Article

In silico and *in vivo* assessment of L-17, a thiadiazine derivative with putative serotonin reuptake properties

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Abstract: L-17 is a thiadiazine derivative with putative anti-inflammatory, neuroprotective, and antidepressant-like properties. In this study, we applied combined *in silico* and *in vivo* electrophysiology techniques to reveal the potential mechanism of action of L-17. PASS 10.4 Professional Extended software suggested that L-17 might have pro-cognitive, antidepressant, and antipsychotic effects. Docking energy assessment with AutoDockVina predicted that the binding affinities of L-17 to the serotonin transporter (SERT) and serotonin receptors 3 and 1A (5-HT_3 and 5-HT_{1A}) are compatible to the selective serotonin reuptake inhibitor (SSRI) fluoxetine and selective antagonists of 5-HT_3 and 5-HT_{1A} receptors, granisetron and WAY100135, respectively. However, while the binding mechanisms of L-17 to the SERT and 5-HT_{1A} receptor were similar to fluoxetine and WAY100135, its interacting with 5-HT_3 receptor might be substantially different from this of granisetron. Acute administration of L-17 led to dose-dependent inhibition of firing activity of 5-HT neurons of the dorsal raphe nucleus. This inhibition was partially reversed by subsequent administration of WAY100135. Based on both *in silico* and *in vivo* electrophysiology assessments, we suggest that L-17 is a potent 5-HT reuptake inhibitor and a putative partial agonist of 5-HT_{1A} receptors. As such, L-17 in particular and thiadiazine derivatives, in general, might be a representative of a new class of antidepressant drugs. Since L-17 also possesses neuro- and cardioprotective properties, it can be useful in affective illness developing due to the general medical condition, such as post-stroke and post-myocardial infarction (MI) depression.

Keywords: thiadiazines; serotonin transporter (SERT); serotonin receptors 3 and 1A (5-HT_3 and 5-HT_{1A}), docking energy, binding affinity, binding mechanisms, electrophysiology *in vivo*

1. Introduction

L-17 (2-morpholino-5-phenyl-6H-1,3,4-thiadiazine, hydrobromide; Fig. 1) is a thiadiazine derivative, synthesized by cyclocondensation of α-bromoacetophenone with the original morpholine-4-carbothionic acid hydrazide [1]:

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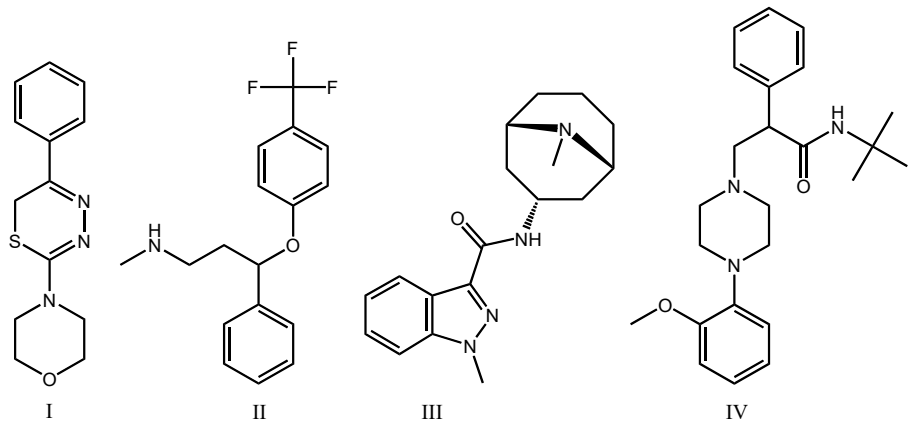

Figure 1. Structural chemical formulas of 2-morpholino-5-phenyl-6H-1,3,4-thiadiazine, hydrobromide (I) (L-17), Fluoxetine (II), Granisetron (III) and WAY100135 (IV).

In our previous studies, L-17 showed a putative therapeutic effect in animal models of myocardial infarction (MI) [2, 3] and pancreatitis [4]. L-17 was also reported to attenuate the immune system response to the immobilization stress in rats, suggesting an immunostimulatory effect of this compound during the stress copying [5]. Neuroprotective [1] and antidepressant-like [6] effects of L-17 in rats were demonstrated as well.

Serotonin (5-HT) is a brain neurotransmitter that is fundamental in the pathophysiology of depression and antidepressant response. The central anti-neuroinflammatory and neuroprotective effects of 5-HT are well established as well. There are also studies reporting the peripheral anti-inflammatory effect of 5-HT in MI [7] and pancreatitis [8]. It is therefore likely that the 5-HT system is a target of L-17 [6].

As in our previous study [9], we used a combination of *in silico* and *in vivo* experimental techniques to test the hypothesis that the anti-inflammatory, neuroprotective, and antidepressant-like effect of L-17 is mediated, at least in part, *via* the targeting of the 5-HT system. The 3-D model of L-17 was constructed, and its interaction with different 5-HT targets, such as serotonin transporter (SERT) and 5-HT receptors 3 and 1A (5-HT_3/5-HT_{1A}) was examined, used *in silico* calculation of the minimum binding energy. Finally, the real-time *in vivo* effect of L-17 administration on the excitability of 5-HT neurons of the rat dorsal raphe nucleus (DRN) was examined using extracellular single-unit electrophysiology under chloral hydrate anesthesia.

2.1. *In silico* predictions

The following pharmacological activities were predicted for L-17 by the PASS 10.4 Professional Extended [10]: Cognition disorders treatment (Pa = 0.489, Pa/Pi = 27.17), Phobic disorders treatment (Pa = 0.703, Pa/Pi = 9.37), Psychotropic (Pa = 0.258, P /Pi = 1.74), Immunostimulant (Pa = 0.191, Pa/Pi = 1.39), and Antidepressant (Pa = 0.162, Pa/Pi = 1.14) one. According to joint predictive evaluations in PASS and experimental data [1, 6], the most likely targeted activities corresponding to the serotoninergic effects of compound L-17 were suggested: 5-HT_3 antagonism (Pa = 0.139, Pa/Pi = 1.17), 5-HT release inhibition (Pa = 0.225, Pa/Pi = 1.01), and 5-HTreuptake blockade (Pa = 0.470, Pa/Pi = 6.35).

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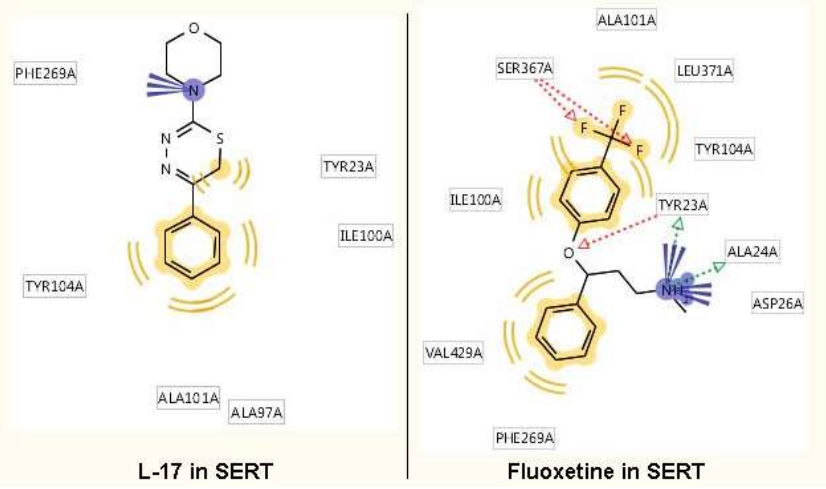
While L-17 compound manifests itself as an atypical mild antipsychotic and antidepressant [1], for the subsequent analysis of its multitarget mechanism of action the serotonin transporter (SERT), the serotonin receptor type 3A (5-HT3A), and the serotonin receptor type 1A (5-HT1A) were chosen as target proteins. Table 1 summarizes the docking energy (ΔE), binding affinity (pK), and relative affinity (RA; L-17th pK as a percent of pK of the corresponding binding compound) of L-17, fluoxetine, granisetron, and WAY100135 with the SERT and 5-HT$_3$ and 5-HT$_{1A}$ receptors, assessed with the AutoDockVina 1.1.2 software [11]:

Table 1. Docking energy, binding affinity, and relevant affinity of L-17 to the SERT and 5-HT$_3$ and5-HT$_{1A}$ receptors, compared with the reference compounds: fluoxetine, granisetron, and WAY100135.

Target	**SERT**			**5-HT$_3$**			**5-HT$_{1A}$**		
Molecule	ΔE, kcal/mol	pK	RA	ΔE, kcal/mol	pK	RA	ΔE, kcal/mol	pK	RA
L-17	-8.1	5.90	0.87	-6.6	4.81	0.94	-7.9	5.78	0.85
Fluoxetine	-9.3	6.78	—	—	—	—	—	—	—
Granisetron	—	—	—	-7.0	5.10	—	—	—	—
WAY100135	—	—	—	—	—	—	-9.3	6.78	—

Figure 2 illustrates the binging mechanisms of L-17, fluoxetine, granisetron, and WAY100135 with the SERT and 5-HT$_3$and 5-HT$_{1A}$ receptors, predicted using the LigandScout 4.2.1 software [12]:



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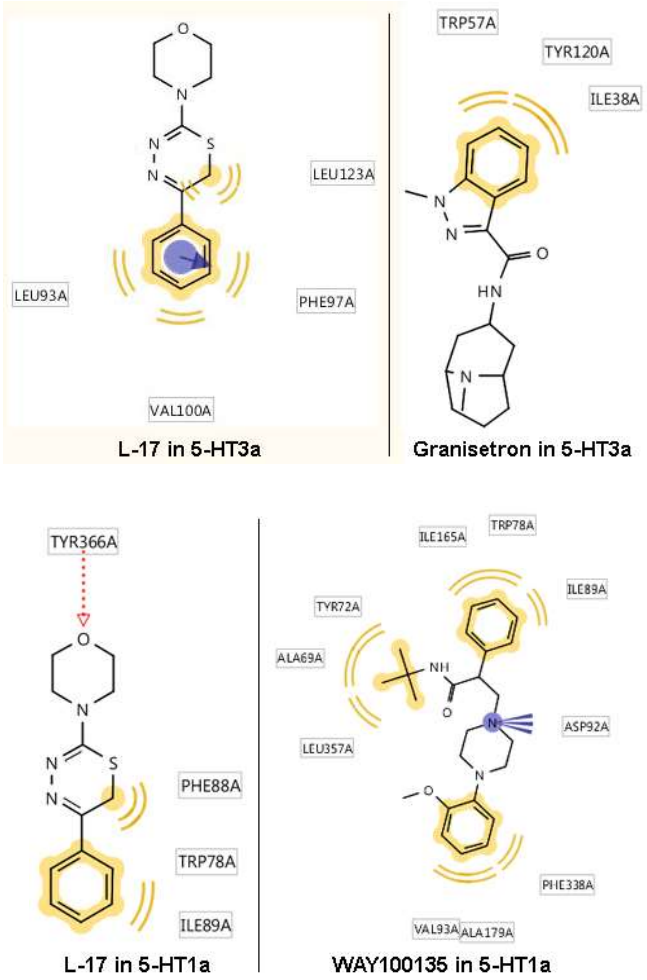

Figure 2. Binging mechanisms of L-17, fluoxetine, granisetron, and WAY100135 with the SERT and 5-HT_3 and 5-HT_{1A} receptors

The morpholine cycle nitrogen atom of L-17 is engaged in a p-π exchange with PHE269 of SERT. If protonated, this nitrogen forms a region of undirected electrostatic interaction. Two more structural elements of L-17 participate in five nonspecific hybrid interactions with TYR23, ALA97, ILE100, ALA101, TYR104 of the SERT. Fluoxetine is being banded to the SERT *via* the electrostatic interaction of protonated NH_2 group. Three additional groups of L-17 form five hydrogen bonds with TYR23, ALA24, ASP26, SER367x. Three more structural elements participate in six nonspecific hybrid interactions with ILE100, ALA101, TYR104, PHE269, LEU371, VAL429.

L-17 might bind to the 5-HT_3 receptor by stacking with PHE97. Two structural elements of L-17 participate in four nonspecific hybrid interactions withLEU93, PHE97, VAL100, LEU123. No stacking is observed in granisetron binding to a 5-HT_3 receptor. Fixation is exerted by three nonspecific hydrophobic interactions with ILE38, TRP57, TYR120.

The morpholine cycle oxygen atom of L-17 forms a hydrogen bond with TYR366 of the binding site of the 5-HT_3 receptor. Two structural elements of L-17 form three nonspecific hydrophobic links with TRP78, PHE88, and ILE89 of the binding site of5-HT_3 receptor. The

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protonated nitrogen atom of the morpholine cycle of WAY100135 exerts electrostatic interaction with TYR366. Two WAY100135 molecule fragments participate in three nonspecific hydrophobic interactions with ALA69, TYR72, TRP78, ILE89, VAL93, ILE165, ALA179, PHE338, LEU357 of the binding site of the 5-HT_3 receptor.

Figure 3 illustrates the poses of L-17, fluoxetine, granisetron, and WAY100135 with the binding sites of SERT and 5-HT_3 and 5-HT_{1A} receptor:

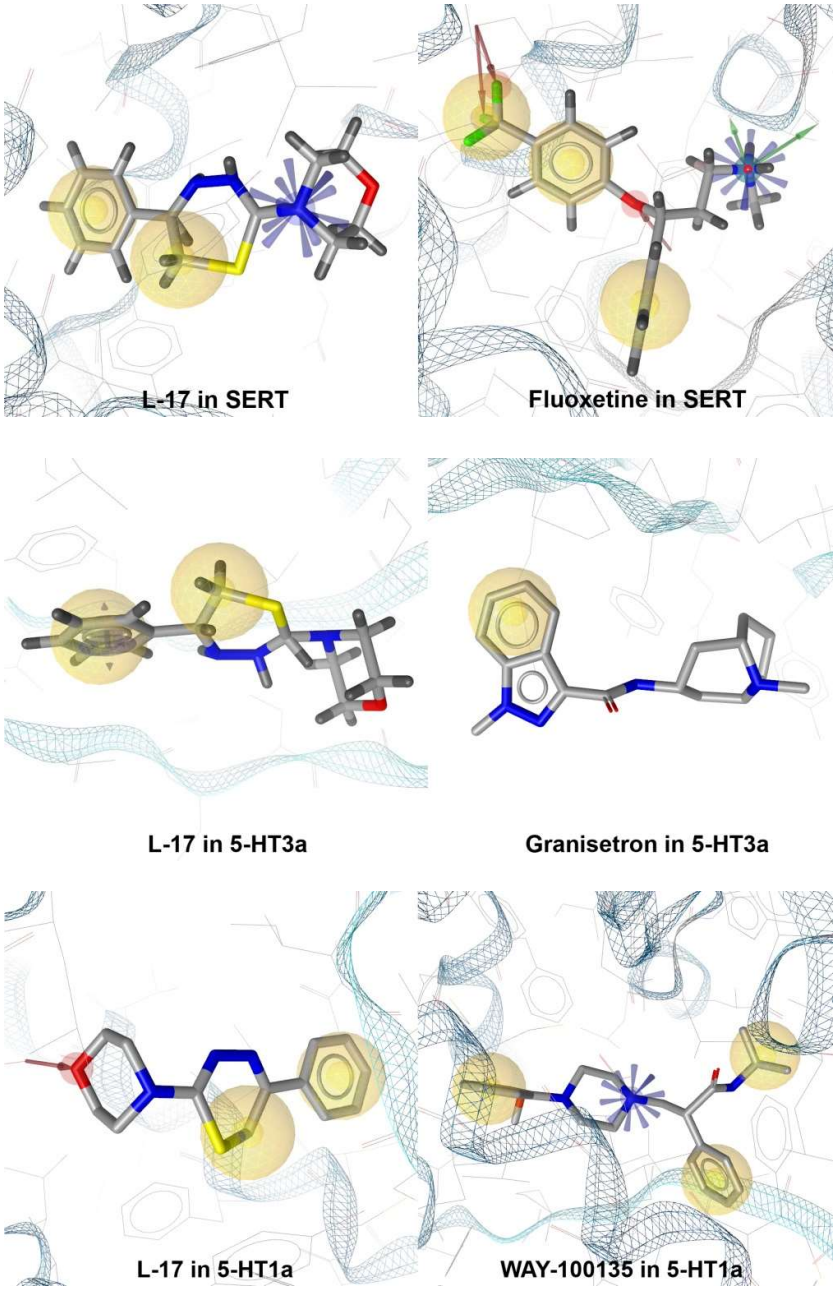

Figure 3. Poses of L-17, fluoxetine, granisetron, and WAY100135 with the binding sites of SERT and 5-HT_3 and 5-HT_{1A} receptor

The pose of L-17 within the SERT binding site is remarkably close to this of fluoxetine. There is also a partial similarity between L-17 and WAY100134 poses within the binding site of the 5-

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HT_{1A} receptor. The poses of L-17 and granisetron within the binding site of the 5-HT3 receptor are remarkably different.

2.2. *In vivo* electrophysiology

The mean basal firing activity was 3.46±0.83 Hz. L-17 (0.1–12 mg/kg, i.v.) significantly and dose-dependently ($F_{8,53}$=4.84, p<0.001, ANOVA for repeated measured) inhibited the firing activity of 5-HT neurons, reaching the maximal 90%-inhibition at 12 mg/kg. WAY100135 partially reversed the L-17-induced inhibition of 5-HT neurons (Fig 3):

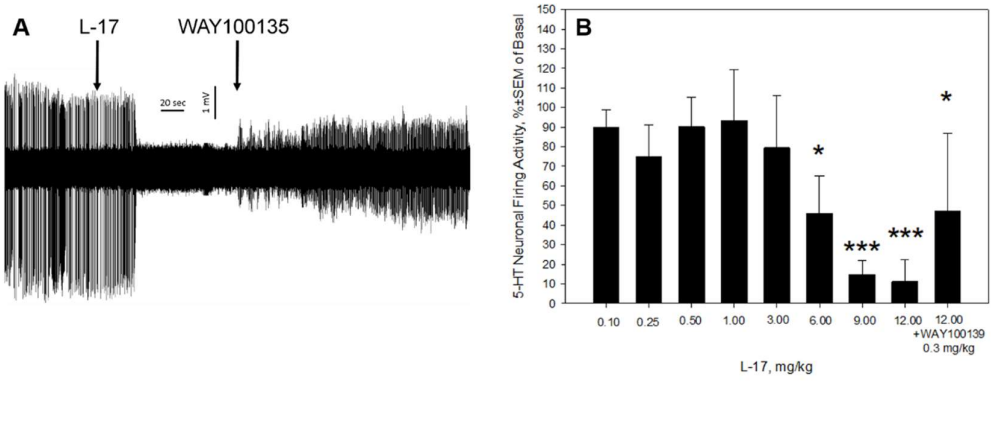

Figure 4: L-17 significantly and dose-dependently inhibited the firing activity of 5-HT neurons. A: representative recording from a 5-HT neuron during L-17 (12 mg/kg) and WAY100135 (0.3 mg/kg) administration. B: Summary effect of L-17 (0.1-12 mg/kg) and WAY100135 (0.3 mg/kg) on the spontaneous firing activity of 5-HT neurons of the DRN(data from 8 neurons from 7 rats).

3. Discussion

In this study, we performed a complex *in silico* assessment of pharmacotherapeutic properties of L-17, as well as in vivo electrophysiological assessment of the effect of this compound on the firing activity of 5-HT neurons of the rat DRN. PASS 10.4 Professional Extended[10] software predicted antidepressant-like properties of L-17. This prediction is consistent with the previously reported antidepressant-like effect of L-17 in rats [6]. PASS 10.4 Professional Extended had also predicted that the antidepressant-like effect of L-17 can be explained, at least in part, *via* its 5-HT reuptake inhibition property.

AutoDockVina 1.1.2 [11] docking energy assessment predicted that L-17 has an affinity to the SERT comparable with this of fluoxetine,5-HT_3 binding affinity similar to this of granisetron, and 5-HT_{1A} binding affinity comparable to WAY100135. The prediction of the molecular interactions between L-17 and the binding sites of SERT and 5-HT_3 and 5-HT_{1A} receptors with LigandScout 4.2.1 software [12] revealed that the mechanisms of L-17 binding to the SERT and 5-HT_{1A} receptor are similar to these of fluoxetine and WAY100135, respectively. On the other hand, L-17 binding to 5-HT_3 receptors is completely different from this of granisetron. Consistently, the topographic poses of L-17 within SERT and 5-HT_{1A} receptor binding sites are remarkably similar to these of fluoxetine and WAY100135, respectively. The prospective pose of L-17 within the binding site of the 5-HT_3 receptor is fundamentally different from this of granisetron. It is thus possible that L-17 is a potent blocker of

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the SERT and an antagonist or a partial agonist of 5-HT$_{1A}$ receptors. Further investigations should be performed to assess the functional character of L-17 interaction with 5-HT$_3$ receptors.

We found that acute intravenous administration of L-17 significantly and dose-dependently inhibited the firing activity of 5-HT neurons of the DRN. Similar inhibitory effects on 5-HT neuronal firing activity were observed with other SSRIs, such as citalopram [13], escitalopram [13, 14], Wf-516 [15], or paroxetine [16]. Similar to what was observed with other SSRIs, the L-17-induced inhibition of 5-HT neuronal firing activity was reversed by WAY100135. It is this likely that L-17 is acting as a potent SERT blocker. Inhibition of the SERT by acute L-17 leads to an increase in extracellular 5-HT levels, activation of 5-HT$_{1A}$ autoreceptors, and reduction of firing activity of 5-HT neurons. The subsequent blockade of 5-HT$_{1A}$ autoreceptors reverses the inhibition of 5-HT neurons.

On the other hand, unlike escitalopram-induced suppression of 5-HT neuronal firing activity, which was completely reversed by 0.1 mg/kg of WAY100135 [13, 14], the L-17-induced inhibition of 5-HT neurons was reversed by WAY100135 only partially, even though WAY100135 was administered at 0.3 mg/kg. It is thus possible that L-17 interacts with a molecular target(s) other than SERTs. One of these targets predicted by *in silico* tests is 5-HT receptors. It is possible that L-17 acts, in addition to its function as a SERT blocker, as a partial agonist of 5-HT$_{1A}$ receptors. The competition between L-17 and WAY100135 on 5-HT$_{1A}$ receptors putatively prevents complete WAY100135-mediated recovery of firing activity of 5-HT neurons. Another possibility might be, however, a direct interaction of L-17 with α_1-adrenoceptors[17].

Summarizing, the antidepressant-like properties of L-17, reported in previous studies, can be explained, at least in part, by the ability of this compound to modulate central 5-HT neurotransmission. The L-17-induced modulation of 5-HT transmission is likely to be mediated, at least in part, *via* the inhibition of 5-HT reuptake and partial antagonism to 5-HT$_{1A}$ receptors. We cannot however exclude the interaction of L-17 with receptors modulation the excitability of 5-HT neurons, such as 5-HT$_{1A}$,5-HT$_3$, and/or $\alpha_{1/2}$-adrenergic receptors. It is also possible that L-17 directly interacts with other neurotransmitter systems, such as norepinephrine, dopamine, and/or histamine. Further electrophysiological and receptor binding studies should be performed to tests these hypotheses. The previously antidepressant-like properties of L-17 and its modulatory effect on the 5-HT system, reported in this study, suggest that L-17 might be an effective antidepressant drug. Since L-17 also possesses neuro- and cardioprotective properties, it can be useful in affective illness developing due to the general medical condition, such as post-stroke and post-MI depression.

4. Materials and Methods

4.1. *In silico* predictions

Using the PASS 10.4 Professional Extended software [10], the presence(Pa) or absence (Pi) probability of 480 systemic types of pharmacotherapeutic activity was calculated. Promising activities were those with Pa ≥0.1 and Pa/Pi ≥1.0.

Further, for a comparative evaluation of the L-17 compound's affinity to the selected biotargets, the docking of the compound to the specific binding sites of these proteins was performed. Five experimental X-ray 3D models of SERT were obtained from Protein Data Bank in Europe[18]. Among these five models, the longest one (PDB code 5I6X), with the maximum resolution including an

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inhibitor, was chosen to allow the unambiguous determination of the binding site position [19]. The experimental 3D models for 5-HT_3 and 5-HT_{1A}receptors were not available in Protein Data Bank in Europe, therefore a search for the best theoretical 3D models from the Database of Comparative Protein Structure Models [20]was conducted. Among the available models, the longest ones, with the highest statistical significance, were selected for 5-HT_3[21]and 5-HT_{1A}[22]receptors.

The 3D models of L-17 and reference molecules were constructed using the molecular mechanics' methods in the MarvinSketch15.6.15software [23], followed by optimization with the semi-empirical quantum chemical method PM7 in the MOPAC2016 software [24]. The docking was performed using AutoDockVina 1.1.2 [11], five times for each compound into each target and then the spectrum of energies were used to determine the minimum binding energies. To reveal the mechanisms of serotoninergic action of the compound L-17, an analysis of its affinity spectrum in comparison with the affinity spectra of reference compounds was performed. The primary information about reference compounds for target proteins was obtained from the UniProtKB base [25]. For each reference compound found in the UniProtKB, the mechanism of its action was clarified using the DrugBank[26]. In the case of the information in UniProtKB and DrugBank datasets was not sufficient, the search for references was performed in the database of pharmacologically relevant proteins and their ligands IUPHAR [27]. The SSRI fluoxetine and the selective antagonists of 5-HT_3(granisetron) and 5-HT_{1A}(WAY100135) receptors were used as reference molecules. Using the obtained energy spectra of ΔE, the pK values were calculated using the formula:

$$pK = -\lg e^{-\Delta E/RT},$$

where R is a universal gas constant (8.314 J×mol^{-1}×K^{-1}) and T is a temperature, set as 300 K. The molecular binging mechanism of L-17, granisetron, and WAT100135 with SERT and with 5-HT_3 and 5-HT_{1A}receptors was furtherly evaluated using the LigandScout 4.2.1 software [12]. The data on binding sites were found in the available literature. For SERT, the key amino acids of the binding site were Gly94, Ala96, Val97, Asn101, Ser336, Asn368, Leu434, Asp437, Ser438 [19]. For 5-HT_3receptor, the key amino acids of the binding site were Tyr229, Phe221, Asn123, Trp85, Trp178, Tyr148, Arg87, Gln146, Tyr138 [21]. For 5HT_{1A}receptor, the key amino acids of the binding site were Tyr56, Gln57, Asp76, Val77, Ser159, Trp318, Phe321, Phe322, Thr339, Gly342, Ala343, Ile345, Asn346 [22].

4.2 *In vivo* electrophysiology

In vivo electrophysiological assessment of the excitability of 5-HT neurons of the DRN was performed as previously described[9, 28-30]. Adult male Wistar rats, weighing 300-350 g, were ordered from the Animal Breeding Facility of The Institute of Experimental Pharmacology and Toxicology, Center of Experimental Medicine, Slovak Academy of Sciences in DobráVoda, Slovakia. Rats were anesthetized with chloral hydrate (Lambda Life s.r.o., Bratislava, Slovakia; 0.4 g/kg, intraperitoneally: *i.p.*) and maintained in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Rat body temperature was maintained between 36 and 37°C with a heating pad (Gaymor Instruments, Orchard Park, NY, USA). The scalp was opened, and a 3 mm hole was drilled in the skull for insertion of electrodes. Glass-pipettes were pulled with a DMZ-Universal Puller (Zeitz-Instruments GmbH, Martinsried, Germany) to a fine tip approximately 1 μm in diameter and filled with 2M NaCl solution. Electrode impedance ranged from 4 to 6 MΩ. The pipettes were lowered into the DRN (7.8-8.3 mm posterior to bregma and 4.5-7.0 mm ventral to brain surface) [31] by David Kopf

Instruments hydraulic micro-positioner. The action potentials generated by 5-HT neurons were recorded using the AD Instruments Extracellular Recording System (Dunedin, New Zealand). The 5-HT neurons were identified by bi- or tri-phasic action potentials with a rising phase of long duration and regular firing rate of 0.5–5.0 Hz [32].

Author Contributions: Conceptualization, O.C., P.V., E.D, and AS; bioinformatics, P.V., A.K. and A.S.; synthesis, L.S. and O.C.; animal experiments, D.G., R.P. and E.D.; writing—original draft preparation, P.V., E.D., M.R., and AS; writing—review and editing, P.S., P.V., E.D., and AS; funding acquisition, E.D, and AS. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
ANOVA	Analysis of variance
DRN	Dorsal raphe nucleus
L-17	2-morpholino-5-phenyl-6H-1,3,4-thiadiazine, hydrobromide
LC	Locus coeruleus
SERT	Serotonin transporter
SSRI	Selective serotonin reuptake inhibitor
VTA	Ventral tegmental area

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