

Structure-Based Discovery of Novel and Selective 5-Hydroxytryptamine 2B Receptor Antagonists for the Treatment of Irritable Bowel Syndrome

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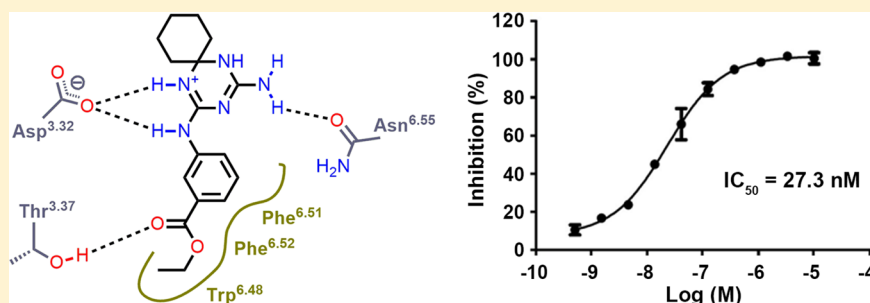
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S Supporting Information



ABSTRACT: Here we employed structure-based ligand discovery techniques to explore a recently determined crystal structure of the 5-hydroxytryptamine 2B (5-HT_{2B}) receptor. Ten compounds containing a novel chemical scaffold were identified; among them, seven molecules were active in cellular function assays with the most potent one exhibiting an IC₅₀ value of 27.3 nM. We then systematically probed the binding characteristics of this scaffold by designing, synthesizing, and testing a series of structural modifications. The structure–activity relationship studies strongly support our predicted binding model. The binding profiling across a panel of 11 5-HT receptors indicated that these compounds are highly selective for the 5-HT_{2B} receptor. Oral administration of compound **15** (30 mg/kg) produced significant attenuation of visceral hypersensitivity in a rat model of irritable bowel syndrome (IBS). We expect this novel scaffold will serve as the foundation for the development of 5-HT_{2B} antagonists for the treatment of IBS.

■ INTRODUCTION

G-protein coupled receptors (GPCRs) represent the most prominent pharmaceutical targets in drug discovery, and over 40% of marketed drugs act through them.¹ GPCR drugs have typically been developed based on the traditional medicinal chemistry approach, which has restricted the diversity of the chemical scaffolds considered as candidate drugs. Recent progress in the determination of GPCR structures has opened up great opportunities for structure-based discovery efforts seeking novel GPCR ligands. Docking screens against these crystal structures have been successfully applied in identification of novel potent ligands.^{2–8}

The 5-HT_{2B} receptor is one of the recently determined GPCR structures.^{9,10} It belongs to 5-HT₂ receptor family, which includes three subtypes: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. Although both the 5-HT_{2A} and 5-HT_{2C} receptors have been widely studied as therapeutic targets, drug discovery efforts focused on the 5-HT_{2B} receptor have been relatively limited. 5-

HT_{2B} agonism has been regarded as a side effect for drug molecules, as its activation is related to cardiac hypertrophy and pulmonary hypertension.^{11,12} More recent studies have focused on the possible application of 5-HT_{2B} antagonists.¹³ Several compounds have been advanced to clinical trials in several disease areas, including migraine disorder,^{14,15} irritable bowel syndrome (IBS),^{16,17} and pulmonary arterial hypertension (PAH).^{18,19}

IBS is a common functional gastrointestinal (GI) disorder worldwide that is characterized by abdominal pain and alterations in bowel habits. Despite these impacts, the pathogenic mechanism of this disease remains incompletely understood and seems to be multifactorial.²⁰ Visceral hypersensitivity is now widely accepted as one of the core characteristics, and this probably plays a major role in the

Received: October 16, 2015

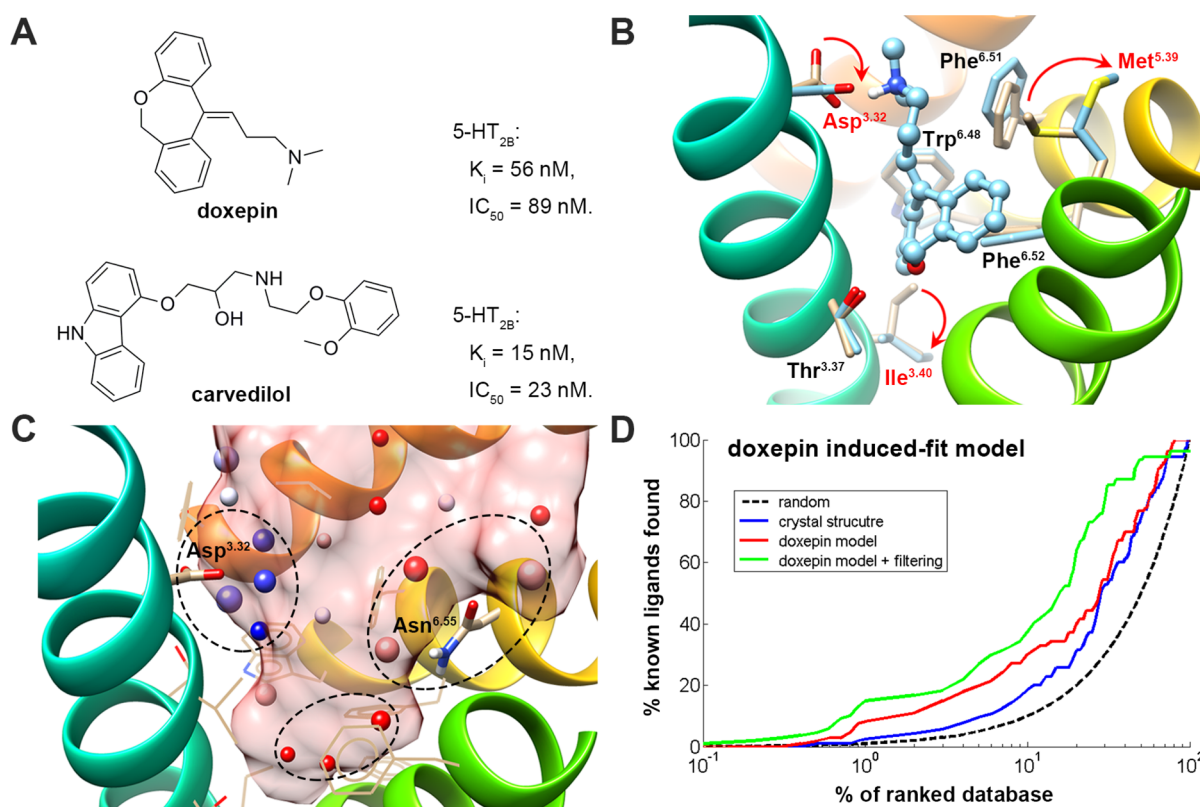


Figure 1. Binding site refinement of the 5-HT_{2B} crystal structure. (A) Chemical structures and biological activities of doxepin and carvedilol. (B) Comparison of the doxepin induced-fit model (cyan) with the 5-HT_{2B} crystal structure (tan, PDB ID: 4IB4). (C) Solvent property analysis of the 5-HT_{2B} binding pocket. Predicted water clusters are shown as spheres. The volume of spheres represents their occupancy in the simulation, and the color represents their free energy. Blue indicates that the water molecule is energy favorable, while red indicates it is energy unfavorable. (D) The enrichment profile of the doxepin induced-fit model. The percentage of ligands found (y-axis) plotted as a function of the percentage of the docking ranked database (x-axis in logarithmic scale).

development of disease symptoms.²¹ Currently, there is no effective cure for IBS. Traditional management has mainly focused on the relief of symptoms. The efficacy of these management approaches has been limited, and the side effects of various drugs used to relieve symptoms have been problematic. For example, the 5-HT₃ antagonist alosetron was approved by the US FDA in 2000 and is used to treat the diarrhea-predominate form of IBS.²² Unfortunately, severe adverse GI reactions were reported soon after its introduction to the market. In 2015, the US FDA approved two new drugs for the diarrhea-predominate form of IBS. The effects of rifaximin, an antibiotic derived from rifampin, are thought to be related to changes in the bacterial content in the GI tract. Eluxadoline, a mixed opioid receptor ligand, acts on the nervous system to reduce bowel contractions. However, the safety of these two drugs is still of great concern given reported side effects that include constipation, nausea, spasm, and increased alanine aminotransferase levels.²³

Recent studies have strongly suggested that the 5-HT_{2B} receptor plays an important role in the pathogenesis of IBS; it is therefore a promising target for the development of novel therapies. The 5-HT_{2B} receptor is widely expressed throughout the human intestinal tract.¹³ It may function in regulating the contraction of smooth muscle to mediate GI motility and visceral perception. The activation of the 5-HT_{2B} receptor was shown to increase the proliferation of interstitial cells of Cajal through protein kinase C γ , a process that may lead to motility disorder and visceral hypersensitivity in the colon.²⁴ In

addition, increased 5-HT_{2B} receptor expression has been reported for rat models of diarrhea-predominant IBS.²⁵ Selective antagonism of the 5-HT_{2B} receptor in IBS rats has been shown to produce significant attenuation of visceral hypersensitivity and improvement with defecation.^{26–29}

Currently, only a few selective antagonists of the 5-HT_{2B} receptor have been reported, due to the high degree of homology with its closely related protein family members.³⁰ Therefore, the discovery of novel and selective 5-HT_{2B} antagonists would be of great interest to further explore the physiological function and therapeutic application of the 5-HT_{2B} receptor. In the present study, we used structure-based virtual screening to explore the opportunity presented by a recently published crystal structure of the 5-HT_{2B} receptor to identify novel antagonists against this protein. One class of compounds with a novel structural scaffold was enriched among the top of the scoring list. Compounds from this class were validated in cellular functional assays and binding profiling assays. We further performed structure–activity relationship (SAR) studies to assess the predicted binding pose of this scaffold, which led to the selection of a promising candidate with high binding affinity and high selectivity for the 5-HT_{2B} receptor. In vivo studies of this drug candidate suggested that this class of 5-HT_{2B} antagonists can serve as a promising starting point for the development of new drugs to treat IBS.

■ RESULTS

Binding-Site Refinement of the 5-HT_{2B} Crystal Structure. GPCR undergoes conformational changes to accommodate different ligands, and these conformational changes are especially pronounced when making comparisons between agonist-bound structures and antagonist-bound structures. The only currently available 5-HT_{2B} crystal structure is bound with a biased agonist (ergotamine), and it has been suggested that this structure exhibits conformational characteristics of both the active and the inactive states (Figure S1A, Supporting Information).⁹ Compared with other inactive aminergic GPCR structures, the conserved anchor residue Asp^{3.32}, which is critical for ligand binding, adopts a different orientation in the 5-HT_{2B} structure (Figure S1B). Accordingly, rigid-receptor docking against this conformation might be of limited utility in identifying efficacious antagonists.⁷ Thus, instead of docking to the crystal conformation directly, we deliberately modified the structure to incorporate the induced-fit effects associated with 5-HT_{2B} antagonists.³¹ To obtain a reliable induced-fit structural model, we selected two promiscuous GPCR ligands: doxepin and carvedilol (Figure 1A). Both of them exhibit potent 5-HT_{2B} antagonist activity;³² they also bind to other GPCRs and have corresponding crystal complex structures available (with histamine H₁ and β_1 -adrenergic receptors, respectively).^{33,34}

Figure 1B illustrates the doxepin induced-fit model, in which doxepin maintains the dominant interactions that form in the histamine H₁ receptor (Figure S2, Supporting Information). The amine moiety forms an anchor salt bridge with Asp^{3.32}. The tricyclic rings of doxepin sit deeply in the hydrophobic binding pocket and are surrounded by the following highly conserved residues: Ile^{3.40}, Trp^{6.48}, Phe^{6.51}, and Phe^{6.52}. The oxygen atom of doxepin (E isomer) forms a hydrogen bond with Thr^{3.37}, which is consistent with that in the histamine H₁–doxepin crystal complex structure. This result significantly bolstered our confidence about the properties of our induced-fit model. Compared with the initial crystal configuration, we found that three residues experience significant conformational changes in the doxepin induced-fit conformation. Asp^{3.32} orientates toward the binding pocket, forming a salt bridge with the ligand, an orientation consistent with that of other inactive monoamine GPCRs. Yet both Met^{5.39} and Ile^{3.40} rotate away from the ligand, resulting in a larger binding pocket. Notably, the conformation of Ile^{3.40} is switched back to the inactive state as shown in other inactive aminergic GPCR structures. We observed similar (but less pronounced) conformational changes in the carvedilol induced-fit model (Figure S3A, Supporting Information).

We further analyzed the binding-site solvent properties of the 5-HT_{2B} receptor. The displacement of water molecules from the binding pocket is a key component of ligand binding. Here we calculated the thermodynamic properties of binding-site water molecules in the 5-HT_{2B} receptor using the Solvent Property Analysis (SPA) program.³⁵ The predictions for occupancy and free energy of the binding-site waters enabled us to generally summarize the potential interaction patterns of 5-HT_{2B} ligands (Figure 1C). First, a cluster of highly energy-favorable waters surround the negatively charged residue Asp^{3.32}, the displacement of which would result in a large enthalpy penalty. Thus, a positively charged amine group is usually required here for the ligand to form a salt bridge interaction with Asp^{3.32} to compensate for the energy penalty.

Second, three less energy-favorable waters form hydrogen bond interactions with Asn^{6.55}, implying a potential hydrogen bond interaction with the ligand in this position. We also identified a group of energy-unfavorable waters bound deeply within the binding pocket that were restricted by the aromatic rings of nearby residues. A nonpolar planar group here would generate significant potency from the entropy gain of the water displacement. The distribution of these binding-site waters represents the chemical composition of an ideal ligand. On the basis of the above analysis, we derived three structural filters for the screening of 5-HT_{2B} antagonists: (a) a salt bridge interaction must be formed between the positively charged amine group of the ligand and residue Asp^{3.32}; (b) the region of unfavorable waters should be occupied by a hydrophobic group of the ligand (here we define the occupation as at least one carbon atom having a distance <3 Å from the oxygen atom of each water molecule); (c) considering the overall hydrophobic nature of the binding site, there should not be many polar atoms that do not form a hydrogen bond interaction. Thus, a ligand with three or more polar atoms buried in the pocket should form at least one hydrogen bond with receptor residues.

We next estimated the ability of our induced-fit models to enrich known antagonists of the 5-HT_{2B} receptor from a background of physicochemically similar, but structurally dissimilar, decoys. The derived structural filters were also applied. The docking results suggested that both of the induced-fit models provide better enrichment performance than does the original crystal structure; this was especially the case when the models were combined with structural filters (Figure 1D and Figure S3B). The EF1 values (enrichment factor at 1% of the ranked database) were 2.4, 8.2, and 5.8 for the crystal structure, the doxepin model, and the carvedilol model, respectively. Structural filtering further improved the results, yielding EF1 values of 4.6, 14.9, and 10.5, respectively. The doxepin induced-fit model combined with structural filtering exhibited the best performance. Thus, we finally selected the doxepin induced-fit model for use in further virtual screening efforts.

Identification of a Novel Scaffold in Prospective Virtual Screening. A structure-based hierarchical virtual screening strategy^{36,37} was applied to identify novel 5-HT_{2B} antagonists. To balance efficiency and accuracy, this protocol combines molecular docking-based high throughput screening and minimization-based MM-GB/SA rescoring (Figure 2). We initially docked our in-house compound library that contains 100 413 diverse drug-like compounds. More than 300 000 binding conformations were generated, and the 10 000 top-ranked poses were further subjected to minimization-based MM-GB/SA refining and rescoring. We then saved the 1000 top-ranked nonredundant compounds for structural analysis; only 253 of the hits passed the structural filters. With the aim of identifying a novel 5-HT_{2B} ligand scaffold, we subjected these surviving molecules to the Similarity Ensemble Approach³⁸ (SEA, <http://sea.bkslab.org>) server in an effort to avoid the selection of compounds that are structurally similar to known GPCR ligands. Finally, we obtained 169 molecules of interest (Table S1, Supporting Information). Among these, one scaffold in particular stood out, as ten molecules with this scaffold were enriched in the hit list, and all ten of these molecules adopted a similar binding mode (Figure 3A and Table S2, Supporting Information).

Cellular functional assays were performed to validate the antagonist activity of the ten selected molecules. Encouragingly,

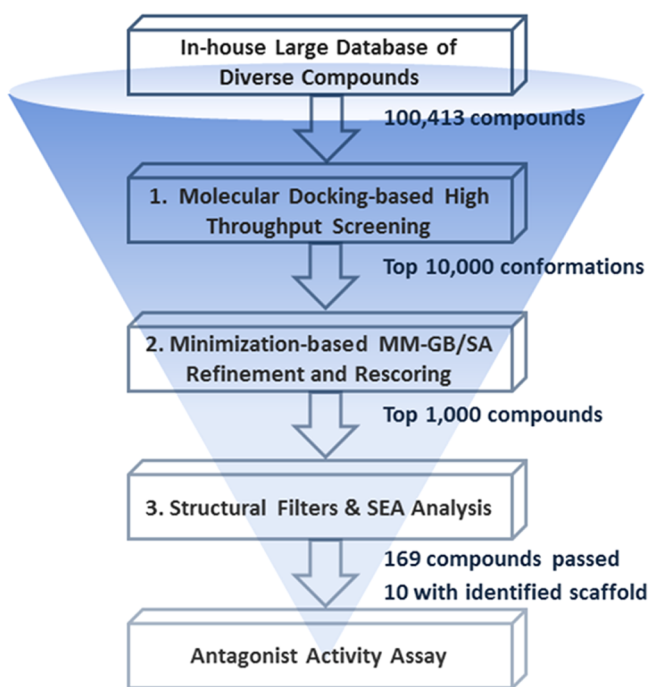


Figure 2. Flowchart of the structure-based hierarchical virtual screening strategy.

seven compounds exhibited >50% inhibition at a 10 μM concentration and five of these seven exhibited >50% inhibition at a 1 μM concentration (Figure 3B), results that clearly suggested that a common mode of action was shared by this series of compounds. We further determined the dose-response inhibition activity of these five compounds (Table S2). The most potent one, compound 9 (Figure 3C), exhibits an IC_{50} value of 27.3 nM (Figure 3D).

Assessment of the Predicted Binding Mode. These compounds represent a novel class of 5-HT_{2B} antagonists. The triazine ring in trizazspiro is basic (with experimental pK_a of 10–11).³⁹ Thus, it is protonated in solution and forms the salt bridge interaction with conserved Asp^{3.32} through N¹ and the nitrogen atom on the 2-amine group (Figure 4A). The nitrogen atom on the 4-amine group forms a hydrogen bond with residue Asn^{6.55}. The saturated cyclohexene group is located in the entrance of the binding pocket and forms hydrophobic contacts with extracellular loop II (ECL II). The phenyl ring is deeply buried into the inner part of the hydrophobic pocket and is surrounded by the aromatic side chains of residues Trp^{6.48}, Phe^{6.51}, and Phe^{6.52}. These interactions are quite consistent with the above solvent property analysis. Specifically, for compound 9, there is an additional hydrogen bond interaction with residue Thr^{3.37} that may further contribute to its strong binding. Compared with the crystal structure of 5-HT_{2B}–ergotamine complex, our compounds occupy the orthosteric binding pocket, without completely overlapping with the biased agonist ergotamine binding site (Figure S4, Supporting Information).

On the basis of this predicted binding mode and potential chemical modifications, we next probed the binding characteristics by envisioning and testing a series of structural analogues (Table 1). To confirm the salt bridge interaction, we synthesized compound 11, in which a methyl group was added to the nitrogen atom on the 2-amine group. As we expected, this modification destabilized the salt bridge with residue Asp^{3.32} and led to a remarkable decrease in potency. Further, compound 12, with a cyclized N¹ nitrogen atom, could not form the salt bridge interaction and lost all activity. Compounds 13 and 14 were designed to probe the hydrogen bond to residue Asn^{6.55}. A single methyl group added on the nitrogen atom of 4-amine group preserves the hydrogen bond.

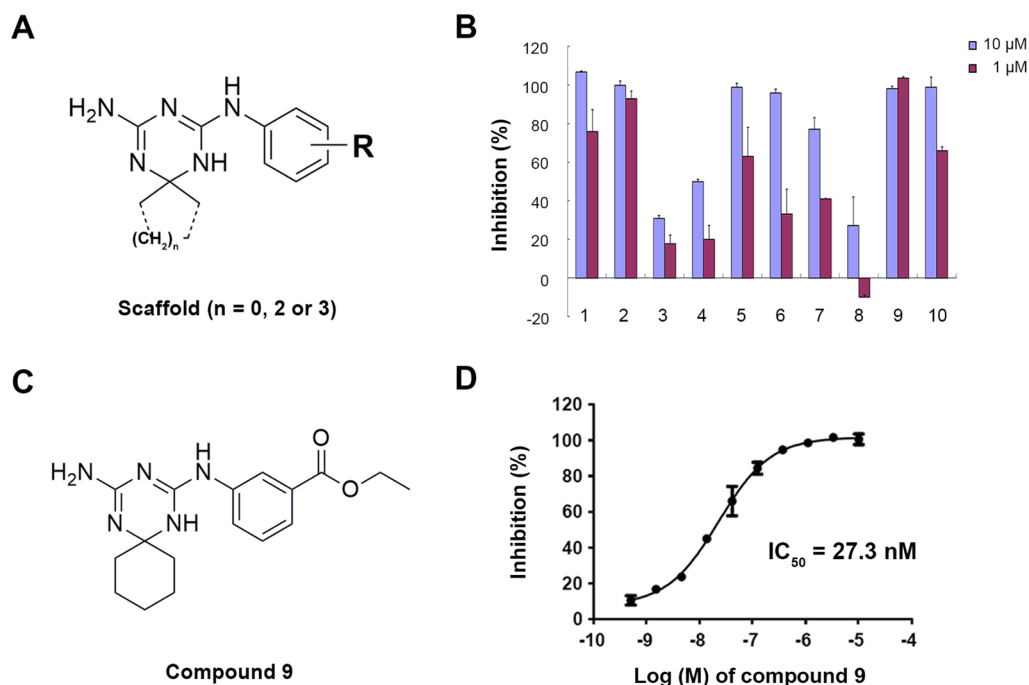


Figure 3. Experimental validation of the identified compounds. (A) Scaffold of the identified compounds. (B) Inhibitory activity on the 5-HT_{2B} receptor at 10 μM and 1 μM concentrations. (C) Chemical structure of compound 9. (D) The dose-dependent inhibitory response curve of compound 9 on the 5-HT_{2B} receptor.

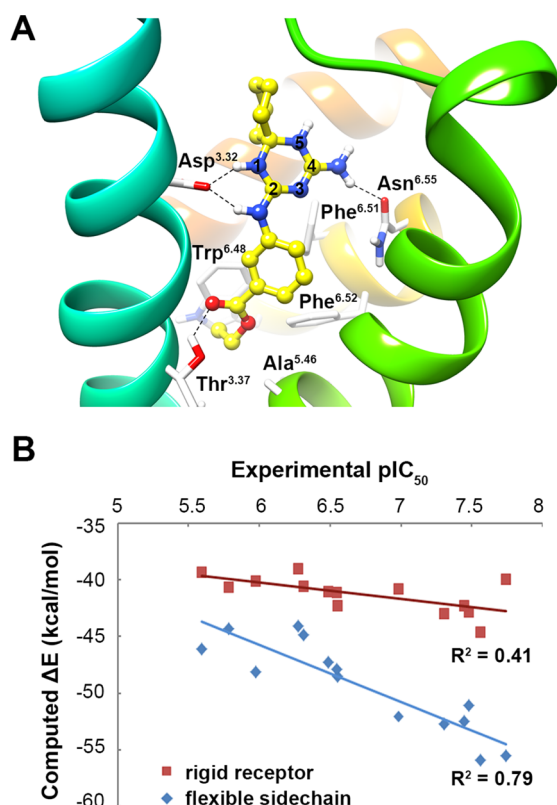


Figure 4. (A) Predicted binding mode of compound 9. (B) Correlation between the experimental pIC_{50} and the computed ΔE based on the predicted binding mode.

Therefore, compound 13 exhibited comparable inhibitory activity with compound 9, with an IC_{50} value of 49.4 nM. However, the substitution of two methyl groups totally abolished the hydrogen bond interaction, resulting in a >10-fold decrease in the potency of compound 14. The saturated cyclohexane ring forms hydrophobic contacts with residues on ECL II. The effects of subtle variations to this region were also explored. Replacing the cyclohexane subunit with a smaller germinal dimethyl or saturated cyclopentane group yielded, respectively, 10-fold and 4-fold decreased potency (compounds 1, 5, 15, and 16).

We then focused on the R-substituents on the phenyl group, which sits deeply in the hydrophobic binding pocket and makes extensive contacts with nearby residues. A series of structural analogues were tested (Table 2). Compound 17, without any modifications on the phenyl ring group, exhibited no inhibition of the 5-HT_{2B} receptor, indicating that the hydrophobic interaction in the binding pocket might be essential for the antagonist activity. The R² position is restricted by the ligand itself or by nearby residues in the protein. Modifications on this site contribute little to binding. Thus, compound 18 and 19 yielded minimal increase in potency. The R³ position is buried deeply into the pocket, where bulky groups are tolerable. We surmised that nonpolar substitutions on this site would introduce favorable hydrophobic interactions. As expected, the potency was significantly enhanced by various monosubstitutions at the R³ position. For example, compounds 15 and 16, modified by a bromine atom or methoxy group, respectively, exhibited potent inhibition; the IC_{50} values for these two compounds were 33.4 nM and 280 nM. Considering the hydrolytic lability of the ester group in compound 9, we

also synthesized and tested its hydrolysis product, compound 20. However, no inhibitory activity was detected. This may be because the negatively charged group is unfavorable for binding in the hydrophobic pocket surrounded by aromatic rings, owing to a large desolvation penalty. This led us to synthesize compounds 21 and 22, with the replacement of the ester group with, respectively, a carbonyl group and an amide group to pursue improved stability; their IC_{50} values were 35.8 nM and 104.0 nM. Compound 23, with an additional methyl group added to compound 9, exhibits a slightly stronger inhibition, with an IC_{50} value of 18.1 nM. Compounds 24 and 25, substituted with relatively bulky groups, exhibited no activity or very low activity, consistent with a narrow and deep cleft in this position in the receptor binding site (Figure S5, Supporting Information). As for the R⁴ position, the space is relatively small (Figure 4A). Only small substitutions such as methyl groups or a chlorine atom (compound 26 and 27) are tolerable. Larger groups here, such as that in compound 28, introduce steric clashes and diminish binding.

The above SAR results strongly support our predicted binding mode. On the basis of this binding mode, we further calculated the binding energies of 14 compounds with experimentally determined IC_{50} values, using the MM-GB/SA rescoring method. In our previous work,³⁶ the target protein is usually kept rigid during the minimization process of the ligand–protein complex. Here we also attempted to include side-chain flexibility during the rescoring stage by simply minimizing side-chain conformations along with the ligand.⁴⁰ There were good correlations between the experimental pIC_{50} and the computed ΔE (Figure 4B). This further supports the assertion that our predicted binding mode is reasonable. Of particular note was the improved correlation ($R^2 = 0.79$) between the experimental pIC_{50} and the computed ΔE that resulted from the flexible treatment of the side chains of the binding-site residues, emphasizing the importance of incorporating protein flexibility into ligand binding energy calculation.

Binding Selectivity over Other 5-HT Receptors. We performed binding profiling tests for five compounds on a panel of 11 5-HT receptors. The results suggested that this class of compounds are relatively specific to the 5-HT_{2B} receptor (Table 3). They strongly bound to the 5-HT_{2B} receptor, with K_i values ranging from 6.2 nM to 38.5 nM, and bound only weakly to the other six 5-HT receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT₅, 5-HT₆, and 5-HT₇); they did not bind to 5-HT_{1E} and 5-HT₃. Even for 5-HT_{2A} and 5-HT_{2C}, which are the two most highly related family members of 5-HT_{2B}, these compounds also exhibited reasonably good selectivity. For example, compound 15, bound to the 5-HT_{2B}, 5-HT_{2A}, and 5-HT_{2C} with K_i values of 6.2, 211, and 156 nM, respectively.

Currently, only crystal structures of 5-HT_{2B} and 5-HT_{1B} receptors are determined in the 5HT receptor family.⁴¹ Structural comparison suggests that most of their binding-site residues are conserved, with the exception of residue 6.55 (Figure 5A). Substitution of Asn^{6.55} for Ser^{6.55} in the 5-HT_{1B} receptor abolishes the hydrogen bond interaction with our identified compounds, which should result in decreased binding affinity. On the basis of the sequence alignment of the 5-HTRs, three additional residues located in the ECL II region of 5-HT_{2B} are unique to the 5-HT_{2B} receptor (Figure 5B). These three additional residues form a helix turn and elevate the ECL II to an upper position, which facilitate the formation of an adjacent binding site in 5-HT_{2B} receptor, consisting of

Table 1. Validation of the Predicted Binding Mode^a

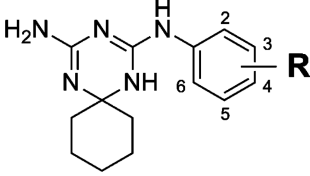
No.	Structure	Inhibition (%) at 1 μ M	IC ₅₀ (nM)
9		107.2 $\pm$ 0.6	27.3
11		50.4 $\pm$ 2.1	N.D.
12		-11.6 $\pm$ 4.5	N.D.
13		107.8 $\pm$ 0.0	49.4
14		91.8 $\pm$ 0.4	284.5
15		97.4 $\pm$ 2.2	33.4
1		75.9 $\pm$ 11.5	327.6
16		84.6 $\pm$ 1.2	280.0
5		63.0 $\pm$ 15.0	1056.0

^aN.D. means not determined.

hydrophobic residues Trp^{3,28}, Leu^{3,29}, Val^{7,39}, Val208, and Leu209 in ECL II. This hydrophobic site may accommodate the cyclohexene group and contribute to the selectivity of this type of compounds (Figure 5A). For the 5-HT_{1B} receptor, only two additional residues are located in the ECL II region and no helix turn is formed. Thus, the cyclohexene group of our compounds may not be accommodated into the reduced pocket created by this short loop region. This supposition is consistent with what is known about other aminergic GPCRs with solved crystal structures. The histamine H₁ and M₂ muscarinic acetylcholine receptors with three additional residues located in the ECL II region, exhibit similar conformations to the 5-HT_{2B} receptor. For the β_2 -adrenergic and dopamine D₃ receptors, however, only one or no residue

located in this region, results in a closed conformation for ECL II and a reduced binding pocket (Figure S6, Supporting Information).

Inhibition of Visceral Hypersensitivity in an IBS Rat Model. We selected one of the most potent compounds from the in vitro binding and functional assays, compound 15, and evaluated its in vivo therapeutic efficiency in an IBS rat model. Compared to its 5-HT_{2B} receptor antagonist activity, compound 15 exhibited relatively weak inhibitory activity against the hERG channel (IC₅₀ > 1 μ M, Figure S7, Supporting Information), and acute oral toxicity evaluation in rat showed no toxic response at a dose of 500 mg/kg. The visceral sensitivity of rats was evaluated by abdominal withdrawal reflex (AWR).⁴² As shown in Figure 6A and 6B, the recorded pain

Table 2. SAR of the R Substituent^a


no.	R	inhibition (%) at 1 μ M	IC ₅₀ (nM)
17	H	-11.1 $\pm$ 3.2	N.D.
18	2-Me	39.9 $\pm$ 3.4	N.D.
19	2-OMe	20.7 $\pm$ 3.6	N.D.
15	3-Br	97.4 $\pm$ 2.2	33.4
16	3-OMe	84.6 $\pm$ 1.2	280.0
9	3-COOEt	107.2 $\pm$ 0.6	27.3
20	3-COOH	4.4 $\pm$ 3.3	N.D.
21	3-CO-n-Pr	106.0 $\pm$ 0.8	35.8
22	3-CONHEt	95.5 $\pm$ 4.8	104.0
23	3-COO-n-Pr	110.5 $\pm$ 2.7	18.1
24	3-CO-c-Pr	4.0 $\pm$ 1.1	N.D.
25	3-CON(Et) ₂	40.7 $\pm$ 1.2	N.D.
26	4-Me	50.2 $\pm$ 8.0	485.9
27	4-Cl	57.1 $\pm$ 5.4	531.0
28	4-N(Me) ₂	17.6 $\pm$ 0.4	N.D.

^aN.D. means not determined.

thresholds significantly decreased in IBS rats compared with control rats (score 3:32 mmHg vs 57 mmHg, $P < 0.001$; score 4:57 mmHg vs 82 mmHg, $P < 0.001$). However, oral administration of compound **15** at 30 mg/kg produced a significant attenuation in visceral hypersensitivity (68% inhibition for score 3, $P < 0.001$ and 63% inhibition for score 4, $P < 0.01$). The mRNA expression level of the 5-HT_{2B} receptor was relatively higher for the IBS rats, in both distal and proximal colons (Figure 6C), further indicating that blocking the 5-HT_{2B} receptor may contribute to the inhibition of visceral hypersensitivity in IBS rats.

DISCUSSION AND CONCLUSION

The great progress in the determination GPCR structures has inspired a surge in structure-based ligand discovery efforts. Previous docking studies have indicated the functional fidelity of the docking screen hits to the conformation of the GPCR targets. The activity of the hits has recapitulated the activity of the cocrystallized ligands.⁷ The only currently available 5-HT_{2B} structure is bound with a biased agonist; it is thought to exhibit conformational characteristics of both its active and inactive states. Our induced-fit models have incorporated the knowledge of antagonist bound induced-fit effects and yielded structures with inactive-like conformations as in other inactive aminergic GPCR structures (Figure 1B, Figure S1). Therefore, on this basis, we propose that our induced-fit models are likely more reasonable for 5-HT_{2B} antagonist screening than using

Table 3. 5-HTRs Binding Profile Results (K_i, nM)

no.	5-HT _{1A}	5-HT _{1B}	5-HT _{1D}	5-HT _{1E}	5-HT _{2A}	5-HT _{2B}	5-HT _{2C}	5-HT ₃	5-HT _{5A}	5-HT ₆	5-HT _{7A}
1	660	1531	2655	>10000	783	20.5	265	>10000	389	696	2083
15	320	>10000	2904	>10000	211	6.2	156	>10000	227	3031	401
9	714	>10000	8590	>10000	174	7.2	851	>10000	457	3587	472
22	6764	>10000	>10000	>10000	1313	27.5	1897	>10000	994	5909	427
14	116	9653	3830	>10000	1541	38.5	1201	>10000	595	4777	516

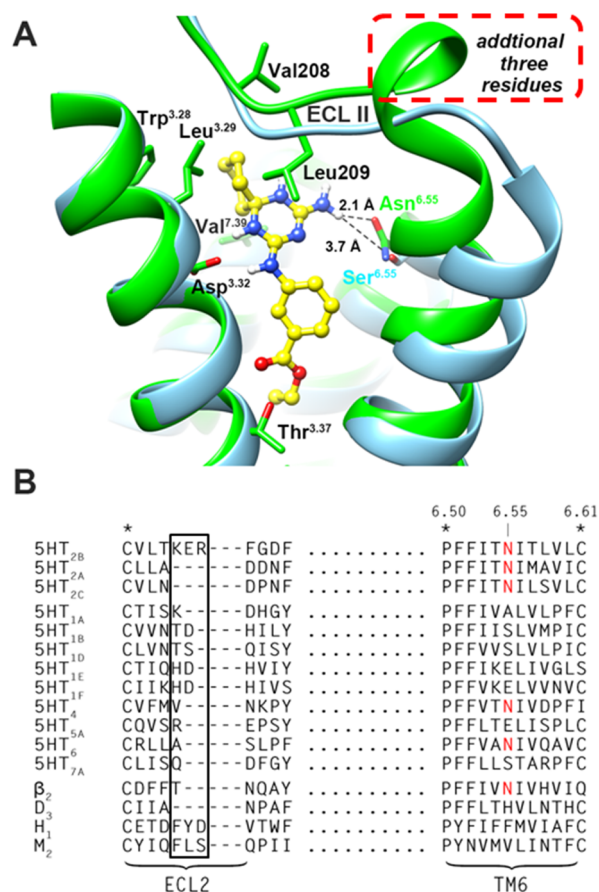


Figure 5. Potential ligand binding selectivity mechanism of the 5-HT_{2B} receptor. (A) Crystal structure comparison between the 5-HT_{2B} (green, PDB ID: 4IB4) and 5-HT_{1B} (cyan, PDB ID: 4IAR) receptors. (B) Sequence alignment of the ECL II and TM6 regions among 5-HTRs and other GPCRs.

the crystal structure bound with a biased agonist. The retrospective docking screens of known 5-HT_{2B} antagonists indicated improved enrichment performance using our induced-fit models. Actually, the prospective docking screen against the crystal structure could not find any of our identified 5-HT_{2B} antagonists in the top 1000 compounds of scoring list (e.g., the rank of compound **9** is 3538); therefore, the optimization of the crystal conformation facilitated the successful identification of novel 5-HT_{2B} antagonists. Besides, we also tested the agonist activity for several of our compounds. None of them increased or decreased the basal-level activity of 5-HT_{2B} receptor at 1 μ M. Thus, our compounds shall be pure antagonist, without possessing partial agonist or inverse agonist activity.

Further, the binding site also experiences subtle conformation changes when binding with different antagonists. Our MM-GB/SA calculation treated the binding site with flexible

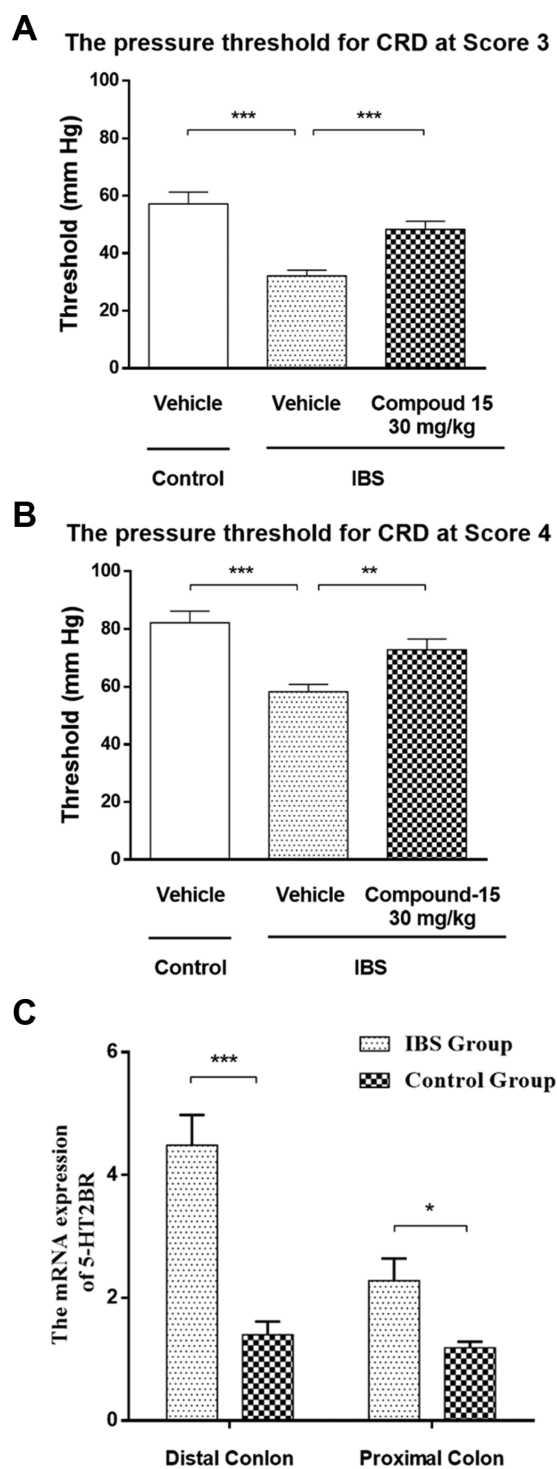


Figure 6. Inhibition of visceral hypersensitivity in IBS rat model. (A and B) Therapeutic effects of compound **15** in IBS rats. The pain threshold decreased in response to CRD (at score 3 and score 4). (C) Overexpression of mRNA for the 5-HT_{2B} receptor in IBS rat colons.

side-chain conformations and yielded improved correlation between the experimental pIC_{50} and computed ΔE compared with the rigid-receptor approximation, a result that emphasized the importance to incorporate binding-site flexibility into binding energy calculations.

As off-target interactions are responsible for the majority of adverse drug effects and failures in clinical trials, the identification of highly selective ligands is a critical aspect in

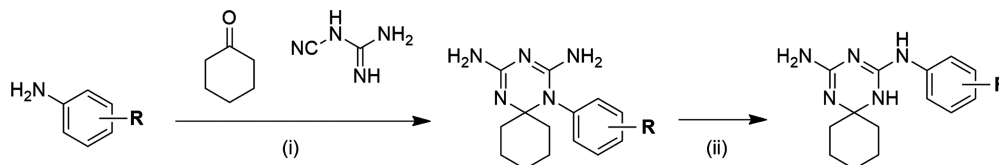
modern drug discovery. Currently, very few 5-HT_{2B} ligands are subtype-selective, and the development of novel 5-HT_{2B} antagonists with better specificity is highly desirable. In this study, we identified a series of potent 5-HT_{2B} antagonists. These compounds represent a novel scaffold with distinct structural characteristics. Instead of containing the classical tertiary amine group, they utilize a protonated triazine ring to form a salt bridge interaction with the carboxylate group of conserved Asp^{3.32} and may also form additional hydrogen bonds with the Asn^{6.55} residue. Encouragingly, these compounds exhibited high selectivity for the 5-HT_{2B} receptor over other 5HTRs. In vivo assessment has further indicated that compound **15** could produce significant attenuation of visceral hypersensitivity in IBS rat models. Thus, this scaffold represents a new avenue for the development of novel and selective 5-HT_{2B} antagonists for the treatment of 5-HT_{2B} receptor-related disease.

EXPERIMENTAL SECTION

Binding Site Refinement and Analysis. Crystal structures of the histamine H₁–doxepin complex (PDB ID: 3RZE) and the β_1 -carvedilol complex (PDB ID: 4AMJ) were utilized to generate the initial binding modes of doxepin and carvedilol to the 5-HT_{2B} receptor. Considering the homology of the transmembrane regions, we assumed that both ligands would adopt a binding mode in the 5-HT_{2B} receptor similar to that in other GPCRs. Thus, we superimposed the ligand-bound GPCR structures on the 5-HT_{2B} structure (PDB ID: 4IB4) and extracted the coordinates of the ligands as their initial binding poses. Subsequently, the conformations of the binding-site residues in the 5-HT_{2B} receptor were sampled along with the placed ligand, using the Protein Local Optimization Program (PLOP).^{43–45} The ligand was minimized first, followed by side-chain sampling of the binding site residues within 10 Å of the ligand. We implemented three rounds of such iterations to fully accommodate the ligand by sampling the surrounding residues.

To analyze the thermodynamic properties of the binding-site waters, we carried out MD simulations of the 5-HT_{2B} structure with the Gromacs v4.5.3 program,⁴⁶ applying the AMBER99SB force field⁴⁷ for the protein and the TIP3P water model.⁴⁸ Water molecules and ligand in the crystal structure of the 5-HT_{2B} receptor were removed. All calculations applied an atom-based truncation scheme that was updated heuristically with a list cutoff of 14 Å, a nonbond cutoff of 12 Å, and the Lennard–Jones (LJ) smoothing function initiated at 10 Å. Long-range electrostatic interactions were computed using the Particle Mesh Ewald (PME) method.⁴⁹ To equilibrate the solvent molecules around the solute, the system was minimized with 500 steps of steepest descent, followed by 200 ps of NVT MD simulation and a subsequent 200 ps of NPT simulation. The 12 ns of NVT MD production simulation was performed at 300 K via the v-rescale temperature coupling scheme. The LINCS algorithm⁵⁰ was applied, with a time step of 2 fs, to constrain all bonds involving hydrogen atoms. All heavy atoms of the protein were harmonically restrained during the equilibration and production simulation with a force constant of 1000 kJ/mol·nm². The trajectories of the last 5 ns of the MD simulations were submitted as input to the Solvent Property Analysis (SPA)³⁵ program to compute the structural and energetic properties of the binding-site water molecules.

The induced-fit models were evaluated based on their ability to enrich known 5-HT_{2B} ligands. In total, 206 5-HT_{2B} antagonists were collected from the GLIDA database.^{51,52} We then further clustered these antagonists by their Bemis–Murcko atomic frameworks⁵³ to obtain 84 structurally diverse ligands (Table S3, Supporting Information). For each ligand, 50 decoy compounds were generated based on the DUD-E protocol,⁵⁴ leading finally to 4096 nonredundant decoys that were physically similar but topologically dissimilar to the annotated ligands (Figure S8, Supporting Information). Enrichment performance is one common metric used to measure docking performance; it represents the prioritization of true ligands among

Scheme 1. General Procedure for Compound Synthesis^a

^aReaction conditions: (i) concd HCl, EtOH, reflux; (ii) pyridine, EtOH, 120 °C.

the top ranking hits of a docking-ordered library, which is calculated as $EF_{\text{subset}} = (\text{ligands}_{\text{selected}}/N_{\text{subset}})/(\text{ligands}_{\text{total}}/N_{\text{total}})$.⁵⁵

Structure-Based Hierarchical Virtual Screening. Molecular docking was performed with the DOCK 3.5.54,^{56,57} using the doxepin-induced model as the receptor structure. Binding-site residues were identified within 12 Å of doxepin. Then the solvent-accessible molecular surface was calculated with the DMS⁵⁸ program using a probe radius of 1.4 Å. Receptor-derived matching spheres were generated with the SPHGEN program,⁵⁹ and the ligand-derived spheres were generated from the positions of the heavy atoms of doxepin. Four sets of grids were generated: an excluded volume grid using DISMAP,⁶⁰ a united atom AMBER-based van der Waals potential grid using CHEMGRID,⁶⁰ an electrostatic potential grid using Delphi,⁶¹ and a solvent occlusion map using SOLVMAP.⁶⁰ All of compounds in the library were prepared in the flexibase format with the latest version of the ZINC protocol.^{62,63} For sampling, the minimum number of graph matching nodes was set to 3, and ligand overlap was set to 0.1. Ligand conformations were scored on the basis of the total energy ($E_{\text{tot}} = E_{\text{vdw}} + E_{\text{ele}} - \Delta G_{\text{lig-solv}}$) after 200 steps of rigid-body minimization, with initial translations of 0.2 Å and initial rotations of 5°.

MM-GB/SA refinement and rescoring was performed using PLOP. Briefly, the docked ligand was subjected to the fully flexible minimization in the receptor and in the solvent with the all-atom OPLS force field and the Surface Generalized Born implicit solvent as described previously.³⁶ The same protein structure used in the docking was used for rescoring. Hydrogen atoms were added in standard geometries as defined by the all-atom OPLS 2005 force field.^{64,65} The protein was kept rigid during protein–ligand complex minimization. The binding energy ($E_{\text{bind}} = E^{\text{R}*L} - E^L - E^R$) was calculated by subtracting the energies of the optimized free ligand in solution (E^L) and the free protein in solution (E^R) from the optimized protein–ligand complex's energy in solution ($E^{\text{R}*L}$). For the flexible rescoring procedure, the binding-site residues within 10 Å of the ligand were subjected to side-chain prediction and energy minimization.

Chemistry. Our in-house screening compound library was constructed from the commercially available ChemDiv compound database (<http://eu.chemdiv.com>) based on maximizing structural diversity.³⁷ Several analogues (**12**, **16–19**, and **26–28**) for SAR studies were purchased from Vitas-M (<http://www.vitasmlab.com>), Chembridge (<http://www.chembridge.com>), and Enamine (<http://www.enamine.net>), respectively. For each of these compounds, the vendor had verified the compound purity with liquid chromatography–mass spectrometry (LC–MS) and/or nuclear magnetic resonance (NMR) experiments. The ¹H NMR spectrum and the MS data for the initially identified active compound **9** are included in the Supporting Information.

The other compounds (**11**, **13–15**, and **20–25**) were prepared with reference to the general procedure described in Scheme 1.⁵⁶ All solvents, chemicals, and reagents were obtained commercially and used without purification. The ¹H NMR spectra were obtained in CDCl₃, d₆-DMSO, CD₃OD, or d₆-acetone at 25 °C at 300 MHz on an OXFORD instrument (Varian), with chemical shift (δ , ppm) reported relative to TMS as an internal standard. HPLC–MS chromatograms and mass spectra were obtained with a Shimadzu LC–MS–2020 system. The prep-HPLC instruments used included a Gilson GX-281 (Gilson) and an Elite P230 Preparative Gradient System (Elite). Chiral Prep-HPLC was performed with the Elite P230 Preparative Gradient System using Thar Prep-80 and Thar SFC X-5 columns. The

microwave instrument used was a CEM Discover SP. All tested compounds had a purity >95%.

Ethyl 3-((4-Amino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-2-yl)(methylamino)benzoate (11). Ethyl 3-aminobenzoate (4.1 g, 24.8 mmol) and di-*tert*-butyl dicarbonate (8.1 g, 37.2 mmol) were dissolved in anhydrous THF (60 mL). Then *N*-ethyl-*N*-isopropylpropan-2-amine (6.4 g, 49.6 mmol) was added. The reaction mixture was stirred at room temperature. After completion of the reaction, it was concentrated in vacuo, and the residue was extracted with ethyl acetate. The organic layer was concentrated and triturated with hexanes to provide ethyl 3-(*tert*-butoxycarbonylamino)benzoate as a white solid (5.6 g, 21.1 mmol, 85%). MS [$M + H$]⁺ calculated for C₁₄H₂₀NO₄: 266.13, found: 266.10.

Ethyl 3-(*tert*-butoxycarbonylamino)benzoate was dissolved in anhydrous THF (20 mL), and NaH (1.7 g, 42.2 mmol) was subsequently added under N₂ atmosphere. The mixture was stirred at room temperature for 15 min. MeI (2.0 mL, 31.7 mmol) was then added slowly in a dropwise manner. After completion, the reaction was quenched with saturated aqueous NH₄Cl and extracted three times with ethyl acetate. The organic layers were combined and concentrated in vacuo to provide a residue; the residue was purified by column chromatography to give ethyl 3-(*tert*-butoxycarbonyl(methylamino)benzoate (2.4 g, 41%).

Ethyl 3-(*tert*-butoxycarbonyl(methylamino)benzoate (2.4 g, 8.59 mmol) was dissolved in HCl (10 mL, 4 M) and THF (10 mL), and the mixture was refluxed overnight. It was then concentrated in vacuo, and the residue was purified by column chromatography to provide the ethyl 3-(methylamino)benzoate (1.2 g, 80%). MS [$M + H$]⁺ calculated for C₁₀H₁₄NO₂: 180.09, found: 180.10.

A mixture of ethyl 3-(methylamino)benzoate (606 mg, 3.4 mmol), cyanoguanidine (313 mg, 3.7 mmol), concentrated HCl (0.28 mL, 3.4 mmol), and cyclohexanone (334 mg, 3.4 mmol) in EtOH (2 mL) was refluxed for 2 days. The reaction mixture was neutralized with saturated NaHCO₃ to pH 8 and then extracted with DCM (50 mL, three times). The organic layers were concentrated in vacuo to provide a crude product, which was purified by column chromatography to give the compound **11** (28 mg, 2.5%). ¹H NMR (400 MHz, DMSO-*d*₆): δ (ppm) 8.77 (s, 1H), 8.05–7.80 (m, 3H), 7.63 (d, *J* = 5.1 Hz, 2H), 7.00 (s, 1H), 4.40–4.29 (m, 2H), 3.38 (s, 3H), 2.02–1.38 (m, 9H), 1.35–1.25 (m, 4H). MS [$M + H$]⁺ calculated for C₁₈H₂₆N₅O₂: 344.20, found: 344.20.

Ethyl 3-(4-(Methylamino)-1,3,5-triazaspiro[5.5]undeca-2,4-dien-2-ylamino)benzoate (13). Sodium dicyanoamide (5 g, 56.2 mmol) and methylamine hydrochloride (3.8 g, 56.2 mmol) were dissolved in *n*-butanol (25 mL) and H₂O (10 mL). The mixture was then refluxed. After completion of the reaction, the mixture was concentrated in vacuo and the residue was purified further by column chromatography to give 1-cyano-3-methylguanidine (1.7 g, 31%). MS [$M + H$]⁺ calculated for C₃H₇N₄: 99.06, found: 99.10.

A mixture of 1-cyano-3-methylguanidine (500 mg, 5.1 mmol), ethyl 3-aminobenzoate (765 mg, 4.63 mmol), and concentrated HCl (0.39 mL, 4.63 mmol) in EtOH (2 mL) was refluxed for 6 h. Then the ethyl 3-(3-(*N*-methylcarbamimidoyl)guanidino)benzoate was obtained by filtration (510 mg, 37%). MS [$M + H$]⁺ calculated for C₁₂H₁₈N₅O₂: 264.14, found: 264.14.

Ethyl 3-(3-(*N*-methylcarbamimidoyl)guanidino)benzoate (510 mg, 1.7 mmol) in cyclohexanone (15 mL) and EtOH (5 mL) was refluxed overnight and then cooled to room temperature. Saturated aqueous NaHCO₃ (25 mL) was then added. The mixture was extracted with

DCM (50 mL, three times) and washed with brine (30 mL). The organic layers were combined and concentrated in vacuo to afford a crude product, which was purified by column chromatography to provide the compound **13** (230 mg, 39%). ¹H NMR (400 MHz, DMSO-*d*₆): δ (ppm) 8.89 (s, 1H), 7.96 (s, 1H), 7.75 (d, *J* = 7.9 Hz, 1H), 7.33 (d, *J* = 7.6 Hz, 1H), 7.22 (t, *J* = 7.8 Hz, 1H), 6.41 (s, 1H), 5.76 (s, 1H), 4.26 (q, *J* = 7.1 Hz, 2H), 2.72 (d, *J* = 4.2 Hz, 3H), 1.73–1.87 (m, 2H), 1.67–1.23 (m, 11H). MS [*M* + *H*]⁺ calculated for C₁₈H₂₆N₅O₂: 344.20, found: 344.20.

Ethyl 3-(4-(Dimethylamino)-1,3,5-triazaspiro[5.5]undeca-2,4-dien-2-ylamino)benzoate (14). Sodium dicyanoamide (5 g, 56.2 mmol) and dimethylamine hydrochloride (4.58 g, 56.2 mmol) were dissolved in *n*-butanol and H₂O, and this mixture was then refluxed. After completion of the reaction, the mixture was concentrated in vacuo and the residue was purified further by column chromatography to obtain the product 2-cyano-1,1-dimethylguanidine (1.5 g, 24%). MS [*M* + *H*]⁺ calculated for C₄H₉N₄: 113.07, found: 113.10.

A mixture of 2-cyano-1,1-dimethylguanidine (500 mg, 4.46 mmol), ethyl 3-aminobenzoate (670 mg, 4.1 mmol), concentrated HCl (0.34 mL, 4.1 mmol), and cyclohexanone (402 mg, 4.1 mmol) in EtOH (2 mL) was refluxed. After completion of the reaction, it was cooled to room temperature. The reaction mixture was then neutralized with saturated NaHCO₃ to pH 8 and then extracted with DCM (50 mL, three times). The organic layers were combined and concentrated in vacuo to afford a crude product, which was purified by column chromatography to give compound **14** (60 mg, 4%). ¹H NMR (400 MHz, DMSO-*d*₆): δ (ppm) 10.63 (s, 1H), 9.14 (s, 1H), 8.46 (s, 1H), 8.22 (s, 1H), 7.69 (d, *J* = 7.6 Hz, 1H), 7.59 (d, *J* = 8.7 Hz, 1H), 7.50 (t, *J* = 7.9 Hz, 1H), 4.31 (q, *J* = 7.1 Hz, 2H), 3.30–2.97 (m, 6H), 1.94–1.61 (m, 9H), 1.36–1.16 (m, 4H). MS [*M* + *H*]⁺ calculated for C₁₉H₂₈N₅O₂: 358.22, found: 358.20.

N⁴-(3-Bromophenyl)-1,3,5-triazaspiro[5.5]undeca-1,3-diene-2,4-diamine (15). A mixture of 3-bromobenzenamine (2 g, 11.6 mmol), cyanoguanidine (1.08 g, 12.8 mmol), concentrated HCl (1.0 mL, 11.6 mmol), and cyclohexanone (1.1 g, 11.6 mmol) in EtOH (2 mL) was refluxed. After completion of the reaction, the solid was filtered to obtain 5-(3-bromophenyl)-1,3,5-triazaspiro[5.5]undeca-1,3-diene-2,4-diamine (1.6 g, 41%). MS [*M* + *H*]⁺ calculated for C₁₄H₁₉BrN₅: 336.07, found: 336.0.

5-(3-Bromophenyl)-1,3,5-triazaspiro[5.5]undeca-1,3-diene-2,4-diamine was dissolved in pyridine (2 mL) and EtOH (3 mL), and the mixture was heated to and held at 120 °C overnight. It was then concentrated in vacuo to dryness. After water (10 mL) was added, the mixture was stirred at room temperature for 30 min. Then the solid was filtered and dried in vacuo to afford compound **15** (310 mg, 62%). ¹H NMR (400 MHz, DMSO-*d*₆): δ (ppm) 10.28 (s, 1H), 8.91 (s, 1H), 8.58 (d, *J* = 4.2 Hz, 1H), 7.82–7.78 (m, 1H), 7.58–7.49 (m, 1H), 7.39 (dd, *J* = 7.6 Hz, *J* = 5.7 Hz, 1H), 7.31–7.27 (m, 2H), 1.88–1.16 (m, 10H). MS [*M* + *H*]⁺ calculated for C₁₄H₁₉BrN₅: 336.07, found: 336.0.

3-(4-Amino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-2-ylamino)benzoic Acid (20). A solution of ethyl 3-(4-amino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-2-ylamino)benzoate (25.0 mg, 0.09 mmol) in MeOH (1.00 mL) was added to NaOH (2.00 mL). Then the reaction mixture was stirred at 30 °C overnight, diluted with water, and slowly acidified with trifluoroacetic acid to adjust the reaction mixture to pH < 4. The mixture was concentrated, and the resulting residue was then purified using reverse-phase chromatography to give a white solid (3.0 mg, 13%). ¹H NMR (400 MHz, DMSO-*d*₆): δ (ppm) 1.37–1.70 (m, 10H), 6.97–7.08 (m, 1H), 7.44 (t, *J* = 8.0 Hz, 1H), 7.65–7.67 (m, 1H), 7.82 (s, 1H), 7.85–7.87 (m, 1H), 8.57–8.71 (m, 2H), 9.69–9.82 (m, 1H). MS [*M* + *H*]⁺ calculated for C₁₅H₂₀N₅O₂: 302.16, found: 302.30.

1-(3-(4-Amino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-2-ylamino)phenyl)butan-1-one (21). A solution of 1-phenylbutan-1-one (2500 mg, 16.9 mmol) was added to HNO₃ (5 mL) at –5 °C dropwise in flask. The resulting suspension was stirred at –5 °C for about 4 h and monitored by TLC until the starting materials were completely consumed. Ice–water was added to the reaction solution and filtered to give the crude product 1-(3-nitrophenyl)butan-1-one as

a yellow solid, which was then used in the next step without further purification (900 mg, 28%). MS [*M* + *H*]⁺ calculated for C₁₀H₁₂NO₃: 194.2, found: 194.2.

A solution of 1-(3-nitrophenyl)butan-1-one (900 mg, 4.7 mmol) in EtOH (50 mL) was added to Zn powder (0.6 g, 9.4 mmol) and concentrated HCl (1.8 g, 18.8 mmol) at 0 °C. The resulting suspension was allowed to warm to room temperature and monitored by TLC until the starting materials were completely consumed. The reaction solution was filtered and the solvent was eliminated under reduced pressure to give the crude product, which was purified by chromatographic column to gain the desired product 1-(3-aminophenyl)butan-1-one (450 mg, 59%). MS [*M* + *H*]⁺ calculated for C₁₀H₁₄NO: 164.2, found: 164.2.

A solution of 1-(3-aminophenyl)butan-1-one (450 mg, 2.8 mmol) in EtOH (50 mL) was added to cyanoguanidine (235 mg, 2.8 mmol) and concentrated HCl (2.8 g, 28 mmol) at room temperature. The resulting suspension was warmed to reflux overnight and monitored by TLC until the starting materials were completely consumed. The solvent was eliminated under reduced pressure to give the crude product 1-(3-(3-(carbamidoyl)guanidino)phenyl)butan-1-one, which was used in the next step without further purification (180 mg, 26%). MS [*M* + *H*]⁺ calculated for C₁₂H₁₈N₅O: 248.3, found: 248.3.

A solution of 1-(3-(3-(carbamidoyl)guanidino)phenyl)butan-1-one (180 mg, 0.7 mmol) in EtOH (50 mL) was added to cyclohexanone (137 mg, 1.4 mmol) and triethyl orthoformate (208 mg, 1.4 mmol) at 0 °C. The resulting suspension was allowed to warm to room temperature overnight and monitored by TLC until the starting materials were completely consumed. The solvent was eliminated under reduced pressure to give the crude product 1-(3-(2,4-diamino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-1-yl)phenyl)butan-1-one, which was used in the next step without further purification (120 mg, 51%). MS [*M* + *H*]⁺ calculated for C₁₈H₂₆N₅O: 328.4, found: 328.4.

A solution of 1-(3-(2,4-diamino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-1-yl)phenyl)butan-1-one (120 mg, 0.37 mmol) in EtOH (15 mL) was added to pyridine (315 mg, 3.7 mmol) at room temperature. The resulting suspension was allowed to warm to reflux overnight and monitored by TLC until the starting materials were completely consumed. The solvent was eliminated under reduced pressure to give the crude product, which was washed with MeOH to afford the compound **21** (27 mg, 22%). ¹H NMR (400 MHz, DMSO-*d*₆): δ (ppm) 10.44 (s, 1H), 9.08 (s, 2H), 8.92 (s, 1H), 8.53 (t, *J* = 8.0 Hz, 1H), 7.99–8.05 (m, 2H), 7.86–7.97 (m, 1H), 7.65–7.75 (m, 1H), 7.46–7.50 (m, 1H), 3.44 (q, *J* = 8.0 Hz, 1H), 3.00 (t, *J* = 8.0 Hz, 2H), 1.60–1.70 (m, 9H), 1.28–1.51 (m, 2H), 1.06 (t, *J* = 8.0 Hz, 2H), 0.94 (t, *J* = 8.0 Hz, 2H). MS [*M* + *H*]⁺ calculated for C₁₈H₂₆N₅O: 328.21, found: 328.2.

3-(4-Amino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-2-ylamino)-*N*-ethylbenzamide (22). A solution of 3-aminobenzoic acid (4.4 g, 32.1 mmol) and di-*tert*-butyl dicarbonate (10.5 g, 48.2 mmol) in anhydrous THF (60 mL) was added to *N*-ethyl-*N*-isopropylpropan-2-amine (8.3 g, 64.2 mmol). The reaction mixture was stirred at room temperature. After completion of the reaction, it was concentrated in vacuo and the residue was extracted with ethyl acetate. The organic layer was concentrated, and the residue was purified by column chromatography to provide 3-(*tert*-butoxycarbonylamino)benzoic acid (6.2 g, 82%). MS [*M* – *H*][–] calculated for C₁₂H₁₄NO₄: 236.10, found: 236.10.

A mixture of 3-(*tert*-butoxycarbonylamino)benzoic acid (2 g, 8.43 mmol), HATU (6.4 g, 16.9 mmol), and *N*-ethyl-*N*-isopropylpropan-2-amine (3.27 g, 25.3 mmol) was dissolved in DMF (40 mL), and then the mixture was stirred at room temperature for 30 min. Then ethanamine hydrochloride (2.1 g, 25.3 mmol) was added. After completion of the reaction, water was added and the mixture was extracted three times with EtOAc. The organic layer was concentrated to give the crude product *tert*-butyl 3-(ethylcarbamoyl)phenylcarbamate (2.24 g, 100%) which was used without further purification.

tert-Butyl 3-(ethylcarbamoyl)phenylcarbamate (3.24 g, 12.3 mmol) was dissolved in dioxane (40 mL), and then concentrated HCl (10 mL) was added. The mixture was stirred at room temperature for 8 h. After completion of the reaction, it was concentrated in vacuo and the residue was purified by column chromatography to give 3-amino-*N*-ethylbenzamide (1.4 g, 70%). MS $[M + H]^+$ calculated for $C_9H_{13}N_2O$: 165.09, found: 165.10.

A mixture of 3-amino-*N*-ethylbenzamide (1 g, 6.1 mmol), cyanoguanidine (563 mg, 6.7 mmol), concentrated HCl (0.51 mL, 6.1 mmol), and cyclohexanone (599 mg, 6.1 mmol) in EtOH (2 mL) was refluxed. After completion of the reaction, the solid was filtered to give the desired product 3-(2,4-diamino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-1-yl)-*N*-ethylbenzamide (1.18 g, 59%) as a white solid. MS $[M + H]^+$ calculated for $C_{17}H_{25}N_6O$: 329.20, found: 329.20.

3-(2,4-Diamino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-1-yl)-*N*-ethylbenzamide (300 mg, 0.91 mmol) was dissolved in pyridine (3 mL), the mixture was heated to 120 °C overnight, and then it was concentrated in vacuo to give the crude product as a salt. Then the solid was neutralized by sat. $NaHCO_3$ to pH = 8. The solid was filtered and dried in vacuo to give the compound **22** (232 mg, 77%) as a pale pink solid. 1H NMR (400 MHz, DMSO- d_6): δ (ppm) 10.17 (s, 1H), 8.90–9.01 (m, 2H), 8.48 (t, J = 5.3 Hz, 1H), 7.76–7.81 (m, 2H), 7.57 (d, J = 7.7 Hz, 1H), 7.41 (t, J = 7.8 Hz, 1H), 7.18 (s, 1H), 3.33–3.24 (m, 2H), 1.89–1.25 (m, 10H), 1.12 (t, J = 7.2 Hz, 3H). MS $[M + H]^+$ calculated for $C_{17}H_{25}N_6O$: 329.20, found: 329.20.

Propyl 3-(4-Amino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-2-ylamino)benzoate (23). 3-Aminobenzoic acid (2 g, 14.6 mmol) was dissolved in propan-1-ol (20 mL), concentrated sulfuric acid (0.5 mL) was added, and the mixture was refluxed overnight. After completion of the reaction, it was concentrated in vacuo to obtain propyl 3-aminobenzoate (2.4 g, 92%) and used in the next step without further purification. MS $[M + H]^+$ calculated for $C_{10}H_{14}NO_2$: 180.09, found: 180.10.

A mixture of propyl 3-aminobenzoate (2 g, 11.2 mmol), cyanoguanidine (1 g, 12.3 mmol), concentrated HCl (0.93 mL, 11.2 mmol), and cyclohexanone (1.1 g, 11.2 mmol) in propan-1-ol (2 mL) was refluxed. After completion of the reaction, the solid was filtered to provide propyl 3-(2,4-diamino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-1-yl)benzoate (1.1 g, 29%). MS $[M + H]^+$ calculated for $C_{18}H_{26}N_5O_2$: 344.20, found: 344.20.

Propyl 3-(2,4-diamino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-1-yl)benzoate (500 mg, 1.46 mmol) was dissolved in pyridine (2 mL) and propan-1-ol (3 mL), and then the mixture was heated to 120 °C overnight. After LC-MS showed that the reaction was completed, the mixture was concentrated in vacuo to give the crude product as a salt. Then the solid was neutralized by sat. $NaHCO_3$ to pH = 8. The solid was filtered and dried in vacuo to give the desired product (410 mg, 82%). 1H NMR (400 MHz, DMSO- d_6): δ (ppm) 10.33 (s, 1H), 8.97 (s, 2H), 8.06–8.02 (m, 1H), 7.88 (s, 1H), 7.70 (d, J = 7.8 Hz, 1H), 7.49 (t, J = 7.9 Hz, 1H), 7.22 (s, 1H), 4.25 (t, J = 6.6 Hz, 2H), 1.78–1.66 (m, 10H), 1.49–1.31 (m, 2H), 0.98 (t, J = 7.4 Hz, 3H). MS $[M + H]^+$ calculated for $C_{18}H_{26}N_5O_2$: 344.20, found: 344.20.

3-((4-Amino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-2-yl)-amino)phenyl(cyclopropyl)methanone (24). A mixture of (3-aminophenyl) (cyclopropyl)methanone (350 mg, 2.0 mmol), cyanoguanidine (185 mg, 2.2 mmol), concentrated HCl (0.21 mL, 2.5 mmol), and cyclohexanone (216 mg, 2.2 mmol) in EtOH (5 mL) was refluxed. After the reaction was complete, the solid was filtered to obtain cyclopropyl(3-(2,4-diamino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-1-yl)phenyl)methanone (160 mg, 25%). MS $[M + H]^+$ calculated for $C_{18}H_{23}N_5O$: 326.1, found: 326.1.

Cyclopropyl(3-(2,4-diamino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-1-yl)phenyl)methanone (80 mg, 0.25 mmol) was dissolved in pyridine (1 mL) and EtOH (1.5 mL), and then the mixture was heated to 120 °C overnight. After the reaction was complete, the mixture was concentrated in vacuo, and water (2 mL) was added. The mixture was stirred at room temperature for 30 min. Then the solid was filtered and dried in vacuo to afford the compound **24** (68 mg, 85%). 1H NMR (400 MHz, DMSO- d_6): δ (ppm) 8.93 (s, 1H), 8.19 (d, J = 7.7 Hz, 1H), 7.95 (d, J = 1.6 Hz, 1H), 7.60–7.69 (m, 3H), 7.38 (s, 1H), 6.44

(s, 1H), 2.93–3.10 (m, 1H), 1.93 (t, J = 12.0 Hz, 2H), 1.56–1.63 (m, 5H), 1.39–1.23 (m, 2H), 1.14–0.88 (m, 5H). MS $[M + H]^+$ calculated for $C_{18}H_{23}N_5O$: 326.1, found: 326.1.

3-(2,4-Diamino-1,3,5-triazaspiro[5.5]undeca-2,4-dien-1-yl)-*N,N*-diethylbenzamide (25). 3-Aminobenzoic acid (4.4 g, 32.1 mmol) and di-*tert*-butyl dicarbonate (10.5 g, 48.2 mmol) were dissolved in anhydrous THF (60 mL). Then *N*-ethyl-*N*-isopropylpropan-2-amine (8.3 g, 64.2 mmol) was added. The reaction mixture was stirred at room temperature. After completion of the reaction, it was concentrated in vacuo and the residue was extracted with ethyl acetate. The organic layer was concentrated, and the residue was purified by column chromatography to provide 3-(*tert*-butoxycarbonylamino)benzoic acid (6.2 g, 82%); MS $[M - H]^-$ calculated for $C_{12}H_{14}NO_4$: 236.10, found 236.10.

A mixture of 3-(*tert*-butoxycarbonylamino)benzoic acid (2 g, 8.43 mmol), HATU (6.4 g, 16.9 mmol), and *N*-ethyl-*N*-isopropylpropan-2-amine (3.27 g, 25.3 mmol) was dissolved in DMF (40 mL), and then the mixture was stirred at room temperature for 30 min. Then diethanamine hydrochloride (2.77 g, 25.3 mmol) was added. After completion of the reaction, water was added and the mixture was extracted with EtOAc for three times. The organic layer was concentrated to give *tert*-butyl 3-(ethylcarbamoyl)phenylcarbamate (2.2 g, 89%) which was used in the next step without further purification.

tert-Butyl 3-(ethylcarbamoyl)phenylcarbamate (2.2 g, 7.52 mmol) was dissolved in dioxane (40 mL), and then concentrated HCl (10 mL) was added. The mixture was stirred at room temperature for 8 h. After completion of the reaction, it was concentrated in vacuo and the residue was purified by column chromatography (silica gel, PE:EA = 4:1) to give 3-amino-*N,N*-diethylbenzamide (1.45 g, 100%); 1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.16 (t, J = 7.7 Hz, 1H), 6.82–6.53 (m, 3H), 3.53 (m, 2H), 3.28 (m, 2H), 1.22 (m, 6H). MS $[M + H]^+$ calculated for $C_{11}H_{16}N_2O$: 193.13, found: 193.20.

A mixture of 3-amino-*N,N*-diethylbenzamide (500 mg, 2.6 mmol), cyanoguanidine (241 mg, 2.86 mmol), concentrated HCl (0.22 mL, 2.6 mmol), and cyclohexanone (255 mg, 2.6 mmol) in EtOH (2 mL) was refluxed for 6 h. LC-MS showed that two isomer products were formed. The desired product compound **25** was obtained by Prep-HPLC (52 mg, 6%). 1H NMR (400 MHz, DMSO- d_6): δ (ppm) 10.24 (s, 1H), 9.17 (s, 1H), 8.83 (s, 1H), 7.82 (s, 1H), 7.71 (m, 1H), 7.49–7.35 (m, 2H), 7.36 (s, 1H), 7.05 (d, J = 7.4 Hz, 1H), 3.43 (m, 2H), 3.19 (m, 2H), 1.33 (m, 16H). MS $[M + H]^+$ calculated for $C_{17}H_{25}N_6O$: 357.23, found: 357.20.

Biological Assays. Detailed descriptions of cellular function assay, binding assay, and animal assay are included in the [Supporting Information](#). Briefly, cellular function assays were performed with the CHO-K1/5-HT_{2B} cell line, using a calcium flux assay method. The binding assays were performed by staff at the National Institute of Mental Health's Psychoactive Drug Screening Program (NIMH-PDSP), following the standard protocol.⁶⁷ The radiolabeled reference compounds ($[^3H]$ 8-OH-DPAT for 5-HT_{1A}; $[^3H]$ GR127543 for 5-HT_{1B} and 5-HT_{1D}; $[^3H]$ 5-HT for 5-HT_{1E}; $[^3H]$ ketanserin for 5-HT_{2A}; $[^3H]$ LSD for 5-HT_{2B} and 5-HT_{2C}; 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT₆, and 5-HT₇; $[^3H]$ LY278584 for 5-HT₃) were used in the K_i determination assays. The PDSP online data entry and analysis system calculates the variance of the quadruplicate measurements (for the total, nonspecific, and test compound binding values); variances greater than 20% are flagged for further inspection, and assays are repeated if necessary. The IBS rat models for the visceral hypersensitivity assays were induced by the injection of 2,4,6-trinitrobenzenesulfonic acid (TNBS) and then treated with chronic unpredictable mild stress (CUMS).⁶⁸ Haematoxylin–eosin (HE) stains of the proximal and distal colon were compared to confirm the lack of histological changes in the IBS rat model (Figure S9, Supporting Information). Behavioral responses to colorectal distension (CRD) were used to assess visceral sensitivity by observing the AWR. The pain threshold to CRD was determined as the minimum pressure required to evoke AWR score 3 and AWR score 4.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01631.

Additional figures and tables illustrating all computational and biological results, detailed experimental procedures, and ¹H NMR spectra and LC-MS spectra data of all synthesized compounds (PDF)

Table illustrating biological results (CSV)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support from the Chinese Ministry of Science and Technology "973" Grant (2014CB849802) and the National Natural Science Foundation of China (81470862) (to N.H.) is gratefully acknowledged. Y.Z. was supported by the AstraZeneca Open Innovation Postdoctoral Research Fellowship, the China Postdoctoral Science Foundation (2015M570948), and the Beijing Postdoctoral Research Foundation (2015ZZ-118). X.P.H. was supported by the National Institute of Mental Health Psychoactive Drug Screening Program (NIMH-PDSP, HHSN-271-2013-00017-C) directed by Bryan L. Roth (MD, Ph.D.) at the University of North Carolina at Chapel Hill and Program Officer Jamie Driscoll at NIMH (Bethesda, MD). Computational support was provided by the Supercomputing Center of the Chinese Academy of Sciences (SCCAS). We also thank the Chemical Center at NIBS for providing the in-house screening compounds.

■ ABBREVIATIONS USED

PAH, pulmonary arterial hypertension; ECL, extracellular loop; TM, transmembrane; TNBS, 2,4,6-trinitrobenzenesulfonic acid; HE, haematoxylin–eosin; AWR, abdominal withdrawal reflex; CRD, colorectal distension; CUMS, chronic unpredictable mild stress

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