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Abstract

Schizophrenia is a disabling mental illness that affects approximately 1% of the world population. The treatment of this disorder is based on two generations of substances, typical antipsychotics, such as haloperidol, and atypical antipsychotics, such as clozapine, which can cause severe adverse effects. Therefore, the development of novel molecules that are safe and efficacious to treat the disease is crucial. PT-31 is a putative α2-adrenoceptor agonist effective against schizophrenia positive and cognitive symptoms in mice. C. elegans is an alternative model that has been successfully used to investigate the toxicity of a variety of substances. The present study aimed to evaluate the potential toxicity of the new molecule PT-31 and the antipsychotics haloperidol and clozapine in C. elegans. The evaluation was carried out based on toxicity endpoint tests, survival, developmental and behavioral assays. The antipsychotics haloperidol and clozapine decreased nematode survival by 30 and 40%, respectively, exposing the potential toxicity of these substances whereas PT-31 was safer based on this parameter. Similar results were obtained in the nematode developmental assay: haloperidol and clozapine significantly reduced nematode body length and area, whereas PT-31 preserved the normal development of the nematodes. The behavioral assessment was based on the frequency of body bends; none of the antipsychotics affected the locomotion rate of the nematodes, and PT-31 also did not compromise this parameter, demonstrating the safety of this new compound and reinforcing the recognized toxicity of antipsychotics.

Keyword: PT-31; C. elegans; alternative model, toxicity evaluation.

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Evaluation of the potential toxicity of haloperidol, clozapine and a new putative antipsychotic molecule, PT-31, in an alternative toxicity model, *C. elegans*

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Abstract

Schizophrenia is a disabling mental illness that affects approximately 1% of the world population. The treatment of this disorder is based on two generations of substances, typical antipsychotics, such as haloperidol, and atypical antipsychotics, such as clozapine, which can cause severe adverse effects. Therefore, the development of novel molecules that are safe and efficacious to treat the disease is crucial. PT-31 is a putative α2-adrenoceptor agonist effective against schizophrenia positive and cognitive symptoms in mice. C. elegans is an alternative model that has been successfully used to investigate the toxicity of a variety of substances. The present study aimed to evaluate the potential toxicity of the new molecule PT-31 and the antipsychotics haloperidol and clozapine in C. elegans. The evaluation was carried out based on toxicity endpoint tests, survival, developmental and behavioral assays. The antipsychotics haloperidol and clozapine decreased nematode survival by 30 and 40%, respectively, exposing the potential toxicity of these substances whereas PT-31 was safer based on this parameter. Similar results were obtained in the nematode developmental assay: haloperidol and clozapine significantly reduced nematode body length and area, whereas PT-31 preserved the normal development of the nematodes. The behavioral assessment was based on the frequency of body bends; none of the antipsychotics affected the locomotion rate of the nematodes, and PT-31 also did not compromise this parameter, demonstrating the safety of this new compound and reinforcing the recognized toxicity of antipsychotics.

Keywords: PT-31; C. elegans; alternative model, toxicity evaluation.

1. INTRODUCTION

Schizophrenia is a complex syndrome that affects approximately 21 million people worldwide [1, 2]. The illness presents a diversity of symptoms categorised as positive (hallucinations and delusions), negative (incapacity to feel pleasure) and cognitive (impaired working memory, altered perception). It is one of the most debilitating diseases, being associated with low employment rates and difficulties in daily life that compromise the patient's autonomy [3].

The pathophysiology of schizophrenia is not well understood. The underlying neurochemical mechanisms hypothesized are the dopaminergic and the glutamatergic; however, it is known that other neurotransmitters are also involved [4]. Most pharmacological treatments for schizophrenia are based on selective dopamine blockade, the mechanism of action of typical antipsychotics, such as haloperidol. Due to their selectivity, these antipsychotics improve only the positive symptoms of the disease, but they fail to treat the negative and cognitive symptoms [5] in addition to triggering extrapyramidal effects, drug-induced Parkinsonism, prolactin elevation, and other less frequent effects, such as hypogonadism leading to sexual dysfunction in men and galactorrhea in women [6, 7]. The second-generation antipsychotics, or atypical antipsychotics, have clozapine as their main representative, a substance also approved for the treatment of resistant schizophrenia [8]. Clozapine targets multiple receptors also causes several adverse effects, such as weight gain, cardiovascular diseases, endocrine disturbances and more serious effects, such as hematological alterations [9, 10, 11].

The noradrenergic system has shown to be a promising target for the development of new neuroleptics. Evidence suggests that norepinephrine plays an important role in cognitive functions in the prefrontal cortex [12]. In this perspective, the Therapeutic Innovation Research Group, from the Federal University of Pernambuco, developed an imidazolidine derivative 3-(2-chloro-6-fluorobenzyl)-imidazolidine-2,4-dione, PT-31 (Figure 1), a putative agonist of alpha-2A adrenergic receptors [13].

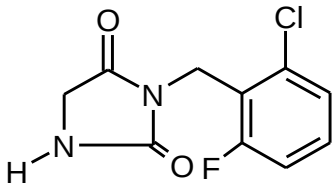


Figure 1. Chemical structure of PT-31.

Studies of the group have demonstrated the potential antipsychotic activity of PT-31 in animal models with positive, cognitive and attentional dysfunction symptoms, without the induction of adverse effects, such as sedation and extrapyramidal symptoms [14]. This molecule also presented neuroprotective effect, demonstrated in an excitotoxicity model [14].

To further contribute to the toxicological evaluation of this molecule, the use of an alternative testing model is of main relevance. *Caenorhabditis elegans* is an alternative model that respects the “three Rs” (reduce, refine and replace) [15] and is widely used for toxicity studies, since this nematode worm provides data representative from a complete animal with fully functioning and metabolically active systems [16]. In addition, its easy maintenance, reproduction and experimentation make it an excellent experimental model. It is also worth mentioning that *C. elegans* has a well-characterized nervous system, presenting the

same neurotransmitters as vertebrates, including acetylcholine, glutamate, γ-amino-butyric acid (GABA), dopamine and serotonin [17, 18].

Within this context, the objective of the present study was to evaluate the potential toxicity of PT-31, as well as the antipsychotics available in the clinic, haloperidol and clozapine, in the alternative model *C. elegans*.

2. Material and Methods

2.1 C. elegans, culture and synchronization

The nematode strain N2 (wild type) was obtained from Caenorhabditis Genetics Center (CGC). The nematodes were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 as food source and kept in a BOD incubator at constant temperature of 20°C [19, 20]. Synchronization, a process to obtain the nematode eggs, was achieved by washing off the gravid nematodes with water from the plates to conical tubes and centrifuging the tubes. Later, the bleaching solution was added: mixture of sodium hypochlorite/ sodium hydroxide (10 M) and water, which was agitated constantly and vigorously for 6 minutes to lyse the worm cuticle. Then, the eggs were separated from the bacterial debris using a 30% sucrose gradient. The eggs obtained were maintained in a BOB® incubator in NGM plates without bacteria to allow hatching overnight (between 13-14 hours all worms were synchronized in larval stage L1) [21, 22].

2.2 Treatment of the nematodes

The antipsychotics haloperidol and clozapine were acquired from Tocris Bioscience®. The molecule PT-31, 3-(2-chloro-6-fluorobenzyl)-imidazolidine-2,4-dione, was synthetized by the Department of Research in Therapeutic Innovation from the Federal University of Pernambuco.

For the treatment of the nematodes, stock solutions containing 10 mM of each compound were prepared in 100% dimethyl sulfoxide (DMSO). From these solutions, the following concentrations were prepared: 80, 160 e 320 µM. Serial dilution from the highest concentration (320 µM) was used to obtain the remaining solutions. A control was prepared containing the highest concentration of DMSO present in the dilutions. 1,500 nematodes synchronized at larval stage L1 were treated for 1 hour in homogenizer with the aforementioned concentrations of haloperidol, clozapine and PT-31, and subsequently seeded onto plates. All assays were conducted in two replicates and repeated in three independent experiments.

2.3 Lethality assay

After 48 hours, the nematodes were counted under dissecting microscope to evaluate the number of live worms per treatment in comparison to control (without treatment). This evaluation aimed to assess the median lethal dose (LD_{50}), which is the dose lethal to 50% of the nematode population, in order to draw a dose-response curve in case of significant mortality [19].

2.4 Developmental assay

After 48 hours of treatment, images of 10 nematodes were taken with help of a dissecting microscope

coupled to a camera. Nematode body length and area were measured using the software ImageJ® [22].

2.5 Behavioral assay

Forty-eight hours after treatment, six nematodes in larval stage L4 of each assayed concentration were transferred to fresh NGM agar plates without E. coli OP50 and kept at 20°C. Nematode locomotion rate was quantified based on the frequency of body bends, the sinusoidal movement of the worm. Each nematode was acclimated for 1 minute, and subsequently, the number of body bends was monitored for 1 minute [23].

2.6 Data analysis

Statistical analysis was carried out on Sigma Stat® using analysis of variance (ANOVA) with post-hoc Student-Newman-Keuls if significant difference, considering $P < 0.05$.

3. Results

Nematode survival rates following treatment with increasing concentrations of haloperidol, clozapine and PT-31 are shown in Figure 2. Clozapine and haloperidol at highest concentration decreased significantly nematode survival rate when compared to control, whereas PT-31 did not impact survival rate. Therefore, the LD_{50} could not be determined.

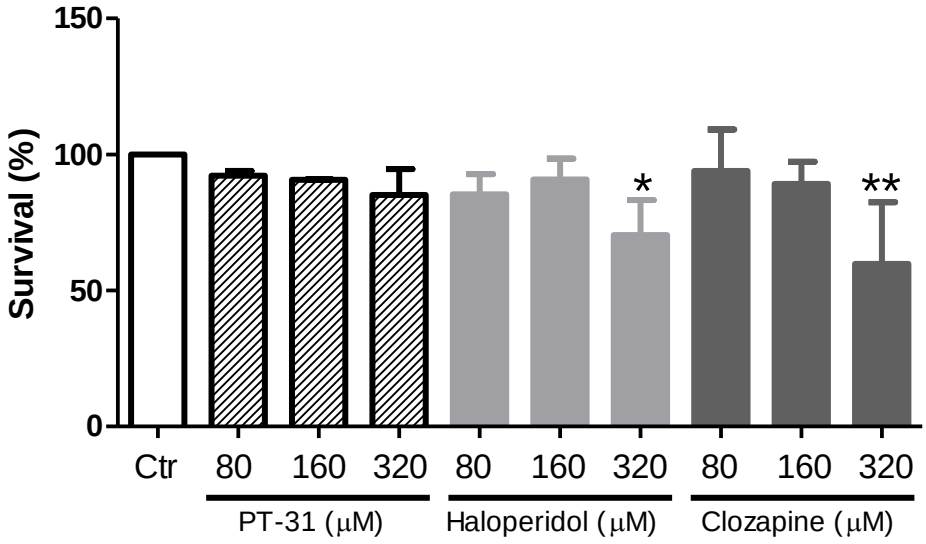
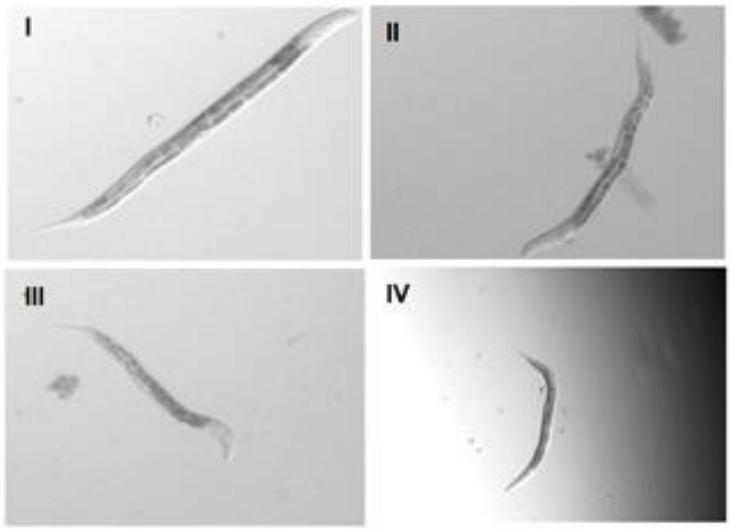


Figure 2. Survival rates of nematodes treated with control (Ctr), PT-31, Haloperidol and Clozapine at concentrations 80, 160 and 320 μM (n = 2 – 4). Data are expressed as mean + S.D. ANOVA with post-hoc Student-Newman-Keuls. F (9,31) = 3.910; P = 0.004. Different from control * P < 0.05.

The development of the nematodes was evaluated based on body length and area. Exposure to increasing concentrations of haloperidol and clozapine reduced significantly nematode length (Figure 3A) and area (Figure 3B) when compared to control; PT-31 did not affect these parameters. Images of *C. elegans*

exposed to the highest concentration (320 µM) of each substance are also presented in Figure 3.



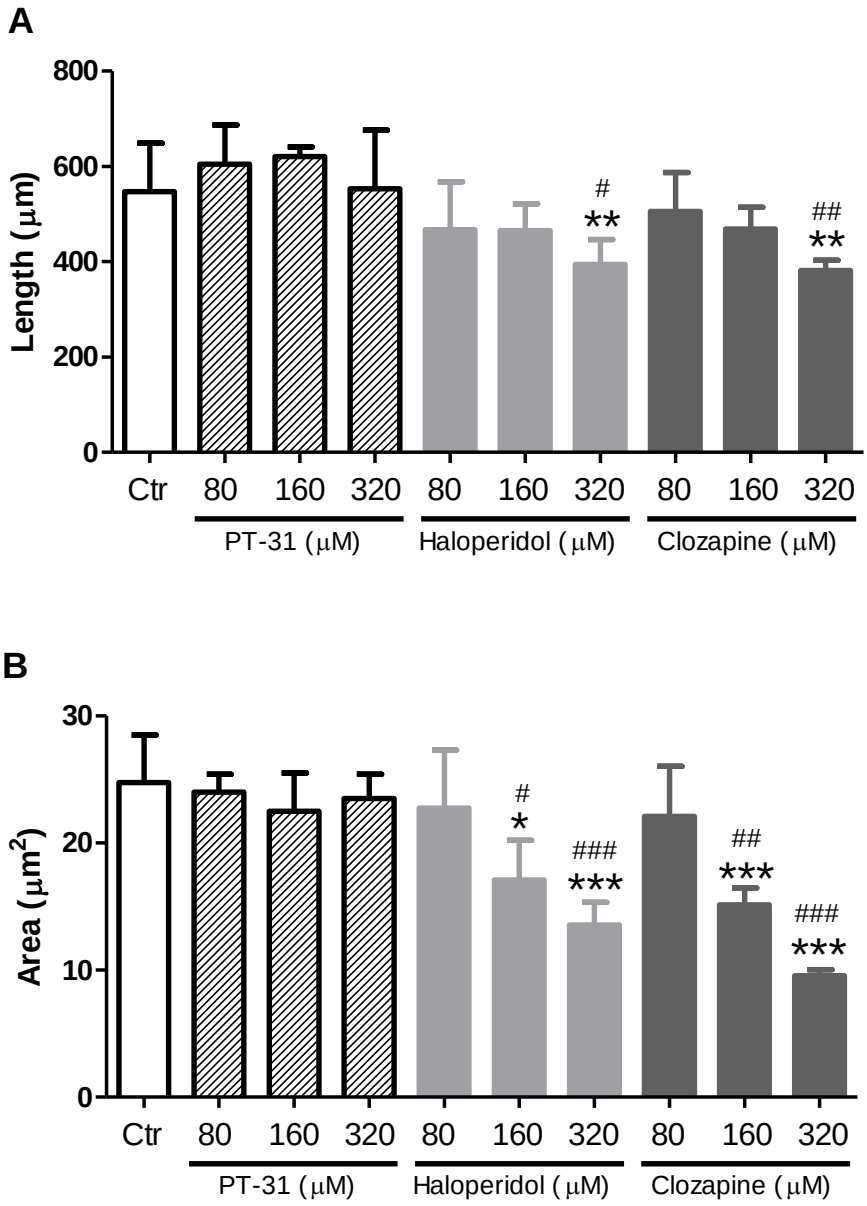


Figure 3. Microscope images of nematodes treated with control (I), haloperidol 320 μM (II), clozapine 320 μM (III) and PT-31 320 μM (IV). Development parameters (A) body length and (B) area of nematodes treated with control (Ctr), PT-31, Haloperidol and Clozapine at concentrations 80, 160 and 320 μM. Data are expressed as mean + S.D. ANOVA with post-hoc Student-Newman-Keuls. $F(9,57) = 5.520$; $P < 0.001$ and $F(9,39) = 13.76$; $P < 0.0001$. Different from control ** $P < 0.01$. Different from PT-31 at the same concentration $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$.

Nematode behavior was monitored based on the frequency of body bends. Following treatment of the nematodes with increasing concentrations of haloperidol, clozapine and PT-31, the usual locomotion behavior was not affected when compared to control (Figure 4).

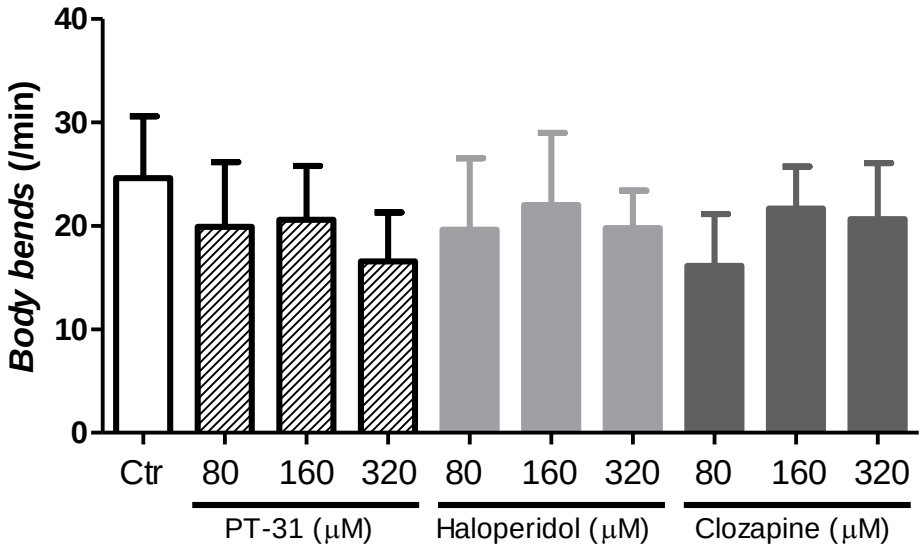


Figure 4. Behavior (body bend frequency) of nematodes treated with control (Ctr), PT-31, Haloperidol and Clozapine at concentrations 80, 160 and 320 μM. Data are expressed as mean + S.D. ANOVA with post-hoc Student-Newman-Keuls. F (9,123) = 2.207; P = 0.023.

4. Discussion

Due to the adverse effects caused by the antipsychotics currently available, it is necessary to develop new drugs with antipsychotic activity, capable of improving the symptoms of schizophrenia with more safety and less side effects. The present study evaluated the toxicity of a new putative antipsychotic molecule, PT-31, and the typical and atypical antipsychotics haloperidol and clozapine in the alternative toxicity testing model *C. elegans*. Based on the evaluations conducted, behavioral, developmental and lethality assays, known as toxicity endpoint tests or toxicity endpoints, it was demonstrated that the antipsychotics haloperidol and clozapine disrupted the normal development and survival of nematodes in the highest concentration tested. In contrast, PT-31 did not compromise these parameters.

Another toxicity endpoint studied was drug-induced lethality, widely assessed to determine the LD_{50} (lethal dose to 50% of test population) of substances through a dose-response curve [24, 22]. Similarly to the present study, Jacques *et al.* [24] and Charão *et al.* [22] also failed to establish an LD_{50}, but assessed the toxicity of different compounds based on nematode survival rate. Charão *et al.* [22] observed a 30% mortality rate in nematodes exposed to paraquat (0.5 mM), suggesting toxicity of this herbicide. Jacques *et al.* [24] demonstrated the toxicity of a solid lipid nanoparticle, which reduced nematode survival rate by 50%. The mortality rates observed in the present study were respectively 30 and 40% following treatment with 320 μM haloperidol or clozapine. By comparison to the aforementioned results of other authors, our data indicated toxicity of the antipsychotics at high concentrations. Contrastingly, PT-31 decreased the survival rate by only 15%, being safer considering this parameter and suggesting absence of toxicity.

The safety of PT-31 was reinforced in the developmental assay, as even the highest concentration of the substance did not reduce significantly nematode body length (552.7 ± 123.5) and area (23.5 ± 1.91) when

compared control (length: 546.9 ± 102.0; area: 24.75 ± 3.77). In contrast, haloperidol and clozapine (320 µM) decreased nematode body length by 28% (394.1 ± 52.93) and 30% (381.3 ± 21.62), respectively. In addition, haloperidol and clozapine respectively decreased nematode body area by 31% (17.10 ± 3.10) and 38% (15.13 ± 1.33) following treatment with 160 µM, and 45% (13.58 ± 1.77) and 61% (9.55 ± 0.48) following treatment with 320 µM. Donohoe *et al.* [25] also observed disruption of development induced by haloperidol and clozapine, with reduction of nematode body area by 45% and 42%, respectively, reinforcing the potential toxicity of these antipsychotics. It is important to highlight that the effects observed by Donohoe *et al.* [25] also occurred at the concentration 160 µM, which impacted nematode body area in the present study.

C. elegans is a testing model that can also be used to evaluate the toxicity of other compounds, such as metals and nanomaterials. Wu *et al.* [26] and Moon et al. [27] evaluated the toxicity of metals based on the length of *C. elegans* worms, showing decrease of 7% in the measurements after nematode treatment with ZnO nanoparticles (50 µg/L) [26] and 22% following treatment with silver nanomaterials [27], therefore suggesting toxicity of these compounds.

Tepper *et al.* [28] proposed that the development of nematodes, assessed through the body length, is related to a conservative genetic regulation pathway. DAF-16 is a transcription factor that activates or inactivates gene regulation. Under normal conditions, DAF-16 allows nematode growth and development; however, stress conditions trigger a modification in DAF-16 generating a stress response that impairs worm development. PT-31 seems to be safer than haloperidol and clozapine, as it did not affect the length and area of the worms in comparison to control.

Another hypothesis raised by Donohoe *et al.* [25] suggests that haloperidol, a D2 dopaminergic antagonist, could compromise worm development through dopaminergic mechanisms. As clozapine does not present preferential inhibition of dopamine, the development of *C. elegans* would be less affected. However, in the present study both antipsychotics disrupted the nematode development to the same extent. The dopaminergic hypothesis is probably based on the fact that the behavior of *C. elegans* is regulated by several neurotransmitters, such as dopamine, serotonin, acetylcholine, glutamate and GABA [29]. Dopamine is responsible for several behavioral functions including locomotion, assessed by means of the frequency of body bends. In addition to dopamine, GABA and acetylcholine are also involved in locomotion: the dorsal and ventral muscles of the worm receive inhibitory GABAergic stimuli for relaxation, as well as excitatory cholinergic stimuli for contraction, creating the “S” movements that drive the worm forward [30].

In the present study, the antipsychotics haloperidol, clozapine and PT-31 did not affect nematode behavior in any tested concentration, suggesting absence of neurotoxicity. Contrastingly, Monte *et al.* [23] observed neurotoxicity following exposure of *C. elegans* to clozapine and haloperidol, with significant reduction of locomotion rate. The referred study was also conducted with wild N2 strains, but the acute exposure assay comprised different concentrations of haloperidol and clozapine: 133 and 459 µM; these concentrations reduced the number of body bends by 15 and 30%, respectively.

The results of our study reinforce the safety of PT-31, based on the first toxicity assessment carried out in this alternative model. This data contrasts with the evidence of toxicity induced by the antipsychotics haloperidol and clozapine, which impacted nematode development and survival in a dose-dependent

manner. In addition to previous studies conducted by the group showing antipsychotic activity of PT-31 in animal models [14], the present study corroborates with the assessment of the safety and efficacy of this molecule.

5. Conclusion

This work reports the first toxicity assessment of PT-31, a new molecule with promising antipsychotic activity, in an alternative animal model, *C. elegans*. PT-31 proved to be non-toxic and safer than haloperidol and clozapine, antipsychotics available in clinical practice, reinforcing the current data available on this molecule for the potential development of a new, safe and effective antipsychotic.

6. Acknowledgements

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