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Investigation of the binding and functional properties of extended length D₃ dopamine receptor-selective antagonists[☆]

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Abstract

The D₃ dopamine receptor represents an important target in drug addiction in that reducing receptor activity may attenuate the self-administration of drugs and/or disrupt drug or cue-induced relapse. Medicinal chemistry efforts have led to the development of D₃ preferring antagonists and partial agonists that are >100-fold selective vs. the closely related D₂ receptor, as best exemplified by extended-length 4-phenylpiperazine derivatives. Based on the D₃ receptor crystal structure, these molecules are known to dock to two sites on the receptor where the 4-phenylpiperazine moiety binds to the orthosteric site and an extended aryl amide moiety docks to a secondary binding pocket. The bivalent nature of the receptor binding of these compounds is believed to contribute to their D₃ selectivity. In this study, we examined if such compounds might also be “bitopic” such that their aryl amide moieties act as allosteric modulators to further enhance the affinities of the full-length molecules for the receptor. First, we deconstructed several extended-length D₃-selective ligands into fragments, termed “synthons”, representing either orthosteric or secondary aryl amide pharmacophores and investigated their effects on D₃ receptor binding and function. The orthosteric synthons were found to inhibit radioligand binding and to antagonize

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Contributors

Cheryse A. Furman, Rebecca A. Roof, Amy E. Moritz, Brittney N. Miller, and Trevor B. Doyle conducted experiments, performed data analysis, and participated in the research design. R. Benjamin Free participated in the research design, performed data analysis, and assisted in writing the manuscript. Ashwini K. Banala, Noel M. Paul, Vivek Kumar, and Christopher D. Sibley synthesized novel compounds and contributed new reagents. David R. Sibley and Amy Hauck Newman oversaw the research project, contributed to research design, and contributed to the writing of the manuscript. All authors contributed to and have approved the final manuscript.

Conflicts of interest

All authors report no conflicts of interest in this study.

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dopamine activation of the D₃ receptor, albeit with lower affinities than the full-length compounds. Notably, the aryl amide-based synthons had no effect on the affinities or potencies of the orthosteric synthons, nor did they have any effect on receptor activation by dopamine. Additionally, pharmacological investigation of the full-length D₃-selective antagonists revealed that these compounds interacted with the D₃ receptor in a purely competitive manner. Our data further support that the 4-phenylpiperazine D₃-selective antagonists are bivalent and that their enhanced affinity for the D₃ receptor is due to binding at both the orthosteric site as well as a secondary binding pocket. Importantly, however, their interactions at the secondary site do not allosterically modulate their binding to the orthosteric site.

Keywords

Dopamine receptor; D₃ receptor; Bivalent; Bitopic; Allosteric; Dopamine antagonists

1. Introduction

Dopamine receptors are a therapeutically relevant subclass of GPCRs that mediate the actions of dopamine, a critically important transmitter in both the central nervous system (CNS) and the periphery. In the CNS, dopamine regulates movement and locomotion, reward and motivation, and also cognition and working memory (Beaulieu and Gainetdinov, 2011). Dysfunctions of CNS dopaminergic systems are involved in the etiology of a number of neuropsychiatric disorders including Parkinson's disease, schizophrenia, and addiction/drug abuse (Beaulieu and Gainetdinov, 2011). The therapeutic link between these seemingly disparate disorders is that they are treated with drugs that either stimulate or block central dopamine receptors (DARs). Five distinct DAR subtypes have been identified and characterized and are divided into two subfamilies based on their structure, pharmacology, and signaling properties (Beaulieu and Gainetdinov, 2011; Missale et al., 1998; Sibley and Monsma, 1992). The D₁-like DARs (D₁ and D₅) are coupled to G_{αs/olf} proteins and activate adenylyl cyclase, thus increasing intracellular cAMP levels. In contrast, the D₂-like DARs (D₂, D₃ and D₄) are coupled to G_{αi/o} proteins and function to inhibit adenylyl cyclase, thus diminishing cAMP levels. As these receptor subtypes have discrete locations in the brain and distinct functions, there is great interest in developing therapeutic agents that selectively target them (Newman et al., 2012b).

Most FDA-approved therapeutics targeting DARs are directed against the D₂ subtype, however, there is reason to believe that the D₃ subtype may also represent an important target for the treatment of a number of neuropsychiatric disorders (Heidbreder and Newman, 2010; Lober et al., 2011; Micheli and Heidbreder, 2013). One of the most promising therapeutic applications for antagonists and partial agonists of the D₃ receptor is in the area of addiction and substance use disorders (Heidbreder and Newman, 2010; Micheli and Heidbreder, 2013; Newman et al., 2012b). Accumulating evidence suggests that reducing D₃ receptor activity may regulate the motivation to self-administer drugs and disrupt drug-associated cue-induced craving and relapse. Further, D₃ receptor blockade may prevent the reinstatement of drug taking, including that of cocaine, methamphetamine, nicotine, and alcohol (Heidbreder and Newman, 2010; Micheli and Heidbreder, 2013; Newman et al.,

2012b; Ross and Peselow, 2009). Antagonism of the D₃ receptor may also be therapeutic in the treatment of schizophrenia or psychosis (Lober et al., 2011). Notably, while all antipsychotic drugs block the D₂ subtype, they also block the D₃ subtype to various degrees (Joyce and Millan, 2005). Interestingly, D₃ receptor antagonism has also been suggested to be highly beneficial in the treatment of certain motor/movement disorders such as L-DOPA-induced dyskinesias, which typically arise during late-stage Parkinson's disease treatment (Kumar et al., 2009; Riddle et al., 2011). Extensive efforts have thus been undertaken towards developing highly selective antagonists and partial agonists of the D₃ receptor for further exploration into therapeutic utility.

The discovery and development of D₃-selective ligands as potential medications was initiated in the late 1990s with the introduction of NGB 2904, BP 897, and SB277011A, followed by an explosion of medicinal chemistry and structure-activity relationship (SAR) information (reviewed in (Boeckler and Gmeiner, 2006; Keck et al., 2014; Lober et al., 2011; Micheli and Heidbreder, 2013)). Through extensive investigation it became apparent that an extended-length molecule that included a tertiary amine on one end and an extended aryl or alkyl amide on the other, linked together with a four atom linking chain was required for the 4-phenylpiperazine class of D₃-selective ligands; one of the most studied. Modifications to this general SAR have led to many high affinity ($K_i < 1$ nM) and D₃-selective (>100-fold vs. the D₂ receptor) molecules that are suitable for in vivo studies. Recently, one of these compounds (GSK 598809) has reached the clinical investigation stage and is being evaluated for smoking cessation (Mugnaini et al., 2013).

Extended-length ligands have the possibility of being bivalent, where each end of the molecule can function as a pharmacophore and bind to separate sites on the same or closely adjacent proteins (Lane et al., 2013b; Valant et al., 2009, 2012). If the pharmacophores are identical, the ligand is homobivalent whereas if they differ, the ligand is described as being heterobivalent. The development of bivalent ligands has proven to be of particular interest as a means of improving the selectivity and/or affinity of ligands for GPCRs as well as an approach to modulate and bias their efficacy (Lane et al., 2013b; Valant et al., 2014). Interestingly, since many GPCRs possess allosteric binding sites that regulate their functional properties (Conn et al., 2009; Lane et al., 2013a; Wootten et al., 2013), the possibility exists for a bivalent ligand to possess both allosteric and orthosteric pharmacophores that concomitantly engage the receptor protein. Such bivalent ligands have been described and termed "bitopic" (Valant et al., 2012, 2009) in recognition of their dualistic nature. In the case of bitopic ligands, the allosteric pharmacophore may modulate the receptor affinity, functional efficacy, or signaling bias of the orthosteric pharmacophore of the molecule (Valant et al., 2009, 2012, 2014).

With the crystallization of the D₃ receptor a high-resolution structure was identified revealing important features of the orthosteric binding pocket and extracellular loops (Chien et al., 2010). Interestingly, docking of an extended-length D₃-selective antagonist, R-22 (also termed PG 648, Fig. 1), confirmed that the 2,3-dichloro-4-phenylpiperazine moiety was the primary pharmacophore that interacted with the orthosteric binding site. In addition, molecular dynamics studies revealed a secondary binding pocket for the aryl amide moiety of R-22 in the D₃ receptor, whereas an equivalent pocket is absent in a structural model of

the D₂ receptor (Michino et al., 2013). These data suggest that extended-length D₃-selective antagonists interact with the receptor in a bivalent binding mode, which may account for their D₃ selectivity versus the D₂ receptor. However, it has also been hypothesized that the secondary binding site on the D₃ receptor may be allosteric and that D₃-selective antagonists like R-22 are bitopic in nature (Chien et al., 2010; Newman et al., 2012a). In this model of bitopic-promoted selectivity, the allosteric pharmacophore in the extended-length ligand may act as a positive allosteric modulator to enhance the affinity of the orthosteric pharmacophore for the receptor. The result would be enhanced affinity and selectivity of the full-length ligand for the D₃ versus the D₂ receptor. In the current study, we evaluate this “bitopic” hypothesis by deconstructing several extended-length D₃ receptor selective ligands into smaller “synthons” representing primary (orthosteric) or secondary pharmacophores and investigating their effects on D₃ binding and function. We also investigate several full-length D₃ selective antagonists for potential bitopic/allosteric properties using different pharmacological approaches.

2. Experimental procedures

2.1. Materials

[³H]-methylspiperone (85.5Ci/mmol) was obtained from PerkinElmer Life Sciences (Waltham, MA). CHO-K1 D₃ cells and CP2 media were purchased from DiscoverX (Fremont, CA). Other cell culture media and reagents were purchased from MediaTech/Cellgro (Manassas, VA). Cell culture flasks and materials and all assay plates were purchased from Greiner Bio-One (Monroe, NC). Dopamine and sulpiride were purchased from Tocris Bioscience/RD Systems (Minneapolis, MN). Batches of SB 269652 were gifts from Dr. Jonathan A. Javitch and Arthur Christopoulos. All other compounds and buffer components were purchased from Sigma-Aldrich (St Louis, MO), except where indicated. All synthons and full length D₃-selective ligands were synthesized using published procedures or modifications thereof (Grundt et al., 2005, 2007; Newman et al., 2009, 2012a, 2003) and were characterized as being >95% pure.

2.2. Cell culture and transfection

CHO-K1 cells expressing hD₃ receptor (DiscoverX) were maintained according to the DiscoverX protocol in F12 media supplemented with 10% FBS, 50 U/ml penicillin/50 µg/ml streptomycin, and antibiotics for D₃ receptor and β-galactosidase expression maintenance. Cells were grown at 37 °C in 5% CO₂ and 90% humidity.

2.3. Radioligand binding assays

The D₃ receptor-expressing CHO-K1 cell line that was used for the β-arrestin interaction assays (see below) was also used for radioligand binding assays. Membrane binding assays were performed as described previously (Chun et al., 2013). Briefly, cells were harvested by incubation with 5 mM EDTA in EBSS lacking CaCl₂ and MgSO₄ and collected by centrifugation at 300 × g for 10 min. The cells were resuspended in lysis buffer (5 mM Tris, pH 7.4 and 5 mM MgCl₂) at 4 °C and were disrupted using a dounce homogenizer followed by centrifugation at 34,000 × g for 15 min. The resulting membrane pellet was resuspended in binding buffer (50 mM Tris, pH 7.4) and 100 µl of the membrane suspension was added

to assay tubes to initiate the reaction. For non-specific binding, 3 μM (+)-butaclamol was added to appropriate tubes. For all competition assays, K_i values were calculated from observed IC_{50} values using the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

2.4. β -Arrestin recruitment assays

The β -arrestin recruitment assay (DiscoverX) was performed as previously described (Banala et al., 2011; Bergman et al., 2013) with minor changes. Briefly, CHO-K1 cells expressing the D_3 dopamine receptor were seeded into 384-well clear bottom plates using CP2 media (DiscoverX) 24 h prior to the assay. Concentration response curves of various compounds were generated using an Eppendorf epMotion 5070 robot. HBSS containing 0.2 mM sodium metabisulfite was used as the buffer. Multiple and/or single concentrations of the indicated drug(s) were added to cells, followed by further addition of buffer or an EC_{95} dose of DA, and incubated for 90 min at 37 °C. DiscoverX reagent was then added to cells followed by incubation for 60 min at room temperature. Luminescence was measured on a Hamamatsu FDSS μ -cell plate reader. Exposure time ranged from 1 to 5 s. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

2.5. Data analysis

Data are expressed either as a percentage of control values or as raw measurement values as indicated in the figures and legends. For binding and functional dose-response experiments, non-linear regression analyses were conducted to generate IC_{50} or EC_{50} values, and results are expressed as $\text{mean} \pm \text{S.E.M}$ as indicated in the figure legends. Comparison of EC_{50} or IC_{50} values was performed using Student's t -test with a significance of $p < 0.05$, as indicated. For radioligand competition binding assays, K_i values were calculated from observed IC_{50} values using the Cheng–Prusoff equation (Cheng and Prusoff, 1973), and significant differences between test and control values were determined using Student's t -test with a significance of $p < 0.05$. For saturation binding isotherms, data were fit to a non-linear saturation analysis and K_D and B_{max} values were subsequently determined using a linear regression of a Scatchard transformation of the data. For functional curve shift experiments, individual curves were first analyzed by non-linear regression and the data then transformed via Schild analyses to generate Hill coefficients via linear regression of Schild plots. Hill coefficients from multiple experiments were then reported as $\text{mean} \pm \text{S.E.M}$. All regressions and statistical analysis were conducted using GraphPad Prism 6.0d (GraphPad Software, Inc., La Jolla, CA).

3. Results

A series of previously described (Keck et al., 2014; Lober et al., 2011; Micheli and Heidbreder, 2013) D_3 receptor-selective antagonists, or low-efficacy partial agonists of the 4-phenylpiperazine class of molecules used in the present study are shown in Fig. 1A. It has been previously shown that the 4-phenylpiperazine moiety binds to the orthosteric binding site of the receptor (Chien et al., 2010; Michino et al., 2013; Newman et al., 2012a). The terminal aryl amide is linked to the 4-phenylpiperazine by a 4-carbon linker and serves as a secondary pharmacophore that, in the case of PG 648, has been shown to bind to a

secondary binding site on the D₃ receptor (Chien et al., 2010; Michino et al., 2013; Newman et al., 2012a).

Our initial approach to testing the hypothesis that these extended-length ligands might exhibit bitopic properties was to first deconstruct them into smaller synthetic “synthons” that represent putatively functional pieces (pharmacophores) of the full-length molecules. For the sake of simplicity, the synthons that are believed to dock to the orthosteric site will be referred to as “primary” or orthosteric synthons while the synthons that are believed to dock to the secondary binding site will be referred to as “secondary” pharmacophores or synthons.

Fig. 1B, right, shows several “orthosteric” synthons that possess the core substituted 4-phenylpiperazine moiety. When evaluated in radioligand binding assays (Table 1) all of these compounds were indeed found to inhibit binding of the orthosteric antagonist [³H]methyspiperone, albeit with much lower affinities than those observed with the full-length structures. Notably, while a number of orthosteric synthons were synthesized, the ones in Table 1 were selected for their structural diversity and for their relatively low receptor affinity (see Discussion). We also synthesized several diverse arylamide synthons corresponding to the non-orthosteric secondary pharmacophores of the full-length molecules, which would predictably dock to the secondary binding site of the D₃ receptor (Fig. 1B, left). When we evaluated these putatively “allosteric” synthons using radioligand binding assays, none of them appeared to exhibit any ability to affect [³H]methyspiperone binding to the orthosteric site (Table 1).

We next attempted “reconstitution” experiments (Fig. 2) where we took each orthosteric synthon and performed radioligand competition binding assays in the absence or presence of each of the secondary synthons. In the bitopic hypothesis being tested, the allosteric or secondary pharmacophore functions as a positive allosteric modulator to enhance the affinity of the orthosteric or primary pharmacophore, resulting in improved affinity of the full-length ligand for the D₃ receptor. If this mode of binding is correct, and contributes to the high affinity and selectivity of the ligand for the D₃ receptor, then we might see an *increase* in the receptor affinity of an orthosteric synthon through the addition of a secondary synthon to the binding assay. Fig. 2A–E shows full competition curves for each orthosteric synthon in the presence of a high concentration (50 μM) of each secondary synthon. Average inhibitory *K_i* values for each synthon combination are also shown in Table 2. Notably, none of the secondary synthons were found to increase the affinities of the orthosteric synthons for the receptor. In fact, there were no significant effects on any of the orthosteric synthon affinities in the radioligand binding assay. Our conclusion is that the secondary synthons have no observable effects on the binding of the orthosteric synthons to the D₃ receptor.

We also noted that when employed at 50 μM in the competition curve analyses (Fig. 2A–E), the secondary synthon BAK 2-50 consistently appeared to inhibit [³H]methyl-spiperone binding to a small degree. We investigated this further in Fig. 2F where we did a BAK 2-50 radioligand binding competition curve up to 50 μM (higher than previously performed in Table 1). Indeed, at concentrations >10 μM, BAK 2-50 was found to exert a small inhibitory

effect on [3H] methylspiperone binding. It is not clear if this effect is non-specific in nature (see below).

We next performed a similar reconstitution experiment as in Fig. 2, but in this case used a functional assay rather than a binding assay. While there is a paucity of cell-based functional assays for the D₃ receptor, agonist-stimulated β -arrestin recruitment has proven to be quite robust (Banala et al., 2011; Bergman et al., 2013). Preliminary experiments revealed that all of our orthosteric synthons behaved as antagonists in our functional β -arrestin recruitment assay for the D₃ receptor (data not shown). We thus chose two orthosteric synthons, BAK 2-46 and BAK 3-44, and evaluated their ability to antagonize dopamine-stimulated β -arrestin recruitment in the absence or presence of the secondary synthon BAK 2-50. According to the bitopic hypothesis, the secondary synthon/pharmacophore BAK 2-50 might increase the potency of the orthosteric synthons BAK 2-46 or BAK 3-44 for inhibiting the dopamine-stimulated response.

Fig. 3A shows concentration response curves for the orthosteric synthon BAK 2-46 as well as the orthosteric antagonist sulpiride for inhibiting dopamine-stimulated β -arrestin recruitment in the absence or presence of the secondary synthon BAK 2-50. As can be seen, BAK 2-50 has no effect on the functional potency of the orthosteric synthon BAK 2-46 or of sulpiride. Similar results were observed using BAK 2-50 and the orthosteric synthon BAK 3-44 (Fig. 3B). Thus, using a functional assay, the potency for inhibiting the D₃ receptor by orthosteric synthons appears not to be influenced by the addition of a synthon representing the secondary pharmacophore.

We were next interested in seeing if any of the secondary, potentially allosteric synthons exhibited any direct functional effects on *dopamine* signaling at the D₃ receptor. Fig. 4 shows dopamine concentration response curves for stimulating β -arrestin recruitment to the D₃ receptor in the absence or presence of a high concentration of each of the secondary synthons. Clearly, none of these synthons had any effect on either the potency or efficacy of the dopamine response. Thus, none of the synthons used to mimic the secondary pharmacophores of the full-length D₃-selective antagonists appear to exhibit allosteric-like properties at the D₃ receptor.

We next examined the full-length D₃-selective compounds to see if they exhibited any pharmacological properties other than those of competitive ligands (i.e., bitopic/allosteric). Fig. 5 shows radioligand binding competition curve analyses using five of these compounds shown in Fig. 1, along with the reference competitive antagonist sulpiride. Notably, all of the D₃-selective ligands uniformly and completely inhibit [3H] methylspiperone binding to the orthosteric site, as does sulpiride. In some instances, bitopic or allosteric ligands have been demonstrated to exhibit complex competition curves or to only partially inhibit receptor-binding activity (May et al., 2007; Valant et al., 2014), but this was not observed with the D₃-selective ligands tested.

In Fig. 6 we further investigated the interactions of two of the full-length D₃-selective ligands using saturation radioligand binding assays. In this case, we constructed [3H]methylspiperone saturation binding curves in the absence or presence of GCC 3-05, PG

571, or sulpiride using concentrations that would partially inhibit [3H]methyspiperone binding. Fig. 6A shows the untransformed saturation binding data from such an experiment whereas Fig. 6B shows the data plotted in Scatchard coordinates in order to linearize the binding curve data. Using this transformation, the inverse slope of the line represents the apparent K_d whereas the x-axis intercept represents the apparent B_{max} (maximum binding capacity) for the radioligand. As can be seen, the addition of the D₃-selective compounds, and sulpiride, greatly reduced (by 4–10-fold) the apparent K_d of [3H]methyspiperone for the receptor while hardly affecting the B_{max} (5–10% increase). These results suggest that the compounds are acting as competitive antagonists at the D₃ receptor. A non-competitive antagonist or negative allosteric modulator of the receptor might be expected to, at least partially, inhibit binding through decreasing the B_{max} of the radioligand (May et al., 2007).

We next evaluated two of the full-length D₃-selective ligands using Schild (curve-shift) analyses (Christopoulos and Kenakin, 2002; May et al., 2007). In these experiments, we used the β -arrestin recruitment assay with dopamine as the stimulating agonist (Fig. 7). Dopamine dose-response curves were performed in the absence of any additional drug and then again with increasing concentrations of either sulpiride (Fig. 7A), GCC 3-05 (Fig. 7B), or PG 571 (Fig. 7C). The addition of each of the antagonists appeared to promote a parallel shift to the right of the dopamine dose-response curves thus decreasing the apparent potency of the agonist without affecting the efficacy, as would be expected for a competitive antagonist (Christopoulos and Kenakin, 2002; May et al., 2007). Schild transformations (insets) of the data resulted in linear plots with slopes not significantly different from unity, again as expected for competitive antagonists. These results additionally confirm that these extended-length ligands antagonize the D₃ receptor in a purely competitive fashion.

Recently, Silvano et al. (2010) have described an extended-length D₃/D₂ antagonist, SB269,652 (Fig. 8A), that is D₃-preferring and reported to exhibit some properties of a negative allosteric modulator. Interestingly, the aryl amide end of the molecule (Fig. 8A) is identical to that found in the extended-length D₃ receptor-selective antagonists PG 648 and PG 571 (and similar to that in GCC 3-05) as well as the BAK 2-50 synthon (cf. Fig. 1). Given this, we decided to characterize the pharmacological properties of SB269,652 in our systems. Fig. 8B shows competition curves for radioligand binding to the D₃ receptor. As with the 4-phenylpiperazine antagonists (Fig. 5), SB269,652 is able to fully and uniformly compete for [3H] methyspiperone binding to the D₃ receptor. We next performed competitive radioligand binding saturation assays as described in Fig. 6. Similar to the results obtained with the 4-phenylpiperazine antagonists, the addition of SB269,652 to a [3H] methyspiperone saturation binding assay decreased the apparent affinity of the radioligand without affecting its maximum binding capacity to the D₃ receptor. These results suggest that SB269,652 inhibits [3H]methyspiperone binding to the D₃ receptor in a competitive fashion Fig. 9.

In Fig. 10, we evaluated the ability of SB269,652 to functionally inhibit dopamine-stimulated β -arrestin recruitment to the D₃ receptor. SB269,652 potently and fully inhibited this response exhibiting an affinity similar to that of the reference antagonist sulpiride. Given these results, we decided to perform a curve-shift/Schild-type analysis using SB269,652 and the β -arrestin recruitment assay (Fig. 11). As seen previously (Fig. 7),

sulpiride behaved as a competitive antagonist by promoting parallel and proportional shifts in the dopamine concentration response curves which were dose-dependent with the sulpiride concentrations (Fig. 11A). This resulted in a linear Schild plot with a slope of approximate unity (Fig. 11A inset). In contrast, while the addition of SB269,652 also promoted a shift in the dopamine concentration response curve, this effect was not proportional to the dose of SB269,652 and had a limited inhibitory effect (Fig. 11B). The resulting Schild plot was curvilinear (Fig. 11B inset) suggesting that the inhibition of the dopamine response by SB269,652 was not completely competitive in nature and, indeed, saturable (Keov et al., 2011). These results appear to replicate those of Silvano et al., 2010 using SB269,652, albeit with a different functional assay, and suggest that SB269,652 may have negative allosteric effects on the D₃ receptor (Keov et al., 2011).

Notably, SB269,652 has a structural similarity to the other extended-length antagonist compounds tested in this study (cf. Figs. 1 and 8), including possessing an aryl amide moiety at the end of the molecule that may dock to the secondary binding site of the D₃ receptor. This structure in SB269,652 is similar, but not identical to that found in the secondary synthon BAK 2-50, which, importantly, did not appear to possess allosteric properties (Fig. 2A–E, Figs. 3 and 4), but did appear to modestly inhibit [³H]methylspiperone binding at high concentrations (Fig. 2F). The latter effect could possibly be due to weak activity as a negative allosteric modulator. In order to investigate this further, we synthesized the synthon, CS 01-12 (Fig. 12A), corresponding to an aryl amide structure found in both SB269,652 (Fig. 8) and BAK 2-50 (Fig. 1).

We examined the ability of CS 01-12 to exert functional effects, potentially allosteric, on the D₃ receptor in two ways. First, we tested the ability of CS 01-12 to inhibit [³H]methylspiperone binding to the receptor. This could occur through blockade of the orthosteric binding site or through negative allosteric interactions at a secondary binding site. Using competition binding analysis, however, Fig. 12B shows that CS 01-12 has no effect on [³H]methylspiperone binding to the D₃ receptor. Second, we tested CS 01-12 for its effects on dopamine-stimulated β -arrestin recruitment to the D₃ receptor. Fig. 12B shows dopamine dose-response curves for stimulating β -arrestin recruitment in the absence or presence of a high concentration of CS 01-12. Clearly, CS 01-12 was without effect on modulating this functional activity, thus replicating the results observed with the related synthon BAK 2-50 (cf. Fig. 4). Thus, the aryl amide moiety of SB269,652 *per se* appears to lack allosteric effects at the D₃ receptor.

4. Discussion

The main goal of this investigation was to investigate the binding of selected high affinity, extended-length D₃ receptor-selective antagonists to see if the nature of their interactions with the D₃ receptor might be bitopic in nature. It has previously been shown, using the crystal structure of the D₃ receptor, and molecular docking analyses, that extended-length D₃ receptor antagonists dock to two sites on the receptor. One is the orthosteric site, where dopamine binds to activate the receptor. The other is a secondary binding site consisting of extracellular loops 1 and 2, and the junctions of transmembrane helices I, II, III, and VII (Chien et al., 2010; Michino et al., 2013; Newman et al., 2012a). This secondary binding

pocket is structurally unique to the D₃ receptor generating the hypothesis that the basis for the D₃ selectivity of these compounds is their bivalent mode of binding to the receptor. It has been further hypothesized, however, that these compounds might also possess bitopic properties such that the binding of the non-orthosteric pharmacophore to the secondary binding site allosterically enhances binding of the orthosteric pharmacophore to the receptor (Chien et al., 2010; Newman et al., 2012a). This would have the effect of increasing the overall affinity of the ligand even further for the D₃ receptor. In order to test this hypothesis, we deconstructed several high affinity extended-length 4-phenylpiperazine-based D₃ selective antagonists into smaller “synthon” compounds and then “reconstituted” them in binding and functional assays. This approach has previously proven fruitful for the characterization of bitopic muscarinic cholinergic receptor ligands (Keov et al., 2013; Valant et al., 2008).

Using the crystal structure of the D₃ receptor, and the associated docking of the antagonist PG 648 (aka R-22) (Chien et al., 2010), we designed synthons that correspond to the predicted primary (orthosteric) and secondary (potentially allosteric) pharmacophores of several extended-length D₃ selective antagonists, including PG 648. All of the extended-length D₃ selective antagonists exhibit nM potency for the receptor and, in general, are about 100-fold selective vs. the D₂ receptor (Keck et al., 2014; Lober et al., 2011; Micheli and Heidbreder, 2013). When tested in radioligand binding assays, the orthosteric synthons exhibited about 100-fold lower affinity compared to the corresponding full-length ligands, thus providing a sufficient signal window for potential affinity improvement by the secondary pharmacophores. In contrast, the secondary pharmacophores did not inhibit radioligand binding to the orthosteric site of the receptor when initially tested up to a 10 μ M concentration.

Our reconstitution experiments initially consisted of performing full dose-response competition curves of the orthosteric synthons in radioligand binding assays in the absence and presence of each secondary synthon in order to assess their affinities for the D₃ receptor. As previously discussed, the secondary synthons might function as positive allosteric modulators and increase the affinities of the orthosteric synthons. Notably, however, none of the secondary synthons had any effect on the orthosteric synthons' abilities to compete for radioligand binding to the orthosteric site of the receptor. Our initial conclusion from these series of experiments is that the secondary synthons do not function as allosteric pharmacophores to modulate the affinities of the orthosteric pharmacophores for the receptor.

We tested this further using a limited set of synthons and a functional assay where the orthosteric synthons were found to exhibit antagonist activity on dopamine-stimulated β -arrestin recruitment to the D₃ receptor. We performed such assays in the presence or absence of a secondary synthon to see if the antagonist dose-response curve might be shifted to greater potency, but, as with the radioligand binding assays, there was no effect. Taking the radioligand binding and functional data together, our results do not support the hypothesis that the secondary pharmacophores of the extended-length D₃ selective antagonists shown in Fig. 1 are allosteric in nature. Rather, our results suggest that the receptor binding of 4-phenylpiperazine-based D₃-selective antagonists is bivalent in nature with the probability

that the overall increased affinity for the D₃ receptor, relative to the D₂ receptor, is simply due to additional free energy obtained from docking to a secondary binding site rather than due to allosteric effects. In agreement with this hypothesis, Michino et al. (2013) used molecular modeling to show that the binding of the secondary pharmacophore of a 4-phenylpiperazine antagonist to the secondary binding pocket is energetically more favorable for D₃ vs. D₂ receptors. They further showed that a divergent glycine residue in the first extracellular loop of the D₃ receptor was critical for forming the secondary binding pocket as well as interacting with the secondary pharmacophore (Michino et al., 2013).

During the course of the radioligand binding reconstitution assays, however, we did note that one of the secondary synthons, BAK 2-50, appeared to inhibit [³H]methyspiperone binding on its own, but by less than 50% and only at very high concentrations (>10 μM), a phenomenon that we did not observe during our initial screen using a single concentration of 10 μM (Table 1). This effect could simply be non-specific in nature, or could involve an interaction with the orthosteric site, although the latter seems unlikely given the structure of BAK 2-50 and the fact that this synthon is related to the pharmacophore of PG 648 that was shown to dock to the secondary binding site of the D₃ receptor (Chien et al., 2010). A third possibility is that BAK 2-50 may be acting as a negative allosteric modulator for [³H]methyspiperone binding to the D₃ receptor. If this is the case, then BAK 2-50 may be exhibiting “probe dependency” in allosterically modulating the binding of the structurally unrelated ligand, methyspiperone, to the D₃ receptor, but not the other primary pharmacophore probes derived from the full-length D₃ selective compounds, or dopamine. Probe dependency of allosterism, where a modulator can affect the receptor binding of one ligand, but not another, is a frequently observed phenomenon with allosteric modulators (Valant et al., 2012, 2009).

In a further attempt to assess potential allosteric activity of the secondary synthons, we evaluated their ability to modulate dopamine-stimulated β-arrestin recruitment to the D₃ receptor. In this case, we used dopamine as a functional probe of the receptor. An allosteric modulator might be expected to promote positive or negative modulation of the potency or efficacy of the response, or some combination of these effects (Conn et al., 2009; Lane et al., 2013a; Wooten et al., 2013). In contrast, we found that none of the secondary synthons, including BAK 2-50, exerted any effect on dopamine signaling through the D₃ receptor using the β-arrestin recruitment assay as the functional output. Thus, using these synthetic probes of the secondary binding site on the D₃ receptor, we were unable to demonstrate any allosteric effects on dopamine signaling.

We also decided to take a different approach to this question and investigate the pharmacological properties of some of the full-length D₃ receptor-selective ligands. In several different pharmacological assays, we found these ligands to behave strictly as competitive antagonists. These included radioligand binding competition curves, competitive radioligand binding saturation analyses, and, perhaps most importantly, functional curve-shift, Schild-type analyses (Keov et al., 2011). Our conclusion is that these compounds are unlikely to act in a bitopic or allosteric manner, but rather function as competitive antagonists through the docking of their primary pharmacophores to the orthosteric site on the D₃ receptor.

Interestingly, Silvano et al. (2010) recently described a D₃-selective antagonist, SB269,652, that was reported to have negative allosteric properties at both D₃ and D₂ receptors. One end of this molecule has an aryl amide structure similar to the termini of the D₃ antagonists PG 648 and PG 571 as well as synthon BAK 2-50 (Fig. 1). As such, we decided to characterize SB269,652 in our assay systems. Our results were mixed. It appeared to be a potent antagonist of the D₃ receptor using both radioligand binding and β -arrestin recruitment functional assays. Using radioligand binding assays, its interactions with the receptor appeared to be competitive in nature, whereas using the β -arrestin recruitment assay, its functional antagonism appeared to be that of a negative allosteric modulator (Keov et al., 2011). The latter results replicated those of Silvano et al. (2010), albeit with a different functional assay. It should be noted that, like the experiments with BAK 2-50, these two different assays used two different probes of the orthosteric site of the receptor – [³H]methyspiperone in the first instance and dopamine in the second instance – which could account for probe-dependence-like results.

Given the structure of SB269,652 (Fig. 8A), it is conceivable that the aryl amide moiety may dock to the secondary binding site of the D₃ receptor. This raises the hypothesis that this is what accounts for its apparent allosteric properties. However, if this were true, one would expect that the extended-length D₃-selective antagonists PG 648 and PG 571 to act similarly and that the secondary synthon BAK 2-50 would function as a small allosteric modulator, as they all possess the same aryl amide moiety of SB269,652. As noted above, BAK 2-50 did inhibit [³H]methyspiperone binding at concentrations >10 μ M, however, it did not modulate dopamine signaling in the manner of SB269,652. One possibility is that BAK 2-50 is not a perfectly matched structural synthon of SB269,652. In order to test this, we synthesized another small molecule synthon, CS 01-12 that replicates the aryl amide end of SB269,652. Importantly, we found that CS 01-12 had no effect on [³H] methyspiperone binding when tested up to 100 μ M and had no effect on dopamine-stimulated β -arrestin recruitment when tested at 50 μ M. Based on these observations, our conclusion is that it is unlikely that the aryl amide end of SB269,652 *per se* is what accounts for the apparent negative allosteric properties of SB269,652 at the D₃ receptor, but that this property must be imparted by other structural components of the molecule. Another possibility is that the presumed orthosteric and/or the linker moieties of SB269,652 uniquely position the secondary pharmacophore such that it can make allosteric contacts with yet-to-be defined sites on the D₃ receptor. Preliminary evidence for a primary pharmacophore, or linking chain, affecting the docking of a secondary pharmacophore of 4-phenylpiperazine antagonists has been observed (Michino et al., 2013; Newman et al., 2012a).

In conclusion, our data supports the notion that the 4-phenylpiperazine D₃-selective antagonists studied herein are bivalent and that their enhanced affinity for the D₃ vs. the D₂ receptor is due to their binding at both the orthosteric site as well as a secondary binding pocket that has been identified in the crystal structure of the D₃ receptor. Importantly, however, their docking at the secondary site does not allosterically modulate their docking to the orthosteric site (i.e., they are not bitopic). In the course of this investigation, we synthesized several small molecule probes of the secondary binding pocket of the D₃ receptor, which were based on the structure of the extended-length antagonists and also

corresponded to a similar moiety in SB269,652. None of these small molecule probes exhibited any allosteric effects on the D₃ receptor as evaluated using our assays. It should be noted, however, that these data do not eliminate the possibility that the interaction of structurally different ligands within this binding pocket might lead to functional allosteric effects (Lane et al., 2013b).

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Fig. 1.
Structures of extended-length D₃ receptor-selective ligands (A) and derived synthons (B) used in this study.

Fig. 2. Effect of secondary synthons on the ability of primary orthosteric synthons to compete for D_3 receptor binding

Radioligand competition binding assays were performed as described in the Experimental Procedures. Competition isotherms of primary synthons (A) BAK 3-33; (B) BAK 3-44; (C) BAK 2-46; (D) BAK 3-48 or (E) BAK 3-51 in the absence (●) or presence of 50 μ M of secondary synthons BAK 2-50, BAK 2-51, BAK 3-21, or NMP 8-073, as indicated. Data in (A–E) are plotted as mean $\pm$ SEM, $n=3$, performed in triplicate. Results are expressed as a percentage of specific [3H]methylspiperone binding in the absence of any competitor (% control). K_i values were calculated from observed IC_{50} values from individual experiments using the Cheng–Prusoff equation and are displayed in Tables 1 and 2. (F) Competition isotherm of BAK 2-50 conducted using [3H]methylspiperone and the indicated concentrations of BAK 2-50. Results are representative of two independent experiments performed in triplicate.

Fig. 3. The effect of a secondary synthon on the inhibition of dopamine-stimulated β -arrestin recruitment by orthosteric synthons

β -arrestin recruitment assays were performed as described in the Experimental Procedures. Cells were stimulated with an EC_{90} concentration of dopamine (2 μ M) and the indicated concentrations of test compound. Data are plotted as a percentage of the response seen with 2 μ M dopamine in the absence of compound as mean $\pm$ SEM, $n=3$. (A) Inhibitory concentration response curves of BAK 2-46 or sulpiride alone or in the presence of 50 μ M BAK 2-50, as indicated. IC_{50} (95% CI) values obtained from mean graphs of BAK 2-46 and BAK 2-46+BAK 2-50 were 9.7 μ M (3.5–27) and 4.3 μ M (2.1–8.7) respectively, and from mean graphs of sulpiride and sulpiride+BAK 2-50 were 0.5 μ M (0.4–0.7) and 0.5 μ M (0.3–1) respectively. (B) Inhibitory concentration response curves of BAK 3-44 alone or in the presence of 50 μ M BAK 2-50, as indicated. Sulpiride data is re-plotted here from panel A for data comparison. IC_{50} (95% CI) values obtained from mean graphs of BAK 3-44 and BAK 3-44+BAK 2-50 were 6.0 μ M (2.4–15) and 11.0 μ M (3.5–35), respectively. No

significant difference between IC_{50} values of each compound upon the addition of BAK 2-50 was observed (Student's t -test ($p < 0.05$)).

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Fig. 4. The effect of secondary synthons on dopamine-stimulated β -arrestin recruitment to the D₃ receptor

β -arrestin recruitment assays were performed as described in Section 2. Cells were stimulated with the indicated concentrations of dopamine either alone or presence of 50 μ M BAK 2-50, BAK 2-51, BAK 3-21, or NMP 8-073, as indicated. Data are representative of three independent experiments run in triplicate and plotted as average relative luminescence units (RLU) $\pm$ SEM. Dopamine EC₅₀ values represented as means $\pm$ SEM are 7.7 ± 2.5 nM, 12 ± 2 nM, 8.1 ± 1.1 nM, 9.6 ± 2.5 nM, and 4.1 ± 2.4 nM, respectively. Comparison of EC₅₀ values using a Student's *t*-test failed to show any significant ($p < 0.05$) differences between dopamine alone or in the presence of any secondary synthon.

Fig. 5. Radioligand binding competition analyses with full-length D₃ receptor-selective antagonists

Radioligand binding competition assays were performed as described in Section 2. Binding was conducted using [³H]methylspiperone plus the indicated concentrations of (A) sulpiride, (B) PG 571, (C) GCC 3-05, (D) PG 648, (E) JJC 4-077, or (F) NGB 2904. Data are mean graphs expressed as percent specific binding and plotted as mean ± SEM, *n* = 3–4. *K_i* values were calculated from observed IC₅₀ values using the Cheng–Prusoff equation and are displayed in Table 1.

Fig. 6. Radioligand binding saturation analyses in the absence or presence of competing ligands Radioligand binding assays were performed as described in Section 2. (A) Saturation binding isotherm of [3H]methylspiperone in the absence of any compound (control) or in the presence of 2 nM GCC 3-05, 1 μ M sulpiride, or 0.5 nM PG571, as indicated. (B) Scatchard transformation of data in A. K_D and B_{max} values were determined using GraphPad Prism: control, 0.21 nM, 5,910 fmol/mg; GCC 3-05, 0.91 nM, 6,590 fmol/mg; sulpiride, 1.13 nM, 6,190 fmol/mg, and PG 571, 2.0 nM, 6,580 fmol/mg. The data shown are from a representative experiment of two independent experiments with similar results, each performed in triplicate.

Fig. 7. Curve-shift analyses of antagonist inhibition of dopamine-stimulated β -arrestin translocation to the D_3 receptor

β -arrestin recruitment assays were performed as described in Section 2. Dopamine concentration response curves were generated in the absence or presence of the indicated concentrations of sulpiride (A), GCC 3-05 (B), or PG 571 (C). The experiment shown is representative of three independent experiments each performed in triplicate and is plotted as the mean relative luminescence units (RLU) $\pm$ SEM. Representative Schild plots (insets) of the data are shown and Hill slopes were determined using GraphPad Prism from each individual experiment (A–C). Slopes did not differ from unity and were 1.04 ± 0.05 , 1.12 ± 0.10 , and 1.01 ± 0.10 respectively ($n=3-5$), expressed as mean $\pm$ SEM.

Fig. 8. SB269,652 competition analysis for [³H]methylspiperone binding to the D₃ receptor (A) Structure of SB269,652. (B) Radioligand binding competition assays were performed as described in Section 2 using [³H]methylspiperone plus the indicated concentrations of SB269,652. Data are representative of three experiments run independently and are expressed as a percentage of the specific binding and plotted as mean±SEM. The mean K_i value was calculated from observed IC_{50} values from individual experiments using the Cheng–Prusoff equation and determined to be 1.5 ± 0.3 nM.

Fig. 9. Radioligand binding saturation analyses in the absence or presence of SB269,652
Radioligand binding assays were performed as described in Section 2. Saturation binding of [3H] methylspiperone in the presence of no drug (control) or 50 nM SB269,652, as indicated. Data are presented in Scatchard coordinates. K_D and B_{max} values are: control, 1.2 nM, 14 pmol/mg; SB269652, 2.2 nM, 14.5 pmol/mg. The experiment shown is representative of three independent experiments each performed in triplicate.

Fig. 10. SB269,652 antagonism of dopamine-stimulated β -arrestin translocation to the D₃ receptor

β -arrestin recruitment assays were performed as described in Section 2. Cells were stimulated with an EC₉₀ concentration of dopamine (2 μ M) and the indicated concentrations of test compound. Inhibitory concentration response curves for SB269,652 and sulpiride are shown as indicated. The experiment shown is representative of three independent experiments each performed in triplicate and are plotted as mean relative luminescence units (RLU) $\pm$ SEM. IC₅₀ values were determined from non-linear regressions of individual experiments and found to be 41.8 nM $\pm$ 7.0 nM for sulpiride and 92.4 $\pm$ 17 nM SB269,652, $n=3$.

Fig. 11. Curve-shift analysis of SB269,652 inhibition of dopamine-stimulated β -arrestin translocation to the D_3 receptor

β -arrestin recruitment assays were performed as described in Section 2. Dopamine concentration response curves were generated in the absence or presence of the indicated concentrations of sulpiride (A), or SB269,652 (B). The experiment shown is representative of three independent experiments each performed in triplicate and are plotted as mean relative luminescence units (RLU) $\pm$ SEM. Representative Schild plots of the data are shown (insets), and Hill slopes were determined using GraphPad Prism from each individual experiment. The Hill slope was found to be 1.04 ± 0.05 for sulpiride and found to be curvilinear for SB269,652.

Fig. 12.

Effect of an SB269,652-related secondary synthon on D₃ receptor radioligand binding and dopamine-stimulated β -arrestin translocation. (A) Structure of CS 01-12. (B) Radioligand binding competition assays were performed as described in Section 2. Competition binding was conducted using the indicated concentrations of CS 01-12. Data are representative of three experiments run independently and are expressed as a percentage of the specific binding. (C) β -arrestin recruitment assays were performed as described in Section 2 Cells were stimulated with the indicated concentrations of dopamine either alone, or in the presence of 50 μ M CS 01-12, as indicated. Data represent mean $\pm$ SEM values from three

independent experiments run in triplicate and plotted as average relative luminescence units (RLU). EC₅₀ values were determined from non-linear regressions of individual experiments and found to be 12.1±2.3 nM for dopamine alone, and 12.9±3 nM for dopamine +CS 01-12. Comparison of EC₅₀ values using the Student's *t*-test failed to show a significant ($p<0.05$) difference between dopamine alone or in the presence of CS 01-12.

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Table 1

Affinities of ligands and derived synthons for the D₃ receptor as determined via radioligand binding competition assays.

Compounds	$K_i \pm \text{SEM}$ (nM)
Extended-length antagonists ^{a,b}	
PG 571	0.44±0.05
PG 01042 ^c	0.47±0.09
CJB 090 ^d	0.6±0.2
PG 648	1.4±0.2
JJC 4-077	1.8±0.1
NGB 2904	1.7±0.3
GCC 3-05	3.3±0.3
“Primary” Orthosteric Synthons ^{a,e}	
BAK 2-46	370±66
BAK 3-44	2,150±858
BAK 3-48	299±63
BAK 3-51	57.2±23
BAK 3-33	294±58
“Secondary” Synthons ^a	
BAK 2-50	>10,000
BAK 2-51	>10,000
BAK 3-21	>10,000
NMP 08-73	>10,000

Radioligand competition binding assays were performed using [³H]methylnspiperone as described in Section 2. K_i values were calculated using the Cheng–Prusoff equation (Cheng and Prusoff, 1973) and all values are expressed as means±SEM of 3–5 individual experiments.

^aStructures in Fig. 1.

^b K_i values extrapolated from Fig. 5, unless indicated otherwise.

^cBinding data previously reported in Riddle et al. (2011). (Exhibits low partial agonist activity).

^dBinding data previously reported in Newman et al. (2003). (Exhibits low partial agonist activity).

^eStructures in Fig. 1 and K_i values extrapolated from Fig. 2.

Affinities of the primary orthosteric synthons as determined from radioligand binding assays in the absence or presence of the secondary synthons.

Table 2

Primary synthon	Secondary synthon $K_i \pm \text{SEM}$ (nM)				
	Vehicle	BAK 2-51	BAK 3-21	BAK 2-50	NMP 8-073
BAK 2-46	370 $\pm$ 65.6	262 $\pm$ 30.7	520 $\pm$ 67.0	505 $\pm$ 121	377 $\pm$ 52.1
BAK 3-44	2150 $\pm$ 858	2280 $\pm$ 1120	3720 $\pm$ 1780	3780 $\pm$ 1340	1860 $\pm$ 684
BAK 3-48	299 $\pm$ 62.6	368 $\pm$ 132	555 $\pm$ 150	982 $\pm$ 287	537 $\pm$ 76.1
BAK 3-51	57.2 $\pm$ 22.9	88.6 $\pm$ 32.4	74.4 $\pm$ 26.1	145 $\pm$ 36.3	103 $\pm$ 36.9
BAK 3-33	294 $\pm$ 57.7	212 $\pm$ 19.6	405 $\pm$ 83.7	793 $\pm$ 132	440 $\pm$ 46.2

Data are extrapolated from individual experiments ($n=3$) represented in Fig. 2. K_i values were calculated from observed IC₅₀ values using the Cheng-Prusoff equation and all values are expressed as mean $\pm$ SEM. Secondary synthons were added to the assays at a final concentration of 50 μ M. All K_i values obtained in the presence of secondary synthons were statistically insignificant from control values obtained in their absence, as determined using Student's t -test with a significance level of $p<0.05$.