Bioorganic & Medicinal Chemistry Letters 22 (2012) 4273–4280

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Antagonists of 5-HT$_6$ receptors. Substituted 3-(phenylsulfonyl) pyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidines and 3-(phenylsulfonyl) pyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidines—Synthesis and 'structure–activity' relationship

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ARTICLE INFO

Article history:
Received 12 March 2012
Revised 4 May 2012
Accepted 8 May 2012
Available online 17 May 2012

Keywords:
Serotonin receptor 5-HT$_6$
Antagonists
Phenylsulfonyl-pyrazolo-pyrido-pyrimidines
Specificity
hERG channel

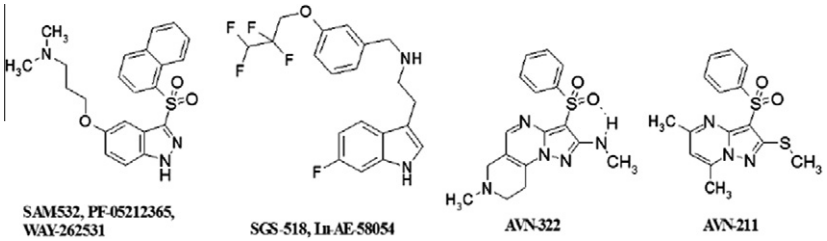
ABSTRACT

Synthesis and biological evaluation of a new series of structurally unrestricted and intramolecular hydrogen bond restricted derivatives of 3-(phenylsulfonyl)pyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidines (angular tricyclics) and 3-(phenylsulfonyl)pyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidines (linear tricyclics) are described. Structurally restricted derivatives are highly potent and selective blockers of 5-HT$_6$ receptors with little difference between angular or linear shape of the tricyclic core, the angular species being only slightly more potent. The angular representative of 3-(phenylsulfonyl)pyrazolo[1,5-*a*]pyrido[3,4-*e*] pyrimidines, **5**, can be considered as more favorable candidate for further development as it shows only weak 5-HT$_{2B}$ blocking activity (IC$_{50}$ = 6.16 μM as compared with IC$_{50}$ = 1.8 nM for 5-HT$_6$ receptors) and very low hERG potassium channel blocking potency (IC$_{50}$ = 54.2 μM). The linear analog, **11**, is less favorable as while showing no binding to the 5-HT$_{2B}$ receptor at concentrations of up to 10 μM, it exhibits quite a high potency to block the hERG channel (IC$_{50}$ = 0.5 μM).

Serotonin receptors of 5-HT$_6$ subtype (5-HT$_6$R) are attractive targets for development of new medicines to treat different diseases and impairments of the central nervous system (CNS). The impressive number of publications in scientific journals as well as a growing number of patents covering new inhibitors of the 5-HT$_6$R[1–4] reflects the high interest in the research and pharmaceutical industry communities.

Currently, several 5-HT$_6$R antagonists, SAM-531 (PF-5212365, Pfizer),[5,6] SGS-518 (Lu-AE-58054, Lundbeck, Lilly)[7–9] and AVN-322 (Avineuro Pharm.),[10–13] are in different phases of clinical trials for treatment of Alzheimer's disease. Another antagonist, AVN-211 (Avineuro Pharm.) has successfully finished phase IIa clinical trials to treat schizophrenia.[14–16]

Earlier, we studied the synthesis and SAR of 5-HT$_6$R ligands embodied by a 3-(phenylsulfonyl)pyrazolo[1,5-*a*]pyrimidine template.[17–20] In the current work, we describe the synthesis and evaluation of 3-(phenylsulfonyl)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido [3,4-*e*]pyrimidines **1**–**6** and 3-(phenylsulfonyl)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidines **7**–**12** as potent and selective 5-HT$_6$R antagonists.



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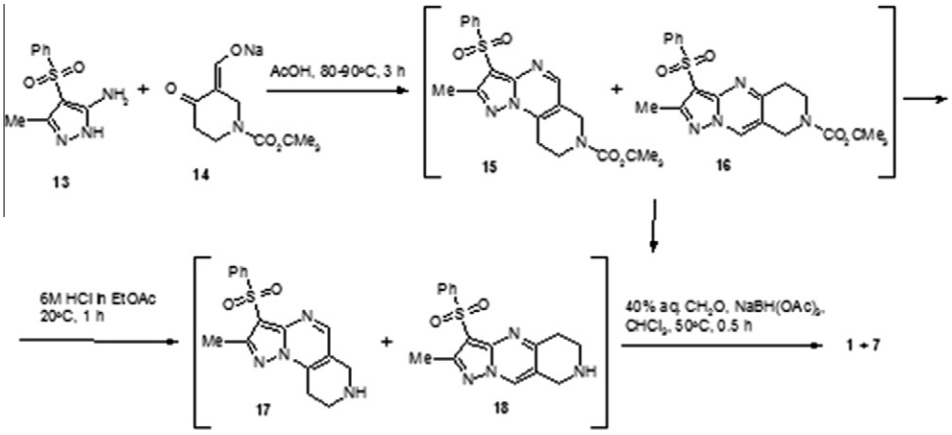
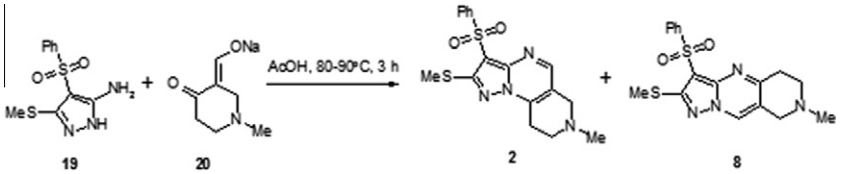
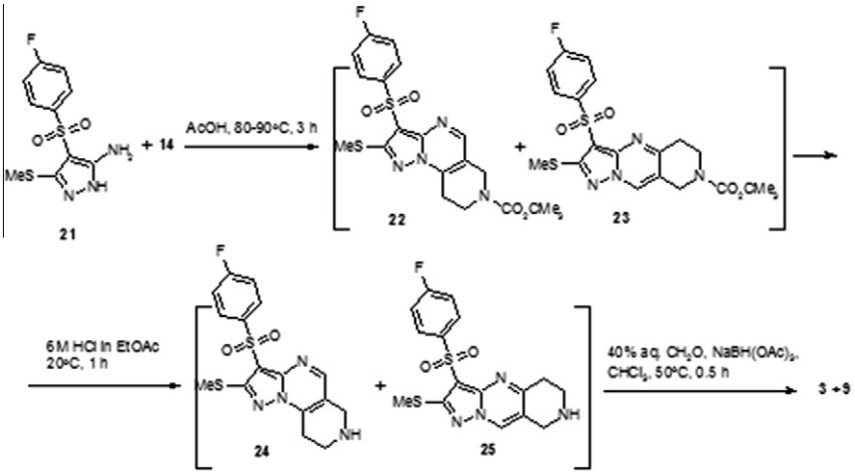
http://dx.doi.org/10.1016/j.bmcl.2012.05.036



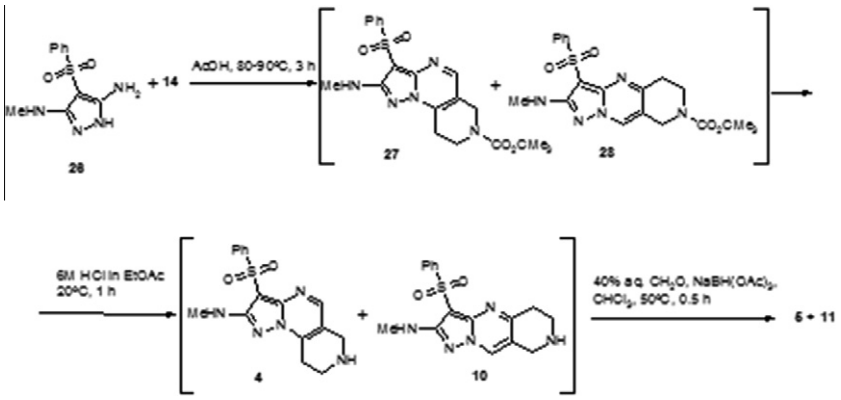
Scheme 1. Synthesis of compounds **1** and **7**.[21]



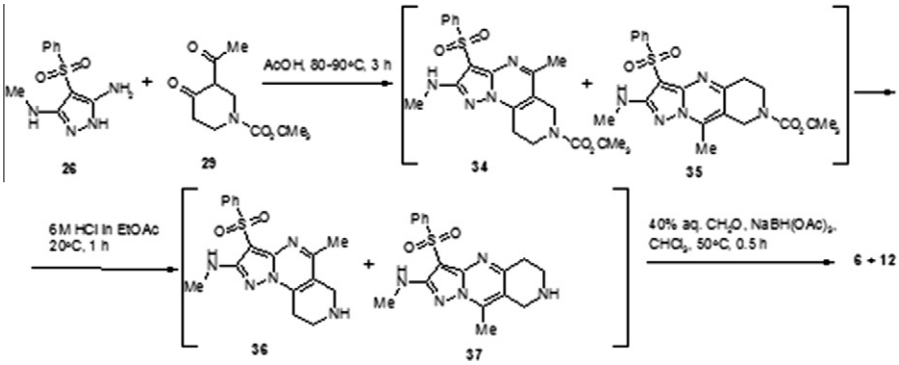
Scheme 2. Synthesis of compounds **2** and **8**.[22]



Scheme 3. Synthesis of compounds **3** and **9**.[23]

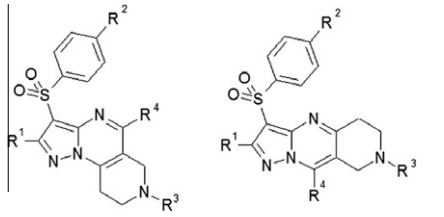


Scheme 4. Synthesis of compounds **4** and **10**, **5** and **11**.[24]



Scheme 5. Synthesis of compounds **6** and **12**.[25]

The reaction of 2*H*-pyrazol-3-ylamines (**13**, **19**, **21**, **26**) with sodium enolate of either 1-Boc-4-oxopiperidine-3-carbaldehyde (**14**) or 3-acetyl-1-Boc-4-oxopiperidine (**29**) or 3-[1-hydroxy-meth-(*E*)-ylidene]-1-methylpiperidin-4-one (**20**) in AcOH, yields mixtures of angular (**1**–**6**) and corresponding linear (**7**–**12**) tricyclic isomers in ratios ranging from 1:1 to 1:3.



1 - 6 **7 - 12**

1, 7: R^1 = Me, R^2 = H, R^3 = Me, R^4 = H;
2, 8: R^1 = SMe, R^2 = H, R^3 = Me, R^4 = H;
3, 9: R^1 = SMe, R^2 = F, R^3 = Me, R^4 = H;
4, 10: R^1 = NHMe, R^2 = H, R^3 = H, R^4 = H;
5 (AVN322), 11: R^1 = NHMe, R^2 = H, R^3 = Me, R^4 = H;
6, 12: R^1 = NHMe, R^2 = H, R^3 = Me, R^4 = Me.

Scheme 1 demonstrates the reaction of 4-(phenylsulfonyl)-5-methyl-2*H*-pyrazol-3-ylamine **13** with sodium enolate of 1-Boc-4-oxopiperidine-3-carbaldehyde **14** yielding a mixture of isomers **15** and **16**, which were difficult to separate. The Boc group was then removed from the compounds in the mixture, which led to formation of a likewise inseparable mixture of isomers **17** and **18**. Reductive amination of the mixture with formaldehyde led to a mixture of isomers **1** and **7**, which then were separated by HPLC (Shimadzu LC-8A) using 50 mm × 20 mm Reprosil-Pur-18-AQ 10 μm pre-column and 250 mm × 20 mm Reprosil-Pur-18-AQ 10 μm column at a flow rate 25 mL/min in a gradient mode with mobile phase MeCN/water + 0.05% CF_3COOH.

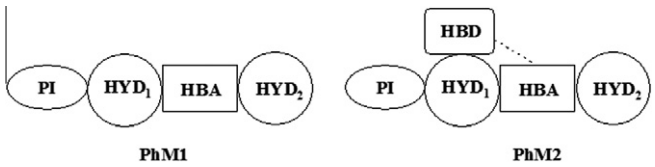


Figure 1. Conceptual pharmacophore models, PhM1 (adapted from Holenz et al.[32] and López-Rodrigues et al.[33]) and PhM2.[20] The HYD1 and HYD2 groups (circles) represent aromatic and/or heterocyclic hydrophobic moieties. HBA (square) is a hydrogen bond acceptor[33] or double electron acceptor[21] group, represented by either a sulfonamide or sulfonyl group. HBD (round cornered square) is a hydrogen bond donor capable of forming intramolecular hydrogen bond (dotted line) with the HBA.[20] PI (oval) is either a positively ionizable atom[33] or proton donor,[32] mainly represented by an amine group.

Similarly, a mixture of 7-methyl-2-(methylthio)-3-(phenylsulfonyl)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidine **2** and 7-methyl-2-(methylthio)-3-(phenylsulfonyl)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidine **8** was obtained in the reaction of 4-(phenylsulfonyl)-5-(methylthio)-2*H*-pyrazol-3-ylamine **19** with sodium enolate of 1-methyl-4-oxopiperidine-3-carbaldehyde **20** (Scheme 2). The compounds **2** and **8** were then separated using HPLC.

3-(4-Fluorophenylsulfonyl)-7-methyl-2-(methylthio)-6,7,8,9-tetr-ahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidine **3** and 3-(4-fluorophenylsulfonyl)-7-methyl-2-(methylthio)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidine **9** were obtained upon reaction of 4-(4-fluorophenylsulfonyl)-5-(methylthio)-2*H*-pyrazol-3-ylamine **21** with sodium enolate of 1-Boc-4-oxopiperidine-3-carbaldehyde **14**. The synthetic conditions were analogous to the ones used for synthesis of **1** and **7**, also without separation of the interim mixtures of isomers **22, 23** and **24, 25** (Scheme 3).

N-Methyl-3-(phenylsulfonyl)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidin-2-amine **4** and *N*-methyl-3-(phenylsulfonyl)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidin-2-amine **10**, as well as *N*,7-dimethyl-3-(phenylsulfonyl)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidin-2-amine **5** and *N*,7-dimethyl-3-(phenylsulfonyl)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidin-2-amine **11** were obtained in the reaction of N^3-methyl-4-(phenylsulfonyl)-1*H*-pyrazole-3,5-diamine **26** with sodium enolate of 1-Boc-4-oxopiperidine-3-carbaldehyde **14**, similar to the synthesis of compounds **1** and **7** without separation of intermediate isomers **27** and **28** (Scheme 4).

N,5,7-Trimethyl-3-(phenylsulfonyl)-6,7,8,9-tetrahydro-pyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidine-2-amine **6** and *N*,7,9-trimethyl-3-(phenylsulfonyl)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidine-2-amine **12** were obtained by employing the same sequence, but starting with N^3-methyl-4-(phenylsulfonyl)-1*H*-pyrazole-3,5-diamine **26** with 3-acetyl-1-Boc-4-oxopiperidine **29** in AcOH. The Boc group was removed without separation of isomers **34** and **35** and finally, **36** and **37** were transformed using conditions of reductive amination, into a mixture of **6** and **12** (Scheme 5). The final compounds **6** and **12** were the separated by HPLC.

Structures of the synthesized compounds were confirmed with LC/MS, HRMS, ^1H and ^{13}C NMR spectroscopy and by X-ray analysis (see Experimental section).

Conceptually, the compounds **1**–**3** and **7**–**9** correspond to a pharmacophore model PhM1 while the compounds **4**–**6** and **10**–**12** can be described by the pharmacophore model PhM2 both shown in Figure 1.

The main difference between the two models is that in accordance with PhM2, the hydrogen bond donor (HBD) represented in these series by the methylamino group, forms an intramolecular hydrogen bond with a hydrogen bond acceptor (HBA) represented by the sulfonyl group.

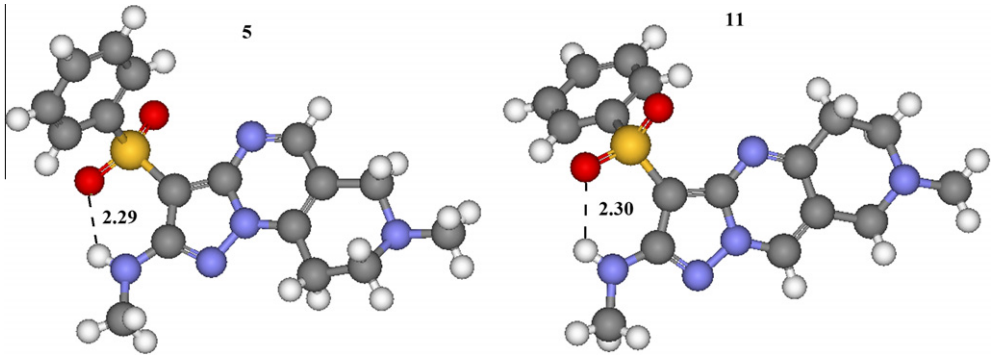
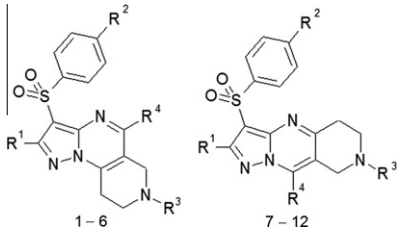


Figure 2. 3D X-ray structures of isomers **5** (*CCDC 880442*) and **11** (*CCDC 880441*) were plotted with software package Mercury 3.0 (*CCDC 880441* and *CCDC 880442* contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif).

Table 1
Functional potencies (K_i antagonism) and binding affinities (K_i binding) of 3-(phenylsulfonyl)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidines 1 – 6 and 3-(phenylsulfonyl)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidines **7**–**12**



Compds	Tricyclic shape	R^1	R^2	R^3	R^4	5-HT_6R, K_i[a] (nM)		IC_{50}[a] (μM)	
						Binding[b]	Antagonism[c]	5-HT_{2B}[d]	hERG[e]
1	Angular	Me	H	Me	H	60.4			
2	Angular	SMe	H	Me	H	7.7	14.8		
3	Angular	SMe	F	Me	H	35.7			
4	Angular	NHMe	H	H	H		1.9		
5	Angular	NHMe	H	Me	H	0.39	1.8	6.16	54.2
6	Angular	NHMe	H	Me	Me	0.68			
7	Linear	Me	H	Me	H	>10,000			
8	Linear	SMe	H	Me	H	372	492		
9	Linear	SMe	F	Me	F		1155		
10	Linear	NHMe	H	H	H		2.1		
11	Linear	NHMe	H	Me	H	0.82	14.3	NA	0.5
12	Linear	NHMe	H	Me	Me	0.66			

NA—not active.
[a] Values are geometric means of three independent experiments. Variations of K_i and IC_{50} values were less than twofold from the mean values. K_i values were calculated as indicated.[26]
[b] Radioligand competitive binding with 5-HT_6R was performed by Ricerca Biosciences (Taiwan) as described.[27]
[c] 5-HT_6 receptor functional activity was determined as described.[28]
[d] 5-HT_{2B} receptor functional activity was determined as described.[29]
[e] hERG functional activity was determined as described.[30]

X-ray analysis (Fig. 2) confirms formation of the intramolecular hydrogen bond between methylamine and sulfonyl moieties of both the angular **5** and linear **11** isomers (Fig. 2).

The ligand affinities to the receptors were measured by concentration-dependent competition with radiolabled ligand.[27] Potencies of the compounds **1**–**12** to block serotonin-induced responses were investigated in a cell-based assay using HEK 293 cells stably expressing recombinant human 5-HT_6 receptor.[28] The inhibition constants, K_i, were calculated from IC_{50} values[26] using the Cheng–Prusoff equation.[34] The data are shown in Table 1.

Among both series of the derivatives, angular and linear, the highest affinity is observed for compounds **4**–**6** and **10**–**12**, irrespective of the tricyclic shape (PhM2). These compounds are capable of forming intramolecular hydrogen bonds between the methylamino group (R^1) and sulfonyl group (Fig. 2). The methylthio R^1-substituted compounds, **2** and **8** (PhM1), exhibit substantially lower affinities than those of their structurally restricted methylamino analogs, **5** and **11** (PhM2). Thus, in the group of angular tricyclic 5-HT_6R ligands, a 20-fold increase in the K_i (binding) value is observed upon transition from **5** to **2**. In the group of linear tricyclic compounds, such transition from methylamino, **11**, to methylthio-substituted compound, **8**, led to even higher, 450-fold increase in the binding K_i. Methyl substitution in the R^1 position, led to further decrease in the affinity (compare **1** with **2** and **7** with **8**). Addition of a methyl group in the R^4 position has no substantial effect on the binding affinity (compare K_i values for **5** and **6** in angular and **11** and **12** in linear tricyclic groups).

To determine the functional consequences of the ligand interaction with the 5-HT_6R in cells, we tested selected compounds in a

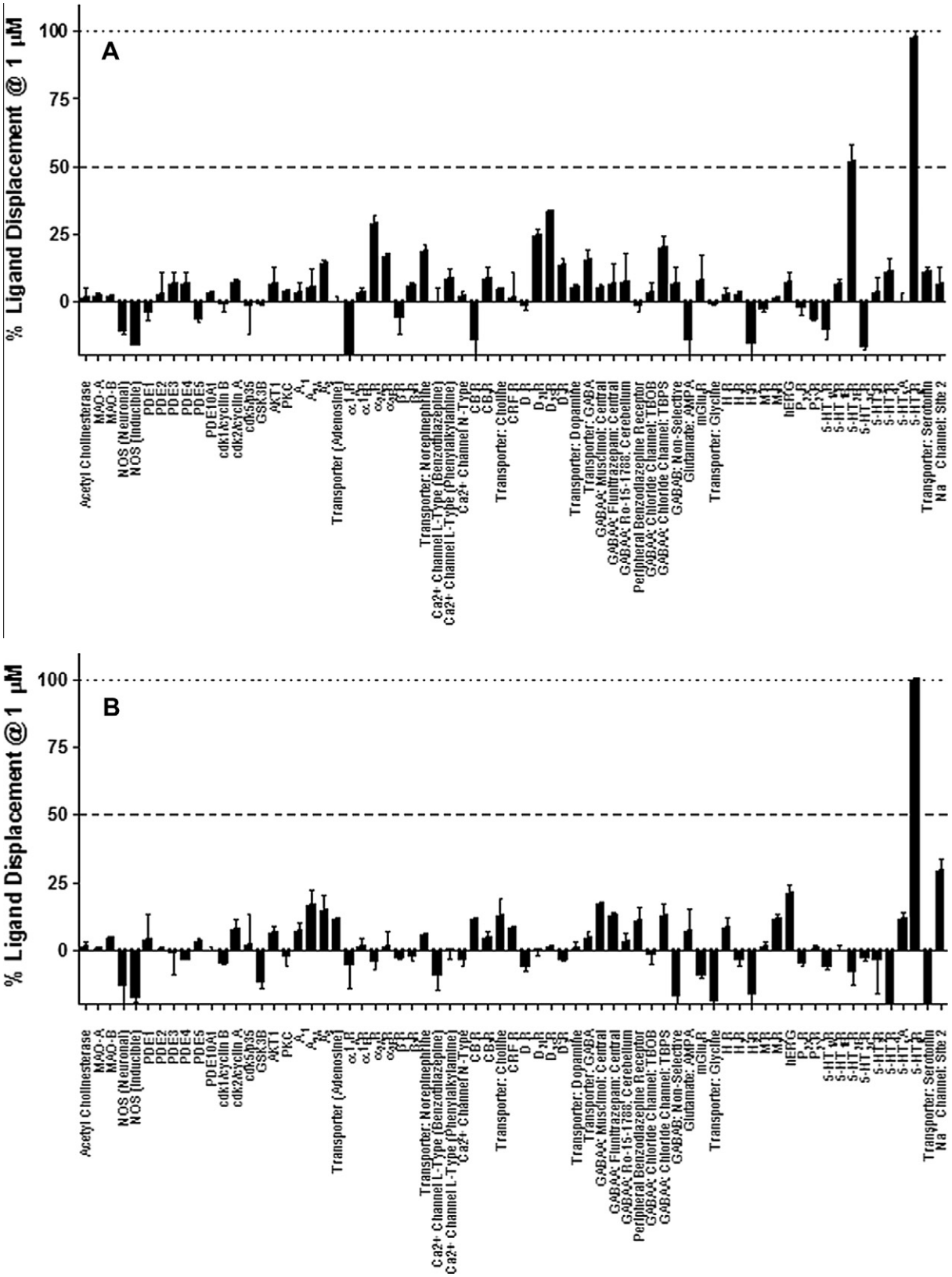


Figure 3. Specificity profiles of (**A**) angular **5** and (**B**) linear **11** antagonists of 5-HT_6R. Shown are percentages (±SD) of a radio labeled ligand displacements from corresponding targets at the antagonist concentration of 1 μM. Measurements were performed by Ricerca Biosciences (Taiwan) in duplicates.[31]

functional cell-based assay by their ability to either stimulate or block serotonin-induced accumulation of intracellular cAMP in cells exogenously expressing 5-HT_6R.[28] All of the tested compounds showed antagonistic activity with potencies (K_i antagonism) roughly corresponding to their affinities (K_i binding) measured in the equilibrium radioligand binding assay[27] (Table 1).

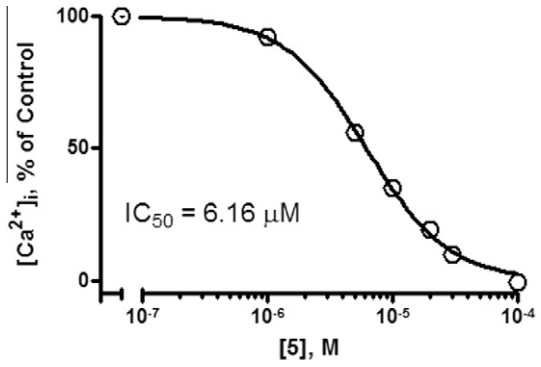


Fig. 4. Concentration-dependent inhibition of agonist-induced $[Ca^{2+}]_i$ mobilization in HEK-293 cells expressing h5-HT_{2B} receptor with **5**. Intracellular concentration of free calcium ions was measured using calcium sensitive dye Fluoro-4. The calcium ion concentration spike in the presence of different concentrations of **5** is expressed as a percentage of the spike induced by 5 nM αMe-5-HT.[29]

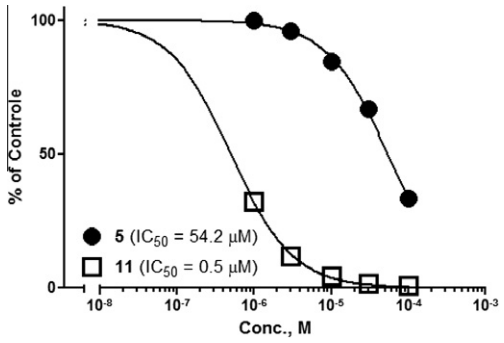


Fig. 5. Blockade of hERG potassium channel in patch clamp assay with angular (**5**) and linear (**11**) 5-HT_6R antagonists.[30]

Representative angular and linear tricyclic 5-HT_6R antagonists, **5** and **11**, were evaluated on a panel consisting of 69 related (other serotonergic) and unrelated (non-serotonergic) therapeutic targets, by their ability to compete with corresponding radio-labeled ligands[31] (Fig. 3).

The data in Figure 3 shows that both the angular **5** and linear **11** analogs are highly selective and specific 5-HT_6R ligands. **11** displays no substantial interaction with all but 5-HT_6R tested targets. compound **5** exhibits a low affinity competition with the radioligand for the 5-HT_{2B} receptor (~50% [^{3}H]lysergic acid diethylamide displacement at 1 μM). It has been shown[35] that activation of 5-HT_{2B}R with fenfluramine and other serotonergic medications can lead to valvular heart disease and hence the agonistic activity could represent a potential liability. On the other hand, antagonists of the receptor are being tested for treatment of chronic heart disease.[36,37] Therefore, it was important to clarify if the interaction of **5** with the 5-HT_{2B}R as measured in the radio-ligand binding assay, exhibits an agonistic or antagonistic mode of action. We studied the effect of **5** on 5-HT_{2B}-induced calcium mobilization in HEK293 cells with stably expressed human recombinant 5-HT_{2B} receptor.[29] When added directly to the cells, **5** caused no effect on the intracellular free calcium concentration but concentration-dependently suppressed the calcium spike induced by subsequent addition of the agonist, αMe-5-HT, with IC_{50} of 6 μM (Fig. 4). Thus, **5** is unlikely to cause problematic effect through the interaction with the 5-HT_{2B} receptor.

We have also tested **5** and **11** in cell-based patch clamp experiments[30] to assess their ability to functionally block exogenously expressed hERG (human Ether-à-go-go-Related Gene) potassium channel associated with a long QT syndrome, which can lead to sudden death. The data are shown in Figure 5. Remarkably, while exhibiting practically equal affinities to 5-HT_6R (Table 1), the angular **5** and linear **11** methylamino-substituted compounds showed a starkly different ability to inhibit human hERG potassium channel, **5** (IC_{50} = 54.2 μM) being 100-fold less potent than its analog **11** (IC_{50} = 0.5 μM). In this regard, while both compounds block the hERG channel at much higher concentrations than those necessary to block 5-HT_6R, **5** as the 5-HT_6R antagonist, can be considered substantially safer than **11**. The effective concentration of **5** to block hERG is four orders of magnitude higher than that to block 5-HT_6R. **11** exhibits the 'safety window' of only two orders of magnitude between the 5-HT_6R and hERG channel efficacy.

In the conclusion, we have shown that structurally restricted derivatives are highly potent and selective blockers of 5-HT_6 receptors with little difference between angular or linear shape of the tricyclic core. The angular species though not substantially, are slightly more potent than their linear analogs. The most potent angular representative of 3-(phenylsulfonyl)pyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidines, **5** (IC_{50} = 1.8 nM, Table 1), can be considered as more favorable candidate for further development as a drug candidate for treatment of such mental disorders as Alzheimer's disease and schizophrenia. **5** is highly potent and very selective against a panel of 68 other therapeutic targets. Though it shows a weak interaction with 5-HT_{2B} receptor (Fig. 3A), functionally, it is an antagonist with low potency (IC_{50} = 6.16 μM, Fig. 4), which is not associated with any therapeutic liability. **5** also exhibits very low hERG potassium channel blocking potency (IC_{50} = 54.2 μM, Fig. 5). Compound **11**, the linear analog of **5**, while showing better specificity (no binding to the 5-HT_{2B} receptor at concentrations of up to 10 μM, data not shown) is less favorable as it is one order of magnitude less potent to block 5-HT_6R (14.3 nM, Table 1) and exhibits quite a high potency to block the hERG channel (IC_{50} = 0.5 μM), which could potentially be associated with QT elongation syndrome.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.05.036. These data include MOL files and InChiKeys of the most important compounds described in this article.

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21. Synthesis of 2,7-dimethyl-3-(phenylsulfonyl)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidine **1** and 2,7-dimethyl-3-(phenylsulfonyl)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidine **7**. A mixture of 3-methyl-4-(phenylsulfonyl)-1*H*-pyrazole-5-amine **13** (1 mMol), sodium enolate of 1-Boc-4-oxopiperidine-3-carbaldehyde **14** (1.4 mMol) and AcOH (3 mL) was stirred at 80–90 °C for 3 h (or at ambient temperature overnight), precipitate (mixture of **15** and **16**) was filtered, washed with *i*-PrOH, dried, and dissolved in $CHCl_3$. This solution was treated with excess of 6 M HCl in EtOAc, stirred at ambient temperature for 1 h, and treated with Et_2O. The precipitate was separated and washed sequentially with Et_2O, EtOAc, and then EtOH. Once dried, analysis revealed a mixture of isomers **17**·HCl and **18**·HCl. The free base was obtained by treating an aqueous solution with 2 N NaOH, filtering the precipitate, washing with water, and re-crystallizing from *i*-PrOH. The solid mixture (89 mg) of isomers **17** and **18** was mixed with $NaBH(OAc)_3$ (200 mg), $CHCl_3$ (4 mL), and 40% aq CH_2O (0.4 mL), stirred at 50 °C for 0.5 h, cooled, washed with 10% aq NaOH, water, dried, and concentrated. The obtained mixture of isomers **1** and **7** was purified by silica gel flash chromatography and separated by HPLC into individual isomers **1** and **7**. The compounds **1** and **7** were dissolved in $CHCl_3$ (2 mL) and treated with 6 M solution of AcCl in EtOH (0.5 mL). The compounds were concentrated, filtered off, washed twice with *i*-PrOH, hexane, and dried. Compound **1**: ^{1}H NMR (400 MHz, $CDCl_3$), δ: 8.45 (s, 1H); 8.12 (d, J = 8.0 Hz, 2H); 7.40–7.53 (m, 3H); 3.62 (s, 2H); 3.22 (t, J = 5.9 Hz, 2H); 2.83 (t, J = 5.9 Hz, 2H); 2.73 (s, 3H); 2.52 (s, 3H). MS-ESI calculated for $C_{17}H_{18}N_4O_2S$ (M+H) 343, found m/z 343. LC–MS (UV-254) purity: 98%. **7**: ^{1}H NMR (400 MHz, DMSO-d_6), δ: 8.25 (s, 1H); 8.13 (d, J = 8.0 Hz, 2H); 7.38–7.54 (m, 3H); 3.59 (s, 2H); 3.21 (t, J = 5.9 Hz, 2H); 2.82 (t, J = 5.9 Hz, 2H); 2.69 (s, 3H); 2.48 (s, 3H). ^{13}C NMR (DMSO-d_6, 75 MHz) δ 155,90, 151,50, 146,42, 142,83, 141,16, 133,49, 129,44, 126,05, 112,58, 106,62, 49,02, 47,90, 41,08, 21,10, 12,87. MS-ESI calculated for $C_{17}H_{18}N_4O_2S$ (M+H) 343, found m/z 343. LC–MS (UV-254) purity: 98%.
22. Synthesis of 7-methyl-2-(methylthio)-3-(phenylsulfonyl)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidine **2** and 7-methyl-2-(methylthio)-3-(phenylsulfonyl)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidine **8.** Reactions were performed similar to those providing **1** and **7** but starting with 3-(methylthio)-4-(phenylsulfonyl)-1*H*-pyrazole-5-amine **19** and sodium enolate of 1-methyl-4-oxopiperidine-3-carbaldehyde **20**. Compound **2**: ^{1}H NMR (400 MHz, DMSO-d_6), δ: 11.76 (br.s, 1H), 8.69 (s, 1H), 8.01 (m, 2H), 7.64 (m, 1H), 7.58 (m, 2H), 4.60 (br.m, 1H), 4.37 (br.m, 1H), 3.76 (br.m, 1H), 3.47 (br.s, 2H), 3.34 (br.m, 1H), 2.94 (s, 3H), 2.61 (s, 3H). ^{13}C NMR (DMSO-d_6, 75 MHz) δ 155.90, 151.50, 146.42, 142.83, 141.16, 133.49, 129.44, 126.05, 112.58, 106.62, 49.02, 47.90, 41.08, 21.10, 12.87. MS-ESI calculated for $C_{17}H_{18}N_4O_2S_2$ (M+H) 375, found m/z 375. LC–MS (UV-254) purity: 98%. Compound **8**: ^{1}H NMR (400 MHz, DMSO-d_6), δ: 11.59 (br.s, 1H), 9.25 (s, 1H), 8.01 (m, 2H), 7.65 (m, 1H), 7.59 (m, 2H), 4.51 (br.m, 1H), 4.34 (br.m, 1H), 3.75 (br.m, 1H), 3.54 (br.m, 1H), 3.35 (br.m, 2H), 2.95 (s, 3H), 2.57 (s, 3H). ^{13}C NMR (DMSO-d_6, 75 MHz) δ 157.89, 156.89, 146.41, 143.27, 133.84, 133.17, 129.19, 126.22, 113.36, 105.44, 50.65, 49.83, 41.78, 29.02, 12.95. MS-ESI calculated for $C_{17}H_{18}N_4O_2S_2$ (M+H) 375, found m/z 375. LC–MS (UV-254) purity: 98%.
23. Synthesis of 3-(4-Fluorophenylsulfonyl)-7-methyl-2-(methylthio)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidine hydrochloride **3** and 3-(4-fluorophenylsulfonyl)-7-methyl-2-(methylthio)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidine hydrochloride **9**. Reactions were performed similar to those providing **1** and **7** but starting with 4-(4-fluorophenylsulfonyl)-3-(methylthio)-1*H*-pyrazole-5-amine **21** and sodium enolate of 1-Boc-4-oxopiperidine-3-carbaldehyde **14**. Compound **3**: ^{1}H NMR (400 MHz, DMSO-d_6), δ: 11.69 (br.s, 1H), 8.69 (s, 1H), 8.07 (m, 2H), 7.43 (m, 2H), 4.60 (br.m, 1H), 4.38 (br.m, 1H), 3.75 (br.m, 1H), 3.47 (br.s, 2H), 3.29 (br.m, 1H), 2.94 (s, 3H), 2.61 (s, 3H). MS-ESI calculated for $C_{17}H_{17}FN_4O_2S_2$ (M+H) 393, found m/z 393. LC–MS (UV-254) purity: 98%. Compound **9**: ^{1}H NMR (400 MHz, DMSO-d_6), δ: 11.59 (br.s, 1H), 9.26 (s, 1H), 8.07 (m, 2H), 7.44 (m, 2H), 4.52 (br.m, 1H), 4.34 (br.m, 1H), 3.75 (br.m, 1H), 3.53 (br.m, 1H), 3.40 (br.s, 2H), 2.95 (s, 3H), 2.57 (s, 3H). MS-ESI calculated for $C_{17}H_{17}FN_4O_2S_2$ (M+H) 393, found m/z 393. LC-MS (UV-254) purity: 98%.
24. Synthesis of *N*-methyl-3-(phenylsulfonyl)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidin-2-amine **4**, *N*-methyl-3-(phenylsulfonyl)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidin-2-amine **10**, *N*,7-dimethyl-3-(phenylsulfonyl)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidin-2-amine **5**, and *N*,7-dimethyl-3-(phenylsulfonyl)-5,6,7,8-tetrahydropyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidin-2-amine **11**. Reactions were performed similar to those providing **1** and **7**, but starting with N^3-methyl-4-(phenylsulfonyl)-1*H*-pyrazole-3,5-diamine **26** and sodium enolate of 1-Boc-4-oxopiperidine-3-carbaldehyde **14**. Compound **4**: ^{1}H NMR (DMSO-d_6): 9.81 (br.s, 2H); 8.45 (s, 1H); 7.98 (d, J = 8.0 Hz, 2H); 7.45–7.63 (m, 3H); 6.53 (br.s, 1H); 4.28 (s, 2H); 3.45 (t, J = 6.1 Hz, 2H); 3.22 (t, J = 6.6 Hz, 2H); 2.90 (s, 3H). ^{13}C NMR (DMSO-d_6, 75 MHz) δ 157.79, 149.35, 146.20, 143.57, 140.72, 132.90, 129.17, 125.58, 111.79, 91.24, 40.04, 38.97, 29.01, 21.08. HRMS calculated for $C_{16}H_{17}N_5O_2S$ (M+H) 344.11812, found 344.1179. MS-ESI calculated for $C_{16}H_{17}N_5O_2S$ (M+H) 344, found m/z 344. LC–MS (UV-254) purity: 99%. Compound **10**: ^{1}H NMR (DMSO-d_6): 9.68 (br.s, 2H); 8.95 (s, 1H); 7.98 (d, J = 8.0 Hz, 2H); 7.47–7.63 (m, 3H); 6.49 (br.s, 1H); 4.22 (s, 2H); 3.45 (t, J = 6.1 Hz, 2H); 3.13 (t, J = 6.6 Hz, 2H); 2.89 (s, 3H). ^{13}C NMR (DMSO-d_6, 75 MHz) δ 158,35, 156,07, 145,98, 143,61, 133,23, 132,87, 129,15, 125,61, 112,07, 89,73, 41,07, 40,20, 28,97, 28,53. HRMS calcd for $C_{16}H_{17}N_5O_2S$ (M+H) 344.11812, found 344.1183. MS-ESI calculated for $C_{16}H_{17}N_5O_2S$ (M+H) 344, found m/z 344. LC–MS (UV-254) purity: 98%. Compound **5**: ^{1}H NMR (400 MHz, DMSO-d_6), δ: 11.73 (br.s, 1H), 8.44 (s, 1H), 8.00 (m, 2H), 7.61 (m, 1H), 7.56 (m, 2H), 6.54 (q, J = 4.8 Hz, 1H), 4.37 (br.m, 2H), 3.55 (br.m, 2H), 3.36 (br. m, 2H), 2.94 (d, J = 4.8 Hz, 3H), 2.89 (s, 3H). ^{13}C NMR (D_2O, 75 MHz) δ 158.08, 148.89, 146.34, 141.33, 140.26, 133.28, 128.94, 124.81, 110.50, 89.81, 50.10, 48.97, 42.02, 28.14, 21.24. ^{13}C NMR (DMSO-d_6, 75 MHz) δ 157.99 (2-C), 149.25 (5-C), 146.48 (3a-C), 143.69 (*ipso*-Ph), 140.20 (9a-C), 133.05 (и-Ph), 129.29 (м-Ph), 125.74 (*o*-Ph), 111.11 (5a-C), 91.53 (3-C), 49.47 (6-C), 48.35 (8-C), 41.30 (2-$NHCH_3$), 29.14 (7-NCH_3), 21.35 (9-C). HRMS calcd for $C_{17}H_{19}N_5O_2S$ (M+H) 358.13377, found 358.1336. MS-ESI calcd for $C_{17}H_{19}N_5O_2S$ (M+H) 358, found m/z 358. LC–MS (UV-254) purity: 99%. Compound **11**: ^{1}H NMR (400 MHz, DMSO-d_6), δ: 11.60 (br.s, 1H); 8.97 (s, 1H); 8.00 (d, J = 8.0 Hz, 2H); 7.50–7.62 (m, 3H); 6.51 (br.q, J = 4.7 Hz, 1H); 4.35–4.49 (br.m, 1H); 4.16–4.30 (br.m, 1H); 3.62–3.76 (br.m, 1H); 3.40–3.52 (br.m, 1H); 2.90 (s, 3H); 2.86 (d, J = 4.7 Hz, 3H). ^{13}C NMR (DMSO-d_6, 75 MHz) δ 158.58, 155.39, 146.28, 143.72, 133.33, 132.99, 129.28, 125.76, 111.62, 89.96, 50.39, 49.69, 41.56, 29.11, 28.67. HRMS calcd for $C_{17}H_{19}N_5O_2S$ (M+H) 358.13377, found 358.1335. MS-ESI calculated for $C_{17}H_{19}N_5O_2S$ (M+H) 358, found m/z 358. LC–MS (UV-254) purity: 98%.
25. Synthesis of *N*,5,7-trimethyl-3-(phenylsulfonyl)-6,7,8,9-tetrahydropyrazolo[1,5-*a*]pyrido[3,4-*e*]pyrimidine-2-amine **6** and *N*,7,9-trimethyl-3-(phenylsulfonyl)-5,6,7,8-tetrahydro-pyrazolo[1,5-*a*]pyrido[4,3-*d*]pyrimidine-2-amine **12**. Reactions were performed similar to those providing **1** and **7**, but starting with N^3-methyl-4-(phenylsulfonyl)-1*H*-pyrazole-3,5-diamine **26** and 3-acetyl-1-Boc-4-oxopiperidine **29**. Compound **6**: ^{1}H NMR (400 MHz, $CDCl_3$), δ: 8.12 (d, J = 8.4 Hz, 2H); 7.40–7.50 (m, 3H); 5.96 (br. q, J = 5.1 Hz); 3.44 (c, 2H); 3.11 (t, J = 6.0 Hz, 2H); 3.01 (d, J = 5.1 Hz, 3H); 2.76 (d, J = 6.0 Hz, 2H); 2.51 (s, 3H); 2.46 (c, 3H). MS-ESI calculated for $C_{18}H_{21}N_5O_2S$ (M+H) 372, found m/z 372. LC–MS (UV-254) purity: 98%.
26. *Curve fitting and statistical analysis:* The concentration curves were fitted with Prism 5 (GraphPad, CA) using built-in 4-parametric equation to calculate IC_{50} values. All experiments were performed in duplicate. Standard deviations (SD) were calculated with Prism built-in statistical package. K_i values for functional 5-HT_6 receptor inhibition assays were calculated using Cheng-Prusoff's[34] modified equation, $K_i = IC_{50}/(1 + [Ag]/EC_{50})$. Where IC_{50} is the concentration of antagonist causing 50% inhibition of serotonin-induced cell response; [Ag] is a concentration of serotonin (10 nM), at which inhibition was measured and EC_{50} is the serotonin concentration causing 50% stimulation of the cell response, measured simultaneously with the test compounds on the same plates. The mean EC_{50} value for serotonin-induced cAMP production in 5-HT_6R-HEK cells was 1.91 ± 0.13 nM as determined from 4 independent experiments with three to five repeats (separate plates), each in quadruplicates.
27. *Competitive radioligand binding with 5-HT_6 receptor:* The assays were performed by Ricerca Biosciences in accordance with their internal protocols. In short, the membranes of HeLa cells expressing human recombinant 5-HT_6R were used to determine competitive displacement of the radio-labeled [^{3}H]lysergic acid diethylamide with the tested compounds. The membrane samples were incubated with the mixture of the label (1.5 nM) and a compound at specified concentrations for 120 min at 37 °C in the buffer consisting of (in mM): Tris–HCl (50, pH 7.4), NaCl (150), ascorbic acid (2), BSA (0.001%). After the incubation, the samples were vacuum-filtered through Whatman® grade GF/F glass microfiber filters with subsequent 3× wash with cold media. For determination of non-specific binding, 5 μM serotonin was added into control wells.
28. *Determination of 5-HT6 receptor functional activity:* The 5-HT6R was sub-cloned into T-Rex system (Invitrogen, Carlsbad, CA) and expressed into HEK (5-HT6R-HEK) cells. The cells were grown in DMEM supplemented with 10% FBS, 1%AAS, blasticidine S, and zeocin (all from Invitrogen, Carlsbad, CA) in a T-175 cell culture flask. T-Rex/5-HT6 receptor expression was activated by addition of tetracycline (1 μg/mL), as recommended by the T-Rex system manufacturer (Invitrogen, Carlsbad, CA), a day before the experiments. On the day of the experiment, the cells were harvested from the flask using 6 mM EDTA/HBSS solution, gently triturated by passing through a pipette tip several times to break down cell aggregates, washed with Serum Free Medium, and counted. The cells were re-suspended to 0.67×10^6 cells/mL in SB2 buffer, HBSS, supplemented with 5 mM HEPES, pH 7.4, 0.05% BSA, and 1 mM IBMX (Sigma–Aldrich, St. Louis, MO) containing Alexa Fluor 647-anti cAMP antibody (from LANCE cAMP 384 kit, PerkinElmer, Waltham, MA). 6 μL (~4000 cells/well) aliquots were then transferred into 384-well assay plates (PerkinElmer White OptiPlates). The test compounds at different concentrations were premixed with serotonin hydrochloride (Sigma, MO) and added to the cells (final serotonin concentration-10 nM, final DMSO concentration-0.32%, final IBMX concentration-500 mM). Each assay plate contained serotonin (to define 100% activation signal) and cAMP standard calibration curves (to transform fluorescent signal into cAMP concentrations). After 2 h of incubation with

the mixture of compound/serotonin, cells were treated as described in the cAMP LANCE assay kit protocol (PerkinElmer, Waltham, MA). The LANCE signal was measured using multimode plate reader VICTOR 3 (PerkinElmer, Waltham, MA) with built-in settings for the LANCE detection.

29. *Determination of 5-HT$_{2B}$ receptor functional activity:* The 5-HT$_{2B}$R was sub-cloned into T-Rex system (Invitrogen, Carlsbad, CA) and expressed into HEK (5-HT$_{2B}$R-HEK) cells. The cells were grown in T-175 flasks at 37 °C in atmosphere of air/CO_2 (95:5) in DMEM (Sigma, MO) supplemented with 10% FBS, 1%AAS, blasticidine S and phleomycin (Invitrogen, Carlsbad, CA). The T-Rex/5-HT$_{2B}$ receptor expression was activated by addition of tetracycline, as recommended by the manufacturer, a day before the experiments. The cells were dissociated with TrypLE™ Express (Invitrogen, Carlsbad, CA), washed twice with PBS and loaded with 4 mM calcium-sensitive dye, Fura-2AM (Invitrogen, Carlsbad, CA) at room temperature for 30 min. After loading, the cells were washed once with PBS, re-suspended into protein-free Hybridoma media without phenol red (Sigma, St. Louis, MO) and allowed to incubate for an additional 30 min with gentle shaking at room temperature. All loading procedures were performed in the dark. The loaded cells were washed twice with PBS and re-suspended into the Hybridoma media at a cell density of 3–4 × 10^6 cells/mL for subsequent experiments. Fura-2 ratiometric fluorescence signal was registered at 510 nm upon alternate excitation at 340 nm and 380 nm using spectrofluorometer RF5301PC (Shimadzu, Columbia, MD). In a square (1 cm) optical cuvette with a magnetic stirring bar, 100 mL aliquots of the loaded cells were diluted into 2.4 mL buffer containing (mM): NaCl (145), KCl (5.4), $MgSO_4$ (0.8), $CaCl_2$ (1.8), HEPES (30), D-glucose (11.2). The fluorescence signal was allowed to stabilize for 20–30 s before addition of a test compound or vehicle to assess potential agonistic activity of the compounds, with subsequent addition of serotonin (2.5 mL, 10 mM) to assess the compounds blocking activity.
30. *Determination of hERG channel functional activity:* The experiments were performed by AVIVA Biosciences (San Diego, CA). Human recombinant hERG channel was stably expressed in HEK-297 cells. Intracellular solution consisted of 130 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 10 mM HEPES, 5 mM Na-ATP, pH 7.2. Extracellular solution consisted of 137 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 11 mM dextrose, 10 mM HEPES, pH 7.4. Voltage clamp measurements were performed at 25 °C using PatchXpress 7000A (Molecular Devices, Sunnyvale, CA). hERG channels were activated by 2 s pulses to +20 mV from a holding potential of −80 mV, and peak tail currents were recorded upon re-polarization to −50 mV. This voltage-clamp pulse protocol was performed continuously during the experiment (vehicle control, test compound, washout, and positive control additions). An inter pulse interval of 15 s allowed recovery from any residual inactivation. Test compounds were incubated with cells until the current reached a steady state level (3–8 min). After the final test compound concentration was tested, test compound was washed out with continuous perfusion of extracellular solution for 3 min, followed by application of positive control (10 μM cisapride). Data were analyzed using DataXpress software. Percent of control values were calculated on the basis of current peak at each compound concentration relative to maximal tail current in the presence of vehicle control.
31. *Target specificity.* The assays were performed by Ricerca Biosciences in accordance with their internally optimized protocols for each target. The radioligand displacement was assessed in the presence of 1 μM of the tested compounds in duplicates.
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