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Article

Cytotoxicity evaluation of haloperidol, clozapine and a new molecule with antipsychotic potential, PT-31, in NIH-3T3 cells

Juliana Machado Kayser[1], Gabriela Zimmermann Prado Rodrigues[2], Carlos Henrique Thomazi[1,2], Alana Witt Hansen[2], Marina Griebeler Moreira[2], Marina Galdino da Rocha Pitta[3], Ivan da Rocha Pitta[3], Ana Luiza Ziulkoski[2], Andresa Heemann Betti[1*]

[1]Bioanalysis Department, Institute of Health Sciences, Feevale University, Novo Hamburgo, Rio Grande do Sul, Brazil, [2]Cytotoxicity Laboratory, Institute of Health Sciences, Feevale University, Novo Hamburgo, Rio Grande do Sul, Brazil, [3]Center for Research in Therapeutic Innovation (NUPIT), Federal University of Pernambuco (UFPE), Recife, Brazil

Schizophrenia is an illness that affects 26 million people worldwide. However, conventional antipsychotics present side effects and toxicity, highlighting the need for new antipsychotics. We aimed to evaluate the cytotoxicity of haloperidol (HAL), clozapine (CLO), and a new molecule with antipsychotic potential, PT-31, in NIH-3T3 cells. The neutral red uptake assay and the MTT assay were performed to evaluate cell viability and mitochondrial activity, morphological changes were assessed, and intracellular reactive oxygen species (ROS) detection was performed. HAL and CLO (0.1 μM) showed a decrease in cell viability in the neutral red uptake assay and in the MTT assay. In addition, cell detachment, content decrease, rounding and cell death were also observed at 0.1 μM for both antipsychotics. An increase in ROS was observed for HAL (0.001, 0.01 and 1 μM) and CLO (0.01 and 1 μM). PT-31 did not alter cell viability in any of the assays, although it increased ROS at 0.01 and 1 μM. HAL and CLO present cytotoxicity at 0.1 μM, possibly through apoptosis and necrosis. In contrast, PT-31 does not present cytotoxicity to NIH-3T3 cells. Further studies must be performed for a better understanding of these mechanisms and the potential risk of conventional antipsychotics.

Keywords: Cytotoxicity. Clozapine. Haloperidol. NIH-3T3 cells. PT-31.

INTRODUCTION

Schizophrenia affects 1% of the world population. Schizophrenic patients have their life expectancy reduced by 20 to 30 years compared to the general population, which is not only due to the disease, but also to its consequences, with suicide and cardiovascular problems being the main contributors to mortality (McGrath *et al.*, 2008; Stahl, 2013). This psychiatric disorder affects more men than women (ratio 1.4:1), but also has hereditary causes (Aleman, Kahn, Selten, 2003).

The disease is treated with typical and atypical antipsychotics. Typical antipsychotics, such as haloperidol, are only effective against schizophrenia positive symptoms, being responsible for sedation and extrapyramidal symptoms, e.g., Parkinsonism, tardive dyskinesia, akathisia, and dystonia. Atypical antipsychotics, such as clozapine, are effective against positive, negative, or cognitive symptoms of schizophrenia. However, it is noteworthy that there is no antipsychotic available on the market that is effective for all symptoms, highlighting the complexity of the disease. Although the atypical antipsychotics have a few adverse effects related to extrapyramidal symptoms, they can cause other relevant side effects or toxicity, including agranulocytosis, the development of cardiovascular diseases, weight gain and

***Correspondence:** A. H. Betti. Departamento de Bioanálises. Instituto de Ciências da Saúde. Universidade Feevale, Campus II. RS 239 2755, 93525-075, Novo Hamburgo, Rio Grande do Sul, Brazil. Phone: +55 51 99291-0680. E-mail: andresa@feevale.br. ORCID: https://orcid.org/0000-0002-7116-5465

metabolic alterations, such as dyslipidemia and diabetes (Hilal-Dandan, Knollmann, Brunton, 2017; Ritter *et al.*, 2018).

Taking into consideration the adverse and toxic effects known for the available antipsychotics, there is a need to search for new molecules with a lower incidence of adverse effects and, primarily, no toxicity. From this perspective, the molecule with antipsychotic potential, PT-31 (Figure 1), a putative α_2-adrenergic agonist, has been studied by our group. Previous studies have shown the efficacy of PT-31 in animal models predictive of positive, cognitive, and attentional symptoms of schizophrenia, with the latter being the major challenges in the treatment of schizophrenia (Betti *et al.*, 2019). In addition, the molecule did not cause extrapyramidal effects or sedation. The first *in vitro* assays, using cerebellar primary cell cultures, demonstrated the protective effect of PT-31 against excitotoxicity (Betti *et al.*, 2019). In addition, the potential toxicity of PT-31 was evaluated in an alternative model of toxicity, against two antipsychotics: haloperidol and clozapine. The evaluation was carried out based on toxicity endpoint tests, such as survival, developmental and behavioral assays. PT-31 was shown to have fewer adverse effects than antipsychotic drugs in all evaluated parameters (Bigolin *et al.*, 2020).

In this context, the aim of this study was to evaluate cell viability and mitochondrial activity as well as analyze the morphological changes caused by the potential antipsychotic PT-31 in the NIH-3T3 cell line, compared to haloperidol and clozapine, which are antipsychotics already available for treatment. Moreover, the effect of PT-31, haloperidol, and clozapine on ROS levels over time was also evaluated.

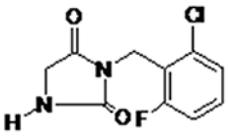

FIGURE 1 - Chemical structure of PT-31, the molecule with antipsychotic potential.

MATERIAL AND METHODS

Drugs and treatments

The compounds haloperidol and clozapine were purchased from Tocris Bioscience (TOCRIS, UK). The compound 3-(2-chloro-6-fluorobenzyl)-imidazolidine-2,4-dione (PT-31) was prepared by the Center for Research in Therapeutic Innovation (NUPIT), of the Federal University of Pernambuco (UFPE), in Recife, Brazil (Sudo *et al.*, 2010).

Cell culture

NIH-3T3 cell lines (mouse embryonic fibroblasts) from the Rio de Janeiro cell bank (BCRJ) were cultured in DMEM (Sigma®) supplemented with 10% fetal bovine serum (FBS) (Gibco®) and maintained at 37ºC in a semi-open system with a humidified atmosphere with 5% carbon dioxide (CO_2). The assays were performed using a density of 1.8 x 10^4 cells per well in 96-well polystyrene microplates, incubated at 37°C for 24 hours.

Cell viability assays

For the exposure to the tested drugs (haloperidol, clozapine and PT-31), the culture medium was replaced with the exposure medium, adding different drug concentrations (0.0001 µM, 0.001 µM, 0.01 µM and 0.1 µM) of each molecule in quadruplicate, and the cultures were maintained for 24 hours under the conditions mentioned above. The tested concentrations were chosen based on the study of Betti *et al.* (2019), which refers to previous PT-31 data. For the negative control, the cells were cultured in only DMEM medium, whereas for the positive control, 1% hydrogen peroxide (Synth®) was added 2 hours before the assays.

Evaluation of morphological changes

The morphological alterations were evaluated using an IX73 inverted optical microscope (Olympus®) coupled to a cellSens image capture system and cellSens Standard 1.7 software (Olympus®). The cells maintained in only the

culture medium were used as a standard morphological structure. Changes in cell detachment, size, structure, and cell death were registered.

Neutral red uptake assay

The cytotoxicity evaluation was performed through the neutral red uptake assay, which assesses cell viability through endocytosis and lysosomal integrity.

After 24 hours of exposure to the test substances, the medium was removed and 0.2 mL/well of serum-free DMEM, containing 50 µg/mL of the neutral red dye, was added. After 3 hours of incubation, the medium was removed from each well and the cells were washed with $CaCl_2$ (1%) in a formaldehyde (0.5%) solution (Nuclear/Synth®). Subsequently, 0.2 mL/well of ethanol/glacial acetic acid solution (50%/1%) was added in ultrapure water for dye solubilization. After 10 minutes of agitation, the content was transferred to another plate for absorbance reading in the M3 Microplate Spectrophotometer (Molecular Devices®) at 540 nm (Borenfreund, Puerner, 1985; Repetto, del Peso, Zurita, 2008).

MTT assay

Mitochondrial functionality was evaluated using the tetrazole salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma®). This assay evaluates mitochondrial functionality, based on the reduction of the tetrazole salt by the mitochondrial dehydrogenases, forming an insoluble purple colored derivative. After 24 hours of exposure to the test medium, 8 µL of MTT (5 mg/mL^{-1}) was added to each well.

The procedure was carried out in a room with low illumination and the plate was wrapped in foil and incubated at 37 °C in a humid atmosphere with 5% CO_2 for 2 hours. After the incubation period, the medium was discarded and 200 µL of DMSO was added for the solubilization of formazan crystals. The supernatant was homogenized and transferred to a new plate. The absorbance was read in a M3 Microplate Spectrophotometer (Molecular Devices®) at 570 nm (Mosmann, 1983; Fotakis, Timbrell, 2006).

Intracellular reactive oxygen species (ROS) detection

Intracellular ROS was evaluated using 2',7'-dichlorodihydrofluorescein (DCFH-DA), a fluorescent probe with membrane permeability that is oxidized into the highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS.

Firstly, the medium was removed from each well and the cells were washed with CMF to remove residual medium. Subsequently, the cells were incubated with DCFH-DA at a final concentration of 40 µM at 37ºC for 1 hour in the dark. After the incubation period, the cells were washed again with CMF to remove the extracellular DCFH-DA and then exposed to the tested drugs (haloperidol, clozapine and PT-31) at different concentrations (0.001 µM, 0.01 µM and 1 µM). For the negative control, the cells were cultured in only DMEM medium, whereas for the positive control, 0.01% hydrogen peroxide (Synth®) was added. The fluorescence intensity of intracellular ROS in treated cultures was read in a M3 Microplate Spectrophotometer (Molecular Devices®) at 388 nm (excitation) and 525 nm (emission) wavelengths 15 minutes, 1 hour, 3 hours, 6 hours, and 24 hours after treatment. All the procedures, including the readings, were performed in the absence of light.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.0 software. The data normality was assessed by the Shapiro-Wilk test and $P<0.05$ was considered significant. For cell viability data, one-way ANOVA was used, followed by Dunnett's *post-hoc* test. For intracellular ROS data, two-way ANOVA was used, followed by Tukey's *post-hoc* test. The assays were performed in at least three independent experiments in quadruplicate. MTT and neutral red uptake results were expressed as percentage of cell viability based on the negative control, whereas ROS results were expressed as fluorescence intensity.

RESULTS

The typical antipsychotic haloperidol showed a significant decrease in cell viability at the highest tested concentration (0.1 μM) in the neutral red cell cytotoxicity assay (P<0.0001), whereas 0.01 μM, 0.001 μM and 0.0001 μM did not interfere in cell viability (P>0.05) (Figure 2A). The same result was observed in the MTT assay, in which the highest tested concentration decreased the number of viable cells (P<0.0001), whereas no significative differences were observed for the other concentrations (P>0.05) (Figure 2B).

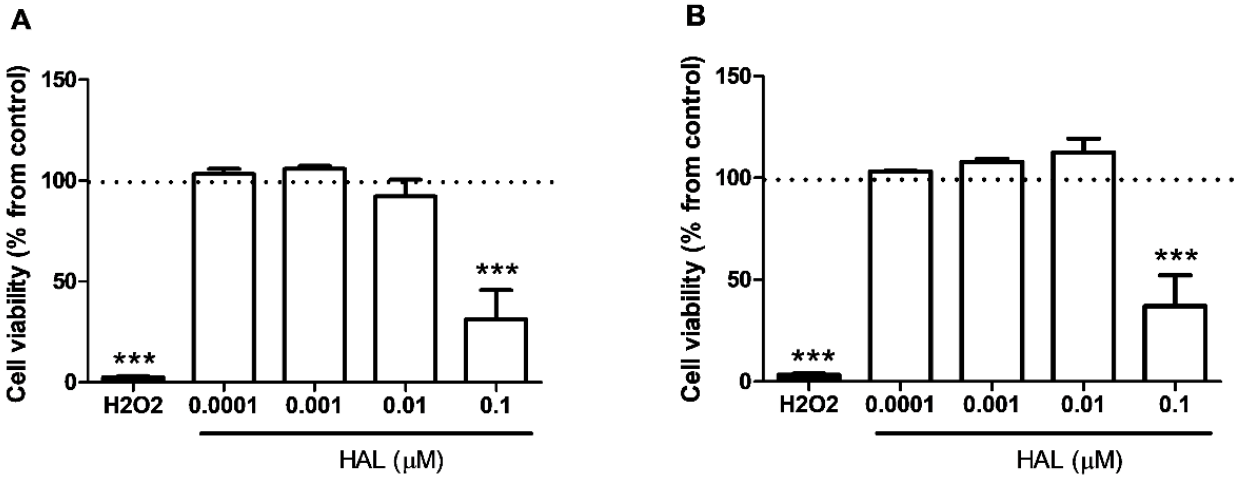

FIGURE 2 - (A) Effect of the typical antipsychotic, haloperidol (HAL), in 3T3 cells in the neutral red uptake cytotoxicity assay (n=3-5, quadruplicate; F(5,23)=30.12; P<0.0001). (B) Effect of the typical antipsychotic, haloperidol (HAL), in 3T3 cells in the MTT assay (n=3-5, quadruplicate; F(5,23)=32.95; P<0.0001). 1% H_2O_2 was used as a positive control. Results were expressed as mean + S.E. One-way ANOVA/Dunnett's *post-hoc* test. Different from the control group (Ctr) *** P<0.001.

Figure 3 illustrates NIH-3T3 culture micrographs, in which A represents the negative control, B the positive control, and C, D, E and F the culture exposed to the different concentrations of the typical antipsychotic haloperidol. The 0.0001 to 0.01 μM (Figure 3C-E) concentrations did not show any morphological difference in relation to the negative control (Figure 3A), maintaining the fusiform aspect of the cells and their adherent characteristics. However, we can observe morphological changes promoted by 0.1 μM of haloperidol (Figure 3F), such as content decrease, cell detachment, rounding and death, as seen in the positive control (Figure 3B).

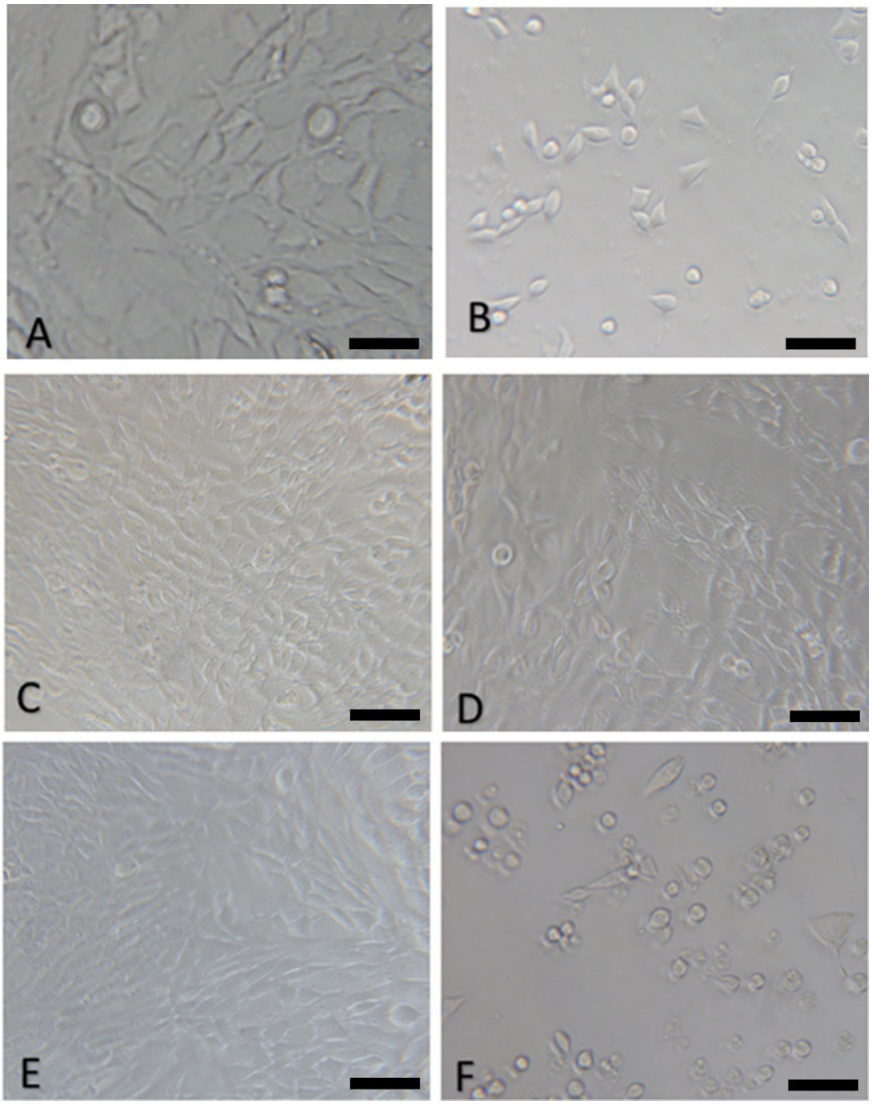

FIGURE 3 - Morphology of NIH-3T3 cells after different treatments (200x magnification). (A) negative control; (B) positive control (H_2O_2); (C) HAL (haloperidol) 0.0001 µM; (D) HAL 0.001 µM; (E) HAL 0.01 µM; (F) HAL 0.1 µM. Scale bars: 50 µm (A) and 100 µm (B - F).

Cellular ROS production after exposure to different concentrations of haloperidol (0.001 µM, 0.01 µM and 1 µM) is shown in Figure 4. Haloperidol significantly increased ROS generation even at the lowest tested concentration (0.001 µM), as observed for different evaluation times: 3, 6 and 24 hours (P<0.0001). ROS levels also increased significantly at 0.01 µM haloperidol at 3, 6 and 24 hours (P<0.0001). A similar pattern was observed for the highest tested concentration, as ROS generation was significantly increased in cells exposed to 1 µM haloperidol (P<0.0001). No significant differences were observed at 15 minutes and 1 hour for any of the tested concentrations (P>0.05).

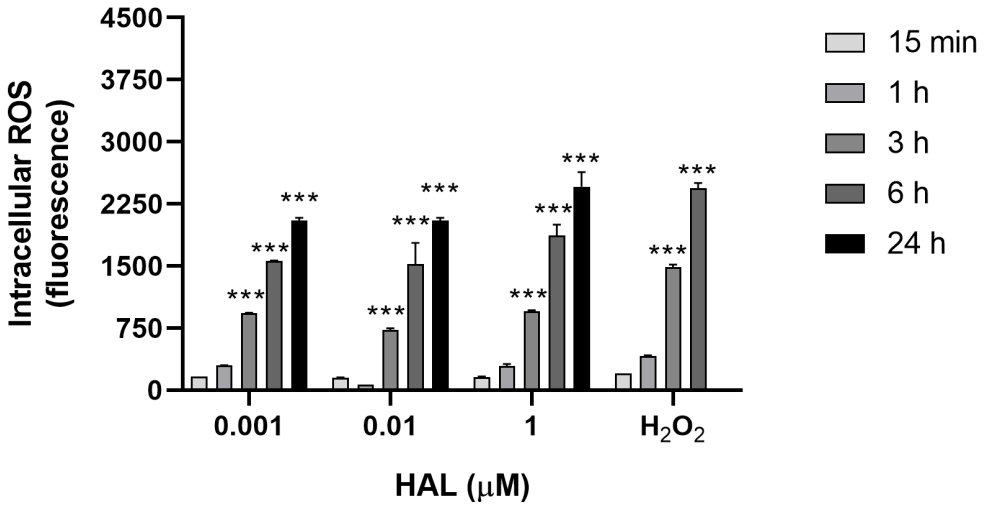

FIGURE 4 – Effect of the typical antipsychotic haloperidol (HAL) on the intracellular ROS production in NIH-3T3 cells over time (n=3). 0.01% H_2O_2 was used as a positive control. Results were expressed as mean + S.E. Two-way ANOVA/Tukey's *post-hoc* test. Different from the control group (Ctr) *** P<0.0001

The atypical antipsychotic clozapine showed a similar profile to haloperidol regarding cell viability. It also decreased cell viability in the neutral red uptake assay at 0.1 μM (P<0.0001) (Figure 5A), whereas the other tested concentrations (0.01 μM, 0.001 μM and 0.0001 μM) did not change cell viability (P>0.05). The same effect was observed in the MTT assay, as the mitochondrial activity only changed at the highest tested concentration of clozapine (P=0.0077), whereas no significative differences were observed for the other concentrations (P>0.05) (Figure 5B).

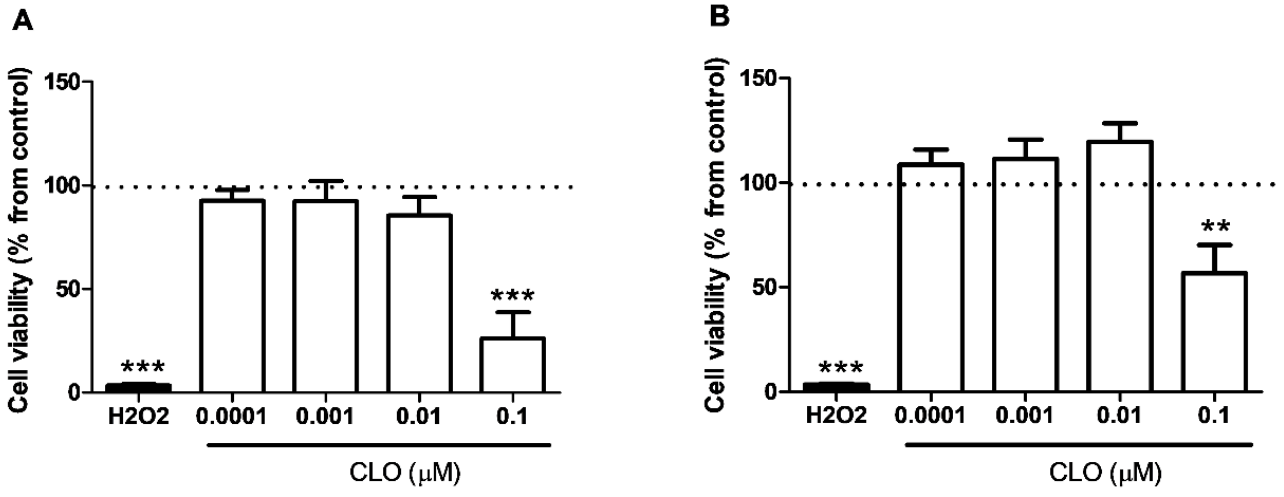

FIGURE 5 - (A) Effect of the atypical antipsychotic clozapine (CLO) in 3T3 cells in the neutral red cell cytotoxicity assay (n=4, quadruplicate; F(5,23)=25.37; P<0.0001). (B) Effect of the atypical antipsychotic clozapine (CLO) in 3T3 cells in the MTT assay (n=5, quadruplicate; F(5,29)=29.31; P<0.0001). H_2O_2 1% was used as a positive control. Results were expressed as mean + S.E. One-way ANOVA/Dunnett's *post-hoc* test. Different from the control group (Ctr) **P<0.01 ***P<0.001.

Figure 6 shows the 3T3 cell cultures exposed to clozapine at different concentrations. In Figure 6F, it was possible to observe morphological changes at the 0.1 µM concentration, such as cellular detachment, content decrease, rounding, and death, as observed in the positive control (Figure 6B). At the other tested concentrations (Figures 6C, 6D, 6E), there was no morphological difference to the negative control (Figure 6A), maintaining the fusiform and adherent characteristics.

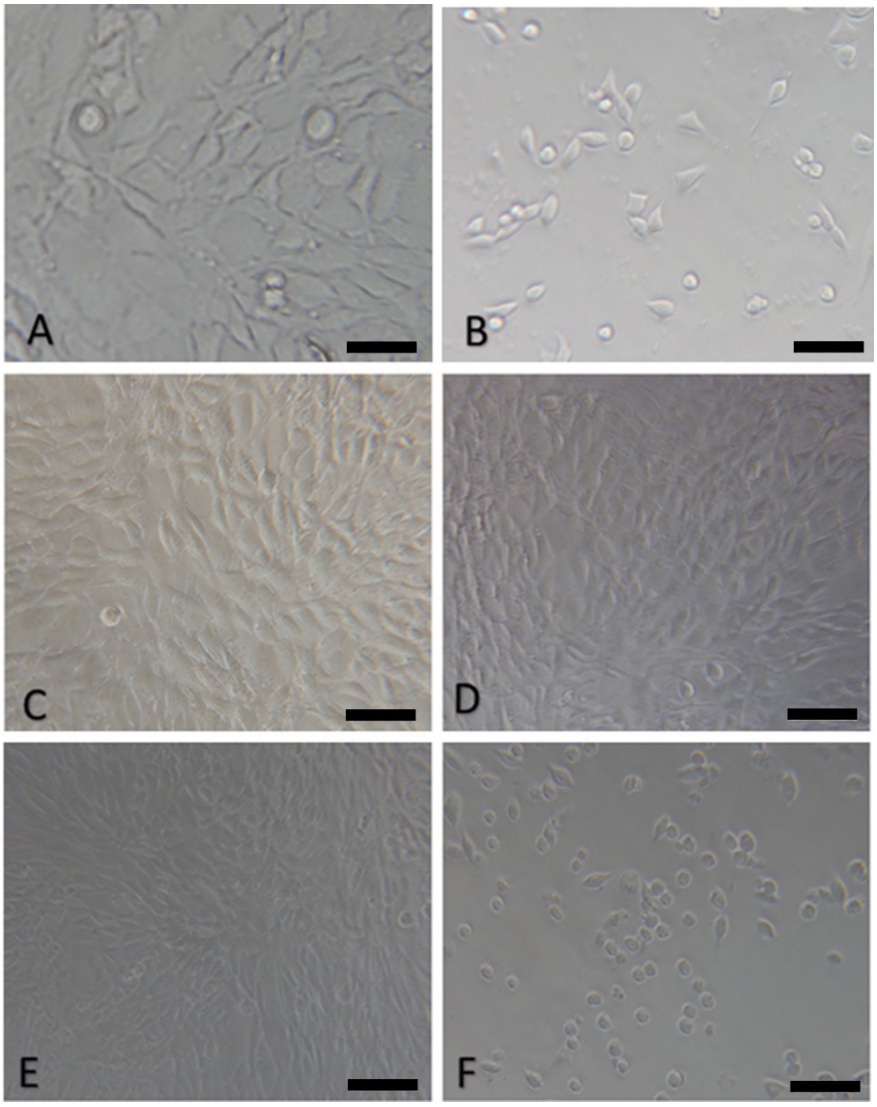

FIGURE 6 - Morphology of NIH-3T3 cells after different treatments (200x magnification). (A) negative control; (B) positive control (H_2O_2); (C) CLO (clozapine) 0.0001 µM; (D) CLO 0.001 µM; (E) CLO 0.01 µM; (F) CLO 0.1 µM. Scale bars: 50 µm (A) and 100 µm (B - F).

ROS production in NIH-3T3 cells exposed to different concentrations of clozapine (0.001 µM, 0.01 µM and 1 µM) is shown in Figure 7. A significant increase in ROS generation was observed for 0.01 µM clozapine at 6 hours (P=0.0019). The highest tested concentration, 1 µM clozapine, also significantly increased ROS levels, now observed at 3, 6 and 24 hours (P<0.0001). However, no significant differences were observed for the lowest tested concentration (0.001 µM) at any of the evaluation times (P>0.05), as well as at 15 minutes and 1 hour at any of the tested concentrations (P>0.05).

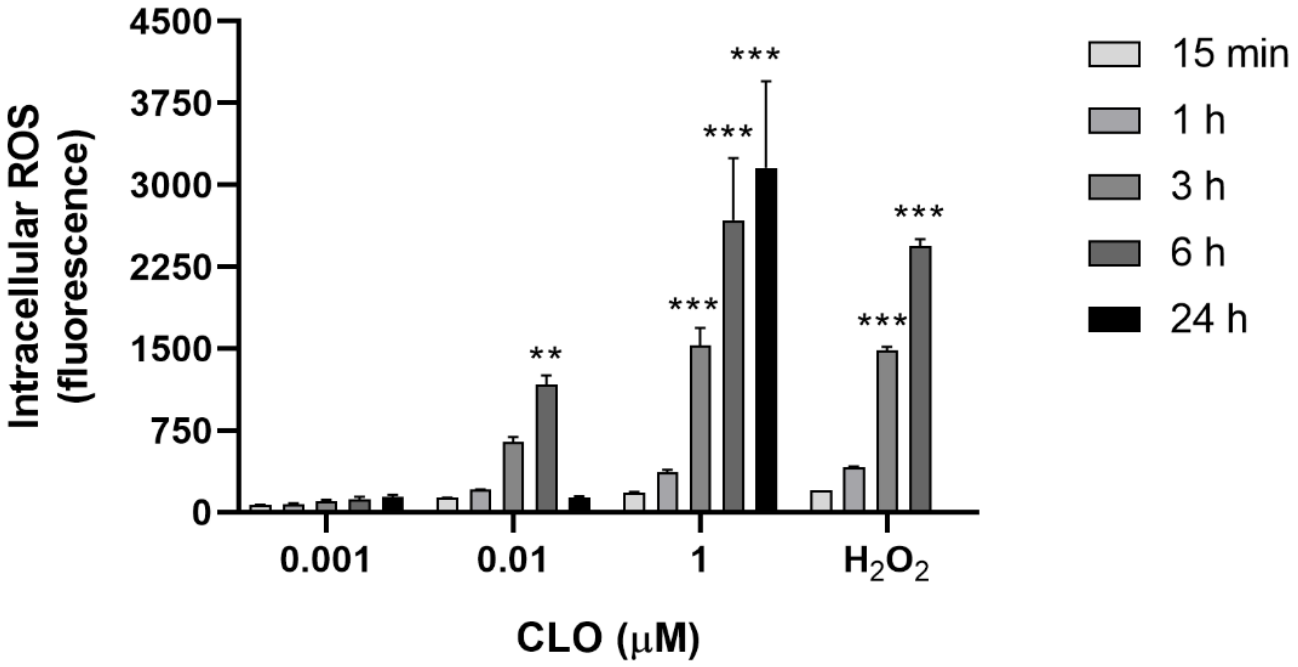

FIGURE 7 - Effect of the atypical antipsychotic clozapine (CLO) on the intracellular ROS production in NIH-3T3 cells over time (n=3). 0.01% H_2O_2 1% was used as a positive control. Results were expressed as mean + S.E. Two-way ANOVA/Tukey's *post-hoc* test. Different from the control group (Ctr) **P < 0.01 ***P < 0.001

In contrast, the new molecule with antipsychotic potential, PT-31, did not alter cell viability (Figure 8A) and mitochondrial activity (Figure 8B) at any of the tested concentrations.

Figure 9 reinforces the absence of cytotoxicity of PT-31, in which no morphological changes were observed at any tested concentration, keeping the cells unaltered.

ROS levels in NIH-3T3 cells after exposure to different concentrations of PT-31 (0.001 μM, 0.01 μM and 1 μM) are presented in Figure 10. A significant increase was observed in ROS production at both 0.01 μM and 1 μM concentrations at 3, 6 and 24 hours (P<0.0001). In contrast, PT-31 did not promote ROS generation at 0.001 μM at any of the evaluation times (P>0.05). Additionally, no significative differences were observed at 15 minutes and 1 hour for any of the tested concentrations (P>0.05).

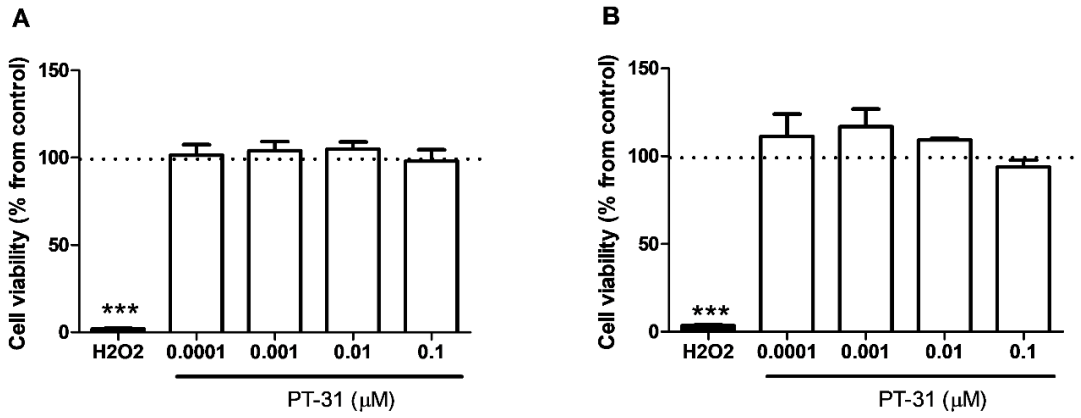

FIGURE 8 - (A) Effect of the new molecule with antipsychotic potential, PT-31, in 3T3 cells in the neutral red cell cytotoxicity assay (n=3, quadruplicate; F(5,17)=76.91; *P*<0.0001). (B) Effect of the new molecule with antipsychotic potential, PT-31, in 3T3 cells in the MTT assay (n=4, quadruplicate; F(5,23)=35.25; *P*<0.0001). H_2O_2 was used as a positive control. Results were expressed as mean + S.E. One-way ANOVA/Dunnett's *post-hoc* test. Different from the control group (Ctr) ***P<0.001.

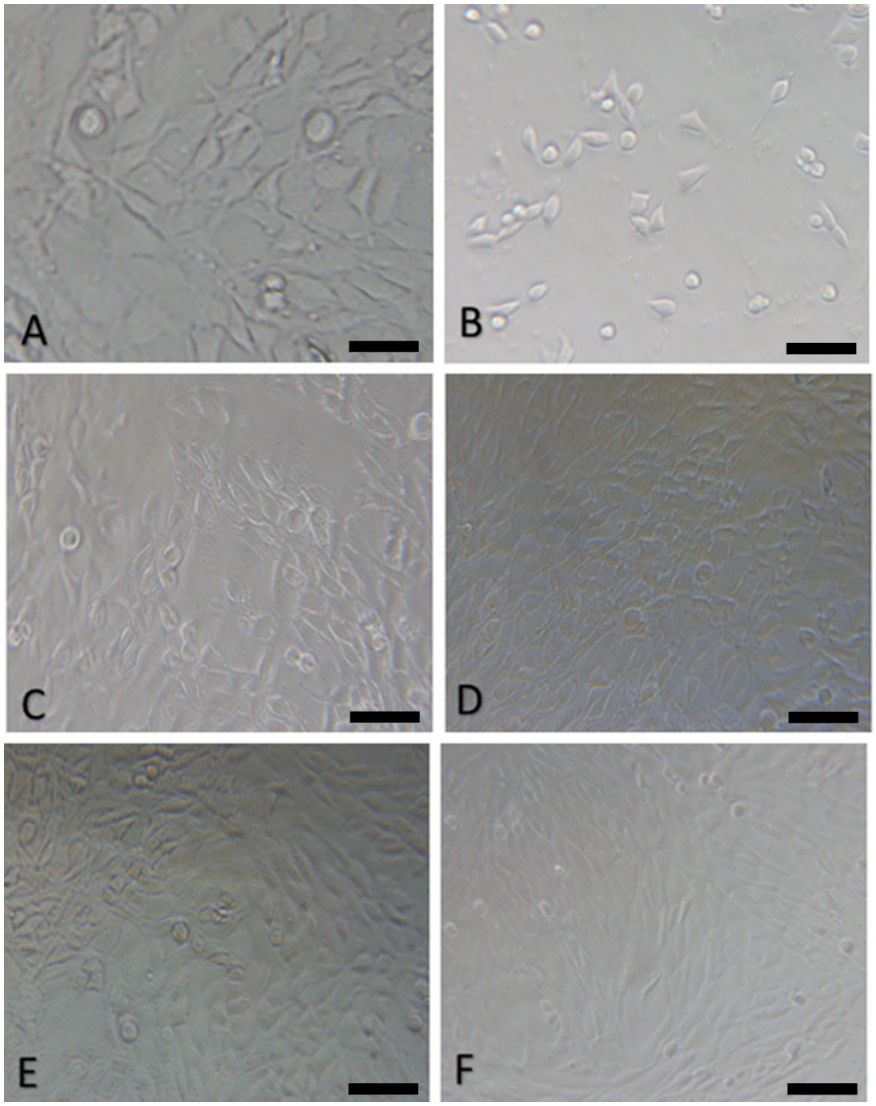

FIGURE 9 - Morphology of NIH-3T3 cells after different treatments (200x magnification). (A) negative control; (B) positive control (H_2O_2); (C) PT-31 0.0001 µM; (D) PT-31 0.001 µM; (E) PT-31 0.01 µM; (F) PT-31 0.1 µM. Scale bars: 50 µm (A) and 100 µm (B - F).

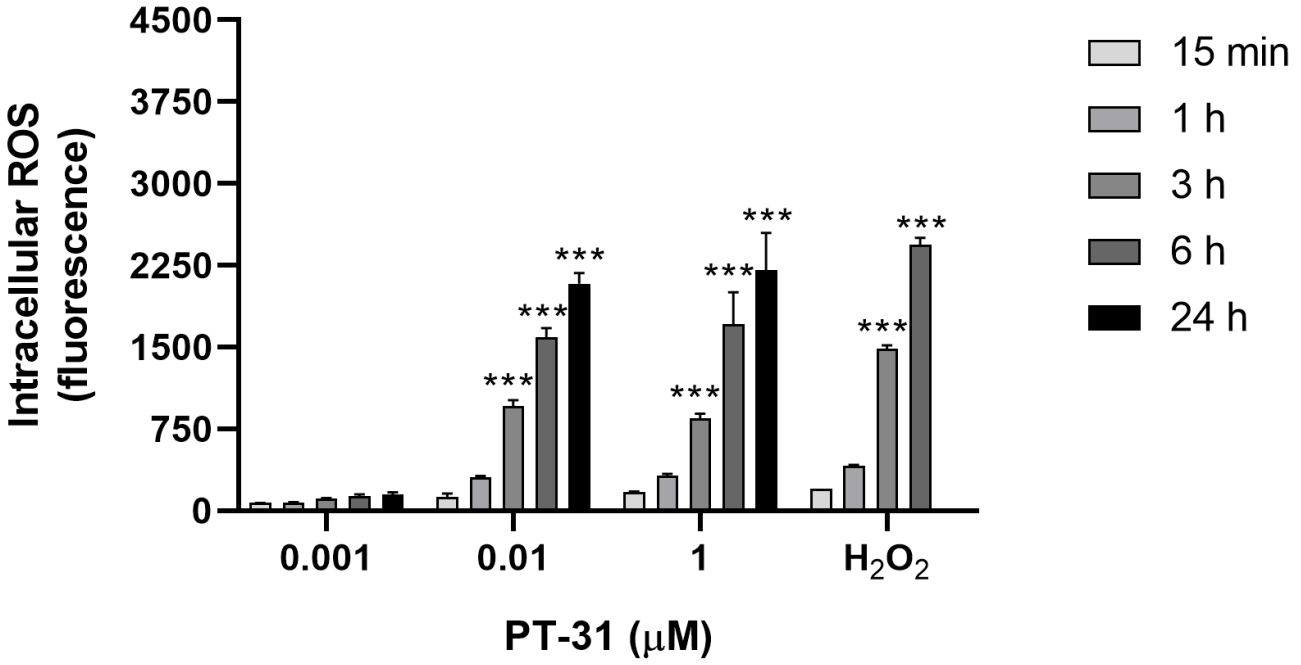

FIGURE 10 - Effect of the new molecule with antipsychotic potential, PT-31, on the intracellular ROS production in NIH-3T3 cells over time (n=3). 0.01% H_2O_2 was used as a positive control. Results were expressed as mean + S.E. Two-way ANOVA/ Tukey's *post-hoc* test. Different from the control group (Ctr) *** $P<0.0001$

DISCUSSION

The present study evaluated for the first time the effect of haloperidol and clozapine over mice embryonic fibroblast cells (NIH-3T3), which are undifferentiated cells that allow toxicity assessments in the first lineage, i.e., before cell differentiation (Todaro, Green, 1963; OECD, 2004; Brasil, 2015). In addition, PT-31 — an innovative molecule with antipsychotic potential — was also evaluated in order to compare it with the conventional treatments. The antipsychotics haloperidol and clozapine were cytotoxic in NIH-3T3 cells at 0.1 μM, unlike PT-31, which did not affect cell viability or mitochondrial activity at any of the tested concentrations.

Antipsychotic drugs have already been studied in different cell lineages. In hepatocyte cells, the typical phenothiazine antipsychotics chlorpromazine, trifluprom azine and thioridazine indicated a potential cytotoxic effect (De Faria *et al*., 2015), as verified for haloperidol in the present study. De Faria *et al.* (2015) suggested that mitochondria are the main target for toxicity of this class of drugs. The involvement of mitochondria in cell death is well established: mitochondrial permeability transition is an intrinsic process associated with necrosis and apoptosis due to energy metabolism, resulting from disruption of adenosine triphosphate (ATP) (Golstein, Kroemer, 2007) or the release of pro-apoptotic proteins, such as cytochrome C in the cytosol (Kawai *et al*., 2009).

Haloperidol is also known for its cytotoxic and genotoxic potential in human peripheral blood lymphocytes, highlighting the importance of patient follow-up in haloperidol treatment to minimize the risk of adverse events (Gajski, Gerić, Garaj-Vrhovac, 2014). Raudenska *et al.* (2013) verified the neurotoxicity and cardiotoxicity of haloperidol and its metabolites, as well as its extrapyramidal symptoms and cardiac effects. The authors suggest that these alterations can be produced by the oxidative stress induced by haloperidol through lipid peroxidation and subsequent membrane changes responsible for cell death, since cells resistant to oxidative stress were also resistant to the toxic effects of haloperidol (Raudenska *et al.* 2013).

Antipsychotics have also been evaluated in tumoral cell lines since several of them seem to have cytotoxic

effects on cancer cells (Yin *et al.*, 2015). Clozapine induced autophagic cell death in lung carcinoma cell lines (A549 and H1299), providing an insight into the relationship between the use of clozapine and the lower incidence of lung cancer among patients with schizophrenia. Clozapine inhibited A549 and H1299 proliferation and increased p21 and p27 gene expression, leading to cell cycle arrest (Yin *et al.*, 2015).

Tan *et al.* (2007) evaluated the cytotoxicity of haloperidol and the atypical antipsychotics risperidone, olanzapine and quetiapine in PC12 cells, a rat adrenal medulla pheochromocytoma derived cell line. Even in low concentrations, haloperidol reduced cell viability and caused apoptotic alterations, whereas the atypical drugs did not show these alterations. In this same cell line, there is another study evaluating the neuroprotection of antipsychotics through the MTT reduction test. The typical antipsychotic haloperidol enhanced cell loss, whereas the atypical ones, clozapine, olanzapine, quetiapine and risperidone, significantly reduced cell loss, attenuating MPP^+ induced cell death and preventing MPP^+ induced apoptosis and DNA fragmentation (Wei *et al.*, 2003; Qing *et al.*, 2003). Dwyer, Lu and Bradley (2003), in turn, evaluated the cytotoxicity of antipsychotics in PC12 cells, regarding the effects of typical (chlorpromazine, fluphenazine and pimozide) and atypical antipsychotics (clozapine, quetiapine and risperidone) on glucose metabolism. In general, the typical ones were more toxic, and only olanzapine demonstrated a neuroprotective effect. A correlation between the cytotoxicity of antipsychotics and their ability to block the transport of glucose was suggested, since typical antipsychotics also affected the expression of glucose transport proteins (Dwyer, Lu, Bradley, 2003).

Human neuroblastoma cells (SK-N-SH) were also used to study antipsychotic neurotoxicity, which appears to be associated with neurological side effects, such as extrapyramidal symptoms, whereas neuroprotective effects may soften or decrease degenerative and progressive structural changes in the brain, leading to improved symptoms of schizophrenia (Heiser *et al.*, 2007; Park *et al.*, 2009; Mas *et al.*, 2012). Since typical and atypical antipsychotics may differ in their neurotoxic and neuroprotective properties, the neurotoxic and neuroprotective activity of haloperidol, risperidone and paliperidone were evaluated in terms of cell viability, caspase-3 enzyme activity and cell death. Haloperidol significantly decreased cell viability and increased caspase-3 activity and cell death. Risperidone and paliperidone did not affect cell viability or cell death and decreased caspase-3 activity. In coadministration with excess dopamine, only paliperidone induced a slight improvement in cell viability. Haloperidol potentiated the activity of caspase-3 and induced apoptosis, whereas risperidone and paliperidone reduced this effect, suggesting a neuroprotective effect (Mas *et al.*, 2012). PT-31 also demonstrated a neuroprotective effect in coincubation with excess glutamate (Betti *et al.*, 2019).

Another human neuroblastoma cell line (SH-SY5Y) was used to compare the effect of aripiprazole and haloperidol over the brain-derived neurotrophic factor (BDNF). Aripiprazole significantly increased BDNF levels in cells, but haloperidol did not, suggesting haloperidol toxicity and a neuroprotective effect for aripiprazole (Park *et al.*, 2009). However, the concentrations of aripiprazole required to produce beneficial effects (5 or 10 μM) were higher than the normal plasma concentrations observed in humans, ranging from 0.9 to 1.1 μM (Citrome *et al.*, 2007).

Heiser *et al.* (2007) evaluated the cytotoxic effects of typical (haloperidol) and atypical (clozapine and olanzapine) antipsychotic drugs on human neuroblastoma (SH-SY5Y) and leukemia monocytes (U937). The authors observed that haloperidol and clozapine caused a significant decrease in metabolic activity in both cell systems, U937 and SH-SY5Y, at high concentrations (25 and 50 μg mL^{-1}), which was also detected after treatment with clozapine (12.5 μg mL^{-1}) in U937 cells. In contrast, olanzapine induced a significant increase in the metabolic activity of SH-SY5Y cells at all tested concentrations (1.6, 3.13, 6.25, 12.5, 25 and 50 μg mL^{-1}), whereas the metabolic activity in U937 cells was increased at concentrations of 1.6 and 6.25 μg mL^{-1}. Regarding the ATP content, haloperidol significantly decreased the levels in both cell systems compared to the control, suggesting that antipsychotic drugs of different classes exert distinct metabolic effects in both neuronal and immunological systems (Heiser *et al.*, 2007).

Regarding the above, data from the literature suggest that atypical antipsychotics present neuroprotective effects, whereas typical antipsychotics induce toxicity. However, our data suggest that both haloperidol and clozapine are cytotoxic in NIH-3T3 cells since morphological changes confirmed the cytotoxicity of these antipsychotics. Considering that the decrease in cell viability was observed in both assays, we suggest that haloperidol and clozapine induce cell death through different mechanisms: apoptosis and necrosis. Apoptosis is a genetically controlled death characterized by cell shrinkage, whereas necrosis is a random and uncontrolled process represented by cell and organelle swelling (Hotchkiss *et al*., 2009; Jan, Chaudry, 2019). Despite these differences, apoptosis and necrosis may occur simultaneously or successively depending on the intensity of the stimuli, ATP concentration and the cell type (Elmore, 2007; Chen, Kang, Fu, 2018).

The present study also demonstrated that overproduction of reactive oxygen species (ROS) is an important mediator of toxicity induced by haloperidol and clozapine in NIH-3T3 cells. Small amounts of ROS, such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxides (H_2O_2), are essential for several biochemical processes of cell metabolism in all aerobic organisms (Zeni *et al*., 2004). However, excess formation and/or insufficient removal of these highly reactive entities characterizes oxidative stress, a phenomenon that results in damage to proteins, lipids, cell membranes and nucleic acids, which ultimately leads to cell death and tissue injury (Heiser *et al*., 2010).

In this context, Post, Holsboer and Behl (1998) evaluated the oxidative neurotoxicity of haloperidol in the clonal mouse hippocampal cell line (HT22) and observed that exposure to haloperidol promoted an increase in intracellular ROS accumulation after 6 hours, in addition to morphological changes and a decrease in cell viability. Similar results were found by Quincozes-Santos *et al.* (2010), who reported an increase in ROS production in C6 astroglial cells exposed to 10 μM of haloperidol. Regarding the atypical antipsychotic, Elmorsy *et al.* (2017) observed that clozapine increased ROS generation and decreased cell viability in rat ovarian interstitial cells (TICs) at the 50% inhibitory concentration (IC_{50}), which was also observed for haloperidol in the same study. The increase in intracellular ROS generation promoted by haloperidol and clozapine, added to the decrease in cell viability and morphological changes, may be related to the imbalance in the oxidant/antioxidant system caused by conventional typical and atypical antipsychotics (Heiser *et al*., 2010). Both haloperidol and clozapine have previously been shown to promote a decrease in antioxidant gluthatione peroxidase (GPx) levels (Post, Holsboer, Behl; 1998; Elmorsy *et al*., 2017). Furthermore, it is also known that mitochondria are the main source of ROS generation through respiratory chain disruption, which can ultimately lead to dysfunctional ATP synthesis, loss of MMP, and cell death, reinforcing the results obtained in the present study (Zou *et al*., 2017).

In addition to previous results of our group, which demonstrated an absence of neurotoxicity in cerebellar primary cultures of neurons and neuroprotection in a model of excitotoxicity (Betti *et al*., 2019), the PT-31 molecule did not show toxic effects on cell viability regarding lysosomal and mitochondrial activity, which reinforces the conclusion that this molecule has great potential for the development of new antipsychotics. Although an increase in intracellular ROS was also observed for PT-31, this was probably not sufficient to significantly affect the cell viability and morphology of NIH-3T3 cells, or perhaps PT-31 has a distinct mechanism of ROS generation compared to haloperidol and clozapine. It is important to highlight that the DCFH-DA probe, used for the detection of intracellular ROS production, does not allow for deduction of the exact reactive species responsible for the oxidative stress, nor does it help to infer the response of the antioxidant system. Therefore, further studies investigating the response of the antioxidant system after exposure to PT-31 would contribute to elucidating the mechanisms involved.

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