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2-Substituted 5,6-dimethyl-3-phenylsulfonyl-pyrazolo[1,5-*a*]pyrimidines: New series of highly potent and specific serotonin 5-HT$_6$ receptor antagonists

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ABSTRACT

Syntheses, biological evaluation, and structure–activity relationships for a series of novel 2-substituted 3-benzenesulfonyl-5,6-dimethyl-pyrazolo[1,5-*a*]pyrimidines are disclosed. In spite of a wide, four orders of magnitude, SAR range (K_i varied from 260 pM to 2.96 μM), no significant correlation of 5-HT$_6$R antagonistic potency was observed with major physiochemical characteristics, such as molecular weight, surface polar area, cLogP, or number of rotatable bonds. Statistically significant trend was only observed for size of substitute group, which was not enough to explain the deep SAR trend. Besides with the substitute group size, another factor that presumably plays a role in defining the compound potencies is a relative position of the heterocycle and sulfophenyl moieties. Among all synthesized derivatives, (3-benzenesulfonyl-5,7-dimethyl-pyrazolo[1,5-*a*]pyrimidin-2-yl)-methyl-amine **18** is the most potent (K_i = 260 pM) and extremely selective, 5000 to >50,000-fold relative to 55 therapeutic targets, antagonist of the 5-HT$_6$ receptor.

1. Introduction

Serotonin 5-HT$_6$ receptors (5-HT$_6$R) have attracted considerable interest [1] due to high affinity to a wide range of psychiatric drugs and its specific distribution in the brain. Development of highly potent and selective antagonists would help clarifying the role of the 5-HT$_6$R in brain functions as well as in etiology of the central neural system (CNS) diseases. At present, there are only few selective antagonists that have been developed and which have progressed to Phase I and Phase II clinical trials as potential drugs for treatment of various CNS diseases [2–7].

Recently, we have reported [8,9] on the synthesis and biological activity of novel 5-HT$_6$R antagonists, 3-phenylsulfonyl-cycloalkano [e and d]pyrazolo[1,5-*a*]pyrimidines I and II (Fig. 1), which posses both the picomolar range affinity and excellent selectivity profiles.

Unlike Ro-65-7674, the new highly potent and selective 5-HT$_6$R antagonists **I** and **II** either did not possess a positive ionizable group (PI) at all [8] or the PI was located in R^1 position [9]. Though in accordance with the pharmacophore model suggested based on analyses of 45 structurally diverse 5-HT$_6$R antagonists, PI was considered as an essential recognition group [10] our data indicated that it might not be necessary determinant of the high antagonist affinity. We have suggested [9] that R^1 substitute group likely served to constrain the phenylsulfo moiety in an appropriate conformation that affected the binding affinity.

2. Chemistry

In this paper, we attempted to evaluate the role of the R^1 substituent group in defining the 5-HT$_6$R antagonistic activity compounds. We have synthesized a series of new substituted 5,7-dimethyl-3-phenylsulfonyl-pyrazolo [1,5-*a*] pyrimidines (DMPSPP) **13**–**23** (Scheme 1) and assessed their serotonin 5-HT$_6$R activity. The DMPSPPs **13**–**23** were synthesized by cyclocondensation of 3-aminopyrazoles **1**–**11** with acetylacetone **12** in high yields (Scheme 1).

In addition, 2-{[5,7-dimethyl-3-(phenylsulfonyl)pyrazolo[1,5-*a*] pyrimidin-2-yl]oxy}-*N*,*N*-dimethylethanamine **24** (Scheme 2) was synthesized in 60% yield by one-pot procedure starting from alcohol **15** that reacted with trifluoromethylsulfonic acid anhydride

Abbreviations: 5-HT$_6$R, serotonin 5-HT$_6$ receptor; 5-HT$_{2B}$R, serotonin 5-HT$_{2B}$ receptor; DMPSPP, 5,7-dimethyl-3-phenylsulfonyl-pyrazolo [1,5-*a*] pyrimidine; TPSA, topological polar surface area; CNS, central neural system; PI, positive ionizable group; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; IBMX, 1-methyl-3-isobutylxanthine.

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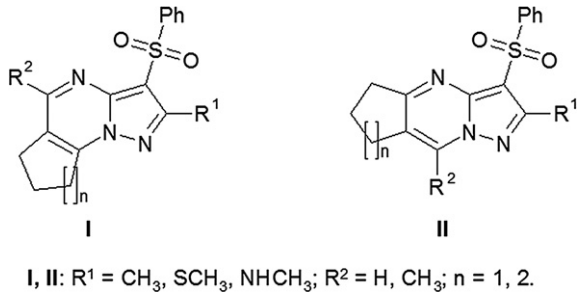


Fig. 1. 3-Phenylsulfonyl-cycloalkano[*e* and *d*]pyrazolo[1,5-*a*]pyrimidines **I**, **II**.

in $CHCl_3$ for 1 h at a temperature between −5 °C and 0 °C followed by treatment of intermediate triflate with excess of 40% aq. Me_2NH and stirring the resulted mixture at 0–20 °C for 2 h.

Cleavage of BOC group in compound **23** by treatment with 6 M solution of AcCl in ethanol at room temperature afforded 2-(piperazin-1-yl)-DMPSPP **25** in 95% yield (Scheme 3).

2-Methylsulfinyl-DMPSPP **26** was synthesized in the 87% yield by oxidation of 2-methythio-derivative **16** with H_2O_2 in acetic acid at 80 °C (Scheme 4).

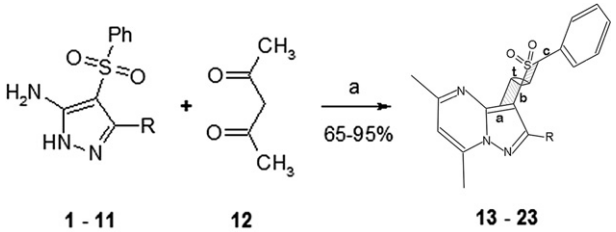
Starting 3-aminopyrazoles **1**–**6** were prepared according to previously described procedures [9,11,12]. Diaminopyrazoles **7**–**11** were synthesized in high yields by reaction of 3,3-bis(methylthio)-2-(phenylsulfonyl)acrylonitrile **27** [13] with corresponding amines (Scheme 5) followed by cyclocondensation of intermediate 3-(methylamino)-3-(methylthio)-2-(phenylsulfonyl)acrylonitriles **28**–**32** with hydrazine hydrate.

The structures of synthesized DMPSPPs were confirmed with LC-MS, HRMS, and NMR spectra. According to the content of molecular ions in LC-MS and HRMS spectra, all synthesized compounds had a purity of 98% and higher. The NMR spectra of the compounds were in a good agreement with their structures.

3. Results and discussion

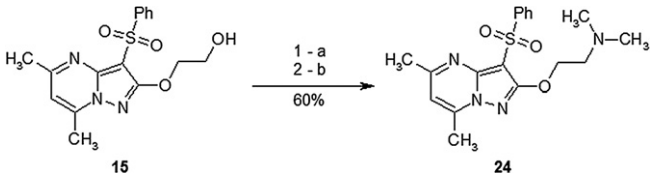
Potencies of the synthesized 2-substituted DMPSPPs to block 5-HT_6R were assessed in a cell-based assay by their ability to diminish serotonin-induced cAMP production in HEK-293 cells stably expressing human recombinant 5-HT_6R (Table 1). For a few of the compounds, we have also measured affinity to the receptor based on competition with the radioactively labeled 5-HT_6 receptor ligand, [^{3}H] lysergic acid diethylamide. The data are summarized in Table 1.

As one can see, the nature of the 2-substituted group attached to the DMPSPP heterocycle, greatly affects ability of the compounds to

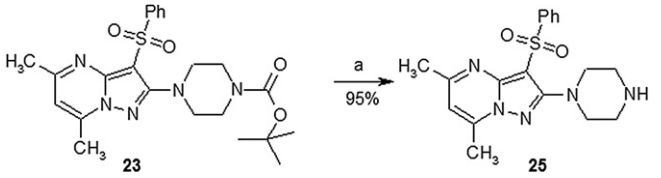


1, **13**: R = H,
2, **14**: R = CH_3,
3, **15**: R = OCH_2CH_2OH,
4, **16**: R = SCH_3,
5, **17**: R = SC_2H_5,
6, **18**: R = $NHCH_3$,
7, **19**: R = $NHCH_2CH_2N(CH_3)_2$,
8, **20**: R = $NHCH_2CH_2CH_2N(CH_3)_2$,
9, **21**: R = $N(CH_3)_2$,
10, **22**: R = $CH_3NCH_2CH_2N(CH_3)_2$,
11, **23**: R = 1-Boc-piperazin-4-yl

Scheme 1. Synthesis of 2-substituted 5,7-dimethyl-3-phenylsulfonyl-pyrazolo[1,5-*a*] pyrimidines **13**–**23**. Reagents and conditions: (a) AcOH, reflux, 3 h *t* – torsion angle formed by bonds *ab* and *bc*.



Scheme 2. Synthesis of [2-(5,7-dimethyl-3-phenylsulfonyl-pyrazolo[1,5-*a*]pyrimidin-2-yloxy)-ethyl]-dimethyl-amine **24**. Reagents and conditions: (a) Tf_2O, $CHCl_3$, -5–0 °C, 1 h; (b) 40% aq. $(CH_3)_2NH$, 0–20 °C, 2 h.

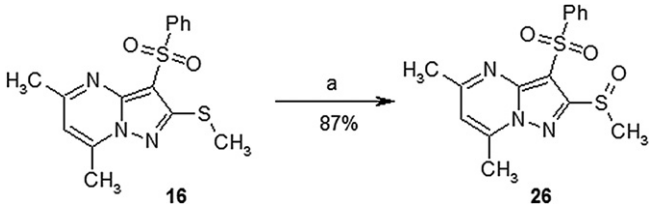


Scheme 3. Synthesis of 5,7-dimethyl-3-phenylsulfonyl-2-piperazin-1-yl-pyrazolo[1,5-*a*]pyrimidine **25**. Reagents and conditions: (a) 6 N AcCl in EtOH, ambient temp.

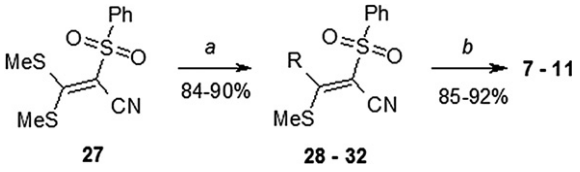
antagonize 5-HT_6R. K_i values vary in a broad, four orders of magnitude, range of potencies, from K_i = 0.26 nM (**18**) to K_i = 2.96 μM (**26**). When relative potencies, K(H), are considered, the negative K(H) values reflect increase, while positive K(H) values reflect decrease in the substituted compound potencies relative to the unsubstituted DMPSPP **13**.

No significant correlation was evident between the 5-HT_6R relative inhibition potency, K(H) and either LogP, polar surface area, or volume of the molecules (Fig. 2A–C). When studying a relationship between the compound potencies to block 5-HT_6 receptor-activated cell response and the same physiochemical parameters calculated for the R-substitute groups, the group volume seemed to have statistically significant effect on the compound potency (Fig. 2F, P = 0.01). Neither the group hydrophobicity, cLogP (Fig. 2D), nor its polar surface area, TPSA (Fig. 2E), significantly affected the potency. Molecule flexibility (function of number of rotatable bonds) was not a factor determining their potency either. For example, while both the 16 and 26 have equal number of rotatable bonds, their respective 2-substitute groups affected the compound potency relative to the 13 (R = H) in opposite directions. Thus 16 exhibited more than 450-fold higher potency over 13 while 26 was 5-fold less potent. Compound 13, which has 2 rotatable bonds, showed K_i value similar to those of compounds 22 and 20, which have six and seven rotatable bonds, correspondingly.

Energy minimization performed with DS ViewerPro 6.0 (Accelrys, San Diego CA) [15], revealed that the 2-substituent group affects both the value and directionality of the molecule dipole as well as angle at which the sulfophenyl moiety is positioned relative to the heterocycle plane. Neither molecule polarizabilities nor dipole moments seem to correlate with the 5-HT_6 receptor antagonistic activity (data not shown). The DMPSPPs with minimized 3D conformations could be separated into two groups, those with potencies higher than that of **13** are on the left side and those with lower potencies are on the right side from the DMPSPP **13** (Fig. 3). It



Scheme 4. Synthesis of 5,7-dimethyl-2-methylsulfinyl-3-phenylsulfonyl-pyrazolo[1,5-*a*]pyrimidine **26**. Reagents and conditions: (a) H_2O_2, AcOH, 80 °C, 7 h.



Scheme 5. Preparation of substituted 3,5-diaminopyrazoles **7**–**11**. Reagents and conditions: (a) $Me_2NCH_2CH_2NH_2$, $Me_2NCH_2CH_2CH_2NH_2$, Me_2NH, $Me_2NCH_2CH_2NHMe$ or 1-Boc-piperazine; *i*-PrOH, reflux for 1 h then ambient temp overnight; (b) $N_2H_4 \cdot H_2O$, *i*-PrOH, reflux, 0.5 h.

Table 1
SAR of the 2-substituted DMPSPPs. Compound potencies to block serotonin-induced cAMP production in HEK-293 cells expressing recombinant human 5-HT_6R and several physiochemical parameters of the molecules.

Cpd	*R*	K_i, nM		K(H)[c]
		Functional[a]	Binding[b]	
18	MeNH	0.26	0.2	−3.33
16	MeS	1.20	2.0	−2.67
17	EtS	1.91		−2.47
21	Me_2N	14.5		−1.59
14	Me	88.8	68.4	−0.80
15	$HOCH_2CH_2O$	283	22.2	−0.30
22	$Me_2NCH_2CH_2NMe$	489		−0.06
13	H	561	237.0	0.00
20	$Me_2NCH_2CH_2CH_2NH$	764		+0.13
25	Piperazin-1-yl	1380		+0.39
24	$Me_2NCH_2CH_2O$	2200		+0.59
19	$Me_2NCH_2CH_2NH$	2600		+0.67
26	MeS(O)	2960		+0.72

[a] Potency to antagonize serotonin-induced cAMP production in HEK cell heterologously expressing 5-HT_6R.
[b] Affinity by competitive displacement of [^{3}H]LSD from its complex with 5-HT_6R.
[c] $K(H) = \mathrm{Log}(K_i^{R(i)}/K_i^{R(H)})$ reflects change in potency (functional assay) of the *R*(*i*)-substituted compounds relative to the DMPSPP **13** with *R* = H.

is interesting to note that for the compounds with R-groups supporting higher than **13** potencies, negative K(H) values, the torsion angles, *t* (Scheme 1), between the heterocycle plane and the plane formed by two bonds, *b* and *c*, connecting serum with the pyrozol and phenyl rings, respectively, are between +0.31° and −52.5°. For the compounds with lower relative to **13** potencies, positive K(H) values, excluding **19**, the characteristic torsion angles substantially deviate from those of the first group (Fig. 4).

Hydrogen replacement in compound **13** with methyl group (compound **14**) led to 6.3-fold increase in the potency, whereas its replacement with methylamino group (compound **18**) led to more than three order of magnitude increase in the potency (K(H) = −3.33). However, substitution with dimethylamino group (**21**) was substantially less effective than that with methylamino group (**18**) in increasing the compound potency (K(H) = −1.59 and −3.33, correspondingly). Almost 2 orders of magnitude difference in the 5-HT_6R antagonistic potencies between the DMPSPPs **21** and **18** is most probably due to the formation of intramolecular hydrogen bond in **18** (Fig. 3), which restricts molecular flexibility and secures the molecule in a conformation that better fits the receptor binding site. Notably, with the bulky R-groups, formation of the intramolecular H-bond does not improve the compound potency. For example, though such bond forms in both **19** and **20** (Fig. 3), their potencies are 3.4-fold apart (K_i values are 2.6 μM and 0.76 μM, respectively) and the potency of **22** (K_i = 0.49 μM), which does not have such bond, is 5.3 times as high as that of its corresponding homolog **19**.

Alkylthio-derivatives **16** and **17** were 4-fold–7-fold less potent than the **18**. Comparable potencies of the DMPSPPs **16** and **17** imply some limited tolerance of the 5-HT_6R binding site to the size of the R-group. However, hydrogen substitution with more bulky 2-hydroxyethoxy group (**15**), *N*-(2-dimethylamino-ethyl)-*N*-methyl-amino group (**22**) or (3-dimethylamino-propyl)-amino group (**20**) practically led to no change in the compound potency (K(H) = −0.3, −0.06, and +0.13, correspondingly). This seemingly controversial behavior could be due to a presence of a rather narrow recognition pocket in the receptor, which accommodates R-groups of molecules **18**, **16**, **17**, **21**, and **14** and orients the molecule to form a tighter binding. Absence of the 2-substitute group in **13** or too bulky substitutes in **15**, **22**, and **20**, do not fit the pocket and thus prevent this "orientation constriction". The relatively low potency of DMPSPPs **26**, K(H) = +0.72, seems to indicate that the receptor small binding pocket does not tolerate electron-withdrawing entity in the group. Alternative explanation could be that this group

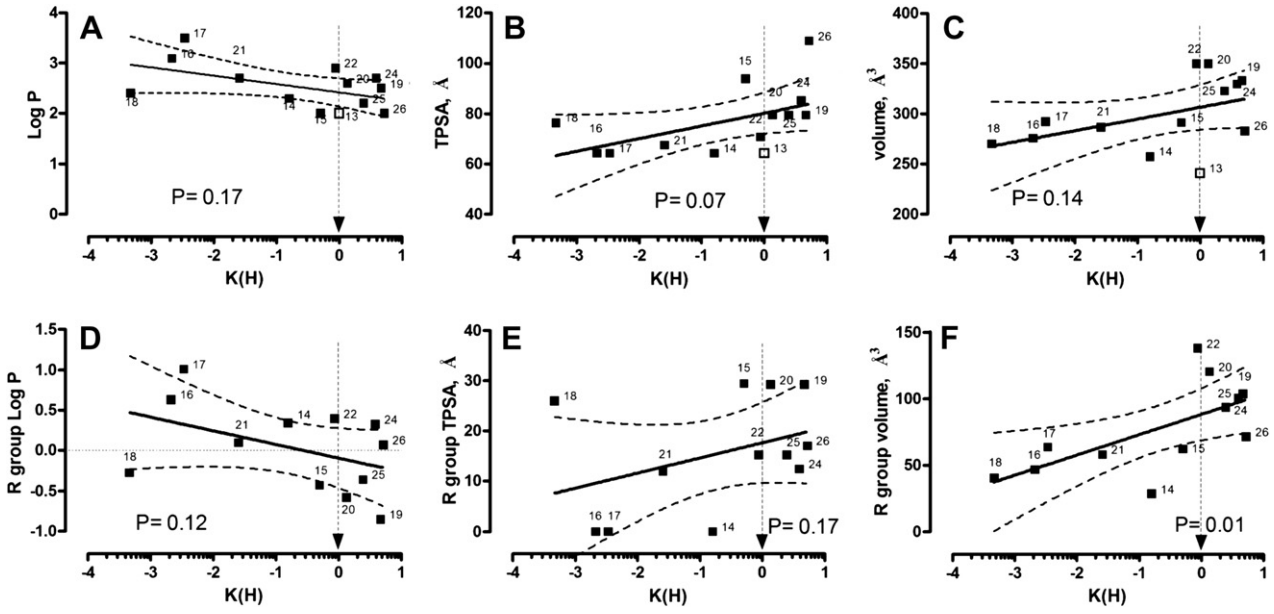


Fig. 2. Correlation between relative potencies of the compounds to block 5-HT_6 receptors in cell-based assay and their physiochemical parameters. cLogP, TPSA, and molecule volume values were calculated using MolInspiration on-line calculator [14] for whole molecules (A, B, and C) and for R-substitute groups (D, E, and F). Dotted lines represent 95% confidence intervals. Dotted arrows show position of **13** on the K(H) scale.

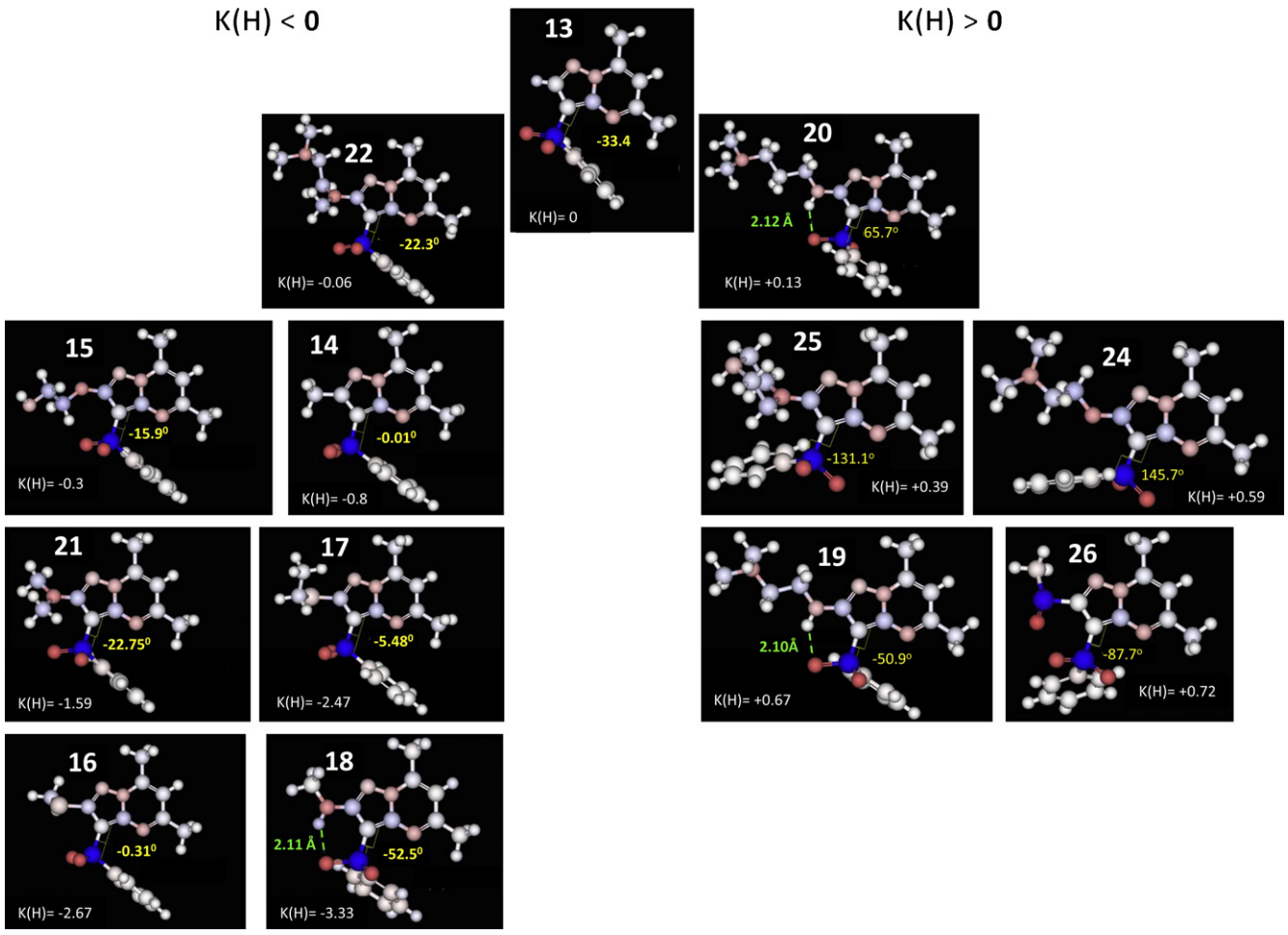


Fig. 3. Free energy minimized conformations of the 5-HT$_6$R antagonists. Atoms are colored in accordance with their partial charges (red/reddish − negative, blue/bluish-positive). Energy minimization was performed using DS ViewerPro 6.0. Top center panel shows unsubstituted molecule **13**. Compounds with increased potencies relative to the compound **13** (negative K(H) values) are grouped on the left side and those with decreased potencies (positive K(H) values) on the right side. $K(H) = \mathrm{Log}(K_i^{R(i)}/K_i^{R(H)})$; where K_i is a functional inhibition constant, superscript R denotes specific substitute group (i), and R_H reflects R = H (compound **13**). For each compound, the conformation with the lowest local free energy minimum is shown. The torsion angles between the heterocyle plane and the plane formed by two bonds connecting serum with the pyrozol and phenyl rings, respectively, are shown in yellow and in green, the H-bond distance is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

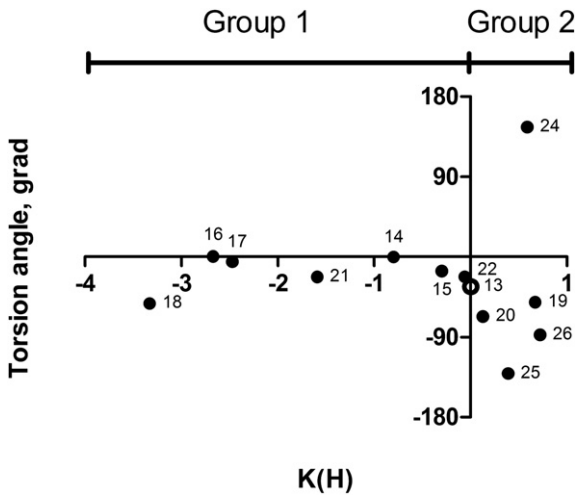


Fig. 4. Relation between relative 5-HT$_6$R blocking potency, K(H), of the R-substituted compounds and the torsion angles between the heterocycle plane and the plane formed by two bonds connecting serum with the pyrozol and phenyl rings.

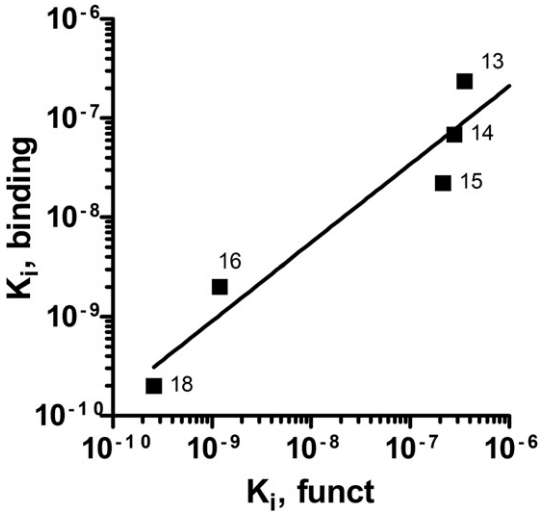


Fig. 5. Correlation between DMPSPP potencies (K_i, functional) to inhibit 5-HT$_6$R-induced cell response and their affinities (K_i, binding) measured in a competitive radioligand binding assay.

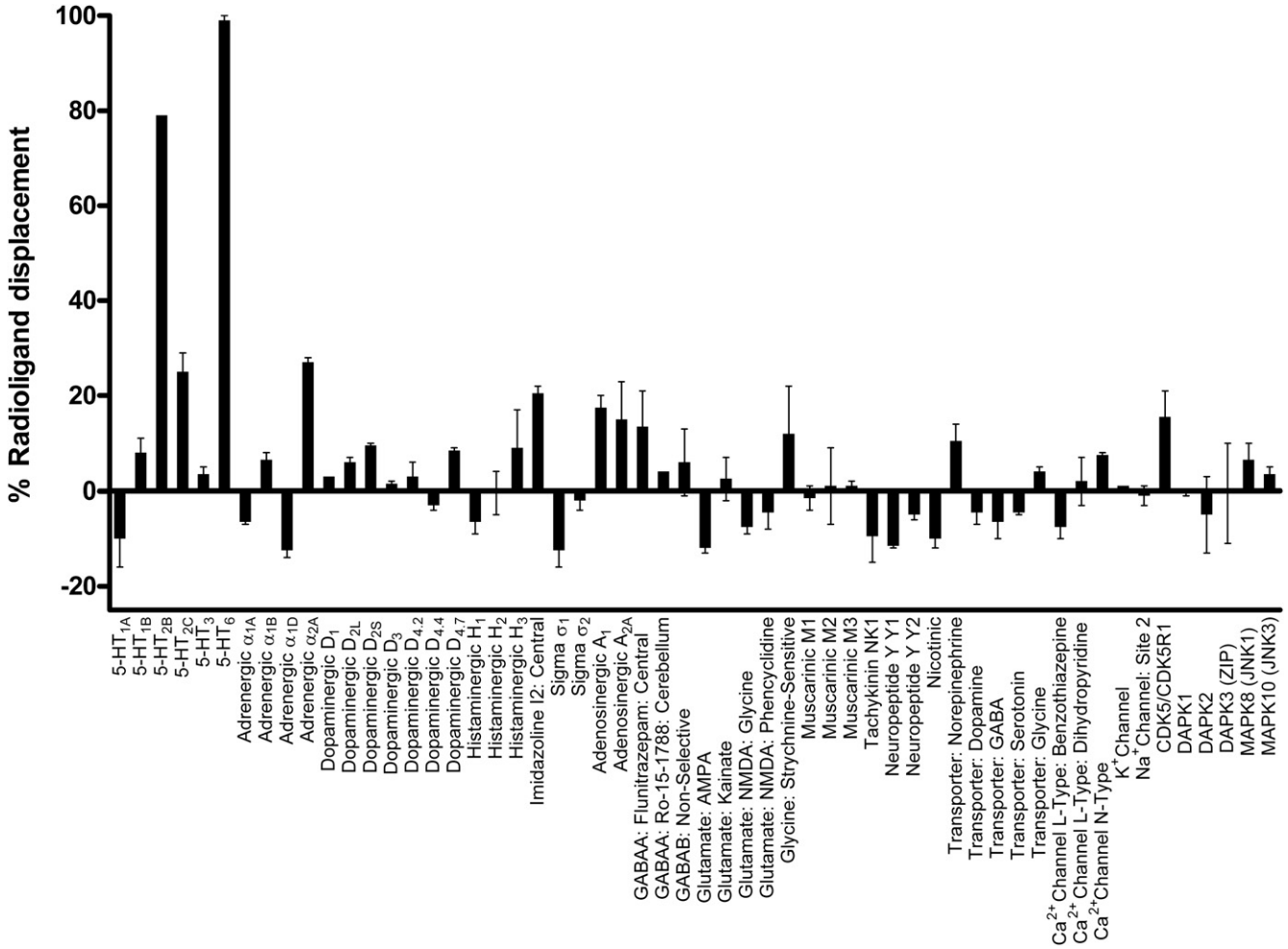


Fig. 6. Target-specific profile determined for DMPSPP **18** (10 μM) using competitive radioligand binding assays. Measurements were performed in duplicates. Mean ± SD values are presented.

causes the torsion angle, *t* (see Scheme 1), of the sulfophenyl moiety (−87.75°) that is incompatible with proper accommodation of this part of the molecule by the binding site of the receptor. Indeed the other two low potency compounds **24** and **25** both have torsion angles, +145.71° and −131.09°, respectively, which substantially deviate from those characteristic of compounds with the higher potencies (Figs. 3 and 4). It seems plausible to suggest that orientation of the sulfophenyl moiety relative to the core heterocyclic plane of the molecules acts concurrently with the presence of rather small 2-substitute group (presumably with some electron-donor characteristics) to define their 5-HT_6 receptor antagonistic potency. Further work to determine crystal structures of these molecules is underway to confirm this preliminary conclusion.

The antagonistic potency values of the compounds were very similar to and correlated well ($r = 0.96$) with their affinities measured in a competitive radioligand binding assay (Fig. 5). This allows one to conclude that the effect of the 2-substituted group on the compounds' functional potency to block serotonin-evoked

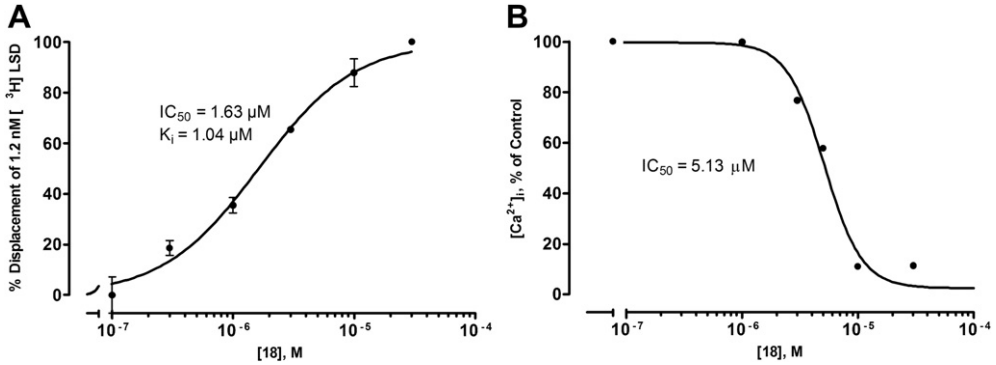


Fig. 7. The DMPSPP **18** concentration-dependent displacement of radio-labeled ligand (1.2 nM [^{3}H]LSD) from its complex with 5-HT_{2B}R (A) and blockage of 5 μM α-methyl serotonin-induced calcium ion mobilization in HEK cells heterologously expressing 5-HT_{2B}R (B). Shown are mean ± SD values of typical experiments performed in duplicates.

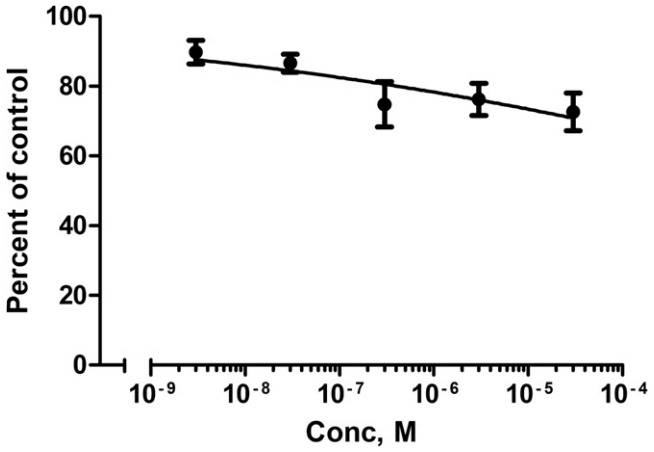


Fig. 8. The DMPSPP 18 is extremely week blocker of the hERG potassium channels. Patch clamp measurements were performed in triplicates (mean ± SD).

cAMP synthesis in 5-HT_6-HEK cells, directly relates to their binding affinities to the receptor.

We then studied a specificity profile of the most potent DMPSPP, **18**, using a panel of 56 therapeutic targets, including GPCRs, ion channels, and transporters. The interaction of **18** with the targets was assessed by its ability to compete with corresponding target-specific radio-labeled ligands (Fig. 6). Except for the 5-HT_6 and 5-HT_{2B} receptors, **18** does not practically bind to any other targets tested (the radioligand displacement at 10 μM is significantly lower than 50%). Except for 5-HT_{2B} receptors, this represents more than 50,000-fold specificity of **18** over all the other targets studied.

We have assessed the affinity of DMPSPP **18** to the 5-HT_{2B}R (Fig. 7A) and its potency to inhibit the receptor-induced intracellular calcium ion mobilization (Fig. 7B). The data shows that affinity of **18** to bind to 5-HT_{2B}R (K_i = 1.04 μM) is 5000 times as low as that for 5-HT_6R (Table 1). Potency to inhibit 5-HT_{2B}R-induced cell response (IC_{50} = 5.13 μM) in 5-HT_{2B}R-HEK cells was 1000-fold lower than that for 5-HT_6R-HEK cells (Table 1). No agonistic activity of **18** towards 5-HT_{2B}R was detected (data not shown). 5-HT_{2B} receptor is considered as a potential liability target as its agonists were linked to cardiac valvulopathy [16]. On the other hand, the antagonists of the receptor are considered as possible candidates for treatment of chronic heart diseases [17,18]. Fig. 6 manifests exceptionally high selectivity of **18** towards 5-HT_6R with practically no potential cardiac liability pertinent to activation of 5-HT_{2B} receptors (Fig. 7).

We then assessed if **18** could inhibit potassium channel, hERG (product of human analog of *Drosophila* ether-a-go-go gene). Blockage of this channel by a diverse group of drugs leads to a prolongation of QT interval in a cardiac electric cycle and a concomitant risk of sudden death [19]. In the patch clamp experiments (Fig. 8) performed for us by MDS Pharma (currently Ricerca), we have found that 18 showed a very weak antagonistic activity with IC_{50} > 30.0 μM, that was more than six orders of magnitude higher than the IC_{50} of the 5-HT_6R blockage with the DMPSPP 18.

4. Conclusion

We have described syntheses and biological evaluations of small-molecule DMPSPPs **13**–**26**, representing novel chemotype of the serotonin 5-HT_6R antagonists. The antagonistic potency dramatically depends on the nature of a substituent group in position 2 of the heterocycle with a SAR in the series having depth of 4 orders of magnitude. The potency does not correlate with either LogP or polar surface area of the molecules, though statistically significant correlation was observed with the volume of substitute group. The 2-substituents affect relative topology between the heterocycle plane and the sulfophenyl ring, which is presumably one of defining factors of the compound affinities. The affinity of most potent antagonist of the 5-HT_6R, 2-methylamino-DMPSPP **18**, is in a mid picomolar range (K_i = 0.2 nM) and the compound has shown remarkable specificity/selectivity characteristics on a panel consisting of 56 therapeutic targets. Based on the large "safety window" between its effect on 5-HT_6R (K_i = 260 pM) and that on 5-HT_{2B}R (IC_{50} = 5.13 μM) and hERG channel (IC_{50} > 30.0 μM), we conclude that **18** is a perspective molecule for further development as clinical candidate to treat associated with 5-HT_6R CNS diseases.

5. Experimental protocols

5.1. General

^{1}H and ^{13}C NMR spectra of the investigated compounds were recorded in DMSO-d_6 or $CDCl_3$, respectively, with spectrometer Bruker DPX-400 (400 MHz, 27 °C). HRMS spectra (ESI-TOF, positive mode) were obtained with a Waters Qt of API US instrument.

Purities of all synthesized compounds (more than 98%) were assessed by LC-MS spectra with Shimadzu HPLC instrument equipped with PE SCIEX API 150EX mass-, Alltech 2056 ELS-, and Shimadzu UV- (254 and 215 nm) detectors. Separation was performed with a Phenomenex Luna 3μ C18 (4.6 × 150 mm) column in a gradient flow of 5–95% acetonitrile in water (both with 0.05% TFA) over 12 min at 0.8 mL min^{-1}.

Free local minima energies of 3D conformations were calculated using DS ViewerPro 6.0 (Accelrys, San Diego CA) [19] using convergence criterion of 0.00001 and 50,000 to 200,000 iterations of energy minimizing. For each structure, energy minimization cycles were performed starting with different initial conformations.

Competitive radioligand displacement experiments were performed by MDS Pharma (currently Ricerca) in accordance with their internally developed protocols briefly described elsewhere [20].

5.2. Chemistry

5.2.1. 5,7-Dimethyl-3-phenylsulfonyl-pyrazolo[1,5-a]pyrimidines *13*–*23* (general procedure)

A mixture of aminopyrazole **1**–**11** (1 mmol), acetylacetone **12** (0.5 g, 5 mmol), and AcOH (2 mL) was heated under reflux for 3 h, the solvent was removed under reduced pressure, the residue was treated with i-PrOH, the obtained mixture was kept in ultrasonic bath for 0.5 h at 0 °C. Formed precipitate was separated by centrifugation, washed twice with fresh portions of cooled i-PrOH, hexane, and dried to provide 65–95% of colorless compound **13**–**23**.

5.2.1.1. 5,7-Dimethyl-3-phenylsulfonyl-pyrazolo[1,5-a]pyrimidine *13*.

^{1}H NMR (DMSO-d_6, 400 MHz), δ: 8.60 (s, 1H); 8.02–8.06 (m, 2H); 7.53–7.63 (m, 3H); 7.18 (q, J = 1 Hz, 1H); 2.66 (d, J = 1 Hz, 3H); 2.57 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.38, 147.43, 145.11, 144.40, 142.94, 133.13, 129.27, 126.56, 111.61, 109.15, 24.69, 16.54. MS-ESI m/z 288 (M + H). LC-MS (UV-254) purity: 98%.

5.2.1.2. 2,5,7-Triimethyl-phenylsulfonyl-pyrazolo[1,5-a]pyrimidine *14*.

^{1}H NMR (DMSO-d_6, 400 MHz), δ: 8.00–8.04 (m, 2H); 7.52–7.62 (m, 3H); 7.10 (s, 1H); 2.62 (s, 3H); 2.61 (s, 3H); 2.55 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 162.79, 153.30, 146.60, 146.50, 143.59, 132.99, 129.22, 126.29, 111.18, 105.86, 24.65, 16.48, 14.09. MS-ESI m/z 302 (M + H). LC-MS (UV-254) purity: 99%.

5.2.1.3. 2-{[5,7-Dimethyl-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidin-2-yl]oxy}ethanol ***15***. ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 7.97–8.01 (m, 2H); 7.50–7.61 (m, 3H); 7.05 (s, 1H); 4.91 (t, J = 5.3 Hz, 1H, exch. with D_2O); 4.36 (t, J = 4.9 Hz, 2H); 3.74 (m, 2H); 2.55 (s, 3H); 2.52 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 162.72, 162.67, 146.70, 146.43, 143.93, 132.82, 129.09, 126.24, 110.73, 92.91, 71.00, 59.19, 24.59, 16.53. MS-ESI m/z 348 (M + H). LC-MS (UV-254) purity: 98%.

5.2.1.4. 5,7-Dimethyl-2-(methylthio)-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidine ***16***. ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 8.21–8.23 (m, 2H), 7.45–7.54 (m, 3H), 6.67 (c, 1H), 2.68 (c, 3H), 2.64 (c, 3H), 2.62 (c, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 162.92, 154.74, 147.08, 146.33, 143.11, 133.05, 129.13, 125.94, 110.73, 104.76, 24.53, 16.26, 12.62. MS-ESI m/z 334 (M + H). LC-MS (UV-254) purity: 98.5%.

5.2.1.5. 5,7-Dimethyl-2-(ethylthio)-3-phenylsulfonyl-pyrazolo[1,5-a]pyrimidine ***17***. ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 8.01 (m, 2H), 7.60 (m, 3H), 7.11 (s, 1H), 3.18 (q, J = 7.6 Hz, 2H), 2.64 (s, 3H), 2.57 (s, 3H), 1.38 (t, J = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 162.49, 153.73, 146.56, 145.91, 142.79, 132.70, 128.78, 125.62, 110.41, 104.40, 24.18, 23.65, 15.92, 13.86. MS-ESI m/z 347 (M + H). LC-MS (UV-254) purity: 98%.

5.2.1.6. N,5,7-Trimethyl-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidin-2-amine ***18***. ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 8.14 (d, J = 8.5 Hz, 2H); 7.40–7.51 (m, 3H); 6.51 (s, 1H); 5.99 (br. q, J = 5.1 Hz, 1H); 3.02 (d, J = 5.1 Hz, 3H); 2.57 (s, 3H); 2.53 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz), δ: 161.2; 160.3; 158.2147.8; 145.2; 144.0; 132.3; 128.6; 128.4; 109.1; 29.0; 24.8; 17.0. MS-ESI m/z 317 (M + H). LC-MS (UV-254) purity: 99%.

5.2.1.7. N′-[5,7-Dimethyl-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidin-2-yl]-N,N-dimethylethane-1,2-diamine ***19***·*HCl*. ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 10.07 (br.s, 1H); 8.02 (d, J = 8.0 Hz, 2H); 7.52–7.62 (m, 3H); 6.95 (s, 1H); 6.72 (br.t, J = 5.7 Hz, 1H); 3.69–3.76 (m, 2H); 3.29–3.35 (m, 2H); 2.80 (s, 6H); 2.54 (s, 3H); 2.47 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.36, 156.29, 146.94, 145.94, 143.79, 132.89, 129.14, 125.96, 109.93, 90.74, 55.50, 42.54, 37.36, 24.37, 16.57. MS-ESI m/z 374 (M + H). LC-MS (UV-254) purity: 98%. HRMS calculated for $C_{18}H_{23}N_5O_2S$ (M + H) 374.165070, found 374.165.

5.2.1.8. N′-[5,7-Dimethyl-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidin-2-yl]-N,N-dimethylpropane-1,3-diamine ***20***·*HCl*. ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 10.30 (br.s, 1H); 8.01 (d, J = 8.0 Hz, 2H); 7.52–7.62 (m, 3H); 6.92 (s, 1H); 6.52 (br.t, J = 6.1 Hz, 1H); 3.38–3.44 (m, 2H); 3.02–3.08 (m, 2H); 2.72 (s, 6H); 2.52 (s, 3H); 2.46 (s, 3H); 1.92–2.06 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.08, 156.61, 146.95, 145.81, 143.91, 132.84, 129.19, 125.80, 109.80, 90.28, 54.53, 41.97, 24.33, 23.89, 16.63. MS-ESI m/z 388 (M + H). LC-MS (UV-254) purity: 99%. HRMS calculated for $C_{19}H_{25}N_5O_2S$ (M + H) 388.18072, found 388.1813.

5.2.1.9. N,N,5,7-Tetramethyl-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidin-2-amine ***21***. ^{1}H NMR ($CDCl_3$, 400 MHz), δ: 8.15 (dd, J = 8.0 Hz, J = 1.8 Hz, 2H); 7.41–7.51 (m, 3H); 6.60 (s, 1H); 3.07 (s, 6H); 2.60 (s, 3H); 2.55 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.14, 160.29, 148.20, 145.60, 144.25, 132.64, 128.94, 126.29, 110.31, 94.93, 42.14, 24.43, 16.50. MS-ESI m/z 331 (M + H). LC-MS (UV-254) purity: 99%.

5.2.1.10. N-[5,7-Dimethyl-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidin-2-yl]-N,N′,N′-trimethylethane-1,2-diamine ***22***·*HCl*. ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 10.46 (br.s, 1H); 8.01 (d, J = 8.0 Hz, 2H); 7.52–7.62 (m, 3H); 7.04 (s, 1H); 3.73 (t, J = 6.6 Hz, 2H); 3.36 (t, J = 6.6 Hz, 2H); 3.03 (s, 3H); 2.78 (s, 6H); 2.58 (s, 3H); 2.48 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.64, 159.13, 147.92, 145.87, 143.84, 132.84, 129.02, 126.44, 110.64, 95.55, 53.05, 48.54, 42.54, 41.02, 24.48, 16.51. MS-ESI m/z 388 (M + H). LC-MS (UV-254) purity: 98.5%. HRMS calculated for $C_{19}H_{25}N_5O_2S$ (M + H) 388.1807, found 388.1811.

5.2.1.11. tert-Butyl 4-[5,7-dimethyl-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidin-2-yl]piperazine-1-carboxylate ***23***. ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 8.14 (d, J = 7.7 Hz, 2H); 7.42–7.54 (m, 3H); 6.64 (s, 1H); 3.59–3.65 (m, 4H); 3.43–3.49 (m, 4H); 2.63 (s, 3H); 2.58 (s, 3H); 1.49 (s, 9H). MS-ESI m/z 472 (M + H). LC-MS (UV-254) purity: 98%.

5.2.2. (2-{[5,7-Dimethyl-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidin-2-yl]oxy}ethyl) dimethylamine ***24***

5.2.2.1. ***24***·*HCl.* Trifluoromethanesulfonic anhydride (0.33 mL, 1.8 mmol) was added to a stirred solution of alcohol 15 (520 mg. 1.5 mmol) in $CHCl_3$ (2 mL) at 0 °C, the mixture was stirred for 1 h, then 40% aq. solution of Me_2NH (2 mL) was added. The mixture was allowed to warm to ambient temperature and stirred for additional 2 h. Organic layer was separated, washed with water three times, and extracted with 6 N aq. HCl, aq. layer was washed with $CHCl_3$, neutralized with 20% aq. NaOH, and extracted with $CHCl_3$. Organic layer was washed with H_2O, brine, dried (Na_2SO_4) and concentrated. The residue was dissolved in CHCl3 (2 mL) and treated with 6 M solution of AcCl in EtOH (0.5 mL). The mixture was concentrated, the residue was treated with i-PrOH, formed precipitate was filtered off, washed twice with i-PrOH, hexane, and dried to afford 369 mg (60%) of compound ***24***·HCl.

5.2.2.2. ***24*** *(free base).* ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 7.95–7.99 (m, 2H); 7.50–7.61 (m, 3H); 7.04 (s, 1H); 4.42 (t, J = 5.5 Hz, 2H); 2.63 (t, J = 5.5 Hz, 2H); 2.55 (s, 3H); 2.52 (s, 3H); 2.20 (s, 6H). ***24***·*HCl*: ^{1}H NMR (DMSO-d_6, 400 MHz), δ: 11.01 (br.s, 1H); 7.96–8.00 (m, 2H); 7.52–7.63 (m, 3H); 7.12 (s, 1H); 4.74–4.79 (m, 2H); 3.55–3.60 (m, 2H); 2.88 (s, 6H); 2.59 (s, 3H); 2.54 (s, 3H). MS-ESI m/z 375 (M + H). LC-MS (UV-254) purity: 98%.

5.2.3. 5,7-Dimethyl-3-(phenylsulfonyl)-2-piperazin-1-ylpyrazolo[1,5-a]pyrimidine hydrochloride ***25***·*HCl*

A solution of compound **23** (141 mg, 0.3 mmol) in CHCl3 (1 mL) was treated 6 M solution of AcCl in EtOH (1 mL), reaction mixture was stirred at ambient temperature for 1 h, and treated with Et_2O. Formed precipitate was separated by centrifugation, washed three times with EtOAc, twice with acetone, hexane, and dried. Yield 111 mg (91%) of colorless solid compound ***23***·HCl.

^{1}H NMR (DMSO-d_6, 400 MHz), δ: 9.57 (br.s, 2H); 8.01 (d, J = 8.0 Hz, 2H); 7.49–7.62 (m, 3H); 7.06 (s, 1H); 3.57–3.65 (m, 4H); 3.16–3.26 (m, 4H); 2.57 (s, 3H); 2.50 (s, 3H). MS-ESI m/z 372 (M + H). LC-MS (UV-254) purity: 98.5%. HRMS calculated for $C_{18}H_{21}N_5O_2S$·HCl (M + H) 372.14942, found 372.1491.

5.2.4. 5,7-Dimethyl-2-(methylsulfinyl)-3-(phenylsulfonyl)pyrazolo[1,5-a]pyrimidine ***26***

A mixture of compound 16 (333 mg, 1 mmol), AcOH (10 mL), and 35% aq. solution of H_2O_2 (88 μL) was stirred at 80 °C for 7 h, concentrated under reduced pressure, and residue was purified by silica column chromatography (eluent $CHCl_3$/EtOAc 5:1). Yield 304 mg (87%) of compound **26**.

^{1}H NMR (DMSO-d_6, 400 MHz), δ: 8.22 (m, 2H), 7.58 (m, 1H), 7.51 (m, 2H), 6.88 (s, 1H), 3.24 (s, 3H), 2.83 (s, 3H), 2.71 (s, 3H). MS-ESI m/z 350 (M + H). LC-MS (UV-254) purity: 99%.

5.2.5. 4-(Phenylsulfonyl)-1H-pyrazole-3,5-diamines ***7***–***11*** *(general procedure)*

A mixture of 2-phenylsulfonyl-3,3-bis-methylsulfanyl-acrylonitrile **27** (2.67 g, 9.35 mmol), a corresponding amine (9.35 mmol),

and i-PrOH (15 mL) was stirred and heated under reflux for 1 h and then stirred overnight at ambient temperature. Formed precipitate was filtered off, washed with i-PrOH, suspended in i-PrOH (15 mL), and $N_2H_2 \cdot H_2O$ (0.48 g, 9.5 mmol) was added into the suspension. The resulted mixture was heated under reflux for 0.5 h, cooled, and poured into a mixture of ice and H_2O (150 mL). Formed precipitate was filtered off, washed with ice water, cooled i-PrOH, and dried. Obtained in yields 85–92% compounds **7**–**11** were used for further reactions without additional purification.

5.3. Biological assays

5.3.1. Cell-based functional assays

5.3.1.1. 5-HT_{2B} receptor functional assay. 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 at room temperature with 4 μM calcium-sensitive dye, Fura-2AM (Invitrogen, Carlsbad, CA) 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 additional 30 min with gentle shaking at room temperature. All loading procedures were performed in dark conditions. The loaded cells were washed twice with PBS and re-suspended into the Hybridoma media at a cell density of $3–4 \times 10^6$ 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 μL 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 μL, 10 mM) to assess the compounds' blocking activity.

5.3.1.2. 5-HT_6 receptor functional assay. The 5-HT_6R 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-HT_6 receptor expression was activated by addition of tetracycline (1 ug/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 0.67×106 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 uL (~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 μM). Each assay plate contained serotonin and cAMP standard concentration curves. 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.

5.3.1.3. Radioligand binding assays. The assays were performed by Ricerca (former MDS Pharma) in accordance with their optimized protocols for each target. The procedures are described in Ref. [20].

5.4. Curve fitting and statistical analysis

The concentration curve data 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 [21] 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 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.

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