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Homobivalent Ligands of the Atypical Antipsychotic Clozapine: Design, Synthesis, and Pharmacological Evaluation

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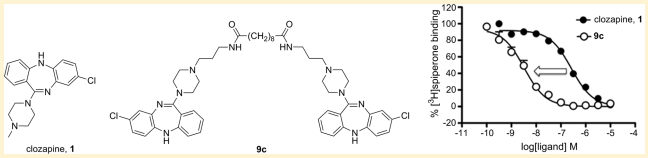
Supporting Information

ABSTRACT: To date all typical and atypical antipsychotics target the dopamine D_2 receptor. Clozapine represents the best-characterized atypical antipsychotic, although it displays only moderate (submicromolar) affinity for the dopamine D_2 receptor. Herein, we present the design, synthesis, and pharmacological evaluation of three series of homobivalent ligands of clozapine, differing in the length and nature of the spacer and the point of attachment to the pharmacophore. Attachment of the spacer at the N4′ position of clozapine yielded a series of homobivalent ligands that displayed spacer-length-dependent gains in affinity and activity for the dopamine D_2 receptor. The 16 and 18 atom spacer bivalent ligands were the highlight compounds, displaying marked low nanomolar receptor binding affinity (1.41 and 1.35 nM, respectively) and functional activity (23 and 44 nM), which correspond to significant gains in affinity (75- and 79-fold) and activity (9- and 5-fold) relative to the original pharmacophore, clozapine. As such these ligands represent useful tools with which to investigate dopamine receptor dimerization and the atypical nature of clozapine.



INTRODUCTION

The neurotransmitter dopamine mediates its physiological effects through interaction with five G protein-coupled receptor (GPCR) subtypes that can be divided into two distinct subfamilies: D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4). GPCRs coupled to G_s and $G_{i/o}$ G proteins, respectively.[1] In accordance with the dopamine hypothesis, which links positive psychotic symptoms with hyperactivity of dopaminergic neurons in the mesolimbic region of the brain, antagonism of D_2 receptors (D_2R) is usually considered to be responsible for the neuroleptic effect of antipsychotic drugs.[2] Clozapine (**1**), a dopamine D_2 receptor antagonist, is an atypical antipsychotic with unparalleled efficacy against refractory schizophrenia, although the dibenzodiazepine structure of clozapine has been implicated in the potentially fatal blood disorder agranulocytosis, which limits its use clinically.[3−6] Compared to typical antipsychotics such as haloperidol, clozapine has a relatively weak affinity for the D_2 receptor that, combined with its affinity for other neuroreceptors, is proposed to relate to the reduced extrapyramidal side effects associated with clozapine. However, this hypothesis has yet to be explored with analogues of clozapine with higher D_2 receptor affinity. Indeed, given clozapine's clinical efficacy, it is surprising to note the relative lack of studies exploring the structure−activity relationship (SAR) of clozapine, particularly with respect to improvements of affinity at the dopamine D_2 receptor.[7−11] Our own preliminary studies[8−10] revealed that the introduction of an aryl moiety at the distal N4′ piperazine nitrogen atom via a variable alkyl spacer has a modest effect on ligand affinity and in vivo activity. More recently, a paper by Su et al.[11] broadly explored the binding affinity resulting from modifications at the N5 and N4′ positions of clozapine using high-throughput parallel synthesis. Their findings report a significant gain in D_2 affinity upon the introduction of substituted arylmethyl groups at the N4′ position.

An alternative approach to modulation of GPCR affinity, efficacy, and selectivity is the generation of bivalent ligands. Bivalent ligands are compounds that consist of two pharmacophores covalently tethered by an appropriate spacer.[12,13] There are two general classes of bivalent ligands: homobivalent ligands, containing two identical pharmacophores, and heterobivalent ligands, wherein the two pharmacophores are different. Most bivalent ligands have been developed with a dual aim: (a) to improve affinity, by providing additional interactions, and (b) to improve selectivity, if these additional interactions involve less conserved regions across a family of receptors.[12] Much of the pioneering work describing bivalent ligands for GPCRs was led by the group of Portoghese,[12,14−16] targeting opioid receptor subtypes. Subsequently, homo- and heterobivalent ligands have also been developed to target a number of GPCRs including adenosine,[17−19] adrenergic,[18] cannabinoid,[20] dopamine,[17,21−23] muscarinic,[24,25] and serotonin[26,27] receptors. A number of mechanisms can account for the increase in affinity observed for a bivalent ligand as compared to the corresponding monovalent ligand.[28] The first possibility is that the local concentration of the pharmacophore is increased in the vicinity of the receptor binding site, which increases the probability of a productive binding event. Second, that one pharmacophore of

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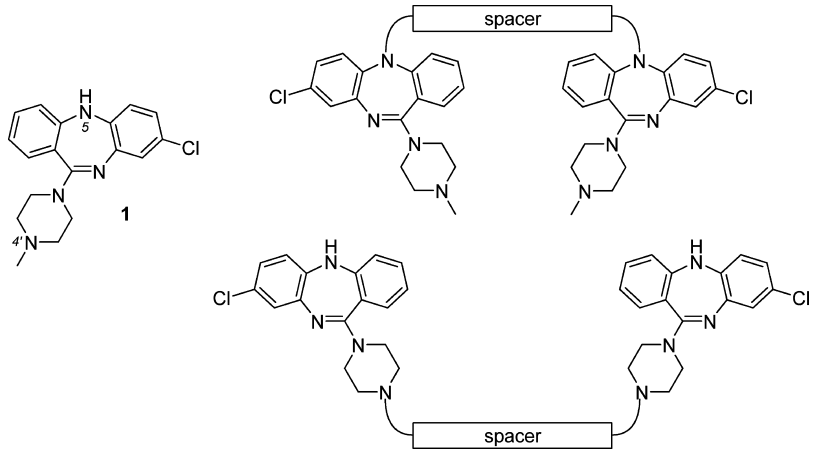
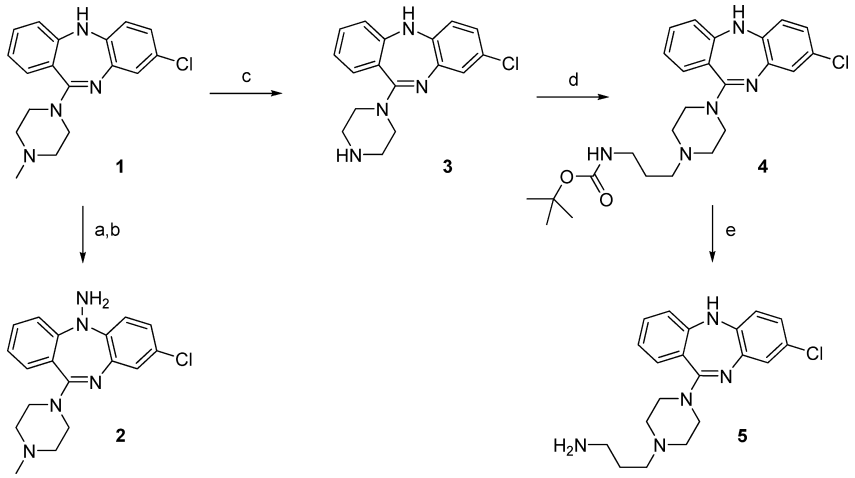
Chart 1. Structure of Clozapine (1) and General Structures of Homobivalent Ligands of Clozapine



Scheme 1. Synthesis of Key Intermediates Clozapine Hydrazine (2), *N*-Desmethylclozapine (3), and Clozapine Propylamine (5) from Clozapine (1)[a]



[a]Reagents and conditions: (a) isoamyl nitrite, CH_2Cl_2; (b) zinc powder, HOAc, 15 °C, yield 48%; (c) 1,2-dichloroethane, α-chloroethyl chloroformate, 0 °C to reflux, followed by CH_3OH, 50 °C, yield 69%; (d) *tert*-butyl 3-bromopropylcarbamate, NaI, DIPEA, CH_3CN, reflux, yield 79%; (e) TFA, CH_2Cl_2, followed by base, yield 94%.

the bivalent ligand binds to the orthosteric site, while the second pharmacophore binds to a neighboring (allosteric) site within the same receptor. Ligands exploiting this mode of interaction have recently been termed bitopic ligands.[29] The third possibility is that the bivalent ligand targets a dimeric or oligomeric complex of GPCRs, binding simultaneously at adjacent orthosteric sites and thus leading to increased affinity and (potentially) selectivity.[12,29] With an increasing amount of evidence from biochemical and biophysical studies suggesting GPCRs may exist in oligomeric complexes, bivalent ligands have emerged as useful tools to investigate this concept.[12,30]

A number of studies have explored bivalent ligands as a method to improve the affinity and selectivity of known pharmacophores targeting D_2R.[21−23] Abadi et al.[21] developed a series of bivalent azecine derivatives, with the six-carbon methylene spacer displaying the best activity. More recently, Gmeiner and co-workers synthesized two series of bivalent ligands to target D_2R: 1,1′-disubstituted ferrocenes[22] and 1,4-disubstituted aromatic piperazines.[23] In all of these studies, however, no significant gain in affinity was observed for the bivalent compound when compared to the appropriate monovalent control. Herein we report the design, synthesis, and pharmacological evaluation of three series of homobivalent ligands of the atypical antipsychotic clozapine (**1**), using two distinct attachment points and a series of simple dicarboxylic acid spacers, with the aim of producing clozapine derivatives with increased affinity, selectivity, efficacy, or activity for D_2R.

LIGAND DESIGN RATIONALE

Both the N5 and the distal piperazine nitrogen (N4′) positions of clozapine were synthetically attractive points for the attachment of spacers for the preparation of homobivalent ligands. Clozapine analogues with attachments on the N4′ [8−10] and the N5 position[11,31] have both been previously synthesized, and modifications at these positions were well tolerated.

The most significant side effect associated with clozapine is the potentially fatal blood disorder agranulocytosis. Formation of a reactive nitrenium ion intermediate resulting from oxidation of the NH group of clozapine has been postulated as a possible cause.[32,33] It is hypothesized that removal of the oxidatively susceptible NH functionality by developing homobivalent ligands of clozapine at the N5 attachment point may result in the reduction or abolishment of any drug-induced dyscrasia. However, directly acylating the N5 position

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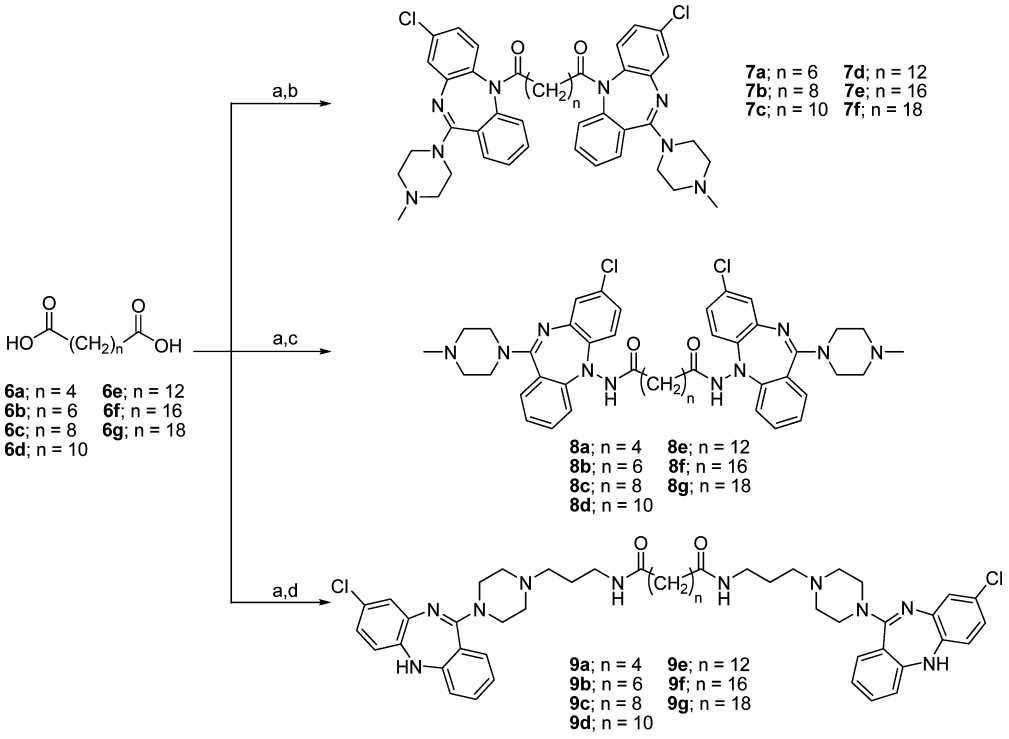
Scheme 2. Synthesis of Clozapine Homobivalent Ligands via Clozapine (1) and Key Clozapine Intermediates (2 and 5)[a]



[a]Reagents and conditions: (a) oxalyl chloride, DMF, CH_2Cl_2; (b) **1**, pyridine, DIPEA, CH_2Cl_2, yield 27−37%; (c) **2**, pyridine, DIPEA, CH_2Cl_2, yield 38−79%; (d) **5**, pyridine, DIPEA or K_2CO_3, CH_2Cl_2, yield 37−66%.

of clozapine may affect the conformational and electronic properties of the tricyclic nucleus. Therefore, in addition to acylation at this position, converting the N5 position to the hydrazine functionality, so that the spacer attachment point was not directly attached to the tricyclic ring system, was also investigated. Formation of a hydrazide at the N5 position has also been demonstrated to be well tolerated at this position.[11]

The other attachment point investigated was the distal piperazine nitrogen (N4′). The N4′ nitrogen is the ionizable nitrogen that interacts with the key aspartate residue on helix 3 (Asp 114)[3.32] at the entrance of the D_2 receptor orthosteric binding site.[34] However, directly acylating at this position would significantly change the pK_a of the ionizable nitrogen and interfere with the critical electrostatic interaction with the receptor. Therefore, a propylamine linker group between the ionizable nitrogen and the spacer was introduced. Compounds have been developed previously by alkylation of the N4′ nitrogen that exhibit activity in dopamine-related in vitro assays and in vivo behavioral models, thereby demonstrating that attachments at this position are well tolerated.[8−10] Representative monovalent ligands of the pharmacophore were also prepared for comparison of pharmacological activity against the corresponding synthesized homobivalent ligands.

Simple dicarboxylic acids were selected as the spacers for the synthesis of homobivalent ligands of clozapine, as they possess the desired functionality to form a stable amide bond to the pharmacophore. These were used to determine the appropriate spacer length for the bivalent ligands. More complex dicarboxylic acids, incorporating heteroatom-rich functionalities, were also explored to improve any solubility issues that may arise from the inclusion of a polymethylene chain.

RESULTS AND DISCUSSION

Chemical Synthesis. Three series of homobivalent ligands of clozapine were synthesized, at two distinct pharmacophore attachment points. Synthesis of the clozapine-based pharmacophores commenced with the preparation of clozapine (**1**) by a previously described procedure.[7,10]

Scheme 1 depicts the synthesis of the key clozapine intermediates (**2**, **3**, and **5**) from clozapine that were incorporated into the target homobivalent ligands. Following the procedure described by Su et al.,[11] clozapine was converted to the clozapine hydrazine (**2**) intermediate, first by N-nitrosylation with isoamyl nitrite, followed by reduction with zinc metal in acetic acid to form **2** in moderate yield (48%). This intermediate was used to form a hydrazide linkage with the designated spacer dicarboxylic acids. Clozapine was also N-demethylated by use of α-chloroethyl chloroformate,[35] yielding *N*-desmethylclozapine (**3**), in respectable yield (69%). Compound **3** was further alkylated with *tert*-butyl 3-bromopropylcarbamate in the presence of sodium iodide and *N*,*N*-diisopropylethylamine, to furnish **4**, and subsequently deprotected (with trifluoroacetic acid, TFA), to yield the clozapine propylamine intermediate (**5**).

The dicarboxylic acids (**6a−g**) were converted to their corresponding diacid chlorides by use of oxalyl chloride and *N*,*N*-dimethylformamide and were reacted, without further purification, with **1**, **2**, or **5** to yield the target homobivalent ligands as white (**7a−f**, yield 27−37%), off-white (**8a−g**, yield 38−79%), and yellow foams (**9a−g**, yield 37−66%), respectively, in moderate to good yields (Scheme 2).

In addition to the simple dicarboxylic acid spacers, more complex *N*,*N*′-disubstituted piperazinyloxocarboxylic acid (**11a,b**, Scheme 3) and dioxodioic acid (**13**, Scheme 4) spacers,

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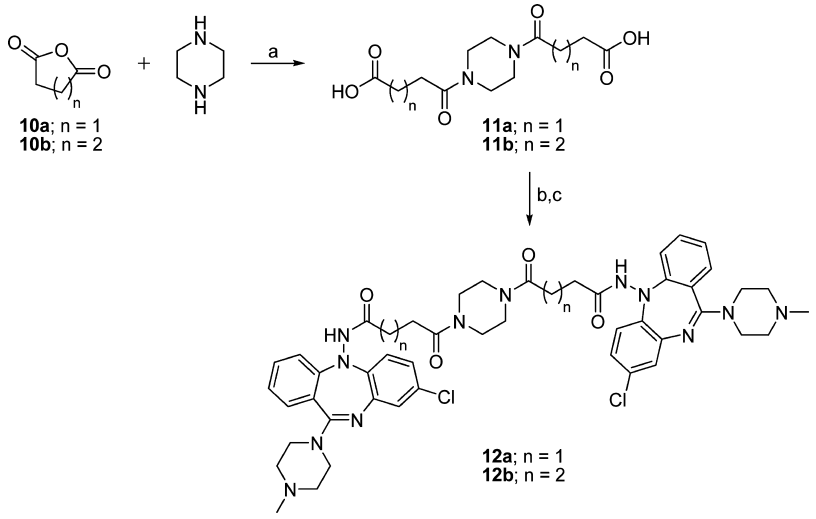
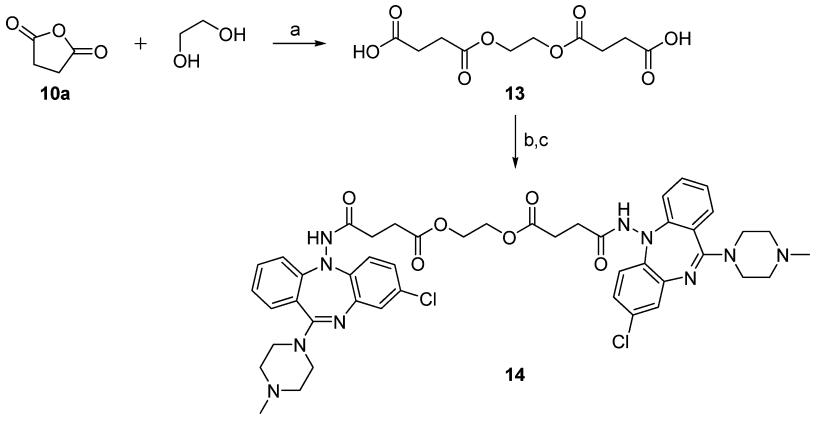
Scheme 3. Synthesis of Homobivalent Ligands of 2 Containing More Complex *N,N′*-Disubstituted Piperazinyloxocarboxylic Acid Spacers[a]



[a]Reagents and conditions: (a) toluene or 1,4-dioxane, reflux, yield 62% (**11a**) or 82% (**11b**); (b) oxalyl chloride, DMF, CH_2Cl_2; (c) **2**, pyridine, CH_2Cl_2, yield 24% (**12a**) or 36% (**12b**).

Scheme 4. Synthesis of Homobivalent Ligand of 2 Containing a Dioxodioic Acid Spacer[a]



[a]Reagents and conditions: (a) toluene, reflux, yield 27%; (b) oxalyl chloride, DMF, CH_2Cl_2; (c) **2**, pyridine, CH_2Cl_2, yield 46%.

with two spacer lengths of 12 and 14 atoms, were synthesized. Through developing spacers with additional functionalities, we were aiming to tune the hydrophobicity of the spacer, by incorporation of additional heteroatoms. Compounds **11a** and **11b** afforded white microcrystalline solids[36] in good yields (62−82%) by reaction of piperazine with 2 equiv of the desired cyclic anhydride (**10a,b**) at reflux. Compound **13** was synthesized by heating ethylene glycol and 2 equiv of succinic anhydride under Dean−Stark conditions and isolated in reasonable yield (27%).[37] These spacers were converted to their corresponding diacid chlorides by use of oxalyl chloride and *N,N*-dimethylformamide and subsequently reacted, without further purification, with **2** to yield the corresponding homobivalent ligands as off-white foams, in moderate yields (24−36%, **12a,b**; 46%, **14**). Interestingly, the target compounds **12a,b** and their corresponding precursors **11a,b** displayed the existence of a mixture of cisoid and transoid amide rotamers by NMR spectroscopy.[36,37] Further investigation into varying spacer lengths was abandoned due to difficulties in synthesizing longer spacers via this procedure, generally resulting in polymerization. Examples of these homobivalent ligands were synthesized only for the clozapine hydrazine intermediate (**2**), as a proof of concept.

Monovalent ligands were also synthesized (Scheme 5) for all three attachment points by reacting clozapine and clozapine intermediates with decanoyl chloride in the presence of base to yield the corresponding monovalent ligands (**15**, **16**, and **17**) in moderate yields. These compounds were designed for comparative purposes in pharmacological assays.

Functional Assays. The biological activity of clozapine and the homobivalent and monovalent compounds were assessed by an AlphaScreen plate-based assay of D_2 receptor-mediated phosphorylation of ERK1/2 (pERK1/2) in intact FlpIn-CHO cells stably expressing the D_{2L} receptor. Dopamine caused a concentration-dependent increase in pERK1/2 with a pEC_{50} of 8.4 ± 0.08 (EC_{50} = 4.0 nM, n = 9). To allow an estimation of the inhibitory potency of homobivalent ligands, monovalent ligands, and clozapine, we tested the ability of increasing concentrations of these ligands to antagonize an EC_{80} concentration (10 nM) of dopamine. IC_{50} values for all compounds were determined from the functional assay. Clozapine (**1**) displayed a submicromolar inhibitory potency

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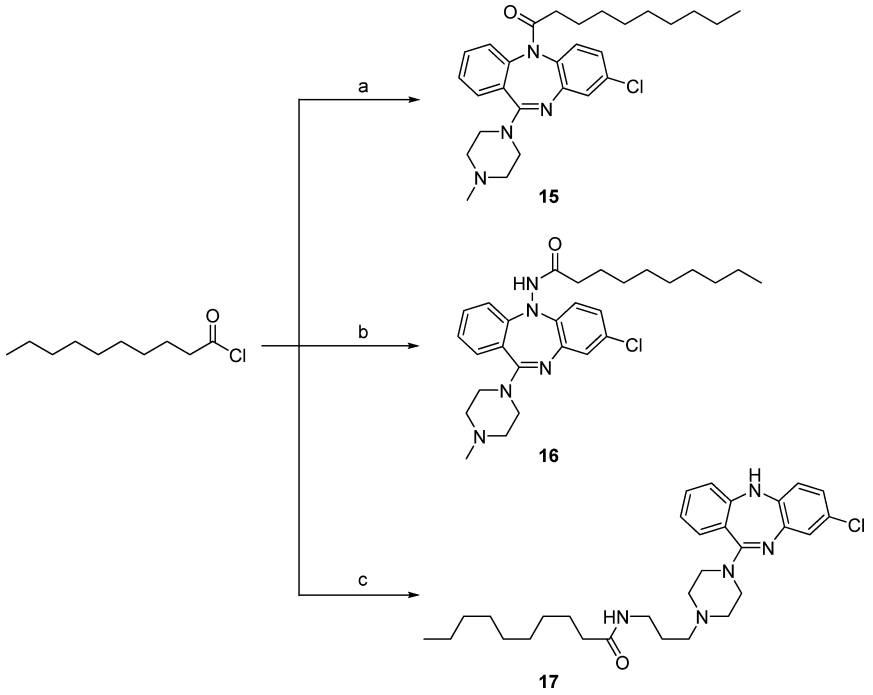
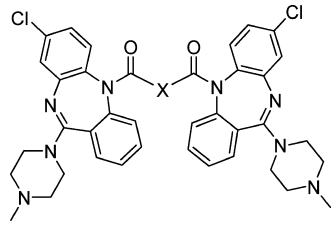
Scheme 5. Synthesis of Monovalent Ligands for Clozapine (1) and Key Clozapine Intermediates (2 and 5)[a]



[a]Reagents and conditions: (a) **1**, pyridine, DIPEA, CH_2Cl_2, yield 36%; (b) **2**, pyridine, CH_2Cl_2, yield 79%; (c) **5**, pyridine, CH_2Cl_2, yield 63%.

Table 1. Potency of Clozapine N5 Homobivalent (7a–f) and Monovalent (15) Ligands to Inhibit the Effect of 10 nM Dopamine[a]



compd	spacer length	spacer type (X)	pIC_{50} ± SEM	IC_{50}, nM
clozapine (**1**)			6.69 ± 0.20	206
15			6.11 ± 0.12	776
7a	8	$(CH_2)_6$	5.75 ± 0.22	2662
7b	10	$(CH_2)_8$	<5	>10 000
7c	12	$(CH_2)_{10}$	<5	>10 000
7d	14	$(CH_2)_{12}$	<5	>10 000
7e	18	$(CH_2)_{16}$	<5	>10 000
7f	20	$(CH_2)_{18}$	<5	>10 000

[a]Measured in an ERK1/2 phosphorylation assay using FlpIn CHO cells stably expressing D_2R. Data represent the mean ± SEM of three separate experiments performed in duplicate.

with a pIC_{50} of 6.69 ± 0.2 (IC_{50} = 206 nM). The clozapine N5 derivatives (**7a–f** and **15**, Table 1) displayed diminished antagonistic activity. Compound **15** showed some antagonistic activity (IC_{50} = 720 nM), which was approximately 4-fold less potent than clozapine (**1**). This result indicated that minor substitutions at this position could be tolerated and was in agreement with other results of N5-acylated clozapine analogues.[38] However, homobivalent ligands with significantly larger substitutions at the N5 position due to the attachment of the second pharmacophore (**7a–f**) displayed negligible activity in the functional assay, which suggests that there is some degree of size limitation to the substitutions that can be made at this position.

A similar trend was observed for the hydrazide-linked homobivalent ligands (**8a–g**, Table 2), with marginal antagonistic activity, displaying at best low micromolar activity in the functional assay. This observation was also applicable to the compounds containing the more complex dicarboxylic acid spacers (**12a,b** and **14**).

For the clozapine propylamine derivatives (**9a–g** and **17**), a spacer-length-dependent effect on inhibitory potency was observed (Figure 1, Table 3). The monovalent ligand (**17**, IC_{50} = 1.46 μM) was approximately 7-fold less active than clozapine (**1**, IC_{50} = 206 nM). The 14-atom spacer ligand (**9a**, IC_{50} = 87 nM) showed notably more activity than clozapine and the monovalent ligand (**17**) (2.4-fold and 17-fold increase

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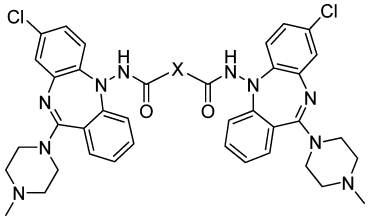
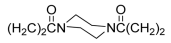
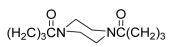
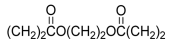
Table 2. Potency of Clozapine Hydrazide Homobivalent (8a–g, 12a,b, and 14) and Monovalent (16) Ligands to Inhibit the Effect of 10 nM Dopamine[a]



compd	spacer length	spacer type (X)	pIC_{50} ± SEM	IC_{50}, nM
clozapine (**1**)			6.69 ± 0.20	206
16			5.05 ± 0.03	8939
8a	6	$(CH_2)_4$	5.69 ± 0.21	2078
8b	8	$(CH_2)_6$	5.61 ± 0.13	2440
8c	10	$(CH_2)_8$	< 5	> 10 000
8d	12	$(CH_2)_{10}$	< 5	> 10 000
8e	14	$(CH_2)_{12}$	< 5	> 10 000
8f	18	$(CH_2)_{16}$	< 5	> 10 000
8g	20	$(CH_2)_{18}$	< 5	> 10 000
12a	12		5.79 ± 0.22	1617
12b	14		5.58 ± 0.20	2633
14	12		5.62 ± 0.16	2412

[a]Measured in an ERK1/2 phosphorylation assay using FlpIn CHO cells stably expressing D_2R. Data represents the mean ± SEM of three separate experiments performed in duplicate.

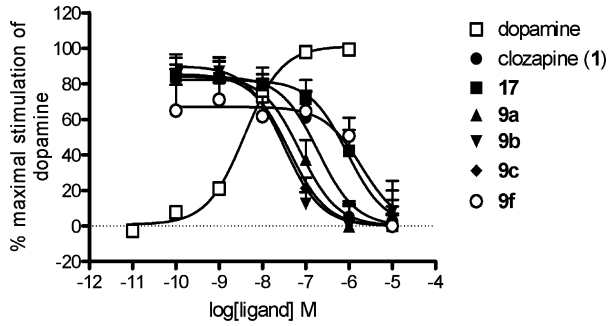


Figure 1. Ability of clozapine propylamine homobivalent (**9a–c,f**) and monovalent (**17**) ligands to inhibit the effect of 10 nM dopamine in an ERK1/2 phosphorylation assay using CHO cells expressing D_2R. Data represent three separate experiments performed in duplicate.

in potency, respectively). The highlight from the series was the 16-atom spacer homobivalent ligand (**9b**, IC_{50} = 23 nM) exhibiting the best activity of all the compounds developed, being 9-fold more potent than clozapine in the functional assay. The 18-atom spacer (**9c**) exhibited slightly less activity (44 nM) compared to the 16-atom spacer but was still 5-fold more active than clozapine. Beyond the 18-atom spacer, we observed a gradual, spacer-length-dependent reduction in activity for the 20-, 22-, 26-, and 28-atom spacers (**9d–g**) compared to the shorter homobivalent ligands.

Radioligand Binding Assays. The pERK1/2 assay represents a useful assay to allow the functional screening of a range of both monovalent and bivalent clozapine-derived ligands at human $D_{2L}R$. To confirm their on-target activity, the most promising clozapine bivalent ligands from the functional assay were further investigated by testing their ability to displace the radiolabeled antagonist [^{3}H]spiperone at human $D_{2L}R$ expressed in FlpIn CHO cell membranes. The most active clozapine propylamine homobivalent ligands described earlier (**9a–c**), the corresponding monovalent ligand (**17**), and clozapine (**1**), as well as a clozapine propylamine homobivalent ligand that displayed poor activity in the functional assay (**9f**), were evaluated (Figure 2, Table 4). The parent compound clozapine (**1**) showed a similar pK_i (6.99 ± 0.08, K_i = 106 nM) in this binding assay as compared to the inhibitory potency (pIC_{50} = 6.69 ± 0.2, IC_{50} = 206 nM) determined in the pERK1/2 functional assay. Furthermore, the order of inhibitory potency observed in the functional pERK1/2 assay was preserved in the radioligand binding assay; the homobivalent ligands with shorter spacers (**9a–c**) displayed significantly greater affinity than the homobivalent ligand with longer spacers (**9f**). Indeed, the bivalent ligands with shorter spacers (14–18 atoms, **9a–c**) displayed 30–79 times greater affinity for $D_{2L}R$ as compared to clozapine, with the two most active compounds demonstrating low nanomolar affinity (**9b** and **9c**, 1.41 and 1.35 nM, respectively). However, one interesting discrepancy between the functional and radioligand binding data should be noted. In the [^{3}H]spiperone binding assay, the monovalent ligand (**17**) displayed markedly enhanced affinity (12-fold, K_i = 9.06 nM) compared to clozapine. By comparison, in the functional assay, this compound displayed a 7-fold decrease in potency as compared to the clozapine (**1**). Importantly, the most active compounds in this series (**9a–c**) still showed increases in affinity from 2.5- to 6.5-fold as compared to the monovalent compound. For all compounds tested, the inhibition curves had Hill slopes not significantly different from unity (Table 4).

The main aim of developing homobivalent ligands of clozapine was to assess the effect on affinity and functional activity of covalently tethering two clozapine pharmacophores by a spacer of a given length and type. Of the three series of clozapine bivalent ligands, the clozapine propylamine series had the spacer attached from the N4′ distal piperazine nitrogen of clozapine; this was identified as the preferred attachment point for the spacer. Importantly, all of the tested clozapine derivatives retained the antagonistic action of the parent compound. It is noteworthy that linking from the ionizable piperazine nitrogen also generated bivalent ligands that retained activity in another series of D_2R targeting homobivalent ligands.[23] This is likely to be a result of positioning the ionizable nitrogen at the entrance of the orthosteric binding site due to formation of the key salt bridge with Asp 114$^{3.32}$. There are distinct similarities between the linking strategy used for our clozapine bivalent ligands and the bivalent ligands developed by Gmeiner and co-workers,[23] as both compound libraries contain an aliphatic spacer associated with a linking alkyl chain from the pharmacophore via a stable connecting motif (amide bond compared to a triazole ring). However, in the case of the clozapine propylamine homobivalent ligands (**9a–g**), the linker is directly attached to the distal piperazine in clozapine, whereas for Gmeiner and co-workers it is attached via an alkoxy benzyl group to the piperazine of the 1,4-disubstituted aromatic piperazines series, thereby slightly increasing the rigidity of the

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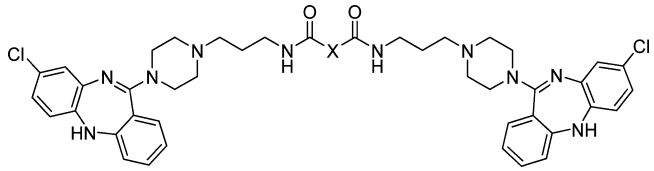
Table 3. Potency of Clozapine Propylamine Homobivalent (9a−g) and Monovalent (17) Ligands to Inhibit the Effect of 10 nM Dopamine[a]



compd	spacer length	spacer type (X)	pIC_{50} ± SEM	IC_{50}, nM
clozapine (**1**)			6.69 ± 0.20	206
17			5.84 ± 0.16	1455
9a	14	$(CH_2)_4$	7.06 ± 0.20	87
9b	16	$(CH_2)_6$	7.63 ± 0.20	23
9c	18	$(CH_2)_8$	7.35 ± 0.12	44
9d	20	$(CH_2)_{10}$	5.96 ± 0.12	1119
9e	22	$(CH_2)_{12}$	4.97 ± 0.18	11 000
9f	26	$(CH_2)_{16}$	5.11 ± 0.14	7800
9g	28	$(CH_2)_{18}$	<5	>10 000

[a]Measured in an ERK1/2 phosphorylation assay using FlpIn CHO cells stably expressing D_2R. Data represents the mean ± SEM of three separate experiments performed in duplicate.

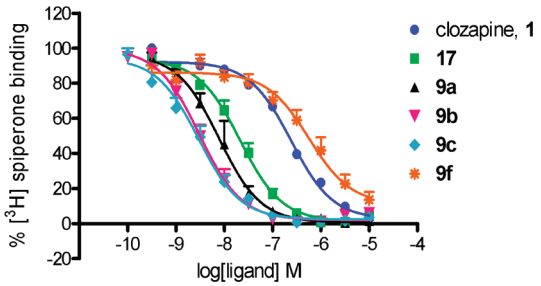


Figure 2. Ability of clozapine propylamine homobivalent (**9a−c,f**) and monovalent (**17**) ligands to inhibit binding of the antagonist [^{3}H]spiperone at D_2R expressed in FlpIN CHO cell membranes. Data represent three separate experiments performed in duplicate.

Table 4. Affinity of Propylamine Homobivalent (9a−c,f) and Monovalent (17) Ligands[a]

compd	spacer length	pK_i ± SEM	K_i, nM	Hill slope ± SEM
clozapine (**1**)		6.99 ± 0.08	106	0.91 ± 0.09
17		8.05 ± 0.06	9.1	0.97 ± 0.12
9a	14	8.50 ± 0.14	3.6	1.04 ± 0.19
9b	16	8.87 ± 0.09	1.41	1.05 ± 0.06
9c	18	8.91 ± 0.14	1.35	0.82 ± 0.10
9f	26	6.61 ± 0.13	269	0.80 ± 0.20

[a]Determined by competition binding experiments using the radiolabeled antagonist [^{3}H]spiperone at D_2R expressed in FlpIN CHO cell membranes. Data represents the mean ± SEM of three separate experiments performed in duplicate.

compound. For the clozapine propylamine bivalent ligands, it was interesting to note that affinity and activity were spacer-length-dependent, with similar trends observed in both functional and radioligand binding studies. Both studies indicated the 16- and 18-atom spacers (**9b** and **9c**) were the most potent/active, followed by a gradual decrease in activity with increasing spacer length. These spacer lengths are within the 15−22-atom spacer length range identified in other bivalent ligand studies targeting GPCR dimers or oligomers, although they are shorter than the 22-atom spacer proposed by Gmeiner and co-workers[23] for D_2R dimer targeting homobivalent ligands.

As postulated by Portoghese,[28] if a bivalent ligand binds simultaneously to two identical binding sites, ideally the binding affinity should be the product of the binding affinities of the two individual pharmacophores. However, in the study by Gmeiner and co-workers,[23] the optimal compound in a 1,4-disubstituted aromatic piperazines series was identified by a change in the Hill slope. These steep Hill slopes were not accompanied by any increase in affinity as compared to the monovalent compound, as would be expected upon simultaneous engagement of two identical binding sites within a D_2 receptor dimer. In contrast, for the clozapine propylamine bivalent ligands (**9b** and **9c**, K_i = 1.41 and 1.35 nM), the Hill slopes were at unity, yet potency gains of 6- and 7-fold relative to the monovalent ligand were observed (**17**, K_i = 9 nM) as well as a 75- and 79-fold increase in affinity compared to clozapine (**1**, K_i = 106 nM). Although this series (**9a−g**) represents the first such homobivalent ligands to achieve significant gains in affinity at dopamine D_2 receptor, such gains are not of the order of magnitude predicted by the product of the affinities. Therefore other binding mechanisms, such as increasing the local concentration of the pharmacophore in the vicinity of the receptor binding site or binding to two topically distinct sites on one receptor, may also explain these gains in affinity.[29] Although there is accumulating evidence to suggest that the D_2R receptor forms homo-oligomers,[39,40] there remains sufficient controversy regarding the ability of class A receptors to dimerize in vivo to give these other mechanisms due consideration.[29,41] As such, these high-affinity clozapine bivalent ligands (**9b** and **9c**) may represent useful pharmacological tools to investigate D_2R dimers in combination with appropriate biochemical or biophysical studies.

The monovalent ligand of the clozapine propylamine series displayed unique activity. Specifically, in the preliminary functional assay the monovalent ligand (**17**, IC_{50} = 1.46 μM) was 7-fold less active than clozapine (**1**, IC_{50} = 206 nM), yet in the radioligand binding assays the monovalent ligand (K_i = 9 nM) displayed 12-fold greater activity than clozapine (K_i = 106 nM). In a recent study using bitopic ligands to target the muscarinic M_2 AChR, Steinfeld et al.[42] observed the seven-

chain pharmacophore spacer itself also promoted an increase in compound affinity, highlighting the importance of including incremental fragments of novel bivalent or bitopic ligands in control experiments. In the present study, introduction of the second pharmacophore (the bivalent ligand) further improved the affinity or inhibitory potency of the compound and implies that the presence of a second pharmacophore engenders this gain of affinity or potency.

It is noteworthy that although the parent compound clozapine had a similar potency in the functional ERK1/2 assay and radioligand binding assay, the bivalent compounds had substantially higher affinity in the radioligand binding assay as compared to their potency in the ERK assay. Interestingly, Abadi et al.[21] noted a similar discrepancy for bivalent azecine derivitives and suggested that altered binding kinetics for the bivalent ligands, as compared to the parent compound, may explain such observations. However, given the biochemical behavior of the monovalent ligand (**17**), the hydrophobic nature of the alkyl chain should be noted. Such a linker may act as a lipid anchor for the ligand and therefore explain the discrepancy between whole-cell functional assays and membrane ligand-binding assays. Future studies should address the role of the linker with the use of more hydrophilic linkers, such as PEG linkers, or linkers with greater rigidity.

It has been hypothesized that the relative lack of extrapyramidal side effects observed for atypical antipsychotics is related to their low affinity and/or fast dissociation kinetics.[2] Considering the significant affinity gains observed for the clozapine bivalent ligands (**9b** and **9c**, 75- and 79-fold) relative to the original pharmacophore, clozapine, it would also be of great interest to investigate how the covalent tethering of two clozapine pharmacophores affects the atypical nature of these compounds compared to clozapine.

CONCLUSION

We describe the design, synthesis, and pharmacological evaluation of homobivalent ligands of the atypical antipsychotic clozapine (**1**), differing in the nature and length of the spacer and point of attachment to the pharmacophore. The best attachment point for the synthesis of clozapine homobivalent ligands was identified as the N4′ position, which also incorporated a linking group between the ionizable nitrogen and the spacer. Both functional and binding assays revealed a spacer-length-dependent effect for compounds **9a**−**g**, with the most active compounds (**9b** and **9c**) having spacer lengths of 16 and 18 atoms, respectively. These compounds displayed marked low nanomolar receptor binding affinity (K_i = 1.41 and 1.35 nM) and noteworthy functional activity (23 and 44 nM) compared to the original pharmacophore, clozapine. Overall, significant gains in affinity (75- and 79-fold) and activity (9- and 5-fold) were observed. The clozapine propylamine bivalent ligands developed in this study could be of use to further elucidate the atypical nature of clozapine, as well as being used as pharmacological tools to investigate D_2R dimerization.

EXPERIMENTAL METHODS

General Experimental. All materials were reagent-grade and were purchased commercially from Sigma−Aldrich, Alfa Aesar, Tokyo Chemical Industry, AOKChem, and Merck. Succinic anhydride and glutaric acid were recrystallized from chloroform, and adipic acid was recrystallized from ethyl acetate prior to use. Ethyl acetate and hexane were redistilled prior to use. Dichloromethane was purified by predrying with calcium chloride and freshly distilling from calcium hydride prior to use.

Thin-layer chromatography (TLC) was performed using Merck Silica Gel 60 F_{254} precoated plates (0.25 mm) and visualized by ultraviolet light, as well as staining with iodine or ninhydrin. Flash column chromatography used Merck silica gel 60, 230−400 mesh ASTM, following the method described by Still et al.[43] All compounds were preadsorbed onto coarse silica (70−230 mesh ASTM) prior to column chromatography, unless otherwise stated. Where gradient elution was utilized for column chromatography, the eluent was modified as detailed, in 50−100 mL increments.

^{1}H NMR spectra were routinely recorded at 300.13 MHz on a Brüker Avance DPX-300 spectrometer or at 400.13 MHz on a Bruker Ultrashield-Avance III NMR spectrometer, using TOPSPIN v2.1 software, at 298 K, unless stated otherwise. Chemical shifts (δ_H) for all ^{1}H NMR spectra were reported in parts per million (ppm) with tetramethylsilane (TMS) as the internal standard (δ_H 0.00 ppm) in deuterated solvents, including chloroform ($CDCl_3$), dimethyl sulfoxide-d_6 (DMSO-d_6), methanol-d_4 (CD_3OD), acetone-d_6, and deuterium oxide (D_2O), as indicated. The ^{1}H NMR spectra are reported as follows: chemical shift (δ), multiplicity, coupling constants (J) in Hertz (quoted to one decimal place ±0.2 Hz), peak integration, and assignment. In reporting the spectral data, the following abbreviations have been used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, app = apparent.

^{13}C NMR spectra were routinely recorded at 75.5 MHz on a Brüker Avance DPX-300 spectrometer or at 100.62 MHz or a Brüker Ultrashield-Avance III NMR spectrometer, using TOPSPIN v2.1 software, at 298 K, unless stated otherwise. Distortionless enhancement by polarization transfer (DEPT) experiments were routinely used for ^{13}C NMR spectra. Chemical shifts (δ_C) for all ^{13}C NMR spectra were reported in parts per million (ppm), using the center of the solvent chemical shift as the reference: $CDCl_3$ (77.16), DMSO-d_6 (39.52), CD_3OD (49.00), and acetone-d_6 (29.84), as indicated.[44] ^{13}C NMR signals are assigned as C = quaternary carbon, CH = methine carbon, CH_2 = methylene carbon, and CH_3 = methyl carbon.

Melting points (mp) were determined on a Mettler Toledo MP50 melting point apparatus, except those marked with an asterisk, which were determined on a Kofler hot stage micromelting point apparatus, and are uncorrected.

Mass spectra were acquired in the positive and negative mode by use of an atmospheric pressure (ESI/APCI) ion source on a Micromass Platform II ESI/APCI single-quadrupole mass spectrometer with sample management facilitated by an Agilent 1100 series HPLC system using MassLynx version 3.5 software. Liquid chromatography mass spectra (LCMS) were measured on an Agilent 6100 series single quad LC/MS, Agilent 1200 series HPLC (pump, 1200 series G1311A quaternary pump; autosampler, 1200 series G1329A thermostated autosampler; detector, 1200 series G1314B variable-wavelength detector). Gradient takes 4 min to get to 100% acetonitrile (ACN), maintained for 3 min, and a further 3 min to return to the original 5% ACN.

High-resolution mass spectrometry (HRMS) analyses were obtained on a Waters Micromass LCT premier XE orthogonal acceleration time-of-flight mass spectrometer coupled to an Alliance 2795 separation module using MassLynx version 4.1 software.

Analytical reverse-phase HPLC was carried out on a Waters Millenium 2690 system, fitted with a Phenomenex Luna C8, 100 Å, 5 μm (50 × 4.60 mm i.d.) column. A binary solvent system was used (solvent A, 0.1% TFA/H_2O; solvent B, 0.1% TFA/19.9% H_2O/80% acetonitrile), with UV detection at 214 nm. Method 1 used gradient elution, beginning with 100% solvent A and going to 20% solvent A/80% solvent B over 20 min at a flow rate of 1 mL/min. Method 2 used gradient elution beginning with 80% solvent A/20% solvent B and going to 100% solvent B over 20 min at a flow rate of 1 mL/min. The purity of all tested compounds and key intermediates was determined to be >95%.

Prior to pharmacological testing, all compounds were converted to their hydrochloride salts, by use of hydrogen chloride in diethyl ether (1.0 M).

*tert-Butyl {3-[4-(8-chloro-5H-dibenzo[b,e][1,4]diazepin-11-yl)-piperazin-1-yl]propyl}carbamate (**4**).* *N*-Desmethylclozapine (**3**) (1.02 g, 3.27 mmol), sodium iodide (0.482 g, 3.22 mmol), and *N*,*N*-diisopropylethylamine (670 μL, 3.85 mmol) were combined in acetonitrile (30 mL), under nitrogen. *tert*-Butyl (3-bromopropyl)-carbamate (0.850 g, 3.57 mmol) was dissolved, under nitrogen, in acetonitrile (2 mL) and added to the stirred mixture. The reaction mixture was heated at reflux for 2 h, after which time additional *tert*-butyl (3-bromopropyl)carbamate (0.400 g, 1.68 mmol) was added. After an additional 1 h, the reaction mixture was cooled to room temperature and concentrated in vacuo. The orange oil was purified by column chromatography (5% methanol/chloroform), yielding **4** as a yellow foam (1.22 g, 2.60 mmol, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 (ddd, J = 7.9, 7.4, 1.6 Hz, 1H, H3″), 7.25 (m, 1H, H1″), 7.06 (d, J = 2.4 Hz, 1H, H9″), 7.01 (ddd, J = 7.8, 7.4, 1.1 Hz, 1H, H2″), 6.82 (m, 1H, H4″), 6.81 (dd, J = 8.3, 2.4 Hz, 1H, H7″), 6.61 (d, J = 8.3 Hz, 1H, H6″), 5.26 (br s, 1H, CONH), 4.92 (s, 1H, H5″), 3.45 (m, 4H, H3′ and H5′), 3.20 (app q, J = 6.3 Hz, 2H, H1), 2.53 (m, 4H, H2′ and H6′), 2.45 (t, J = 6.8 Hz, 2H, H3), 1.68 (app p, J = 6.7 Hz, 2H, H2), 1.44 [s, 9H, $C(CH_3)_3$]. ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.8 (C), 156.2 (C), 152.9 (C), 142.0 (C), 140.5 (C), 132.0 (CH), 130.4 (CH), 129.2 (C), 126.9 (CH), 123.6 (C), 123.18 (CH), 123.15 (CH), 120.2 (CH), 120.1 (CH), 79.1 (C), 57.0 (CH_2), 53.3 (CH_2), 47.4 (CH_2), 40.0 (CH_2), 28.6 (CH_3), 26.6 (CH_2). HPLC: t_R 8.01 min, >99% purity (method 1). LCMS (m/z): 470.1 $[M + H]^+$. HRMS (m/z): $C_{25}H_{33}ClN_5O_2^+$ requires $[M + H]^+$ 470.2317; found 470.2330.

*3-[4-(8-Chloro-5H-dibenzo[b,e][1,4]diazepin-11-yl)piperazin-1-yl]propan-1-amine (**5**).* To a stirred solution of *tert*-butyl {3-[4-(8-chloro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-11-yl)piperazin-1-yl]propyl}-carbamate (**4**) (1.22 g, 2.60 mmol) in dichloromethane (5 mL) at room temperature was added trifluoroacetic acid (5 mL, 65.3 mmol). The reaction mixture was stirred for 2 h before being diluted with dichloromethane (50 mL). Aqueous potassium carbonate (50 mL, 1 M) was added pipetwise to the stirred mixture. The aqueous layer was separated and extracted with dichloromethane (3 × 50 mL), and the combined organic layers were washed with water (2 × 50 mL) and saturated brine (50 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to yield **5** as a yellow foam (0.901 g, 2.44 mmol, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 (ddd, J = 7.9, 7.4, 1.6 Hz, 1H, H3″), 7.25 (dd, J = 8.1, 1.6 Hz, 1H, H1″), 7.06 (d, J = 2.4 Hz, 1H, H9″), 7.00 (ddd, J = 7.8, 7.3, 1.1 Hz, 1H, H2″), 6.81 (m, 1H, H4″), 6.80 (dd, J = 8.3, 2.4 Hz, 1H, H7″), 6.60 (d, J = 8.3 Hz, 1H, H6″), 4.97 (s, 1H, H5″), 3.46 (m, 4H, H3′ and H5′), 2.77 (m, 2H, H1), 2.54 (m, 4H, H2′ and H6′), 2.45 (m, 2H, H3), 1.66 (app p, J = 7.0 Hz, 2H, H2), 1.51 (br s, 2H, NH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.9 (C), 152.8 (C), 141.9 (C), 140.6 (C), 131.9 (CH), 130.4 (CH), 129.1 (C), 126.8 (CH), 123.5 (C), 123.09 (CH), 123.08 (CH), 120.2 (CH), 120.1 (CH), 56.6 (CH_2), 53.3 (CH_2), 47.4 (CH_2), 40.9 (CH_2), 30.5 (CH_2). HPLC: t_R 6.26 min, 95% purity (method 2). LCMS (m/z): 370.1 $[M + H]^+$. HRMS (m/z): $C_{20}H_{25}ClN_5^+$ requires $[M + H]^+$ 370.1793; found 370.1788.

Synthesis of Bivalent Ligands. *General Procedure A for Preparation of Clozapine Bivalent Ligands (**7a**–**f**).* All glassware used in the following procedure was flame-dried and cooled under nitrogen. The dicarboxylic acid (**6a**–**f**) (0.342–0.428 mmol) was suspended in dry dichloromethane (2 mL) at room temperature, under a nitrogen atmosphere. Oxalyl chloride (2.2 equiv) and *N*,*N*-dimethylformamide (1 drop) were added to the solution, which was stirred for 1 h at room temperature. To this mixture was added a solution of clozapine (**1**, 1.8 equiv) and pyridine (2.8 equiv), and in some cases *N*,*N*-diisopropylethylamine (2.0–2.5 equivs), in dry dichloromethane (2 mL). The reaction was monitored by TLC, and after 1 h, if a significant amount of **1** remained, an additional 0.5 equiv of diacid chloride in dry dichloromethane (1 mL) was added to the stirred solution. The reaction mixture was stirred overnight at room temperature, under nitrogen. The reaction mixture was partitioned between dichloromethane (10 mL) and aqueous potassium carbonate (10 mL, 1 M). The organic layer was separated and the aqueous layer was extracted with dichloromethane (3 × 10 mL). The combined organic extracts were washed with water (2 × 10 mL) and saturated brine (10 mL), dried over anhydrous sodium sulfate, filtered, and evaporated to dryness in vacuo, yielding a pale brown oil. The product was purified by preadsorption onto coarse silica, followed by flash column chromatography. Compounds were typically purified with 1–3 columns (refer to specific experimental description for details of eluent).

*1,8-Bis[8-chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e]-[1,4]diazepin-5-yl]octane-1,8-dione (**7a**).* 1,8-Octanedioic acid (**6a**) (0.060 g, 0.342 mmol) and oxalyl chloride were reacted, followed by the addition of **1** (0.209 g, 0.639 mmol) and pyridine (0.080 mL, 0.991 mmol) as per general procedure A. Additional 1,8-octanedioyl dichloride (0.037 g, 0.175 mmol) was added. Column chromatography conditions: column 1, 20% methanol/acetone until clozapine eluted and then 10% methanol/chloroform; column 2, gradient elution from 5% to 10% methanol/chloroform, increasing in 1% increments; column 3, 1% ammonia/9% methanol/chloroform. Yield: **7a** as a white foam (0.069 g, 0.087 mmol, 27%). ^{1}H NMR (400 MHz, $CDCl_3$, 320 K) δ 7.49 (ddd, J = 7.9, 7.0, 1.9 Hz, 2H, H3′), 7.41–7.30 (m, 6H, H1′, H2′, and H4′), 7.14 (d, J = 2.4 Hz, 2H, H9′), 7.09 (d, J = 8.4 Hz, 2H, H6′), 6.95 (m, 2H, H7′), 3.70 (m, 4H, H2″a and H6″a), 3.47 (m, 4H, H2″b and H6″b), 2.46 (m, 4H, H3″a and H5″a), 2.36 (m, 4H, H3″b and H5″b), 2.32–2.20 (m, 8H, CH_3, H2a and H7a), 2.09 (m, 2H, H2b and H7b), 1.51 (m, 4H, H3 and H6), 1.18 (m, 4H, H4 and H5). ^{13}C NMR (101 MHz, $CDCl_3$, 320 K) δ 173.8 (C), 160.7 (C), 146.5 (C), 145.2 (C), 134.1 (C), 133.8 (C), 132.1 (CH), 129.2 (CH), 127.93 (CH), 127.87 (CH), 127.1 (C), 126.3 (2 × CH), 123.4 (CH), 55.0 (CH_2), 47.2 (CH_2), 46.19 (CH_3), 33.5 (CH_2), 29.0 (CH_2), 25.0 (CH_2). HPLC: t_R 11.76 min, 98% purity (method 2). LCMS (m/z): 791.1 $[M + H]^+$, 396.2 $[M + 2H]^{2+}$. HRMS (m/z): $C_{44}H_{49}Cl_2N_8O_2^+$ requires $[M + H]^+$ 791.3350; found 791.3354.

*General Procedure B for Preparation of Hydrazide Bivalent Ligands (**8a**–**g**).* All glassware used in the following procedure was flame-dried and cooled under nitrogen. The diacid chloride was prepared by one of two methods. In method A, the dicarboxylic acid (14.8 mmol) was refluxed in neat thionyl chloride (10.0 mL, 138 mmol) for 90 min and then concentrated in vacuo before further use. In method B, the dicarboxylic acid (0.242–0.351 mmol) was suspended in dry dichloromethane (2 mL) at room temperature, under a nitrogen atmosphere. Oxalyl chloride (2.2 equiv) and *N*,*N*-dimethylformamide (1 drop) were added to the solution, which was stirred for 1 h at room temperature. To the diacid chloride from method A or B, was added a solution of 8-chloro-11-(4-methylpiperazin-1-yl)-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-amine (**2**, 1.8 equiv) and pyridine (2.8 equiv), and in some cases *N*,*N*-diisopropylethylamine (2.5 equiv), in dry dichloromethane (5 mL). The reaction was monitored by TLC, and after 1 h, if some **2** remained, an additional 0.5 equiv of diacid chloride in dry dichloromethane (1 mL) was added to the reaction mixture and stirred overnight at room temperature. The reaction mixture was partitioned between dichloromethane (10 mL) and aqueous potassium carbonate (10 mL, 1 M). The organic layer was separated and the aqueous layer was extracted with dichloromethane (3 × 10 mL). The combined organic layers were washed with water (2 × 10 mL) and saturated brine (10 mL), dried over anhydrous sodium sulfate, filtered, and evaporated to dryness in vacuo, yielding a pale brown oil. The product was purified by preadsorption onto coarse silica, followed by flash column chromatography. Compounds were typically purified with 1–3 columns (refer to specific experimental description for details of eluent).

*N^1,N^6-Bis[8-chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e]-[1,4]diazepin-5-yl]hexanediamide (**8a**).* Adipoyl dichloride was prepared according to method A in general procedure B. A portion of adipoyl dichloride (0.065 g, 0.357 mmol) in dry dichloromethane (5 mL) was added to **2** (0.227 g, 0.665 mmol) and pyridine (0.080 mL, 0.991 mmol) in dry dichloromethane (5 mL) according to general procedure B. Additional adipoyl dichloride (0.016 g, 0.090 mmol) was added. The crude product was recrystallized from dichloromethane/hexane to give **8a** as off-white crystals (0.209 g, 0.264 mmol, 79%), mp

185.3−186.4 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.46 (s, 2H, NH), 8.54 (d, J = 2.3 Hz, 2H, H9′), 7.78 (m, 2H, H1′/H4′), 7.44−7.37 (m, 4H, H3′/H2′ and H4′/H1′), 7.36 (d, J = 8.6 Hz, 2H, H6′), 7.15 (m, 2H, H2′/H3′), 7.13 (dd, J = 8.5, 2.4 Hz, 2H, H7′), 3.57 (m, 8H, H2″ and H6″), 2.68 (m, 8H, H3″ and H5″), 2.39 (s, 6H, CH_3), 2.25 (m, 4H, H2 and H5), 1.67 (m, 4H, H3 and H4). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.6 (C), 153.9 (C), 141.9 (C), 133.8 (C), 132.8 (C), 128.3 (CH), 126.9 (C), 124.6 (CH), 123.7 (CH), 122.7 (CH), 121.9 (CH), 120.8 (CH), 116.3 (C), 110.7 (CH), 54.8 (CH_2), 49.4 (CH_2), 46.4 (CH_3), 37.8 (CH_2), 24.8 (CH_2). HPLC: t_R 9.72 min, >99% purity (method 1). LCMS (m/z): 793.1 $[M + H]^+$, 397.2 $[M + 2H]^{2+}$. HRMS (m/z): $C_{42}H_{47}Cl_2N_{10}O_2^+$ requires $[M + H]^+$ 793.3255; found 793.3256.

*4,4′-(Piperazine-1,4-diyl)bis{N-[8-chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepin-5-yl]-4-oxobutanamide} (**12a**).* 4,4′-(Piperazine-1,4-diyl)bis(4-oxobutanoic acid) (**11a**, 0.090 g, 0.316 mmol) and oxalyl chloride were reacted, followed by the addition of **2** (0.191 g, 0.558 mmol) and pyridine (70 μL, 0.867 mmol) as per general procedure B. Additional 4,4′-(piperazine-1,4-diyl)bis(4-oxobutanoyl chloride) (0.052 g, 0.161 mmol) was added. Column chromatography conditions: 1% ammonia/4% methanol/chloroform. Yield: **12a** as an off-white foam (0.063 g, 0.067 mmol, 24%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.70 (s, 2H, NH), 8.56 (s, 2H, H9″), 7.79 (ddd, J = 8.2, 0.9, 0.9 Hz, 2H, H1″/H4″), 7.42−7.36 (m, 4H, H3″/H2″ and H4″/H1″), 7.36 (d, J = 8.5 Hz, 2H, H6″), 7.15 (m, 2H, H2″/H3″), 7.13 (dd, J = 8.5, 2.4 Hz, 2H, H7″), 3.59 (m, 8H, H2‴ and H6‴), 3.55−3.46 (m, 4H, piperazine spacer), 3.42−3.36 (m, 4H, piperazine spacer), 2.69 (m, 8H, H3‴ and H5‴), 2.64 (s, 8H, H2′ and H3′), 2.40 (s, 6H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$, mixture of amide rotamers)[36] δ 170.3 (C), 170.1 (C), 170.0 (C), 153.9 (C), 141.9 (C), 133.9 (C), 132.9 (C), 128.2 (CH), 126.9 (C), 124.8 (CH), 123.7 (CH), 122.6 (CH), 121.8 (CH), 120.7 (CH), 116.4 (C), 110.7 (CH), 54.8 (CH_2), 49.4 (CH_2), 46.3 (CH_3), 45.2 (CH_2), 45.0 (CH_2), 41.53 (CH_2), 41.45 (CH_2), 32.7 (CH_2), 32.6 (CH_2), 28.0 (CH_2). HPLC: t_R 7.22 min, 96% purity (method 2). LCMS (m/z): 933.2 $[M + H]^+$, 467.2 $[M + 2H]^{2+}$. HRMS (m/z): $C_{48}H_{55}Cl_2N_{12}O_4^+$ requires $[M + H]^+$ 933.3841; found 933.3803.

*Ethane-1,2-diyl bis(4-{[8-chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepin-5-yl]amino}-4-oxobutanoate) (**14**).* 4,4′-[Ethane-1,2-diylbis(oxy)]bis(4-oxobutanoic acid) (**13**, 0.090 g, 0.343 mmol) and oxalyl chloride were reacted, followed by the addition of **2** (0.204 g, 0.596 mmol) and pyridine (76 μL, 0.942 mmol) as per general procedure B. Column chromatography conditions: column 1, 0.5% ammonia/2.5% methanol/chloroform; column 2, gradient elution from 2% to 5% methanol/chloroform, increasing in 1% increments. Yield: **14** as an off-white foam (0.124 g, 0.136 mmol, 46%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.64 (s, 2H, NH), 8.52 (d, J = 2.3 Hz, 2H, H9″), 7.78 (ddd, J = 8.3, 0.9, 0.9 Hz, 2H, H1″/H4″), 7.43−7.36 (m, 4H, H3″/H2″ and H4″/H1″), 7.36 (d, J = 8.5 Hz, 2H, H6″), 7.14 (ddd, J = 8.1, 6.3, 1.8 Hz, 2H, H2″/H3″), 7.12 (dd, J = 8.5, 2.4 Hz, 2H, H7″), 4.16 (s, 4H, H1 and H2), 3.58 (m, 8H, H2‴ and H6‴), 2.72 (m, 8H, H3‴ and H5‴), 2.66 (m, 4H, H2′/H3′), 2.57 (m, 4H, H3′/H2′), 2.42 (s, 6H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.3 (C), 169.4 (C), 153.7 (C), 141.8 (C), 133.6 (C), 132.7 (C), 128.3 (CH), 126.9 (C), 124.6 (CH), 123.7 (CH), 122.6 (CH), 121.8 (CH), 120.7 (CH), 116.3 (C), 110.6 (CH), 62.3 (CH_2), 54.6 (CH_2), 49.2 (CH_2), 46.1 (CH_3), 32.2 (CH_2), 28.9 (CH_2). HPLC: t_R 10.17 min, >99% purity (method 1). LCMS (m/z): 909.1 $[M + H]^+$, 455.2 $[M + 2H]^{2+}$. HRMS (m/z): $C_{46}H_{51}Cl_2N_{10}O_6^+$ requires $[M + H]^+$ 909.3365; found 909.3328.

*General Procedure C for Preparation of Clozapine Propylamine Bivalent Ligands (**9a**−**g**).* All glassware used in the following procedure was flame-dried and cooled under nitrogen. The dicarboxylic acid (**6a**−**g**) (0.169−0.184 mmol) was suspended in dry dichloromethane (2 mL) at room temperature, under a nitrogen atmosphere. Oxalyl chloride (2.2 equiv) and *N*,*N*-dimethylformamide (1 drop) were added to the solution, which was stirred for 1 h at room temperature. To this mixture was added a solution of **5** (2.0 equiv) and pyridine (2.8−6.8 equiv), and in some cases *N*,*N*-diisopropylethylamine (2.5 equiv) or anhydrous potassium carbonate (2.0 equiv), in dry dichloromethane (3 mL). The reaction was monitored by TLC, and after 1 h, if any **5** remained, an additional 0.5 equiv of diacid chloride in dry dichloromethane (1 mL) was added to the solution and the reaction mixture was stirred for an additional 2−5 h at room temperature. The reaction mixture was partitioned between ethyl acetate (30 mL) and aqueous sodium hydroxide (30 mL, 1 M). The organic layer was separated and the aqueous layer was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with water (2 × 30 mL) and saturated brine (30 mL), dried over anhydrous sodium sulfate, filtered, and evaporated to dryness in vacuo, yielding a yellow oil. The product was purified by preadsorption onto coarse silica, followed by flash column chromatography. Compounds were typically purified with 1−3 columns (refer to specific experimental description for details of eluent).

*N^1,N^6-Bis{3-[4-(8-chloro-5H-dibenzo[b,e][1,4]diazepin-11-yl)-piperazin-1-yl]propyl}adipamide (**9a**).* 1,6-Hexanedioic acid (**6a**) (0.027 g, 0.184 mmol) and oxalyl chloride were reacted, followed by the addition of **5** (0.130 g, 0.352 mmol) and pyridine (0.040 mL, 0.496 mmol) as per general procedure C. Additional 1,6-hexanedioyl dichloride (0.016 g, 0.089 mmol) was added. Column chromatography conditions: 10% methanol/chloroform. Yield: **9a** as a yellow foam (0.068 g, 0.080 mmol, 46%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 (m, 2H, H3‴), 7.24 (dd, J = 7.7, 1.4 Hz, 2H, H1‴), 7.08 (br t, J = 5.0 Hz, 2H, CONH), 7.05 (d, J = 2.4 Hz, 2H, H9‴), 7.00 (ddd, J = 7.6, 7.5, 1.0 Hz, 2H, H2‴), 6.86 (dd, J = 8.0, 0.6 Hz, 2H, H4‴), 6.82 (dd, J = 8.3, 2.4 Hz, 2H, H7‴), 6.66 (d, J = 8.3 Hz, 2H, H6‴), 5.26 (s, 2H, H5‴), 3.47 (m, 8H, H3″ and H5″), 3.29 (td, J = 5.8, 5.8 Hz, 4H, H1′), 2.58 (m, 8H, H2″ and H6″), 2.52 (t, J = 6.4 Hz, 4H, H3′), 2.17 (m, 4H, H2 and H5), 1.69 (app p, J = 6.5 Hz, 4H, H2′), 1.64 (m, 4H, H3 and H4). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.9 (C), 163.1 (C), 153.1 (C), 141.7 (C), 140.8 (C), 132.2 (CH), 130.3 (CH), 129.1 (C), 126.8 (CH), 123.5 (CH), 123.3 (C), 123.2 (CH), 120.4 (2 × CH), 57.2 (CH_2), 53.0 (CH_2), 47.3 (CH_2), 39.1 (CH_2), 36.5 (CH_2), 25.3 (CH_2), 25.2 (CH_2). HPLC: t_R 7.93 min, >99% purity (method 1). LCMS (m/z): 849.2 $[M + H]^+$, 425.2 $[M + 2H]^{2+}$. HRMS (m/z): $C_{46}H_{55}Cl_2N_{10}O_2^+$ requires $[M + H]^+$ 849.3881; found 849.3881.

Synthesis of Monovalent Ligands. *1-[8-Chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepin-5-yl]decan-1-one (**15**).* Compound **1** (0.103 g, 0.315 mmol) was dissolved in dry dichloromethane (3 mL), at room temperature, under a nitrogen atmosphere. Pyridine (37 μL, 0.459 mmol) and *N*,*N*-diisopropylethylamine (80 μL, 0.459 mmol) were added to the stirred solution, followed by decanoyl chloride (127 μL, 0.612 mmol). After 2 h, the reaction mixture was diluted with dichloromethane (30 mL) and washed with aqueous hydrochloric acid (30 mL, 1 M). The organic layer was separated and the aqueous layer was further extracted with dichloromethane (2 × 30 mL). The combined organic layers were then washed with aqueous sodium hydroxide (3 × 30 mL), water (2 × 30 mL), and saturated brine (30 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The crude yellow oil was purified by flash column chromatography (column 1, 5% methanol/chloroform; column 2, 1% ammonia/4% methanol/chloroform) to give the pure title compound **15** as a pale yellow oil (0.055 g, 0.114 mmol, 36%). ^{1}H NMR (400 MHz, $CDCl_3$, 320 K) δ 7.49 (ddd, J = 8.0, 7.0, 1.8 Hz, 1H, H3′), 7.40−7.30 (m, 3H, H1′, H2′, and H4′), 7.15 (d, J = 2.4 Hz, 1H, H9′), 7.10 (d, J = 8.3 Hz, 1H, H6′), 6.96 (dd, J = 8.3, 2.3 Hz, 1H, H7′), 3.70 (m, 2H, H2″a and H6″a), 3.48 (m, 2H, H2″b and H6″b), 2.46 (m, 2H, H3″a and H5″a), 2.36 (m, 2H, H3″b and H5″b), 2.32−2.24 (m, 4H, NCH_3, H2a), 2.17 (m, 1H, H2b), 1.55 (m, 2H, H3), 1.33−1.17 (m, 12H, H4−H9), 0.87 (t, J = 7.0 Hz, 3H, H10). ^{13}C NMR (101 MHz, $CDCl_3$, 320 K) δ 173.7 (C), 160.5 (C), 146.3 (C), 145.0 (C), 133.9 (C), 133.6 (C), 131.9 (CH), 129.0 (CH), 127.7 (CH), 127.6 (CH), 126.9 (C), 126.1 (2 × CH), 123.1 (CH), 54.9 (CH_2), 46.9 (CH_2), 46.0 (CH_3), 33.5 (CH_2), 31.8 (CH_2), 29.3 (CH_2), 29.21 (CH_2), 29.19 (2 × CH_2), 25.1 (CH_2), 22.6 (CH_2), 14.0 (CH_3). HPLC: t_R 9.10 min, >99% purity (method

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2). LCMS (m/z): 481.2 $[M + H]^+$. HRMS (m/z): $C_{28}H_{38}ClN_4O^+$ requires $[M + H]^+$ 481.2729; found 481.2711.

*N-[8-Chloro-11-(4-methylpiperazin-1-yl)-5H-dibenzo[b,e][1,4]-diazepin-5-yl]decanamide (**16**).* Compound **2** (0.101 g, 0.295 mmol) was dissolved in dry dichloromethane (2 mL) at room temperature under a nitrogen atmosphere. Pyridine (70 μL, 0.869 mmol) was added to the stirred solution, followed by decanoyl chloride (122 μL, 0.588 mmol). After 3 h, the reaction mixture was diluted with dichloromethane (30 mL) and worked up as described in the preparation of **15**. The crude brown oil was purified by flash column chromatography (2% methanol/chloroform) to give the pure title compound **16** as a pale brown oil (0.115 g, 0.232 mmol, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.50 (s, 1H, NH), 8.59 (d, J = 2.3 Hz, 1H, H9′), 7.80 (ddd, J = 8.2, 0.9, 0.9 Hz, 1H, H1′/H4′), 7.44−7.37 (m, 2H, H3′/H2′ and H4′/H1′), 7.36 (d, J = 8.5 Hz, 1H, H6′), 7.15 (m, 1H, H2′/H3′), 7.13 (dd, J = 8.5, 2.4 Hz, 1H, H7′), 3.57 (m, 4H, H2″ and H6″), 2.68 (m, 4H, H3″ and H5″), 2.41 (s, 3H, NCH_3), 2.27 (t, J = 7.5 Hz, 2H, H2), 1.63 (app p, J = 7.3 Hz, 2H, H3), 1.32−1.15 (m, 12H, H4−H9), 0.86 (t, J = 7.0 Hz, 3H, H10). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.4 (C), 153.8 (C), 141.9 (C), 134.0 (C), 132.9 (C), 128.3 (CH), 126.9 (C), 124.7 (CH), 123.6 (CH), 122.7 (CH), 121.8 (CH), 120.7 (CH), 116.3 (C), 110.7 (CH), 54.8 (CH_2), 49.4 (CH_2), 46.4 (CH_3), 38.4 (CH_2), 31.9 (CH_2), 29.49 (CH_2), 29.45 (CH_2), 29.32 (CH_2), 29.25 (CH_2), 25.6 (CH_2), 22.7 (CH_2), 14.2 (CH_3). HPLC: t_R 9.17 min, 98% purity (method 2). LCMS (m/z): 496.2 $[M + H]^+$. HRMS (m/z): $C_{28}H_{39}ClN_5O^+$ requires $[M + H]^+$ 496.2838; found 496.2831.

*N-{3-[4-(8-Chloro-5H-dibenzo[b,e][1,4]diazepin-11-yl)piperazin-1-yl]propyl}decanamide (**17**).* Compound **5** (0.100 g, 0.269 mmol) was dissolved in dry dichloromethane (3 mL) at room temperature under a nitrogen atmosphere. Pyridine (33 μL, 0.410 mmol) was added to the stirred solution, followed by decanoyl chloride (71 μL, 0.342 mmol). After 1.5 h, additional decanoyl chloride was added (20 μL, 0.096 μmol). After 2 h, the reaction mixture was diluted with dichloromethane (30 mL) and worked up as described in the preparation of **15**. The crude yellow oil was purified by flash column chromatography (gradient elution from 2% to 10% methanol/chloroform, increasing methanol in 2% increments) to give the pure title compound **17** as a yellow oil (0.089 g, 0.171 mmol, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (ddd, J = 7.9, 7.4, 1.6 Hz, 1H, H3‴), 7.26 (m, 1H, H1‴), 7.06 (d, J = 2.4 Hz, 1H, H9‴), 7.01 (ddd, J = 7.8, 7.4, 1.1 Hz, 1H, H2‴), 6.83 (ddd, J = 8.0, 1.1, 0.4 Hz, 1H, H4‴), 6.82 (dd, J = 8.3, 2.4 Hz, 1H, H7‴), 6.79 (br t, 1H, NH), 6.62 (dd, J = 8.4, 0.3 Hz, 1H, H6‴), 4.99 (s, 1H, H5‴), 3.45 (m, 4H, H3″ and H5″), 3.35 (td, J = 6.0, 6.0 Hz, 2H, H1′), 2.55 (m, 4H, H2″ and H6″), 2.51 (t, J = 6.3 Hz, 2H, H3′), 2.13 (m, 2H, H2), 1.70 (app p, J = 6.3 Hz, 2H, H2′), 1.61 (app p, J = 7.4 Hz, 2H, H3), 1.33−1.21 (m, 12H, H4−H9), 0.86 (t, J = 6.9 Hz, 3H, H10). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.2 (C), 162.9 (C), 153.0 (C), 141.8 (C), 140.6 (C), 132.1 (CH), 130.3 (CH), 129.2 (C), 126.9 (CH), 123.4 (C), 123.4 (CH), 123.2 (CH), 120.3 (CH), 120.2 (CH), 57.7 (CH_2), 53.3 (CH_2), 47.5 (CH_2), 39.5 (CH_2), 37.2 (CH_2), 32.0 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 26.0 (CH_2), 25.4 (CH_2), 22.8 (CH_2), 14.2 (CH_3). HPLC: t_R 7.29 min, >99% purity (method 2). LCMS (m/z): 524.2 $[M + H]^+$. HRMS (m/z): $C_{30}H_{43}ClN_5O^+$ requires $[M + H]^+$ 524.3151; found 524.3149.

Biological Assays. *Cell Culture.* Chinese hamster ovary (CHO) FlpIn cells were stably transfected with the human D_2(long) dopamine receptor (D_2-CHOFlpIn). Cells were grown and maintained in Dulbecco's modified Eagle medium (DMEM) containing 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 5% fetal bovine serum, and 200 μg/mL hygromycin B. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2/95% O_2. For ERK1/2 phosphorylation assays, cells were seeded into 96-well silicon coated plates at a density of 50 000 cells/well. After 4 h, cells were washed twice with phosphate-buffered saline (PBS) and then maintained in DMEM containing 20 mM HEPES for at least 16 h before assaying.

Radioligand Binding Studies. When cells were approximately 90% confluent, they were harvested and centrifuged (300*g*, 3 min). The resulting pellet was resuspended in assay buffer [20 mM HEPES, 6 mM $MgCl_2$, 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4], and the centrifugation procedure was repeated. The intact cell pellet was then resuspended in assay buffer and homogenized with a Polytron homogenizer for three 10-s intervals on the maximum setting, with 30-s periods on ice between each burst. The homogenate was made up to 30 mL and centrifuged (1000*g*, 10 min, 25 °C), the pellet was discarded, and the supernatant was recentrifuged at 30000*g* for 1 h at 4 °C. The resulting pellet was resuspended in 5 mL of assay buffer and the protein content was determined by the method of Bradford.[45] The homogenate was then separated into 1 mL aliquots and stored frozen at −80 °C until required for radioligand binding assays. Membrane homogenates (5 μg/mL) were incubated in 1 mL total volume of assay buffer containing ascorbic acid (0.1%), bovine serum albumin (BSA) (0.1%), [^{3}H]spiperone (0.1 nM), and a range of concentrations of ligand for 3 h at 37 °C. Nonspecific binding was defined by use of 10 μM butaclamol. Incubation was terminated by rapid filtration through Whatman GF/C filters on a Brandell cell harvester (Gaithersburg, MD). Filters were washed three times with 3 mL aliquots of assay buffer and dried before the addition of 4 mL of scintillation cocktail (Ultima Gold; Packard Bioscience, Meriden, CT). Radioactivity was determined by scintillation counting.

ERK1/2 Phosphorylation. Dose−response experiments in the absence or presence of ligand were performed at 37 °C in 200 μL total volume of DMEM containing 20 mM HEPES and 0.1% ascorbic acid. Dose−response stimulation or inhibition curves were generated by exposure of the cells to antagonist ligand for 120 min and then dopamine for 5 min. Stimulation of cells was terminated by the removal of medium and the addition of 100 μL of SureFire lysis buffer to each well. The plate was agitated for 1−2 min. A 4:1 (v/v) dilution of lysate/SureFire activation buffer was made in a total volume of 50 μL. A 1:100:120 (v/v/v) dilution of AlphaScreen beads/activated lysate mixture/SureFire reaction buffer in 11 μL total volume was then transferred to a white opaque 384-well Proxiplate in the dark. This plate was then incubated in the dark at 37 °C for 1.5 h, after which time the fluorescence signal was measured by a Fusion plate reader (PerkinElmer), with standard AlphaScreen settings.

Data Analysis. Data from radioligand binding experiments were analyzed by the nonlinear regression curve-fitting program Prism 5 (GraphPad, San Diego, CA). For the displacement of [^{3}H]spiperone, data were fit to a one-site model with a variable Hill slope:

$$Y = \frac{(\text{top} - \text{bottom})x^{n_H}}{x^{n_H} + \text{IC}_{50}{}^{n_H}} \quad (1)$$

where Y denotes the percent specific binding; top and bottom denote the maximal and minimal asymptotes, respectively; x denotes the inhibitor potency (midpoint location) parameter; and n_H denotes the Hill slope factor. With the assumption of simple competition, IC_{50} values were converted to K_i values via the Cheng−Prusoff equation.[46] In the functional ERK1/2 assay, agonist concentration−response curves were fitted to the following four-parameter Hill equation by use of Prism 5:

$$\text{response} = \frac{(\text{top} - \text{bottom})}{1 + (10^{\log \text{EC}_{50}/x})^{n_H}} \quad (2)$$

where top represents the maximal asymptote of the concentration−response curves, bottom represents the lowest asymptote of the concentration−response curves, log EC_{50} represents the logarithm of the agonist EC_{50}, x represents the concentration of the agonist, and n_H represents the Hill slope. To determine the inhibitory potency of the various monovalent and bivalent ligands, data were fit to the following equation:

$$\text{response} = \frac{(\text{top} - \text{bottom})}{[1 + 10^{(X - \log \text{IC}_{50})}]} \quad (3)$$

where top represents the maximal asymptote of the concentration−response curves, bottom represents the lowest asymptote of the concentration−response curves, log IC_{50} represents the logarithm of the antagonist IC_{50}, X represents the concentration of the agonist, and the Hill slope is assumed to be unity. Data shown are the mean ± SEM of at least three separate experiments performed in duplicate.

■ ASSOCIATED CONTENT

Supporting Information

Additional text, describing synthesis and spectroscopic data for linkers and bivalent ligands, and HPLC chromatograms for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

GPCR, G protein-coupled receptor; SAR, structure−activity relationships; ERK, extracellular signal-regulated kinases; CHO, Chinese hamster ovary; TLC, thin-layer chromatography

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