7% (n = 4) and at D3 receptors it was 36.6 ± 2.1% (n = 5). The percent maximum mitogenesis is based upon dose response curves (one concentration per decade) using a maximum of 10⁻⁵ M test drug. The numerical values are the mean ± the S.E.M. and the number in the parentheses is the number of independent experiments (n).

Figure 9. Comparison of the Efficacy for Adenylyl Cyclase Inhibition at Human D2L and D3 Receptors for the 2-Methoxy Substituted Phenylpiperazines

A comparison of the mean $\pm$ S.E.M. for the maximal intrinsic efficacy for the inhibition of adenylyl cyclase using human D2 (hatched bars) or D3 (solid bars) dopamine receptors expressed in HEK 293 cells is shown. Mean values ($n = 3$) were normalized relative to the activity observed for the full agonist quinpirole (1000 nM for D2 and 100 nM for D3). The order of the compounds is based upon the rank order of efficacy at D3 receptors.

	PG 848	PG 845
D2 affinity	38.9 ± 2.0 (3)	87.4 ± 12.3 (4)
D3 affinity	0.60 ± 0.06 (3)	3.6 ± 0.5 (3)
D2:D3 ratio	65	24
% Max D2 AC inhibition	39.2 ± 1.5 (3)	27.1 ± 4.5 (3)
% Max D3 AC inhibition	69.9 ± 6.7 (3)	77.2 ± 10.5 (3)
% Max D2 Mitogenesis	0 (1)	0 (1)
% Max D3 Mitogenesis	0 (1)	0 (1)

Figure 10. Structural and Functional Comparison of the 2-Methoxyphenyl Substituted Piperazines PG 848 and PG 845

The affinity (K_i value: nM) for the binding to the low affinity state of human D2 and D3 dopamine receptors was obtained using competitive radioligand binding experiments with ^{125}I -IABN as the radiotracer. Maximal inhibition of AC was achieved using the test ligands at concentrations approximately $10\times$ the K_i values (PG 848, 500 nM for D2 and 10 nM for D3; PG 845, 1000 nM for D2 and 50 nM for D3; quinpirole, 1000 nM for D2 and 100 nM for D3). The maximal percent inhibition (% Max) was obtained by normalizing to the mean maximal inhibition achieved using the full agonist quinpirole (*mean values in parentheses $\pm$ S.E.M.*). For this set of experiments the maximal inhibition at D2 receptors was $90.1 \pm 1.6\%$ ($n = 7$) and at D3 receptors it was $46.5 \pm 3.6\%$ ($n = 3$). The percent maximum mitogenesis is based upon dose response curves (one concentration per decade) using a maximum of 10^{-5} M test drug. The numerical values are the mean $\pm$ the S.E.M. and the number in the parentheses is the number of independent experiments (n).

	PG 582	PG 586
D2 affinity	28.3 ± 4.5 (3)	69.0 ± 9.3 (3)
D3 affinity	0.38 ± 0.06 (3)	2.9 ± 0.8 (3)
D2:D3 ratio	$\overline{75}$	$\overline{24}$
% Max D2 AC inhibition	47.6 ± 6.8 (4)	35.6 ± 3.6 (3)
% Max D3 AC inhibition	34.1 ± 11.1 (5)	42.0 ± 10.1 (5)
% Max D2 Mitogenesis	17.4 (2)	0 (1)
% Max D3 Mitogenesis	0 (1)	0 (1)

Figure 11. Structural and Functional Comparison of the 2-Methoxyphenyl Substituted Piperazines PG 582 and PG 586

The affinity (K_i value: nM) for the binding to the low affinity state of human D2 and D3 dopamine receptors was obtained using competitive radioligand binding experiments with ^{125}I -IABN as the radiotracer. Maximal inhibition of AC was achieved using the test ligands at concentrations approximately 10x the K_i values (PG 582, 500 nM for D2 and 5 nM for D3; PG 586, 1000 nM for D2 and 50 nM for D3; quinpirole, 1000 nM for D2 and 100 nM for D3). The maximal percent inhibition (% Max) was obtained by normalizing to the mean maximal inhibition achieved using the full agonist quinpirole (*mean values in parentheses* $\pm$ S.E.M.). For this set of experiments the maximal inhibition at D2 receptors was $90.1 \pm 1.6\%$ ($n = 7$) and at D3 receptors it was $40.5 \pm 4.4\%$ ($n = 3$). The percent maximum mitogenesis is based upon dose response curves (one concentration per decade) using a maximum of 10^{-5} M test drug. The numerical values are the mean $\pm$ the S.E.M, and the number in the parentheses is the number of independent experiments (n).

	PG 849	PG 844
D2 affinity	92.5 ± 7.1 (3)	78.8 ± 2.2 (3)
D3 affinity	3.8 ± 0.7 (3)	11.3 ± 1.4 (3)
D2:D3 ratio	24	7
% Max D2 AC inhibition	25.7 ± 4.7 (3)	28.4 ± 4.4 (3)
% Max D3 AC inhibition	34.8 ± 6.9 (3)	49.0 ± 6.0 (3)
% Max D2 Mitogenesis	0 (1)	0 (1)
% Max D3 Mitogenesis	0 (1)	0 (1)

Figure 12. Structural and Functional Comparison of the 2-Methoxyphenyl Substituted Piperazines PG 849 and PG 844

The affinity (K_i value: nM) for the binding to the low affinity state of human D2 and D3 dopamine receptors was obtained using competitive radioligand binding experiments with ^{125}I -IABN as the radiotracer. Maximal inhibition of AC was achieved using the test ligands at concentrations approximately 10 $\times$ the K_i values (PG 849, 1000 nM for D2 and 50 nM for D3; PG 844, 1000 nM for D2 and 200 nM for D3; quinpirole, 1000 nM for D2 and 100 nM for D3). The maximal percent inhibition (% Max) was obtained by normalizing to the mean maximal inhibition achieved using the full agonist quinpirole (*mean values in parentheses $\pm$ S.E.M.*). For this set of experiments the maximal inhibition at D2 receptors was $90.1 \pm 1.6\%$ ($n = 7$) and at D3 receptors it was $46.5 \pm 3.6\%$ ($n = 3$). The percent maximum mitogenesis is based upon dose response curves (one concentration per decade) using a maximum of 10^{-5} M test drug. The numerical values are the mean $\pm$ the S.E.M. and the number in the parentheses is the number of independent experiments (n).

	PG 838	PG 841
D2 affinity	37.5 ± 3.0 (3)	59.8 ± 7.0 (3)
D3 affinity	0.45 ± 0.09 (4)	2.6 ± 0.29 (3)
D2:D3 ratio	$\frac{83}{23}$	$\frac{23}{23}$
% Max D2 AC inhibition	20.8 ± 2.3 (3)	14.1 ± 2.5 (3)
% Max D3 AC inhibition	35.3 ± 3.4 (3)	40.2 ± 5.8 (3)
% Max D2 Mitogenesis	0 (1)	0 (1)
% Max D3 Mitogenesis	0 (1)	0 (1)

Figure 13. Structural and Functional Comparison of the 2-Methoxyphenyl Substituted Piperazines PG 838 and PG 841

The affinity (K_i value: nM) for the binding to the low affinity state of human D2 and D3 dopamine receptors was obtained using competitive radioligand binding experiments with ^{125}I -IABN as the radiotracer. Maximal inhibition of AC was achieved using the test ligands at concentrations approximately $10\times$ the K_i values (PG 838, 500 nM for D2 and 5 nM for D3; PG 841, 1000 nM for D2 and 50 nM for D3; quinpirole, 1000 nM for D2 and 100 nM for D3). For this set of experiments the maximal inhibition at D2 receptors was $90.1 \pm 1.6\%$ ($n = 7$) and at D3 receptors it was $46.5 \pm 3.6\%$ ($n = 3$). The percent maximum mitogenesis is based upon dose response curves (one concentration per decade) using a maximum of 10^{-5} M test drug. The numerical values are the mean $\pm$ the S.E.M. and the number in the parentheses is the number of independent experiments (n).

Figure 14. Comparison of the Effect of the Single Bond and the *Trans*-butenyl Double Bond Four Carbon Chain Linker on D3 Adenylyl Cyclase Intrinsic Efficacy

A comparison of the mean $\pm$ S.E.M. for the maximal intrinsic efficacy for the inhibition of adenylyl cyclase of compounds containing either the saturated 4 carbon chain (**solid bars**) or the *trans*-butenyl (**hatched bars**) chain using human D3 dopamine receptors expressed in HEK 293 cells is shown. Mean values ($n = 3$) were normalized relative to the activity observed for the full agonist quinpirole (100 nM). The comparison is presented for both the 2,3-dichloro (*left portion*) and the 2-methoxy substituted phenylpiperazines (*right portion*).

Figure 15. Comparison of the Effect of the 2,3-Dichloro and 2-Methoxyphenyl Substitution on D3 Adenylyl Cyclase Intrinsic Efficacy

A comparison of the mean $\pm$ S.E.M. for the maximal intrinsic efficacy for the inhibition of adenylyl cyclase of compounds containing either the 2,3-dichloro (**solid bars**) or the 2-methoxy substituted phenylpiperazines (**hatched bars**) using human D3 dopamine receptors expressed in HEK 293 cells is shown. Mean values ($n = 3$) were normalized relative to the activity observed for the full agonist quinpirole (100 nM). The comparison is presented for both the saturated 4 carbon chain (*left portion*) or the *trans*-butylene (*right portion*) chain that links the substituted phenylpiperazines with the aryl amide.

A. 2- versus 3-phenylpyridine

B. Benzofuran versus benzothiophen

Figure 16. Comparison of the Effect of the Heterocyclic Amide on D3 Adenylyl Cyclase Intrinsic Efficacy

A comparison of the mean $\pm$ S.E.M. for the maximal intrinsic efficacy for the inhibition of adenylyl cyclase of compounds containing the different heterocyclic groups. (A) A comparison of the effect on intrinsic efficacy of the 2-phenylpyridine (**hatched bars**) and the 3-phenylpyridine (**solid bars**) groups is shown for the saturated 4 carbon chain (*left portion*) or the *trans*-butenyl (*right portion*) chain that links the substituted phenylpiperazines with the heterocyclic amide is shown. (B) A comparison of the effect on intrinsic efficacy of the benzofuran (**solid bars**) and the benzothiophen (**hatched bars**) groups is shown for the saturated 4 carbon chain (*left portion*) or the *trans*- butenyl (*right portion*) chain that links the substituted phenylpiperazines with the heterocyclic amide is shown.