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Research paper

# 5-HT$_2$ receptor affinity, docking studies and pharmacological evaluation of a series of 1,3-disubstituted thiourea derivatives

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ABSTRACT

A series of 10 thiourea derivatives have been synthesized by the reaction of aromatic amine with a substituted aryl (compounds **1**–**3**, **6**–**8**) and alkylphenyl (**4**, **5**, **9**, **10**) isothiocyanates. Their *in vitro* and *in vivo* pharmacological properties were studied. Among the evaluated compounds, two displayed very high affinity for the 5-HT$_{2A}$ receptor (**1**–0.043 nM and **5**–0.6 nM), being selective over the 5-HT$_{2C}$ receptor. Derivatives **3**, **5**, **9**, **10** by 70–89% diminished L-5-HTP-induced head twitch episodes. Compounds **1** and **5** as the 5-HT$_{2A}$ receptor antagonists produced a dose-dependent decrease in the number of DOI-elicited HTR. Compounds **1**–**5** strongly reduced amphetamine-evoked hyperactivity in rodents. In another test, **1** and **2** caused hyperthermia in mice, whereas **9** and **10** led to hypothermia. Antinociceptive and anticonvulsant properties of selected derivatives were demonstrated. Molecular docking studies using a homology model of 5-HT$_{2A}$ revealed a significant role of hydrogen bonds between both thiourea NH groups and Asp155/Tyr370 residues, as well as π–π interaction with Phe339.



## 1. Introduction

The 5-HT$_2$ receptor family comprises of the 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ subreceptors, which are G-protein coupled receptors linked to the inositol phosphate signal transduction system [1]. Since 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors exhibit 46–50% overall sequence identity [2,3], their molecular and pharmacological profiles are similar [4]. Both are involved in a range of physiological functions, such as motor behavior, mood, pain, appetite, sleep, thermoregulation, urea excretion and hormone secretion [1,4]. They are implicated in several psychiatric disorders, including schizophrenia (mixed D$_2$/D$_1$/5-HT$_2$ antagonists), psychosis (mixed D$_2$/5-HT$_{2A}$ antagonists) [5,6], depression, obsessive-compulsive disorders (5-HT$_{2C}$ agonists) [1,7] and generalized anxiety disorders (5-HT$_{2A}$/5-HT$_{2C}$ antagonists) [6]. Type 2 serotonin receptors (5-HT$_2$) mediate the action of large number of psychoactive drugs, including antipsychotics, hallucinogens, anxiolytics and anti-depressants [2,8,9]. The key site for hallucinogen action is the 5-HT$_{2A}$ receptor subtype, which was developed by correlation of the high affinity for that receptor and behavioral activity of hallucinogenic amphetamines [4]. It is known that 5-HT$_{2C}$ rather than 5-HT$_{2A}$ blockade can prevent the extrapyramidal side effects induced by atypical antipsychotics, such as Haloperidol [10].

Several structurally different compounds are known to bind 5-HT$_{2A}$ receptors. Among them, some urea and thiourea derivatives have been extensively studied (Fig. 1). All of them display nanomolar potency at 5-HT$_{2A}$ receptors, but only for Pimavanserin high

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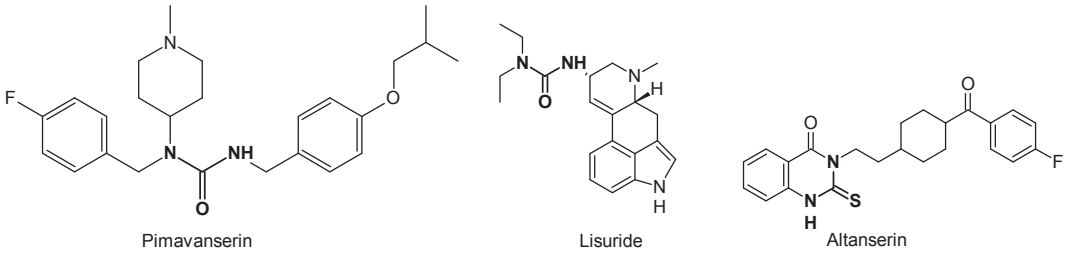



**Fig. 1.** Some representative ligands for 5-$HT_{2A}$ receptors.

selectivity over 5-$HT_{2C}$ is observed [11]. What is more, it lacks other serotonin, as well as dopamine receptors activity, in contrast to Lisurde [12] and Altanserin [7,13], which effectively block dopamine $D_2$ receptors. The mode of action at 5-$HT_{2A}$ receptors among mentioned above (thio)urea derivatives also varies: Pimavanserin is an inverse agonist [14,15], the profile of Lisuride is described as a partial agonism [12], and Altanserin acts as an antagonist [7].

It is supposed that 5-$HT_{2A}$ receptor blockade, without affecting dopamine $D_2$ receptors, represented *e.g.* by pimavanserin, could be the proper target mechanism for non-motor disorders accompanying Parkinson's disease, such as depression, sleep problems, dementia and psychosis, known as Parkinson's disease psychosis [11,16]. Another urea connection, Lisuride, is applied in an initial anti-Parkinsonian therapy, aimed at alleviating movement dysfunction. Additionally, it also lowers prolactin and its low doses prevent migraine attacks [12]. On the other hand, 5-$HT_{2A}$ antagonists and inverse agonists are also being developed as potential anti-insomnia drugs [17], without many of the less desirable side effects of benzodiazepines [14].

The substituted amphetamine hallucinogen, 5-$HT_{2A/2C}$ receptor agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI) has emerged as the most popular pharmacological tool used in HTR studies of hallucinogens. Numerous receptor systems are involved in the behavioral response induced by DOI and an activation of the 5-$HT_{2A}$ receptor is essential for this effect [18]. Some studies suggest considering also the 5-$HT_{2B}$ receptor as a potential modulator of HTRs. What is more, recent investigations have demonstrated that 5-$HT_{2C}$ and 5-$HT_{1A}$ agonists [17,19], chronic administration of serotonin norepinephrine transporter inhibitors (SNRIs), selective AMPA glutamate antagonists, $GABA_A$ agonists, adenosine $A_1$ agonists, $D_1$ receptor antagonists [18], as well as indirect cannabinoid agonists decrease the number of DOI-elicited HTRs [20]. It has also been suggested that head twitch shakes may be useful as a model of Tourette's syndrome [21].

Some reports concerning a strong influence of urea [22–24] and thiourea [24–27] derivatives on the central nervous system (CNS) in rodents have been presented. Several *N*-arylthioureas [26], similarly as atypical opioid analgesic (morphine, Tramadol) and antipsychotics (Clozapine), inhibited L-5-HTP-induced head twitch responses (HTR) in mice, an animal model for the activation of the CNS 5-$HT_{2A}$ receptors [28]. It was demonstrated that also Pimavanserin shared a behavioral characteristics consistent with the atypical antipsychotic drugs, such as potently inhibits head-twitching produced by DOI [15,16]. Both urea and thiourea connections are known of their anticonvulsant properties [24,27], indicating the therapeutic potential in petit mal seizures. Moreover, a sedative effect of thioureas was observed [22,23], making them potentially useful for panic attacks, anxiety and sleep disorders.

There is no data concerning binding modes of (thio)urea derivatives to 5-$HT_2$ receptors so far; what is more, limited docking studies are available for other class of 5-$HT_{2A}$ receptor ligands, as tryptamines, phenylisopropylamines or *n*-alkylpiperidines [5,10,29–31].

This work reports the synthesis, docking studies and evaluation of 1,3-disubstituted thiourea derivatives related with 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors, as well as determination of their *in vivo* properties in rodent behavioral models. The outcome enabled us to identify the key structural features responsible for the serotonergic activity, therefore leading to novel agents with antipsychotic action.

## 2. Results and discussion

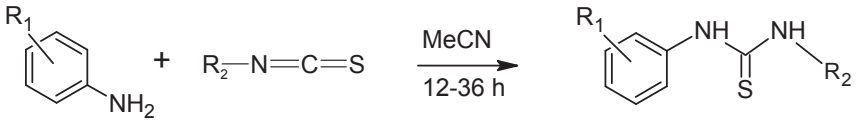
### 2.1. Chemistry

The synthetic route for the preparation of 1,3-disubstituted thiourea derivatives is shown in Scheme 1. The amine compounds were reacted with substituted isothiocyanates in dry acetonitrile medium and gave some 3-(trifluoromethyl)phenyl)thiourea (**1**–**5**) and (4-chloro-3-nitrophenyl)thiourea derivatives (**6**–**10**). The structures of newly obtained compounds were elucidated by spectral analyses, and molecular structure of **2** and **6** was confirmed by an X-ray crystallography (Fig. 2).

The set of substituents at the thiourea nitrogen was selected on the basis of our previous experiences with the substituent impact on the pharmacological properties [25,26,32]. As a result, the representative *N*-arylthiourea and *N*-alkylthiourea derivatives of 3-(trifluoromethyl)aniline (**1**–**5**) and 4-chloro-3-nitroaniline (**6**–**10**) were presented. From the first group, compounds with electron-withdrawing functionalities **1** (3-chloro-4-fluorophenyl), **3** and **7** (2-chlorophenyl) were investigated, as well as 4-methoxyphenylthiourea **2**, that contains an electron-donating substituent on benzene ring. In the *N*-alkylthiourea set, the nitrogen atom of thiourea was linked to phenylethyl (**4, 10**) or benzyl (**5**, **9**) fragment. Unsubstituted phenyl (**1**) and benzoyl (**8**) derivatives were also evaluated. As a result, the tested collection of compounds presented diverse and comparable group.

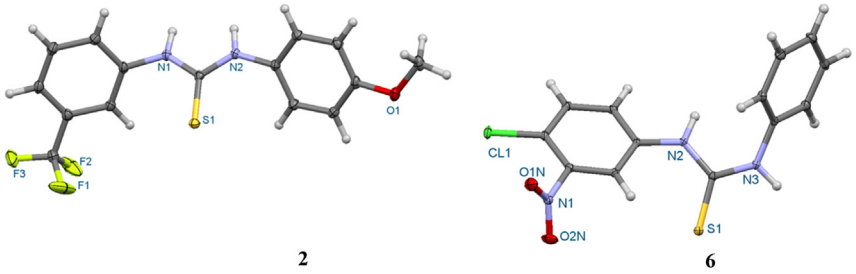
### 2.2. In vitro tests

All the new compounds were tested for their affinity to 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors. Some of the synthesized derivatives showed affinities in the subnanomolar range towards 5-$HT_{2A}$ receptors and no affinity for other relevant 5-$HT_{2C}$ receptors (Table 1). Besides the outstanding 5-$HT_{2A}$ receptor affinity and selectivity of compound **1** ($K_i = 0.043 \pm 0.009$ nM), another interesting $K_i$ value was that of compound **5** ($K_i = 0.60 \pm 0.024$ nM). Although the derivatives **1**–**10** showed different substituents $R_1$ and $R_2$ on the aromatic rings linked to the thiourea scaffold, the $R_2$ functionalities do not seem to be decisive in determining a general trend towards 5-$HT_2$ receptors. The affinity/selectivity profiles towards 5-$HT_{2A}$ receptor are more influenced by the presence of a *m*-$CF_3$ group on the aromatic ring than $R_1$ substituent. In fact, the association of this structural feature to a 3-chloro-4-fluorophenyl group and a phenylethyl fragment on $R_2$ position of the thioureidic scaffold (**1** and **5**), conferred the




**1** $R_1$= 3-$CF_3$, $R_2$= 3-Cl,4-F-Ph
**2** $R_1$= 3-$CF_3$, $R_2$= 4-$OCH_3$-Ph
**3** $R_1$= 3-$CF_3$, $R_2$= 2-Cl-Ph
**4** $R_1$= 3-$CF_3$, $R_2$= $CH_2CH_2$-Ph
**5** $R_1$= 3-$CF_3$, $R_2$= $CH_2$-Ph
**6** $R_1$= 3-$NO_2$,4-Cl, $R_2$= Ph
**7** $R_1$= 3-$NO_2$,4-Cl, $R_2$= 2-Cl-Ph
**8** $R_1$= 3-$NO_2$,4-Cl, $R_2$= -(C=O)-Ph
**9** $R_1$= 3-$NO_2$,4-Cl, $R_2$= $CH_2$-Ph
**10** $R_1$= 3-$NO_2$,4-Cl, $R_2$= -$CH(CH_3)$-Ph

**Scheme 1.** Synthesis of thiourea derivatives **1**–**10**.




**2** **6**

**Fig. 2.** Perspective view of molecules **2** and **6** (conformers observed in solid).

**Table 1**
Affinities values of compounds **1**–**10** for 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors.

| Comp. | $R_1$ | $R_2$ | Receptor affinity Ki ± SD (nM) | | ClogP[a] |
|---|---|---|---|---|---|
| | | | 5-$HT_{2A}$ [$^3$H]Ketanserin | 5-$HT_{2C}$ [$^3$H]Mesulergine | |
| **1** | 3-$CF_3$ | 3-Cl, 4-F-Ph | 0.043 ± 0.009 | > $10^4$ | 4.75 |
| **2** | 3-$CF_3$ | 4-$OCH_3$-Ph | > $10^4$ | > $10^4$ | 3.57 |
| **3** | 3-$CF_3$ | 2-Cl-Ph | > $10^4$ | > $10^4$ | 3.84 |
| **4** | 3-$CF_3$ | −$CH_2CH_2$−Ph | > $10^4$ | > $10^4$ | 4.19 |
| **5** | 3-$CF_3$ | −$CH_2$−Ph | 0.60 ± 0.024 | > $10^4$ | 3.76 |
| **6** | 3-$NO_2$,4-Cl | Ph | > $10^4$ | > $10^4$ | 3.23 |
| **7** | 3-$NO_2$,4-Cl | 2-Cl-Ph | > $10^4$ | > $10^4$ | 3.42 |
| **8** | 3-$NO_2$,4-Cl | −(C=O)Ph | > $10^4$ | > $10^4$ | 3.62 |
| **9** | 3-$NO_2$,4-Cl | −$CH_2$−Ph | > $10^4$ | > $10^4$ | 3.71 |
| **10** | 3-$NO_2$,4-Cl | −$CH(CH_3)$−Ph | > $10^4$ | > $10^4$ | 4.02 |
| Ketanserine | – | – | 0.85 | – | |
| Mesulergine | – | – | – | 1.9 | |

[a] *Calculated* by ChemBioDraw Ultra 14.

highest affinity and selectivity values for the 5-$HT_{2A}$ receptor. Moreover the outstanding affinities and selectivity of compounds **1** and **5** towards 5-$HT_{2A}$ receptors agree with data already reported in literature by Parker et al. [33] and could be explained also in terms of correlation between lipophilicity and binding affinity, as confirmed by molecular docking studies.

### 2.3. Molecular docking studies

In this study binding modes of all synthesized compounds to 5-$HT_{2A}$ receptor using molecular docking approach was analyzed. The 5-$HT_{2A}$ receptor model was created applying comparative modeling methods based on the crystal structure of β2AR. In 2013, two crystal structures of serotonin receptors subtypes 5-$HT_{1B}$ [34] and 5-$HT_{2B}$ [35] were determined. However, in both structures different conformational patterns are observed which show features of an intermediate active-state for the 5-$HT_{1B}$ receptor and a β-arrestin-biased activation-state for the 5-$HT_{2B}$ receptor [35]. Therefore we decided to use crystal structure of β2AR presenting inactive state of the receptor as a template. Moreover, β2AR was also used as a template for homology modeling of 5-$HT_{2A}$ receptor and ligand docking in previous studies [36]. The molecular model of the receptor was refined during 10 ns MD simulation, in the membrane environment, with weak position restraints imposed on the protein backbone. The conducted simulation allowed for relaxation and optimization of the receptor side-chains (see Fig. S1, Supplementary Materials). The obtained model was used in the docking. For each of the 10 compounds, we have obtained a set of 1000 lowest energy conformers during docking procedure. The estimated values of free binding energies were ranging from −5.53 kcal/mol to −7.9 kcal/mol. Final docking conformation was selected by means of structural clustering. Depending on a compound case, the largest cluster consisted from 193 to 970 members (see Table 2 for detail description of docking results). These numbers indicate a high degree of convergence towards a single and favored binding mode for each of the ligands. Additional

**Table 2**
5-$HT_{2A}$ binding data based on docking results for compounds **1**–**10**.

| Comp. | Position | LC/LBE | Ligand surrounding amino acids (d ≤ 3 Å) |
|---|---|---|---|
| **1** | LBM | LC = 327; LBE = −6.05 kcal/mol | TRP151, ILE152, ASP155, VAL156, SER226, PHE222, LEU228, LEU229, VAL235, GLY283, SER239, SER242, PHE339, PHE340, ASN234, VAL366 |
| **2** | UBM | LC = 384; LBE = −5.53 kcal/mol | SER131, THR134, ILE135, GLY138, ARG140, TRP141, TRP151, ILE152, CYS227, LEU228, LEU229, PHE339, ASN363, VAL366, TYR370 |
| **3** | LBM | LC = 311; LBE = −6.18 kcal/mol | ILE152, ASP155, VAL156, SER226, PHE222, LEU228, LEU229, TRP336, PHE339, PHE340, ASN234, VAL366, TYR370 |
| **4** | UBM | LC = 193; LBE = −6.49 kcal/mol | SER131, THR134, ARG140, TRP141, TRP151, ILE152, CYS227, LEU228, ASN363, VAL366, TRP367, TYR370, |
| **5** | LBM | LC = 742; LBE = −5.95 kcal/mol | TRP151, ILE152, ASP155, SER226, PHE222, CYS227, LEU228, LEU229, TRP336, PHE339, PHE340, ASN234, TYR370 |
| **6** | UBM | LC = 970; LBE = −6.69 kcal/mol | SER131, THR134, ILE135, ARG140, TRP141, TRP151, ILE152, ASP155, PHE222, CYS227, LEU228, LEU229, ASN363, TYR370 |
| **7** | UBM | LC = 291; LBE = −7.48 kcal/mol | THR134, ILE135, ARG140, TRP141, TRP151, ILE152, PHE222, SER226, LEU228, LEU229, TYR370 |
| **8** | UBM | LC = 277; LBE = −7.6 kcal/mol | THR134, ILE135, ARG140, TRP141, TRP151, ILE152, ASP155, VAL156, PHE222, SER226, CYS227, LEU228, LEU229 |
| **9** | UBM | LC = 268; LBE = −7.42 kcal/mol | SER131, THR134, ILE135, ARG140, TRP141, TRP151, ILE152, CYS227, LEU228, LEU229, TYR370 |
| **10**[a] | (S′ isomer) UBM | LC = 295; LBE = −7.9 kcal/mol | THR134, ILE135, TRP151, ILE152, ASP155, CYS227, LEU228, LEU229, VAL366, TYR370 |
| | (R′ isomer) LBM | LC = 329; LBE = −7.4 kcal/mol | ILE152, ASP155, VAL156, PHE222, LEU228, LEU229, VAL235, GLY283, SER239, SER242, TRP336, PHE339,ASN234, VAL366, TYR370 |

LBM = lower binding mode.
UBM = upper binding mode.
LC = number of members of the largest cluster calculated for 1000 docking runs using RMSD tolerance = 2 Å
LBE = estimated lowest free energy of binding by AutoDock4 energy function.
[a] Two isomers were investigated.

MD simulations of predicted complexes could be applied for detailed investigation of protein-ligand interaction/interplay. However, they would require an extensive long timescale simulations [37], which is beyond the scope of this study.

Analysis of resulting binding modes revealed that ligands were grouped in two distinct regions of the receptor binding site. Compounds **2**, **4**, **6**, **7**, **8**, **9** and **10** (R′ isomer) preferred upper portion of the receptor binding site, whereas compounds **1**, **3**, **5** and **10** (S′ isomer) were grouped at the bottom of the pocket. Graphical representation of the two distinct binding modes is shown in Fig. 3. Those two distinct binding modes correlate well with binding affinity experimental data. According to experiment, compounds interacting at the lower portion of the binding site show high affinity to the receptor, in contrast to derivatives showing no affinity for the receptor protein that are grouped near the extracellular side of the protein. Two ligands **1** and **5** (exhibiting the highest experimental affinity) bind at the lower portion of the binding site, penetrating deeper into the receptor alpha helical core. The ligands are stabilized mainly by hydrogen bonds with Asp155 residue. In case of **5** additional hydrogen bond is created with Tyr370. That ligand is also engaged in π–π interaction with Phe339 (Fig. 4). Interestingly, the thiourea **5** is the representative of the largest cluster (LC = 742, see Table 2) of the lowest energy conformers localized at lower portion of the binding site. In contrast, two compounds **6** and **8** (showing no experimental affinity) interact at the upper portion of the receptor binding pocket. Both of them are engaged in hydrogen bond network with backbone fragments of the residues located in extracellular loops (ECLs), namely: Trp141 from ECL I and Cys227 from ECL II. In addition, the Trp151 residue formed π–π interactions with the molecule **8** (Fig. 5). What is more, the Clog*P* values for inactive compounds **6**–**8** are the lowest among the series that indicate their higher hydrophilicity (Table 1).

Based on the docking results obtained in this study, a general pharmacophore model for ligands showing high experimental affinity was proposed. The model consists of the two aromatics sites and one hydrogen bond donor (HBD) site (Fig. 6). Similar three-point pharmacophores models have been also generated for 5-$HT_{2A}$ receptor antagonists by other authors [38]. In models for triangular arylpiperazine antagonists developed previously by Mokrosz [39,40] or Klabunde and Evers [5], the distances between groups crucial for activity are different than these described in this study, mainly in the relative position of aromatic groups versus the nitrogen atom. To reach maximum binding affinity, the thiourea NH group has to be placed in the distance 4.3–4.9 Å from both aromatic centers.

The studied structural modifications were focused on the aromatic substituents at thiourea nitrogen atoms. Results of both affinity and molecular modeling studies confirmed the role of the modified fragment for the biological activity. Within the group of (3-trifluoromethyl)phenyl derivatives **1**–**5**, compounds substituted at N3 with 3-chloro-4-fluoro-phenyl (**1**) or benzyl (**5**) ring belong to the class of the most potent ligands, deeply penetrating the binding pocket of the 5-$HT_{2A}$ receptor. This bottom part of the active site was also preferred by the derivative **3**, possessing electron-withdrawing chlorine substituent at the benzene ring (similarly as **1**) and S-enantiomer of phenylethyl compound **10** (close analog of **5**). In the group of weakly bounded ligands, gathered in the upper place of the binding site, derivatives of 4-chloro-3-nitroaniline can be found (**6**–**9**, R-enantiomer of **10**). Within this series, the presence of unsubstituted phenyl ring (**6**), benzoil (**8**) or benzyl (**9**) rings at the end of the molecules critically decreased affinity for the 5-$HT_{2A}$ receptor. Introduction of longer spacer at the terminal phenyl ring (as for phenylethyl compound **4**, homolog of **5**) or an electron-donating functionality (*p*-methoxy in **2**) was definitely unprofitable for serotonin receptor affinity. Comparing two pairs of corresponding thiourea derivatives (**3**–**7** and especially **5**–**9**) it can be concluded that the (3-trifluoromethyl)phenyl containing molecules are more active and/or fit better to the receptor pocket in each pair.

### 2.4. In vivo tests

On the basis of docking and affinity binding results, several thioureas were chosen for *in vivo* experiments. Compounds with

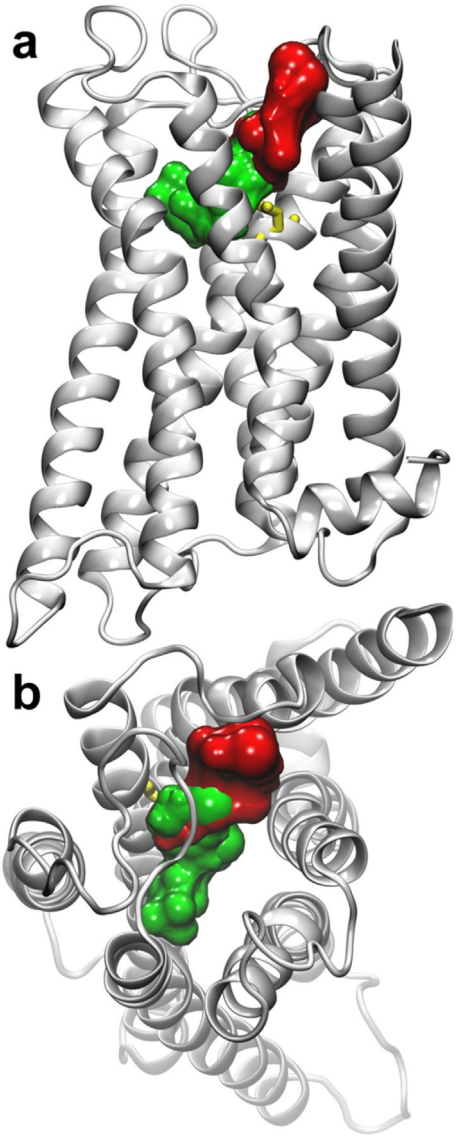



**Fig. 3.** Structure of 5-$HT_{2A}$ receptor model. Colored surface indicates portion of the binding site occupied by ligands: the color green represents lower binding mode for compounds **1** and **5**; the color red represents upper binding mode for compounds: **6** and **8**. Residue Asp155 is shown in yellow. **a)** View parallel to the membrane plain, **b)** View from the extracellular side. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

the highest affinity for 5-$HT_{2A}$ receptor (**1, 5**), derivatives with preferred lower localization in the active site (**3, 10**), as well as their very close structural analogs (**2, 4, 9**) were investigated. The intrinsic activity of structures **6**–**8** was not examined, because they occupy upper, non-preferred place of the receptor pocket that suggested no potential biological potency.

To determine the 5-$HT_2$ binding profile precisely, the tendency to diminish the head twitch responses (HTR), evoked by either 5-hydroxy-L-tryptophan (L-5-HTP) and 2,5-dimethoxy-4-iodoamphetamine (DOI) was assessed. Previously performed the rota-rod and chimney tests allowed to check possible occurrence of drug-induced changes in the muscle relaxant activity of mice, which may have contributed to their behavior. Since already reported structurally related thiourea derivatives strongly influenced the central nervous system in mice [22–28], some common behavioral tests were estimated, such as effects on the spontaneous and drug-evoked locomotor potency, and anticonvulsant or analgesic activities. Additionally, the involvement in alteration of body temperature in mice was studied, as it shows the link with 5-$HT_1$ receptors. The established $ED_{50}$ of tested compounds were the following: 286.2 mg/kg (**1**), 771.4 mg/kg (**2**), 2000 mg/kg (**3**), 709.6 mg/kg (**4**), 731.3 mg/kg (**5**), 909 mg/kg (**9**) and 1031.3 mg/kg (**10**). The progressive doses, calculated as a part of $ED_{50}$, were used in behavioral experiments.

In the first instance, no observable neurotoxic effects were detected in the rota-rod and chimney tests for any of investigated compounds (data not shown). Thus, one can ascertain that tested thiourea derivatives do not disturb motor coordination and do not provoke myoreleaxation.

The head twitch responses (HTR) in rodents induced by 5-HTP, a precursor of 5-HT, is considered as a specific behavioral model for the activation of serotonergic neurons [28]. According to literature data, the attenuating action on HTR reflects an antagonist activity of a compound at 5-$HT_{2A}$ receptors [42]. Early behavioral studies revealed that 5-$HT_{2A}$ receptor antagonists, such as ketanserin and ritanserin, inhibit HTR by more than 80% [43]. However, this test is not considered very specific, because other substances, like adrenergic ligands, can influence HTR [44]. The effect of new compounds (used at the dose of 0.1 $ED_{50}$) on HTR evoked by L-5-HTP (230 mg/kg) is presented in Fig. 7. Compounds **3**, **5, 9** and **10** seem to be the strongest blocking agents of head twitch frequency among the whole series, as compared to the basal activity after administration of L-5-HTP. The head twitch shakes were decreased in the presence of derivatives **3**, **5, 9** and **10** by 72.4%, 73.3%, 70.5% ($p < 0.05$) and 89.0%, respectively ($p < 0.01$). The results for **1**, **2** and **4** did not reach statistical significance, though decrements of the responses were also noticed.

Experimental evidence in animals proved that some hallucinogens like 2,5-dimethoxy-4-iodoamphetamine (DOI) induce hallucinations by activating 5-$HT_{2A}$ receptors, and their properties are blocked by mainly selective 5-$HT_{2A}$ receptor antagonists [6]. According to *in vitro* affinity results, synthesized derivatives **1** and **5** were found as very potent 5-$HT_{2A}$ ligands, therefore their antagonistic properties were assessed in the (±)DOI)-induced head twitch responses in mice (Fig. 8). Studied compounds produced a dose-dependent decrease in the number of (±)DOI-induced head shakes (Table 3), that proved their 5-$HT_{2A}$ receptor antagonistic activities. The thiourea **5** was stronger 5-$HT_{2A}$ antagonist than **1**, inhibiting the HTR by 72.2%, if administrated in the dose equivalent to 0.1 $ED_{50}$ ($p < 0.001$) and by 69.4%, when taken in its lower dose (0.05 $ED_{50}$). These findings are consistent with docking results, since the ligand **5** has formed more interactions in the upper binding site of the receptor protein. Although the binding constant Ki of the derivative **1** is even higher than this estimated for both **5** and Ketanserine, its bioavailability *in vivo* seems to be lower, which could explain weaker inhibitory effect against head twitch responses observed among laboratory animals.

The influence of a thiourea series on spontaneous motility and amphetamine-induced hyperactivity was assessed (Fig. 9). It was noticed that **3,** administered at the dose of 0.1 $ED_{50}$ inhibited the locomotor activity in studied animals ($p < 0.05$), while the other derivatives (**1**, **2**, **4**, **5**, **7**–**10**) did not produce any significant effects. Nonetheless, it was observed that almost all 3-(trifluoromethyl) phenyl derivatives**,** administrated at the same dose, generated a significant decline of the amphetamine-induced hyperactivity of animals ($p < 0.05$ for **2**, **5**, $p < 0.01$ for **1**, **4**, Fig. 10). Derivatives **1** and **4** reduced the amphetamine-induced motility by 47.30% and 42.48%, respectively. This effect was not observed for tested 4-chloro-3-nitrophenylthioureas (**9**, **10**). Literature survey shows that in contrast to the atypical antipsychotic drugs, the urea derivative such as pimavanserin lacks $D_2$ antagonist activity and

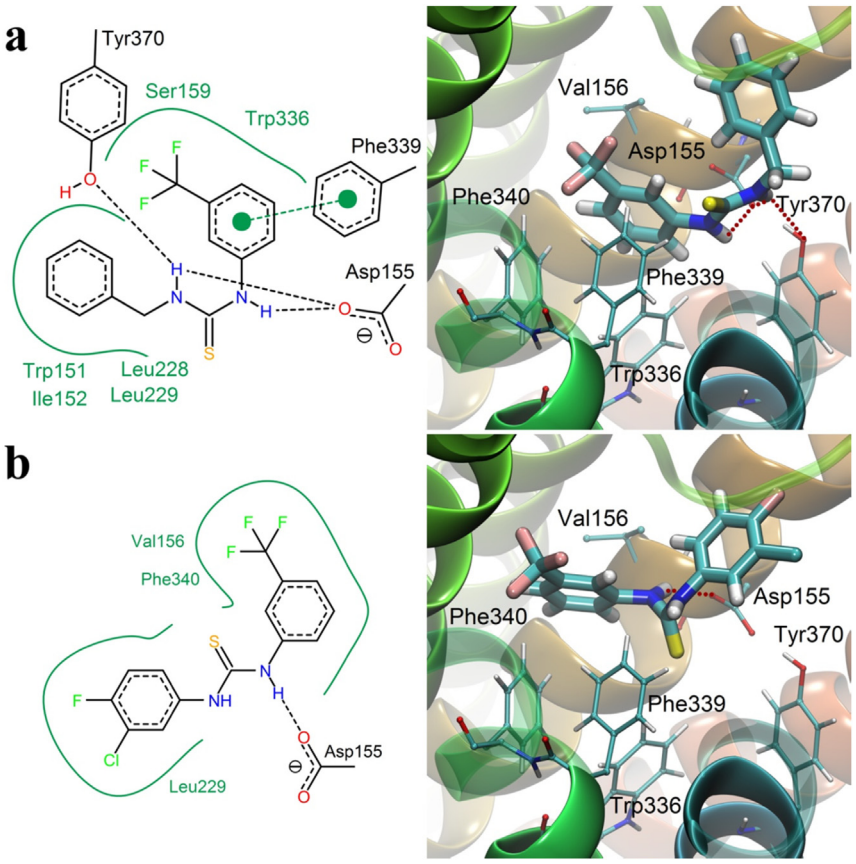



**Fig. 4.** Binding modes of compounds **a) 5** and **b) 1**. The left panel presents receptor–ligand interaction scheme generated using PoseView server [41]. Black dashed lines indicate hydrogen bonds. Green solid lines show hydrophobic interactions and green dashed lines show π–π interactions. The right panel shows ligand position inside the lower portion of binding the site of the receptor protein. Red dotted lines indicate hydrogen bonds. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

therefore does not effectively reverse amphetamine-induced hyperactivity [16]. Comparing studied compounds with that drug we could conclude that mechanism of action of compounds **1**, **2**, **4**, **5** is mediated by catecholamine system, since amphetamine exerts its behavioral effects by modulating monoamine neurotransmission in the brain [45].

The antinociceptive activity was measured as the ability to reduce the number of abdominal writhes induced by *i.p.* injection of acetic acid in mice (Fig. 11). This test, known as "writhing test", is one of the most sensitive methods used to detect even very weak antinociceptive agents. It is also considered as an experimental model closest to the nature of clinical pain. Although it does not allow to determine the length of analgesic action and it is not specific, it allows to evaluate activity of both central and peripheral origin [46,47]. It was observed that substances **3**–**5**, **9**, **10**, used at a dose equivalent to 0.1 $ED_{50}$, were the most potent in this test ($p < 0.01$). When given at half a dose (0.05 $ED_{50}$), statistically significant reduction in the number of writhing episodes was observed for **4**, **5**, **9** and **10** ($p < 0.05$). These derivatives administrated at a dose equivalent to 0.025 $ED_{50}$, still exhibited antinociceptive activity, though without any statistical importance.

In order to check the correlation between the antinociceptive effect of a compound and the opioid system, the writhing test in the presence of nonselective opioid receptor antagonist, Naloxone, was performed (Fig. 12). One-way ANOVA showed significant changes in the number of writhing episodes of mice after the administration of tested derivatives ($F_{(10,96} = 5443$; $p < 0.0001$). Post hoc Dunnett's test showed a significant reduction in the writhing episodes of mice after the administration of the compound **3** at the dose of 0.1 $ED_{50}$ ($p < 0.01$) and after **4**, **5** ($p < 0.05$; $p < 0.01$) and **9**, **10** ($p < 0.01$ and $p < 0.001$) at the dose of 0.05 $ED_{50}$. Pretreatment with Naloxone did not reverse these antinociceptive effects of tested compounds. It leads to the conclusion, that mechanism of their activity is not linked with endogenous opioid system and the compounds do not behave as its agonists.

The anticonvulsant activity of new derivatives was determined by using pentetrazol-induced seizure test (Table 4). This rodent model is widely used as the standard method for predicting protection against tonic-clonic seizures. In PTZ-induced seizures model compound **4** administrated at a dose equivalent to 0.1 $ED_{50}$, significantly protected from tonic seizures (by 46,66%) and death of animals (by 48.33%, $p < 0.05$). Derivative **5** reduced clonic seizures (by 40.0%) and was found effective in preventing from tonic convulsions (by 46.66%, $p < 0.05$); the ability to decrease the mortality by this compound was also observed. Among 4-chloro-3-nitrophenyl compounds, derivative **9** considerably (in 50–43.75%) reduced both tonic and clonic seizures, as well as the mice mortality. Used in half of its dose (0.05 $ED_{50}$), it still diminished death episodes ($p < 0.05$). The obtained results have confirmed other authors findings [24,48], since *N*-alkylphenylthiourea analogues were found to possess higher anticonvulsant activity than *N*-arylthioureas, because of the bulk and lipophilicity.

The results of measurement of the body temperature in normothermic mice may also suggest the link between the serotonergic system and mechanism of action of studied compounds (Figs. 13 and 14). According to literature data, that system is

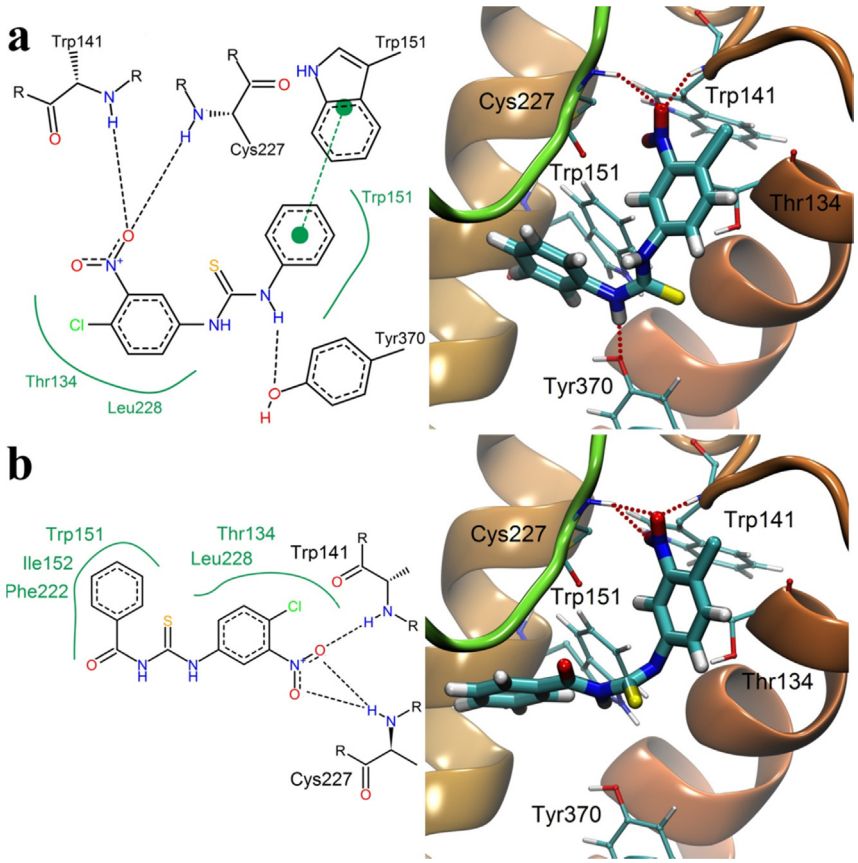



**Fig. 5.** Binding modes of compounds **a) 6** and **b) 8**. The left panel presents receptor–ligand interaction scheme generated using PoseView server [12]. Black dashed lines indicate hydrogen bonds. Green solid lines show hydrophobic interactions and green dashed lines show π–π interactions. The right panel shows ligand position inside the upper portion of the binding site of the receptor protein. Red dotted lines indicate hydrogen bonds. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

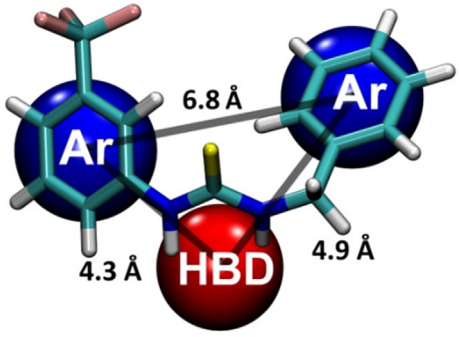



**Fig. 6.** The 5-$HT_{2A}$ receptor pharmacophore model: Ar - aromatic; HBD–hydrogen bond donor. For comparison the graphical representation of **5** in its receptor interacting conformation is superimposed on the pharmacophore model.

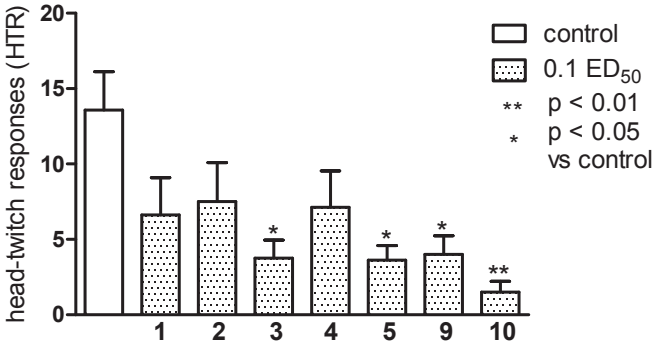



**Fig. 7.** The influence of new derivatives on head-twitch responses (HTR) evoked by L-5-HTP (230 mg/kg, i.p). Compounds were injected 60 min before the test, whereas L-5-HTP immediately before the test. Data are expressed as mean ± SEM values. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. control group (Dunnett's test).

involved in the body temperature regulation in hypothalamus [49,50]; 5-$HT_2$ receptor agonists (*e.g.* DOI) and 5-$HT_{1A}$ antagonists can induce hyperthermia, whereas 5-$HT_2$ antagonists (*e.g.* 8-OH-DPAT, Ketanserine) or 5-$HT_{1A}$ agonists cause hypothermia [51,52]. Bonferroni's post hoc test revealed a significant increase in the body temperature of mice after the administration of the compound **1** at the dose of 0.1 $ED_{50}$ from 30 to 180 min ($p < 0.001$), and after intake of the compound **2** in 30 ($p < 0.01$), 60 ($p < 0.001$) and 90 min ($p < 0.05$). The compound **9**, given in a dose corresponding to 0.1 $ED_{50}$, provoked considerable decrease in the body temperature (in 60 ($p < 0.01$), 90 ($p < 0.001$) and 120 min ($p < 0.01$). The derivative **10** was even more effective – it has decreased the body temperature from 30 to 120 min ($p < 0.001$, 0.1 $ED_{50}$). Other tested compounds did not affect body temperature in a clear, statistically significant manner, as compared to the control. The results of body temperature measurement allow for conclusion that the action of **1**, **2** and **9**, **10** could be an effect of a stimulation of 5-$HT_{1A}$ receptors.

To sum up, derivatives of 3-(trifluoromethyl)aniline (**1**–**5**) regardless of the type of the terminal substituent, have considerably reduced amphetamine-evoked hyperactivity, thus the

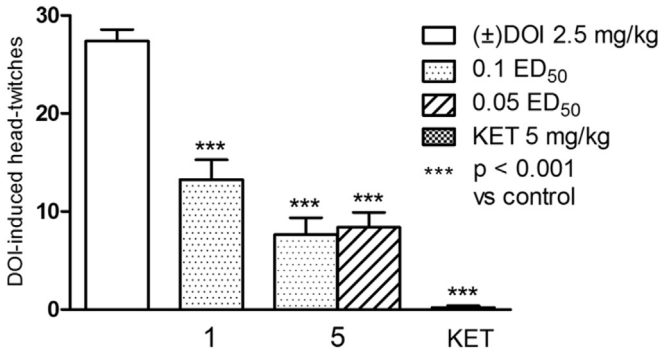



**Fig. 8.** The effect of compounds **1** and **5** on (±)DOI-induced head twitches reaction in mice. The investigated compounds (i.p.) were administrated 60 min before (±)DOI (i.p.). ***p < 0.001 versus (±)DOI treated group (one-way ANOVA followed by Bonferroni's post hoc test).

**Table 3**
Effect of compounds **1** and **5** on (±)DOI-induced head twitches reaction (HTR) in mice.

| Treatment | Dose (mg/kg) | Head twitches/20 min (mean ± SEM) | % of HTR reduction |
|---|---|---|---|
| (±)DOI | 2.5 | 27.38 ± 1.19 | |
| **1** | 28.6 | 13.25 ± 2.03 | 51.6 |
| **5** | 73.1 | 7.62 ± 1.74 | 72.2 |
| | 36.55 | 8.37 ± 1.55 | 69.4 |
| Ketanserin (KET) | 5 | 0.0 ± 0.0 | 100 |

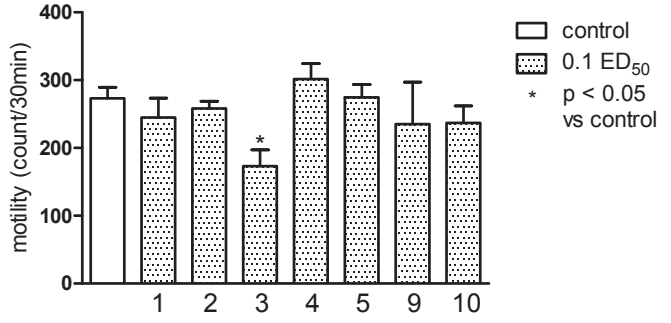



**Fig. 9.** The influence of new derivatives on the spontaneous locomotor activity of mice. Compounds tested were injected 60 min before the test. Locomotor activity was measured for a period of 30 min. Data are expressed as mean ± SEM values. *p < 0.05 vs. control group (Dunnett's test).

involvement between the observed activity and the catecholamine system is supposed. The common feature of behaviorally tested derivatives was also the ability to diminish the number of L-5-HTP-induced head twitch episodes in mice – for derivatives **1** and **5** it was linked with their direct antagonistic impact on 5-$HT_{2A}$ receptors. However, *N*-alkylphenylthiourea derivatives (**4**, **5**, **9**, **10**) were most potent and multidirectional in performed behavioral tests than their aryl analogues. The connection of the analgesic potency of phenylethyl (**4**, **10**) benzyl (**5**, **9**) and also 2-chlorophenyl (**3**) derivatives with endogenous opioid system was confirmed. The presence of an alkylphenyl fragment attached to thiourea in **4**, **5** and **9** was responsible for their anticonvulsant properties. In contrast to other derivatives, the sedative effect of thiourea analogue **3** on spontaneous activity was denoted. The administration of *N*-arylthioureas **1** and **2** induced hyperthermia, however alkylphenyl connections of thiourea **9** and **10** caused decreasing effect on the body temperature in animals. No compounds exerted a depressant activity on the CNS in rodents.

It is worth noting that the presented CNS-activity of derivatives bearing 3-(trifluoromethyl)phenyl moiety is more broad than the potency of 4-chloro-3-nitrophenylthioureas. The first group was dominant by strong 5-$HT_{2A}$ receptor antagonists, as they reduced head twitch evoked by both L-5-HTP and DOI. Compounds of these groups expressed also comparable analgesic activity in writhing test and strongly influenced the body temperature in mice. However, the tendency of 3-(trifluoromethyl)phenylthioureas to diminish drug-induced hyperlocomotion tend to be more evident. All observed effects seem to be connected primarily with serotonergic and/or opioid systems.

## 3. Conclusion

Our study performed for the series of 1,3-disubstituted thiourea derivatives **1**–**10** provided new information in the field of structural properties responsible for affinity and selectivity toward the 5-$HT_{2A}$ receptor, comparing to the 5-$HT_{2C}$. The molecular modeling demonstrated the role of three structural parameters: two terminal aromatic fragments and at least one thiourea NH group. (3-Trifluoromethyl)phenyl ring and 3-chloro-4-fluorophenyl (compound **1**) or benzyl (**5**) moieties were particularly profitable for affinity and selectivity for the 5-$HT_{2A}$ receptor. 4-Chloro-3-nitrophenyl part at the thiourea nitrogen was unfavorable for affinity for both 5-$HT_2$ receptors. Hydrogen bond between NH group and Asp155 residue of the receptor was the main stabilizing interaction. For the derivative **5** also other hydrogen bonds and π–π interactions with the receptor were decisive. These two most active compounds, along with **3** and **10** (S-isomer) occupied upper portion of the receptor binding site. On the other hand, **2**, **4**, **6**–**9**, **10**

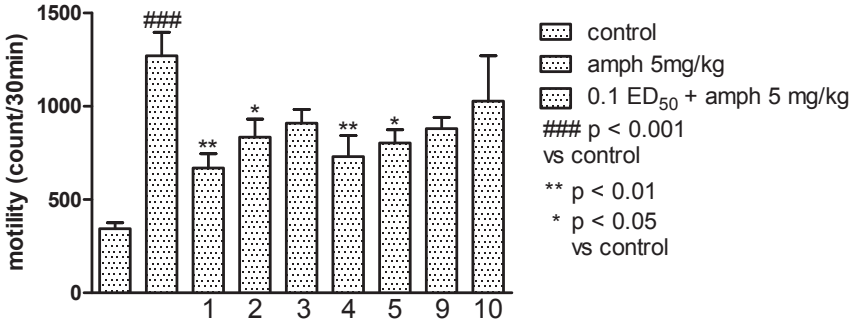



**Fig. 10.** The influence of new derivatives on the amphetamine-induced hyperactivity of mice. Compounds tested were injected 60 min and amphetamine 5 mg/kg 30 min before the test. Locomotor activity was measured for a period of 30 min. Data are expressed as mean ± SEM values. **p < 0.01, *p < 0.05 vs. control group (Dunnett's test).

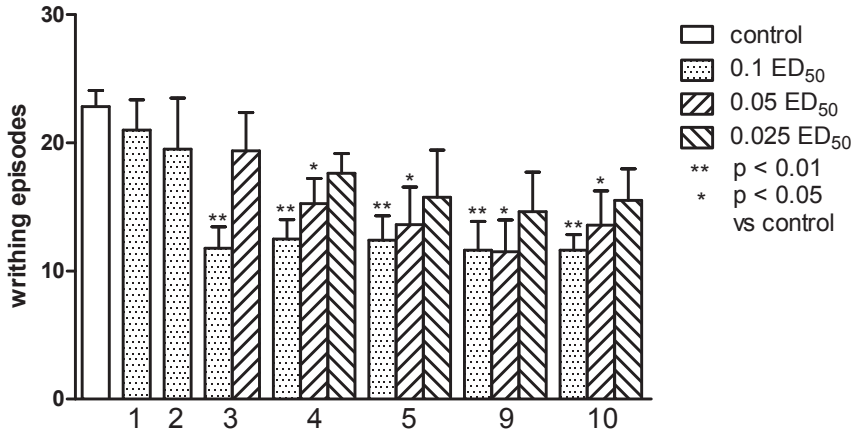



**Fig. 11.** The influence of the new derivatives on nociceptive reactions in the writhing test in mice. Compounds tested were injected 60 min and acetic acid (0.6% solution) 5 min before the test. Data are expressed as mean ± SEM values. *p < 0.05; **p < 0.01 and ***p < 0.001 vs. control group (Dunnett's test).

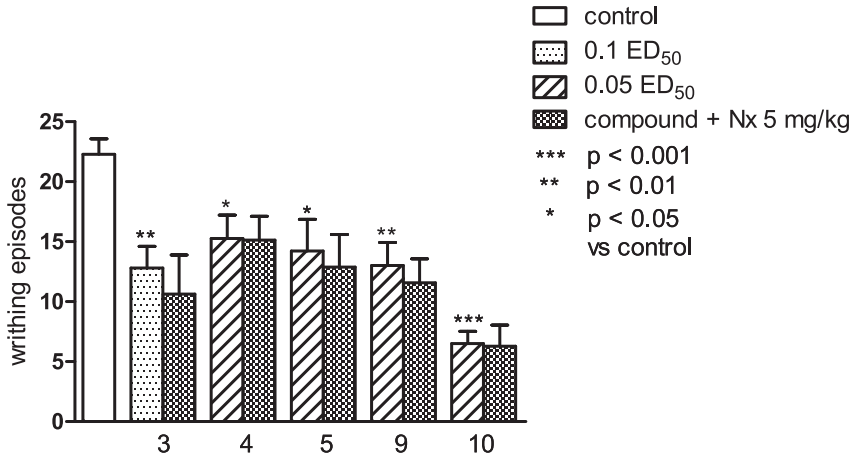



**Fig. 12.** The effect of Naloxone on the antinociceptive effects of compounds **3, 4, 5, 9** and **10** assessed in the writhing test in mice. The results are expressed as mean ± SEM values.

**Table 4**
Effect of new derivatives on the pentylenetetrazole (PTZ)-induced clonic seizures, tonic convulsions and death of mice.

| Comp. | Clonic seizures[a] | Tonic convulsions[a] | Mortality[b] |
|---|---|---|---|
| Control (PTZ 110 mg/kg) | 12/12 | 8/12 | 7/12 |
| **1** (0.1 $ED_{50}$) | 10/10 | 8/10 | 6/10 |
| **2** (0.1 $ED_{50}$) | 10/10 | 5/10 | 5/10 |
| **3** (0.1 $ED_{50}$) | 9/10 | 8/10 | 7/10 |
| **4** (0.1 $ED_{50}$) | 8/10 | 2/10* | 1/10* |
| **4** (0.05 $ED_{50}$) | 10/10 | 4/10 | 4/10 |
| **5** (0.1 $ED_{50}$) | 6/10* | 2/10* | 2/10 |
| **5** (0.05 $ED_{50}$) | 8/10 | 8/10 | 8/10 |
| Control (PTZ 110 mg/kg) | 16/16 | 15/16 | 15/16 |
| **9** (0.1 $ED_{50}$) | 5/10* | 5/10* | 5/10* |
| **9** (0.05 $ED_{50}$) | 8/10 | 6/10 | 5/10* |
| **10** (0.1 $ED_{50}$) | 10/10 | 8/10 | 8/10 |

*$p < 0.05$ vs. control (Fisher's exact test).
[a] Number of mice reacting/animals tested.
[b] Number of dead mice/animals tested.

(R-isomer) were gathered at the bottom of the pocket, that resulted in lack of affinity.

A strict correlation between docking results, binding affinity studies and *in vivo* tests was observed. Compounds **1** and **5** were strong and selective ligands of the 5-$HT_{2A}$ receptor (according to *in vitro* data), deeply penetrating the active site of the receptor protein that was revealed by molecular modeling studies. The *in vivo* evaluation exhibited their distinct antagonistic potency in DOI-evoked HTR test. The accordance between theoretically favorable position in the receptor pocket and head twitch inhibiting potency in 5-HTP-induced HTR test was also proved for derivatives **3** and **10**. Since ligands of the 5-$HT_{1A}$ receptor could also diminish head twitch shakes [17,19]. That serotonergic affinity was confirmed experimentally for **2** and **9** by their influence on the body temperature in laboratory animals. In general, compounds **1**–**5**, **9**, **10** have exerted strong influence on the central nervous system of laboratory animals.

The results of *in vivo* and *in vitro* investigations, along with the molecular docking studies on the 5-$HT_{2A}$ receptor, have provided a valuable basis for further structural design in the search for novel potential antipsychotics based on the structure of 1,3-disubstituted thiourea derivatives. Extended studies concerning affinity and selectivity towards the 5-$HT_{1A}$, α-adrenergic and dopaminergic receptors develop their full pharmacological profile.

## 4. Experimental

### 4.1. Chemistry

#### 4.1.1. General procedure

The starting amines (3-(trifluoromethyl)aniline and 4-chloro-3-

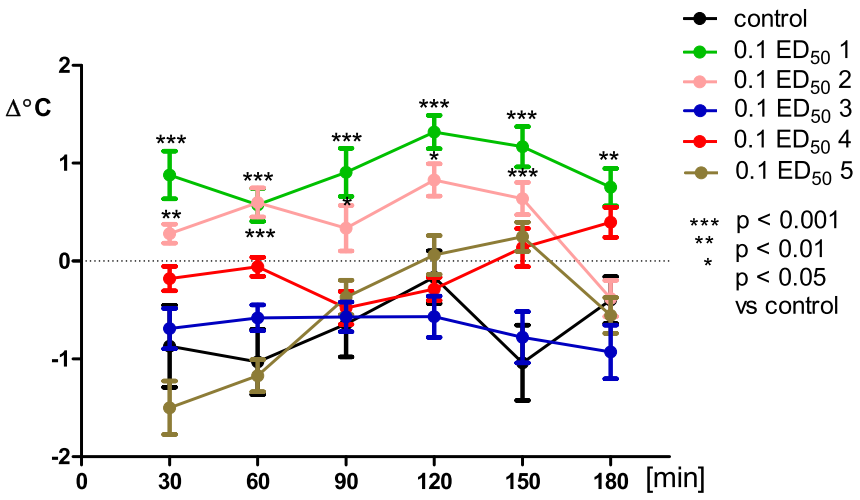



**Fig. 13.** The influence of derivatives **1**–**5** (used at the dose of 0.1 $ED_{50}$) on the mice body temperature. Body temperature was measured over a total period of 240 min (60 min before and 180 min after the tested compound injection). Data are expressed as mean ± SEM values. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control group (Bonferroni's test).

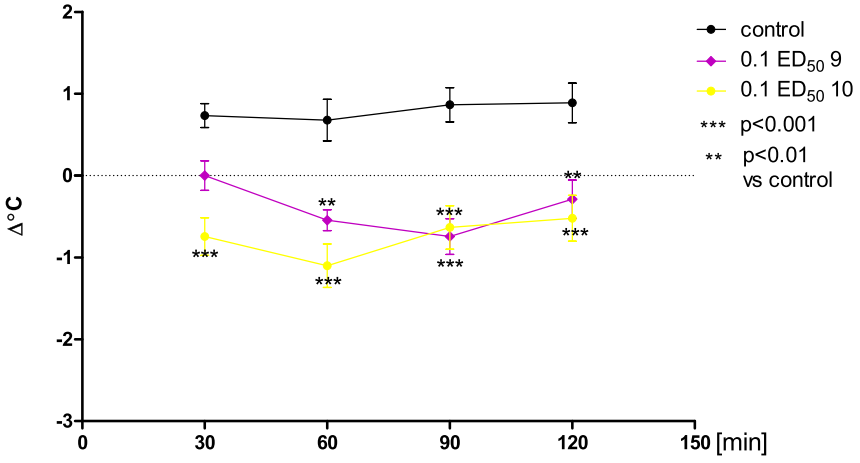



**Fig. 14.** The influence of derivatives **9** and **10** (used at the dose of 0.1 $ED_{50}$) on the mice body temperature. Body temperature was measured over a total period of 180 min (60 min before and 120 min after the tested compound injection). Data are expressed as mean ± SEM values. *p < 0.05 and **p < 0.01 vs. control group (Bonferroni's test).

nitroaniline) were commercially available (Alfa Aesar). Isothiocyanates were supplied from Alfa Aesar or Sigma Aldrich. Organic solvents (acetonitrile, chloroform and methanol) were supplied from POCh (Polskie Odczynniki Chemiczne). All chemicals were of analytical grade and were used without any further purification. Prior usage, dry acetonitrile was kept in crown cap bottles over anhydrous phosphorus pentoxide (Carl Roth). The NMR spectra were recorded on Varian VNMRS 300 Oxford NMR spectrometer, operating at 300 MHz ($^1$H NMR) and 75.4 MHz ($^{13}$C NMR). Chemical shifts (δ) were expressed in parts per million relative to tetramethylsilane used as the internal reference. Mass spectral ESI measurements were carried out on Waters ZQ Micro-mass instruments with quadruple mass analyzer. The spectra were performed in the negative or positive ion mode at a declustering potential of 40–60 V. The sample was previously separated on a UPLC column (C18) using UPLC ACQUITYTM system by Waters connected with DPA detector. Elemental analyses were carried out on a Carlo Erba model 1106. Flash chromatography was performed on Merck silica gel 60 (230–400 mesh) using chloroform eluent. Analytical TLC was carried out on silica gel F254 (Merck) plates (0.25 mm thickness). The diffraction data for **2** and **6** were collected at 120(2) K on a SuperNova diffractometer (Oxford Diffraction) equiped with the microfucus X-ray source (Cu Kα, λ = 1.54184 Å) and CCD detector. The CRYSALIS program system [53] was used for data collection, cell refinement and data reduction. The data were corrected for Lorentz and polarization effects. A multi-scan absorption correction was applied. The structure was solved using direct methods implemented in the SHELXS-97, and refined and refined by the full-matrix least-squares on $F^2$ with the SHELXL-97 program [54]. All non-H atoms were refined with anisotropic displacement parameters. The H-atoms attached to carbon were positioned geometrically and refined using the riding model with $U_{iso}(H) = 1.2U_{eq}(C)$. The nitrogen bonded H-atoms were found in the difference-Fourier map and refined with isotropic displacement parameters.

#### *4.1.2. General procedure for the synthesis of N-substituted 3-(trifluoromethyl)phenyl)thiourea (**1**–**5**) and (4-chloro-3-nitrophenyl)thiourea derivatives (**6**–**10**)*

A solution of commercially available amines: 3-(trifluoromethyl)aniline (0.0031 mol, 0.50 g) or 4-chloro-3-nitroaniline (0.0029 mol, 0.50 g) in anhydrous acetonitrile (10 mL) was treated with appropriate isothiocyanate in 1:1 M ratio. The mixture was stirred at room temperature for 12–36 h. After

solvent evaporating, the residue was either crystallized from acetonitrile (or chloroform) or purified by column chromatography (chloroform).

The synthesis of 1-(3-chloro-4-fluorophenyl)-3-[3-(trifluoromethyl)phenyl]thiourea (**1**), 1-(2-phenylethyl)-3-[3-(trifluoromethyl)phenyl]thiourea (**4**) and 1-benzyl-3-[3-(trifluoromethyl)phenyl]thiourea (**5**) has been described recently [55]. The method of preparation of derivatives 1-(4-chloro-3-nitrophenyl)-3-phenylthiourea (**6**), 1-(4-chloro -3-nitrophenyl)-3-(2-chlorophenyl)thiourea (**7**), 1-benzyl-3-(4-chloro-3-nitrophenyl) thiourea (**9**) and 1-(4-chloro-3-nitrophenyl)-3-(1-phenylethyl) thiourea (**10**) has also been presented [56].

**(1)** Anal. calcd for $C_{14}H_9ClF_4N_2S$: C 48.22, H 2.60, N 8.03, S 9.19; found: C C 48.31, H 2.60, N 8.04, S 9.20.
**(4)** Anal. calcd for $C_{16}H_{15}F_3N_2S$: C 59.25, H 4.66, N 8.64, S 9.89; found: C 59.47, H 4.67, N 8.66, S 9.90.
**(5)** Anal. calcd for $C_{15}H_{13}F_3N_2S$: C 58.05, H 4.22, N 9.03, S 10.33; found: C 58.26, H 4.21, N 9.04, S 10.35.
**(7)** Anal. calcd for $C_{13}H_9Cl_2N_3O_2S$: C 45.63, H 2.65, N 12.28, S 9.37; found: C 45.53, H 2.63, N 12.24, S 9.36.
**(9)** Anal. calcd for $C_{14}H_{12}ClN_3O_2S$: C 52.26, H 3.76, N 13.06, S 9.96; found: C 52.20, H 3.76, N 13.08, S 9.93.
**(10)** Anal. calcd for $C_{15}H_{14}ClN_3O_2S$: C 53.65, H 4.20, N 12.51, S 9.55; found: C 53.48, H 4.19, N 12.48, S 9.56.

*4.1.2.1. 1-(4-Methoxyphenyl)-3-[3-(trifluoromethyl)phenyl]thiourea (**2**).* Yield 70%, white crystals, m.p. 143–144.5 °C. FT-IR (KBr, $cm^{-1}$): 3185.5 (ν N–H); 3025.7, 3004.9 (ν arC–H); 1596.9 (δ N–H); 1548.2 (ν C N); 1449.8 (ν arC–C); 1328.9 (δ arC–H); 1279.4 (ν C–O–C); 1160.6, 1115.4 (δ arC–H); 1069.4 (ν C═S); 1039.5 (ν C–O–C); 724.6 (ν C═S). $^1$H NMR (300 MHz, DMSO) δ (ppm): 9.83 (m, 2H, NH), 7.96–7.93 (m, 1H, Ar–H), 7.75 (d, 1H, $J$ = 8.4 Hz, Ar–H), 7.54 (t, 1H, $J$ = 7.9 Hz, Ar–H), 7.44 (d, 1H, $J$ = 7.8 Hz, Ar–H), 7.35–7.30 (m, 2H, Ar–H), 6.95–6.88 (m, 2H, Ar–H), 3.75 (s, 3H, $OCH_3$). $^{13}$C NMR (75.4 MHz, DMSO) δ (ppm): 179.98, 156.79, 140.59, 131.67, 129.33, 128.64 (q), 127.26, 126.08 (2C), 124.08 (q), 120.37 (q), 119.76 (q), 113.83 (2C), 55.81. ESI MS: $m/z$ = 349.0 $[M+Na]^+$ (100%). Anal. calcd for $C_{15}H_{13}F_3N_2OS$: C 55.21, H 4.02, N 8.58, S 9.83; found: C 55.15, H 4.03, N 8.59, S 9.82.

*Crystal data* for **2:** $C_{15}H_{13}F_3N_2OS$, $M_w$ = 326.33; crystal system orthorhombic, space group $Pna2_1$, unit cell dimensions $a$ = 13.538(3) Å, $b$ = 14.789(2) Å, $c$ = 7.293(1) Å, $V$ = 1460.2(4) $Å^3$, $Z$ = 4; $D_{calc}$ = 1.484 g/$cm^3$, μ = 2.321 $mm^{-1}$, $F$(000) = 672; θ range 4.43–73.73°, reflections collected/independent/observed 20306/2934/, Goodness-of-fit on $F^2$ 0.889, final $R$ indices $[I > 2\sigma(I)]$ $R1$ = 0.0285, $wR2$ = 0.0764, residual electron density max/min 0.25/−0.23 e $Å^{-3}$. CCDC No. 1457555.

*4.1.2.2. 1-(2-Chlorophenyl)-3-[3-(trifluoromethyl)phenyl]thiourea (**3**).* Yield 85%, white powder, m.p. 162–163 °C. FT-IR (KBr, $cm^{-1}$): 3296.1, 3189.6 (ν N–H); 3030.8, 3004.7 (ν arC–H); 1595.2 (δ N–H); 1546.3 (ν C N); 1509.9, 1450.1 (ν arC–C); 1334.5, 1167.6, 1125.3 (δ arC–H); 1069.3 (ν C═S); 870.1 (δ C–Cl); 721.6 (ν C═S). $^1$H NMR (300 MHz, DMSO) δ (ppm): 10.17 (s, 1H, NH), 9.69 (s, 1H, NH), 8.02 (m, 1H, Ar–H), 7.80–7.77 (m, 1H, Ar–H), 7.60–7.46 (m, 4H, Ar–H), 7.39–7.34 (m, 1H, Ar–H), 7.32–7.26 (m, 1H, Ar–H). $^{13}$C NMR (75.4 MHz, DMSO) δ (ppm): 179.69, 140.19, 132.58, 130.06, 129.63, 129.33 (q), 128.41, 126.98, 125.19, 124.22, 123.09 (q), 122.01, 120.79 (q), 119.84 (q). ESI MS: $m/z$ = 353.2 $[M+Na]^+$ (100%). Anal. calcd for $C_{14}H_{10}ClF_3N_2S$: C 50.84, H 3.05, N 8.47, S 9.69; found: C 50.89, H 3.04, N 8.49, S 9.66.

*4.1.2.3. 1-(4-Chloro-3-nitrophenyl)-3-phenylthiourea (**6**).* Yield 56%, pale yellow powder, m.p. 163–165 °C. FT-IR (KBr, $cm^{-1}$): 3323.5, 3161.3 (ν N–H); 3089.6, 2982.8 (ν arC–H); 1592.0 (δ N–H); 1527.6 (ν N–O); 1482.0 (ν arC–C); 1407.5 (δ ar(1,4)C–C); 1362.7 (ν N–O); 1298.8, 1187.6, 1143.4 (δ arC–H); 1047.1 (ν C═S); 863.7 (δ C–Cl); 695.2 (ν C═S). $^1$H NMR (300 MHz, DMSO) δ: 10.15 (s, 2H, NH), 8.35 (d, 1H, $J$ = 2.4 Hz, Ar–H), 7.81 (dd, 1H, $J_1 = J_2$ = 2.4 Hz, Ar–H), 7.70 (d, 1H, $J$ = 9.0 Hz, Ar–H), 7.47–7.44 (m, 2H, Ar–H), 7.37 (t, 2H, $J$ = 7.8 Hz, Ar–H), 7.18 (t, 1H, $J$ = 7.35 Hz, Ar–H). $^{13}$C NMR (75.4 MHz, DMSO) δ: 179.95, 147.09, 140.05, 139.08, 131.53, 128.92, 128.62, 125.31, 124.20, 119.98, 119.65. ESI MS: $m/z$ = 330.0 [M+Na]+ (100%). Anal. calcd for $C_{13}H_{10}ClN_3O_2S$: C 50.73, H 3.28, N 13.65, S 10.42; found: C 50.92, H 3.28, N 13.67, S 10.44.

*Crystal data for* **6**: $C_{13}H_{10}Cl\ N_3O_2\ S$, $M_w$ = 307.75, crystal system triclinic, space group P-1; unit cell dimensions $a$ = 6.836(2) Å, $b$ = 8.012(3) Å, $c$ = 12.542(4) Å, α = 76.88(2)°, β = 88.62(2)°, γ = 89.54(2)°, $V$ = 668.8(4) $Å^3$; $Z$ = 2, $D_{calc}$ = 1.528 g/$cm^3$, μ = 4.041 $mm^{-1}$, $F$(000) = 316; θ range 3.62–73.22°, reflections collected/independent/observed 4269/2589/2363; Goodness-of-fit on $F^2$ 1.071, final $R$ indices $[I > 2\sigma(I)]$ $R1$ = 0.0335, $wR2$ = 0.0864, residual electron density max/min 0.30/-0.33 e $Å^{-3}$. CCDC No. 1457556.

*4.1.2.4. N-[(4-chloro-3-nitrophenyl)carbamothioyl]benzamide (**8**).* The synthesis of **8** was described previously [57], however without spectral data presentation.

Yield 72%, cream powder, m.p. 168–170 °C. FT-IR (KBr, $cm^{-1}$): 3333.7 (ν N–H); 2998.9 (ν arC–H); 1672.8 (ν C═O); 1587.7 (δ N–H); 1524.4 (ν N–O); 1483.4 (ν arC–C); 1327.5 (ν N–O); 1275.6, 1187.7, 1143.1 (δ arC–H); 1042.5 (ν C═S); 881.5 (δ C–Cl); 683.4 (ν C═S). $^1$H NMR (300 MHz, DMSO) δ: 12.67 (s, 1H, NH), 11.79 (s, 1H, NH), 8.60 (d, 1H, $J$ = 2.1 Hz, Ar–H), 8.00–7.96 (m, 3H, Ar–H), 7.81 (d, 1H, $J$ = 9.0 Hz, Ar–H), 7.67 (t, 1H, $J$ = 7.35 Hz, Ar–H), 7.55 (t, 2H, $J$ = 7.5 Hz, Ar–H). $^{13}$C NMR (75.4 MHz, DMSO) δ: 179.64, 167.74, 146.66, 137.75, 132.97, 131.66, 131.31, 129.72, 128.44 (2C), 128.18 (2C), 121.58, 121.12. ESI MS: $m/z$ = 334.0 $[M-H]^-$ (100%). Anal. calcd for $C_{14}H_{10}ClN_3O_3S$: C 50.08, H 3.00, N 12.51, S 9.55; found: C 49.92, H 3.00, N 12.47, S 9.56.

### 4.2. In vitro tests

#### 4.2.1. Binding affinity studies

*4.2.1.1. General procedures.* The synthesized compounds were tested for *in vitro* affinity for serotonin 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors by radioligand binding assays. All the compounds were dissolved in 5% DMSO. The following specific radioligands and tissue sources were used: (a) serotonin 5-$HT_{2A}$ receptor, [$^3$H]ketanserin, rat brain cortex; (b) serotonin 5-$HT_{2C}$ receptor, [$^3$H]mesulergine, rat brain cortex.

Non-specific binding was determined as described below, and specific binding as the difference between total and non-specific binding. Blank experiments were carried out to determine the effect of 5% DMSO on the binding and no effects were observed. Competition experiments were analyzed by PRISM 5 (GraphPadPrism®, 1992–2007, GraphPad Software, Inc., La Jolla, CA, USA) to obtain the concentration of unlabeled drug that caused 50% inhibition of ligand binding ($IC_{50}$), with six concentrations of test compounds, each performed in triplicate. The $IC_{50}$ values obtained were used to calculate apparent inhibition constants ($K_i$) by the method of Cheng and Prussoff [58], from the following equation: $K_i = IC_{50}/(1 + S/K_D)$ where S represents the concentration of the hot ligand used and $K_D$ its receptor dissociation constant ($K_D$ values, obtained by Scatchard analysis [59], were calculated for each labeled ligand).

#### 4.2.2. 5-$HT_{2A}$ and 5-$HT_{2C}$ binding assays

Radioligand binding assays were performed as previously reported by Herndon et al. [60]. Briefly, frontal cortical regions of male Sprague–Dawley rats (180–220 g) were dissected on ice and homogenized (1:10 w/v) in ice-cold buffer solution (50 mM Tris HCl, 0.5 mM EDTA, and 10 mM $MgCl_2$ at pH 7.4) with a Polytron PT10 (setting 5 for 15 s) and centrifuged at 3000 *g* for 15 min. The pellet was resuspended in buffer (1:30 w/v), incubated at 37 °C for 15 min and then centrifuged twice more at 3000 *g* for 10 min (with resuspension between centrifugations). The final pellet was resuspended in buffer that also contained 0.1% ascorbate and $10^{-5}$ M pargyline.

Assays were performed in triplicate in a 2.0 mL volume containing 5 mg wet weight of tissue and 0.4 nM [$^3$H]ketanserin hydrochloride (47.3 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) for 5-$HT_{2A}$ receptor assays, and 10 mg wet weight of tissue and 1 nM [$^3$H]mesulergine (83.1 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) for 5-$HT_{2C}$ receptor assays. Cinanserin (1.0 μM) was used to define nonspecific binding in the 5-$HT_{2A}$ assay. In the 5-$HT_{2C}$ assays, mianserin (1.0 μM) was used to define nonspecific binding, and 100 nM spiperone was added to all tubes to block binding to 5-$HT_{2A}$ receptors. Tubes were incubated for 15 min at 37 °C, filtered on Schliecher and Schuell (Keene, NH, USA) glass fiber filters presoaked in polyethylene imine, and washed with 10 mL of ice-cold buffer. Filters were counted at an efficiency of 50%.

### 4.3. Molecular docking studies

The sequences of human 5-$HT_{2A}$ receptor (UniProt entry: P28223) and the sequence of beta2-adrenergic receptor (β2AR) were aligned using ClustalW program [61] and manually adjusted in order to obtain correct position of corresponding transmembrane regions (see Supplementary material, Fig. S2, Supplementary Materials). Molecular model of 5-$HT_{2A}$ receptor was constructed applying MODELLER program [62] using (PDB ID: 2RH1) as a template. The protein fragments including: N-terminal domain (residues Met1 to His70), second intracellular loop (residues Val269 to Thr311) and C terminal domain (residues Gln398 to Val471) have been omitted during structure prediction, because they have no impact on predicted receptor binding site. In the next step, molecular dynamics simulations (MD) were performed to optimize the geometry and packing of amino acids side chains. The receptor model was inserted into POPC membrane and solvated with water molecules. The simulation box consisted of 115 POPC lipids, 11424 water molecules and 5 $Cl^-$ ions. During the MD simulation lasting 10 ns the weak position restraints were applied on protein backbone atoms in order to preserve its initial conformation. The modified version of GROMOS force field [63] (parameter set 53A6) was used and simulation time step was equal to 2 fs The final step of model refinement included energy minimization using 2000 steps of steepest descent algorithm. All simulation were performed using GROMACS v. 4.6.5 program [64].

Molecular structures for 10 analyzed compounds were constructed using Automated Topology Builder server (ATB Version 2.2) [65]. Ligand docking to receptor model and analysis of resulting ligand-receptor binding modes were conducted using AutoDock4 (v. 4.2) and AutoDockTools4 [66]. For each of 10 ligands 1000 independent docking calculations were performed using Lamarckian Genetic Algorithm (LGA), (see Supplementary materials for detailed description of docking parameters, Fig. S3, Supplementary Materials). Ligand molecules were fully flexible during molecular docking procedure whereas receptor remained rigid. For each of 10 compounds the most favorable binding mode was identified by means of structural clustering (with RMSD 2 Å cutoff) of the set of 1000 lowest energy conformers obtained during docking procedure. The central structure of the largest observed cluster was selected as the final ligand binding conformation. Visual inspection and analysis of resulting complexes and ligand-receptor interactions were performed using VMD program [67].

### 4.4. In vivo tests

The experiments were carried out on male Albino Swiss mice (18–30 g). The animals were kept 8–10 to a cage under standard laboratory conditions (at a temperature of 20 ± 1 °C and a 12 h light/dark cycle) with free access to food (LSM, Motycz, Poland) and water. All experiments were performed between 9:00 a.m. and 4:00 p.m., according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and to the European Community Directive for the Care and Use of Laboratory of 24 November 1986 (86/609/EEC), and approved by the Local Ethics Committee for Animal Experimentation (51/2013 i 73/2015).

The investigated substances in all tests were administered intraperitoneally (i.p.), as a suspensions in aqueous solution of 0.5% methylcellulose (tylose) and were injected 60 min before the tests. All substances were administered in a volume of 10 mL/kg. The control animals received an equivalent volume of the solvent at the respective time before the test. All tests performed, suggested by Vogel and Vogel [47], are generally accepted as basic in investigation of the central activity by behavioral methods. The acute toxicity of the compound was assessed in mice acc. to Litchfield and Wilcoxon method [68] as the $ED_{50}$ calculated as "the loss of righting reflex" within 48 h: after an i.p. administration of tested compounds, an observation of mice was carried out in the following time intervals: in 15-, 30-, 60-, 180-min and 24, 48 h. Each compound in behavioral experiments was injected in doses equivalent to 0.1, 0.05, 0.025, 0.0125 $ED_{50}$. In addition, the activity of compounds was assessed in the following tests:

- locomotor activity was measured for single mice in photoresistor actometers (circular cages, diameter 25 cm, two light beams, Multiserv, Poland; the number of crossed light beams by the mice was recorded) for 30 min as:
  a) spontaneous activity
  b) amphetamine-induced hyperactivity: mice received subcutaneously (s.c.) 5 mg/kg of amphetamine 30 min before the test;
- nociceptive reactions were studied in the acetic acid (0.6%)-induced 'writhing' test [26,69] The number of writhing episodes was measured for 10 min starting 5 min after i.p. administration of acid solution;
- motor coordination was evaluated in rota-rod test [70]; and chimney test [71].
- body temperaturdarmanie in normothermic mice was measured in the rectum by thermistor thermometer during a total period of 180 min (60 min before and 120 min after tested compound injection). The mean value from the first two measurements (60 and 30 min before drug administration) was assumed as initial temperature (ti). The final temperature (tf) was measured 30, 60, 90 and 120 min after the injection of tested compounds at a dose of 0.1 ED50. Body temperature changes (Δt) were calculated according to the formula:

$$\Delta t = tf - ti$$

- pentylenetetrazole (110 mg/kg, s.c.)-induced convulsions were evaluated as the number of mice with clonic seizures, tonic convulsions and dead animals;

- HTR after 5-hydroxytryptophan (5-HTP), acc. to Corne et al. [72]. Mice received 5-HTP (230 mg/kg, i.p.) and the number of HTR was recorded in 6 2-min intervals (4–6, 14–16, 24–26, 34–36, 44–46, 54–56 min).
- head twitches induced by ((±)-DOI (2.5 mg/kg). Immediately after treatment, the head twitches were counted throughout 20 min [73]. The investigated compounds were administered 60 min before ((±)-DOI.

### 4.5. Statistics

Obtained data were calculated by Fisher exact test (PTZ-induced seizures), two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test (body temperature) and one-way ANOVA followed by Dunnett's post hoc test (other tests). Each group of animals consisted of 8–12 mice. All results are presented in the figures as mean ± SEM. A level of $p < 0.05$ was considered as statistically significant. All the figures were prepared by the GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, California, USA), www.graphpad.com.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.03.073.

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