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Brief Article

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Proof of Concept Study for Designed Multiple Ligands Targeting the Dopamine D_2, Serotonin 5-HT_{2A}, and Muscarinic M_1 Acetylcholine Receptors

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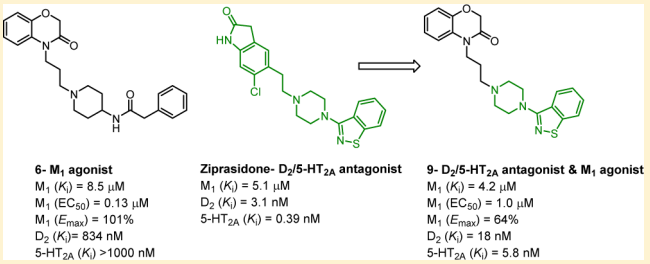
Supporting Information

ABSTRACT: Herein we describe the hybridization of a benzoxazinone M_1 scaffold with D_2 privileged structures derived from putative and clinically relevant antipsychotics to develop designed multiple ligands. The M_1 mAChR is an attractive target for the cognitive deficits in key CNS disorders. Moreover, activity at D_2 and 5-HT_{2A} receptors has proven useful for antipsychotic efficacy. We identified **9** which retained functional activity at the target M_1 mAChR and D_2R and demonstrated high affinity for the 5-HT_{2A}R.



INTRODUCTION

All antipsychotic drugs currently on the market for the treatment of schizophrenia antagonize the dopamine D_2 receptor (D_2R), a member of the G protein-coupled receptor (GPCR) family. They are effective in alleviating the positive symptoms of schizophrenia (hallucinations, delusions) which are postulated to arise from hyperdopaminergia in the mesolimbic pathway of the brain.[1] Antipsychotics such as ziprasidone (**1**) and risperidone (**2**, Figure 1) have also been shown to cause improvements in negative symptoms (social withdrawal, lack of motivation), an effect attributed to their favorable polypharmacology and in particular their action as high affinity antagonists at the serotonin 5-HT_{2A} receptor (5-HT_{2A}R).[2,3] Indeed the favorable polypharmacology that is observed with many atypical antipsychotics such as clozapine was achieved through serendipitous discovery rather than by a rational drug design process.[4] A newer class of clinically used antipsychotics are the D_2R partial agonists, of which aripiprazole (**3**, Figure 1) is the most commonly prescribed antipsychotic in the U.S. for the treatment of schizophrenia and other CNS disorders.[5] Partial agonists act to stabilize dopaminergic signaling rather than exert the complete inhibition associated with D_2R antagonists.[6] Since the success of aripiprazole, other D_2R partial agonists have emerged such as cariprazine[7] and brexpiprazole,[8] which both have the 2,3-dichlorophenylpiperazine motif present in **3**. Another D_2R partial agonist bifeprunox (**4**) exhibits a structurally more diverse heterocycle attached to the piperazine and incorporates a hydrophobic and unfunctionalized biphenyl substituent. There are also high affinity D_2R partial agonists such as **5** that contain a 2-methoxy substituted phenylpiperazine motif rather than 2,3-dichlorophenylpiperazine.[9] However, no current antipsychotic drug addresses the cognitive deficits associated with schizophrenia, which is an equally important component of the etiology of schizophrenia.

There is evidence to support that targeting the M_1 muscarinic acetylcholine receptor (M_1 mAChR), also belonging to the GPCR family, improves the cognitive deficits of patients suffering from schizophrenia and other CNS disorders.[10,11] However, selective targeting of this receptor remains a challenge due to the high conservation of the orthosteric site across the mAChR receptor family, and activity at other

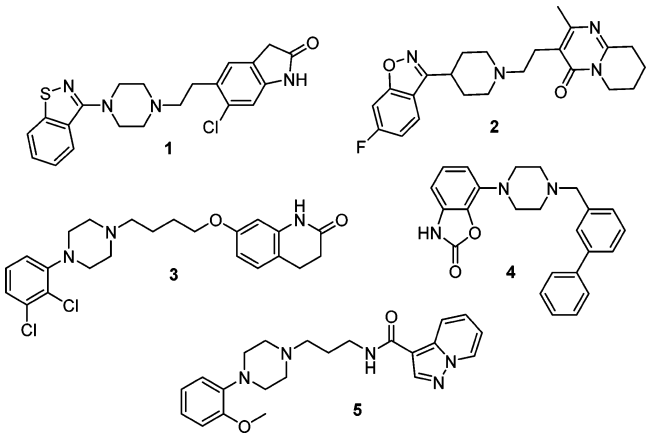


Figure 1. Antipsychotics: ziprasidone (**1**), risperidone (**2**), aripiprazole (**3**), bifeprunox (**4**), and a high affinity D_2 partial agonist (**5**).

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receptors in this family is associated with limiting side effects.[12] Of interest, M_1 allosteric agonists have particularly gained a great deal of research focus.[13] Ligands that act at an allosteric site (a topographically distinct site to the orthosteric site) offer the added benefit of possibly being subtype selective, as the residues are less conserved in an allosteric site versus an orthosteric site.[14] Sams et al. used this rationale to generate the putative M_1 allosteric agonist LuAE51090 (**6**, Figure 2) that has

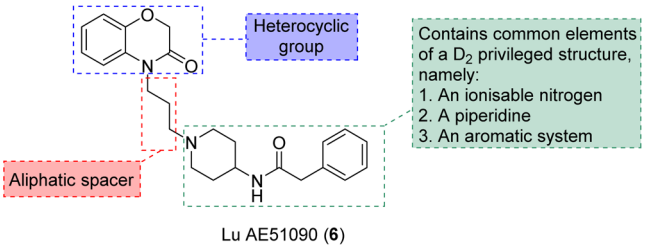


Figure 2. Structure of **6** highlighting common dopaminergic D_2 structural characteristics: a motif containing features of common privileged structures, namely, an aryl system and ionizable nitrogen atom, an aliphatic spacer/linker, and a heterocyclic group.

a favorable M_1 profile (EC_{50} = 61 nM; intracellular Ca^{2+} mobilization assay) but exhibits poor binding affinity at the D_2R and the 5-$HT_{2A}R$ ($K_i \approx 1\ \mu M$).[15] We found this surprising, as **6** displays some key pharmacophoric features of many D_2R ligands, both antagonists and partial agonists as highlighted in Figure 2 and in an earlier publication.[16]

Therefore, utilizing **6** as our primary scaffold, we envisioned that we could design in an enhanced D_2R binding profile using the designed multiple ligand (DML) approach described by Morphy that takes two separate pharmacophores with distinct pharmacology and integrates them into one molecule that has the attributes of both parent molecules. In this approach the degree of integration is systematically increased until the structure becomes merged and essentially more druglike.[17] We made use of privileged structures from known D_2R ligands that covered three distinct classes (Figure 3): (1) phenylpiperazines which are known to be important motifs for D_2R affinity and functional activity; our previous work demonstrated that compounds incorporating the 2,3-dichlorophenylpiperazine and 2-methoxyphenylpiperazine scaffolds are useful in the design of antagonists and partial agonists for the D_2R;[16] (2) using privileged structures from two distinct antipsychotics (ziprasidone and risperidone) that have a piperazine or piperidine moiety followed by similar structural heterocycles;[18] (3) using a privileged structure from a partial agonist (bifeprunox) which is unique compared to the 2,3-dichloro- and 2-methoxyphenylpiperazine family.

As such, rather than embarking on a screening program to identify ligands that display the desired pharmacology for two or more receptors required for activity, we hoped to achieve this polypharmcology through the rational combination of distinct pharmacophores.[19] To characterize the ligands, we pharmacologically evaluated them in radioligand binding assays for all three receptors (D_2R, M_1 mAChR, and 5-$HT_{2A}R$). To evaluate the ligands in functional assays, we tested them in ERK1/2 phosphorylation assays for the D_2R, intracellular Ca^{2+} mobilization assays for the M_1 mAChR and IP_1 accumulation assays for the 5-$HT_{2A}R$.

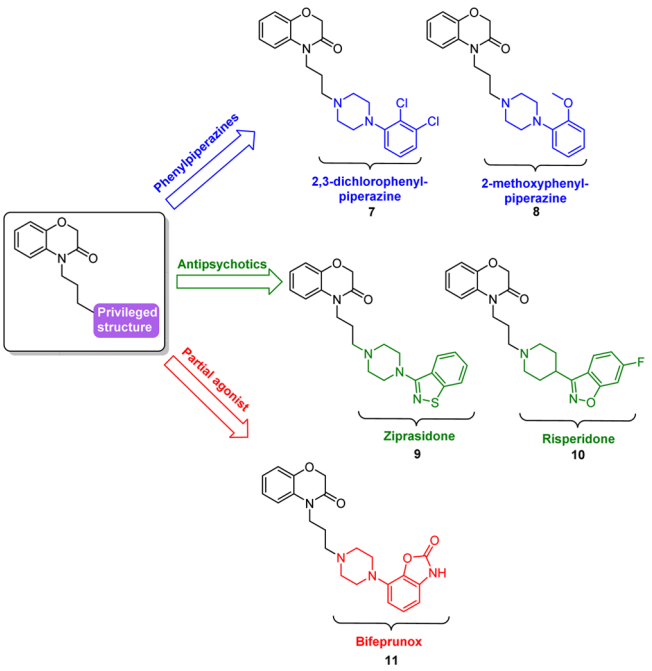
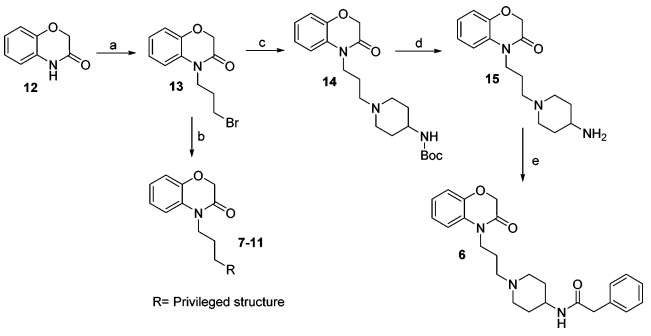


Figure 3. Merged DMLs (**7**–**11**) derived from combining the propylbenzoxazinone moiety from **6** with D_2 privileged structures (colored segments) from parent ligands **1**–**5**.

RESULTS AND DISCUSSION

Chemistry. The syntheses of all DMLs and the reference M_1 ligand (**6**) are outlined in Scheme 1. This initially required

Scheme 1. Synthesis of DMLs (7–11) and 6[a]



[a]Reagents and conditions: (a) dibromopropane, NaH (60%), DMF, N_2, rt, 67%; (b) privileged structures, DIPEA, NaI, CH_3CN, reflux, 5 h or overnight, 17–75%; (c) 4-*N*-Boc-aminopiperidine, K_2CO_3, CH_3CN, 80 °C, 62%; (d) TFA, DCM, rt, overnight, 73%; (e) 2-phenylacetyl chloride, Et_3N, THF, N_2, 0 °C → rt, 1.5 h, 82%.

the installation of a three-carbon atom spacer to the precursor benzoxazinone (**12**). This reaction was performed under alkaline conditions using 60% sodium hydride and a large excess of 1,3-dibromopropane to give the key intermediate **13**. Rapid diversification with **13** and the required privileged structures, as illustrated in Figure 3, furnished the target DMLs (**7**–**11**) in yields of 17–75%. The chemical synthesis of **6** (which is also commercially available) followed similar conditions to that previously published.[15] Compound **13** was refluxed under basic conditions with 4-*N*-Boc-aminopiperidine to afford **14** in respectable yield. The final step was achieved by removal of the Boc protecting group (**15**) and subsequent

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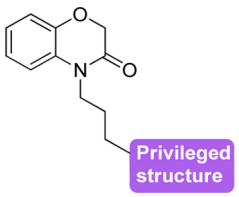
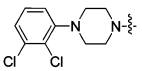
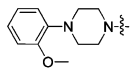
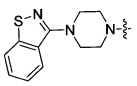
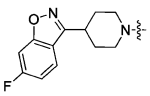
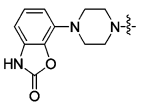
Table 1. Binding and Functional Data at the D_2R, M_1 mAChR, and 5-$HT_{2A}R$ for All DMLs[a]



Compd	Privileged structure	D_2R Binding $pK_i \pm$ SEM (K_i, nM)[b]	D_2R pERK1/2 $pK_B \pm$ SEM (K_B, nM)[c]	M_1 mAChR Binding $pIC_{50} \pm$ SEM (IC_{50}, nM)[b]	M_1 mAChR Ca^{2+} $pEC_{50} \pm$ SEM (EC_{50}, nM)	M_1 mAChR Ca^{2+} $E_{max} \pm$ SEM[d]	5-$HT_{2A}R$ Binding $pK_i \pm$ SEM (K_i, nM)[b]	5-$HT_{2A}R$ IP_1 $pK_B \pm$ SEM (K_B, nM)[e]
6	-	6.08 ± 0.28 (834)	5.68 ± 0.34 (2091)	5.07 ± 0.11 (8525)	6.89 ± 0.14 (129)	101 ± 6	5.98 ± 0.29 (1042)	5.80 ± 0.06 (1574)
7		6.56 ± 0.10 (273)	7.00 ± 0.12 (99.0)	5.73 ± 0.09 (1872)	n/a	n/a	7.12 ± 0.21 (75.8)	5.73 ± 0.03 (1861)
8		7.37 ± 0.10 (42.7)	8.11 ± 0.11 (7.8)	5.29 ± 0.08 (5180)	n/a	n/a	6.56 ± 0.31 (275)	6.24 ± 0.09 (578)
9		7.75 ± 0.10 (17.7)	8.16 ± 0.10 (7.0)	5.38 ± 0.13 (4191)	5.98 ± 0.25 (1042)	64 ± 10	8.24 ± 0.37 (5.8)	6.63 ± 0.05 (235)
10		8.49 ± 0.12 (3.2)	8.91 ± 0.16 (1.2)	5.49 ± 0.07 (3253)	n/a	n/a	8.57 ± 0.16 (2.7)	7.67 ± 0.07 (21.2)
11		8.76 ± 0.14 (1.7)	-	5.37 ± 0.10 (4259)	n/a	n/a	6.25 ± 0.44 (568)	5.94 ± 0.10 (1138)

[a]Data are the mean of three to four experiments ± SEM performed in duplicate. n/a = compound not active. [b]Binding affinity values are obtained using [^{3}H]raclopride (D_{2L}-FlpIn CHO whole cells) or [^{3}H]NMS (M_1 mAChR-FlpIn CHO whole cells) or [^{3}H]ketanserin (5-HT_{2A}-FlpIn CHO cell membranes). [c]Value of functional affinity (K_B) obtained from interaction studies with varying concentrations of dopamine in an assay measuring levels of pERK1/2. Data are fit to the Gaddum–Schild analysis. [d]E_{max} is the percentage of maximal activity relative to the maximal activity of ACh in the intracellular calcium mobilization assay. [e]Value of functional affinity (K_B) obtained from interaction studies with varying concentrations of serotonin in an IP_1 accumulation assay.

reaction with 2-phenylacetyl chloride to generate the target **6** in 82% yield.

Pharmacology. *D_2R Binding and Functional Characterization of DMLs.* The synthesized DMLs were pharmacologically characterized in radioligand binding and functional ERK1/2 phosphorylation (pERK1/2) assays for the D_2R. The pERK assay provides a robust readout for D_2R activation and is medium throughput, thereby allowing efficient screening of a number of compounds. The results of these studies are summarized in Table 1. Compound **6** demonstrated the weakest binding affinity (pK_i), consistent with previously published results.[15] Compounds **10** and **11** (consisting of the motifs from risperidone (**2**) and bifeprunox (**4**), respectively) exhibited the highest binding affinities (K_i of 3.2 and 1.7 nM, respectively) for the D_2R overall. This result is consistent with the high affinity of the parent compounds **2** and **4**.[20]

Indeed the incorporation of D_2R privileged structures conferred a significant increase in D_2R affinity for all compounds as compared to **6** apart from the incorporation of the 2,3-dichlorophenylpiperazine moiety (**7**). The high binding affinity of **10** at the D_2R may be attributed to its bicyclic system of greater molecular size as opposed to **8** (phenylpiperazine) and its piperidine moiety compared to the piperazine of **8** and **9**. Furthermore, the equally high binding affinity of **11** may result from being the only heterocyclic moiety that contains a H-bond donor capable of hydrogen bond interactions with residues such as serines present in transmembrane domain 5 in the orthosteric binding site.

For functional pERK1/2 assays, all ligands were initially tested in time-course assays (data not shown), upon which we identified ligands **7**–**10** to be antagonists at the D_2R and **11** as an agonist. Importantly, this latter result is consistent with the partial agonist action of bifeprunox; the ligand from which the 7-(piperazin-1-yl)benzo[*d*]oxazol-2(3*H*)-one moiety was derived.

To determine the functional affinity (pK_B) of each of the antagonists, we performed interaction studies using varying concentrations of dopamine. The functional affinities correlated well with the binding affinities obtained. Compound **11**, derived from merging the privileged structure of **4** with **6**, was a potent agonist at the D_2R in pERK1/2 and cAMP signaling assays (Table 2; 0.64 nM and 96 pM, respectively). We calculated a bias factor for **11** relative to the full agonist ropinirole (Supporting Information Table 1 and Figure 1) and found that **11** displays a similar bias toward cAMP over pERK1/2 to that previously determined for aripiprazole (fold bias of 448 and 102, respectively).[16] The results from Table 1 show that the D_2 profile of the ligands are significantly

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Table 2. Profiling of 11 in Functional Assays at the D_2R[a]

	ERK1/2 phosphorylation		cAMP	
compd	pEC_{50} ± SEM (EC_{50}, nM)	E_{max}[b] ± SEM	pEC_{50} ± SEM (EC_{50}, nM)	E_{max}[b] ± SEM
11	9.20 ± 0.29 (0.64)	26 ± 3	10.02 ± 0.13 (0.096)	97 ± 2

[a]Data are the mean of four to six experiments ± SEM performed in duplicate. Data are generated via concentration−response curves. [b]E_{max} is the percentage of maximal activity relative to the maximal activity of FBS (pERK1/2) or ropinirole (cAMP).

enhanced with the incorporation of the privileged structures and compounds from all three classes (phenylpiperazines, antipsychotics, and partial agonists) with the benzoxazinone scaffold from **6** at the D_2R.

M_1 mAChR Binding and Functional Characterization of DMLs. As previously indicated, we tested compounds in radioligand binding and functional assays at the M_1 mAChR (Table 1). All compounds displaced [^{3}H]NMS at the M_1 mAChR with relatively weak inhibitory potencies (IC_{50}), with **7** displaying the highest inhibitory potency (IC_{50}= 1.9 μM), as compared to **6** (IC_{50} = 8.5 μM, $p < 0.05$). As a functional assay for the M_1 mAChR, we used an intracellular Ca^{2+} mobilization assay as a measure of coupling to G_q pathways. Only DML **9** showed activity at the M_1 mAChR, displaying a diminished potency (EC_{50} =1.04 μM) as compared to **6** (EC_{50} = 129 nM) equating to an 8-fold loss in potency. The maximal stimulation (E_{max}) of **9** was also reduced to 64% compared to **6** which demonstrated an E_{max} of 101% (defined by the maximal effect of ACh) consistent with an action as a partial agonist. The subtle differences in the D_2R privileged structures used could account for their loss in M_1 mAChR activity. For example the M_1 mAChR may not accommodate the more linear orientation of the phenylpiperazine analogues **7** and **8** as compared to a more flexible structure present in **6**. For the antipsychotics, the ziprasidone and risperidone privileged structures reveal slightly different heterocycles (benzoisothiazole vs benzoisoxazole), in addition to the absence of a fluorine substituent on the aromatic ring, perhaps making **9** more favorable for the M_1 mAChR than **10**, as a fluorine atom is powerfully electron-withdrawing and subsequently deactivates an aromatic system. This makes it partially positive in nature and complementary to relatively electron rich and activated aryl systems of amino acids in a receptor such as tryptophan, tyrosine, and phenylalanine. The results also suggest that it is unclear whether a piperazine or piperidine is more ideally suited for activity at the M_1 mAChR and perhaps the functionality before and after the six-membered ring containing ionizable nitrogen at physiological pH is more detrimental for activity. Compound **11**, as compared to similar structures **9** and **10**, was connected to the flexible piperazine through the phenyl ring as opposed to the five-membered ring being directly connected to the piperazine or piperidine, which possibly affected the binding of the ligand at the M_1 mAChR and therefore attributed to its loss of activity.

5-HT_{2A}R Binding and Functional Characterization of DMLs. There is substantial literature evidence that antagonism of the 5-HT_{2A}R is important for the therapeutic efficacy of atypical antipyschotics such as clozapine.[21] In addition, **9** and **10** stem from antipsychotics **1** and **2**, respectively, that display high affinity for the 5-HT_{2A}R. Therefore, we deemed it prudent to investigate if this attribute was maintained upon integration of these privileged structures into the M_1 mAChR/D_2R DML and tested the ligands in a radioligand binding assay to determine their affinities for this receptor (Table 1). Consistent with the literature,[15] the M_1 mAChR agonist **6** demonstrated poor binding to the 5-HT_{2A}R ($K_i \approx 1$ μM). Both phenylpiperazine analogues **7** and **8** showed no notable enhancements in affinity as compared to **6**. Of note, DMLs **9** and **10** show a strong binding affinity for the 5-HT_{2A}R (K_i of 5.8 and 2.7 nM, respectively). Compound **11**, which as mentioned previously has a slightly different heterocycle following the piperazine, had a diminished binding affinity similar to its M_1 mAChR binding profile. As **9** and **10** maintain their D_2/5-HT_{2A} binding profiles, it is evident that both privileged structures exhibit versatility for use in the design of ligands with favorable polypharmacology. The compounds were also tested in an IP_1 functional assay at the 5-HT_{2A}R. When tested in the absence of serotonin (Supporting Information Figure 2), all compounds showed no activation of the receptor except for compound **7**. As such compounds **8**−**11** are all antagonists at the serotonin receptor. In agreement with the binding data for the 5-HT_{2A}R, **6** demonstrated the weakest functional affinity. DMLs containing the ziprasidone and risperidone privileged structures both showed the highest functional affinities with **10** displaying an 11-fold increase over **9** ($p < 0.05$). As mentioned in the Introduction, there is considerable evidence that antagonism at the 5-HT_{2A}R is useful for antipsychotics; therefore, all DMLs, excluding **7**, are useful starting compounds for further SAR studies and optimization.

Compound **9** was our most promising candidate, as it displayed activity at all three receptor targets. Despite showing strong affinities for the D_2R and 5-HT_{2A}R (Table 1; K_i of 17.7 and 5.8 nM, respectively), there was a significant reduction in potency at the M_1 mAChR (EC_{50} = 1.04 μM, E_{max} = 64%). However, we have shown that we can use a merged DML approach utilizing D_2R privileged structures to confer a D_2R pharmacological profile to a putative M_1 mAChR allosteric agonist. In addition to this, privileged structures derived from parent structures with known D_2/5-HT_{2A} receptor binding profiles were maintained, thus validating their usefulness in a DML approach. It should be noted that it was difficult to maintain activity at the M_1 mAChR, as only one structure was able to show any noteworthy agonism. It is therefore possible that key elements in the structure of **6** that account for its M_1 mAChR allosteric agonist profile were lost as a result of the integration process. As such, new structural analogues may look at incorporating greater elements of the original structure. To expand on this work, it may also be useful to incorporate other benzoxazinones or heterocyclic compounds similar to this scaffold as a way to optimize and possibly enhance the M_1 mAChR profile. Additionally, other selective M_1 mAChR agonists[22,23] may be explored for optimization as a DML.

CONCLUSION

In terms of antipsychotic action, engaging multiple targets has become useful in the drug design process to address the numerous symptom domains of schizophrenia. The DML approach offers a way to selectively design in polypharmacology by using privileged structures that are known to be advantageous toward the targets of interest. Ligands in this study were designed to be D_2 antagonists or partial agonists for the positive symptoms, M_1 mAChR allosteric agonists for the cognitive deficits, and antagonists at the 5-HT_{2A}R to address the negative symptoms and reduce the occurrence of extrapyramidal side

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effects. The privileged structures covered phenylpiperazines in addition to mixed piperazine/piperidine heterocyclic compounds derived from antipsychotics or a clinically developed compound. The final DMLs (**7**−**11**) were generally well-tolerated at the D_2R and 5-$HT_{2A}R$ but are in need of further development at the M_1 mAChR. Despite this, we identified **9**, incorporating a privileged structure derived from the antipsychotic ziprasidone, that retained strong binding and functional activity at the D_2R and 5-$HT_{2A}R$ and weak partial agonism at the M_1 mAChR. Compound **9** represents a useful starting point for further optimization to improve its M_1 mAChR profile.

EXPERIMENTAL SECTION

Chemistry. All solvents and chemicals were purchased from standard suppliers and were used without any further purification. ^{1}H NMR and ^{13}C NMR spectra were acquired at 400.13 and 100.62 MHz, respectively, on a Bruker Advance III 400 MHz UltrashieldPlus NMR spectrometer using TOPSPIN 2.1 software. Chemical shifts (δ) for all ^{1}H spectra are reported in parts per million (ppm) using tetramethylsilane (TMS, 0 ppm) as the reference. The data for all spectra are reported in the following format: chemical shift (δ), (multiplicity, coupling constants J (Hz), integral), where the multiplicity is defined as s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, st = sextet, and m = multiplet. For ^{13}C NMR spectra C = quaternary carbon, CH = methine carbon, CH_2 = methylene carbon, and CH_3 = methyl carbon. The purity and retention time of final products were determined on an Agilent 1260 Infinity analytical reverse-phase HPLC system fitted with a Poroshell 120 SB-C18 4.6 mm × 100 mm 2.7 μm column. The HPLC operates on Agilent OpenLAB CDS, revision C.01.04, software. Solvent A is water + 0.1% TFA, and solvent B is acetonitrile + 0.1% TFA. Samples were run using a gradient method (5−100% solvent B over 10 min). The purities of all compounds are ≥95%. Thin layer chromatography (TLC) was carried out routinely on silica gel 60F_{254} precoated plates (0.25 mm, Merck). Flash column chromatography was carried out using Merck silica gel 60, 230−400 mesh ASTM.

General Procedure for the Synthesis of Merged DMLs. Compound **13** (1 equiv) was dissolved in CH_3CN (10 mL). NaI (1 equiv), DIPEA (1−2 equiv), and the required amine (1 equiv) were added and heated at reflux for 5−6 h. After this time, the CH_3CN was removed in vacuo and the resulting residue dissolved in ethyl acetate (20 mL). Aqueous K_2CO_3 (1 M, 20 mL) was added and the product further extracted with ethyl acetate (2 × 20 mL). The organic layers were combined and washed with water (20 mL) and brine (20 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness to give the crude product. Purification via column chromatography (petroleum spirits/ethyl acetate 1:1) gave the pure product.

4-(3-(4-(Benzo[*d*]isothiazol-3-yl)piperazin-1-yl)propyl)-2*H*-benzo[*b*][1,4]oxazin-3(4*H*)-one (9). Pale yellow oil (208 mg, 69%). ^{1}H NMR ($CDCl_3$): δ 1.91 (m, 2H), 2.51 (t, J = 6.9 Hz, 2H), 2.65−2.68 (m, 4H), 3.55−3.57 (m, 4H), 4.04 (t, J = 7.3 Hz, 2H), 4.60 (s, 2H), 6.98−7.05 (m, 3H), 7.13 (d, J = 7.6 Hz, 1H), 7.35 (t, J = 7.5 Hz, 1H), 7.45 (t, J = 7.5 Hz, 1H), 7.80 (d, J = 8.1 Hz, 1H), 7.90 (d, J = 8.2 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 24.5 (CH_2), 39.5 (CH_2), 50.2 (CH_2), 53.1 (CH_2), 55.6 (CH_2), 67.7 (CH_2), 115.1 (CH), 117.2 (CH), 120.7 (CH), 122.9 (CH), 123.9 (CH),12.9 (CH), 124.0 (CH), 127.6 (CH), 128.1 (C), 128.7 (C), 145.4 (C), 152.8 (C), 164.0 (C), 164.4 (C). HPLC purity (λ = 214 nm): 100%, t_R = 6.19 min. HRMS (ESI)-TOF (m/z): $[M + H]^+$ 409.1698 calcd for $C_{22}H_{24}N_4O_2S$; found $[M + H]^+$ 409.1701.

ASSOCIATED CONTENT

Supporting Information

Synthesis, characterization, and pharmacology for all compounds; bias calculations for **11** at the D_2R and compounds tested in the absence of serotonin at the 5-$HT_{2A}R$. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ERK, extracellular signal-regulated kinase; Boc, *tert*-butyloxycarbonyl; cAMP, cyclic adenosine monophosphate; ACh, acetylcholine

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