

## Antipsychotic drugs inhibit nucleotide hydrolysis in zebrafish (*Danio rerio*) brain membranes

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### ABSTRACT

Haloperidol (HAL), olanzapine (OLZ), and sulpiride (SULP) are antipsychotic drugs widely used in the pharmacotherapy of psychopathological symptoms observed in schizophrenia or mood-related psychotic symptoms in affective disorders. Here, we tested the *in vitro* effects of different concentrations of a typical (HAL) and two atypical (OLZ and SULP) antipsychotic drugs on ectonucleotidase activities from zebrafish brain membranes. HAL inhibited ATP (28.9%) and ADP (26.5%) hydrolysis only at 250  $\mu$ M. OLZ decreased ATPase activity at all concentrations tested (23.8–60.7%). SULP did not promote significant changes on ATP hydrolysis but inhibited ADP hydrolysis at 250  $\mu$ M (25.6%). All drugs tested, HAL, OLZ, and SULP, did not promote any significant changes on 5'-nucleotidase activity in the brain membranes of zebrafish. These findings demonstrated that antipsychotic drugs could inhibit NTPDase activities whereas did not change 5'-nucleotidase. Such modulation can alter the adenosine levels, since the ectonucleotidase pathway is an important source of extracellular adenosine. Thus, it is possible to suggest that changes promoted by antipsychotic drugs in the bilayer membrane could alter the NTPDase activities, modulating extracellular ATP and adenosine levels.

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### 1. Introduction

Schizophrenia has traditionally been treated with the so-called typical antipsychotic drugs, such as chlorpromazine or HAL, which have potent D2 dopamine receptor antagonist properties. These drugs are effective in reducing the positive symptoms of schizophrenia, such as hallucinations and delusions. However, they are less effective at reducing the incidence of negative symptoms such as apathy and poor social functioning, nor do they have significant effects on cognitive deficits of the illness. These drugs are also associated with a high incidence of extrapyramidal symptoms (EPS). Clozapine (CLO), the first drug to be introduced into clinical practice as an atypical antipsychotic drug, maintains efficacy in treating the positive and negative symptoms. CLO also has a low incidence of EPS and it is considered as the first-choice drug for treatment-resistant schizophrenic patients (Kane et al., 1988; Fleischhacker, 1999).

This drug has been classified based on less selective activity across several neurotransmitter receptors (moderately potent antagonists at 5HT<sub>2</sub> receptors with lesser and equal potency at D1, D2, and  $\alpha_1$ -adrenergic receptors) (Kapur et al., 1999; Wadenberg et al., 2001; Seeman, 2002).

The neuromodulator adenosine has been proposed to contribute to the pathophysiology of schizophrenia. This hypothesis postulates that a dysfunction in adenosinergic activity in schizophrenia would lead to putative alterations of dopaminergic and glutamatergic activities. Moreover, the ubiquity of adenosine could also account for some of the systemic alterations reported in schizophrenic patients (Lara and Souza, 2000; Lara et al., 2006). The proposed adenosine dysfunction in schizophrenia, leading to a synaptic adenosinergic deficit, could be due to receptor alterations or altered metabolism, decreased production/release or increased degradation/uptake of adenosine (Lara et al., 2006). ATP and adenosine are important signaling molecules in the central nervous system (Ralevic and Burnstock, 1998). ATP is synthesized, stored and released by the central and peripheral nervous systems upon depolarization (Phillis and Wu, 1981; Bodin and Burnstock, 2001). It has been proposed that extracellular ATP

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evokes responses by two families of P2 receptors, namely, P2X ionotropic ligand-gated ion channel receptors and P2Y metabotropic G protein-coupled receptors (for a review see Burnstock, 2006). The signaling actions induced by extracellular ATP are directly correlated to the activity of ectonucleotidases once they trigger the enzymatic conversion of ATP to adenosine (Zimmermann, 2001; Robson et al., 2006). Ectonucleotidases constitute a highly refined system for the regulation of nucleotide-mediated signaling, controlling the rate, amount and timing of nucleotide degradation and formation. The hydrolysis of ATP to AMP is catalyzed mainly by a family of ectonucleotidases, named nucleoside triphosphate diphosphohydrolases (NTPDases). The nucleotide AMP is hydrolyzed to adenosine, an important neuromodulator, by the action of an ecto-5'-nucleotidase (Zimmermann, 1992, 1996, 2001). The neuromodulator adenosine can mediate different cellular functions by operating G-protein-coupled receptors, named A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>, which can inhibit (A<sub>1</sub> and A<sub>3</sub>) or facilitate (A<sub>2A</sub> and A<sub>2B</sub>) neuronal communication (Fredholm et al., 2001).

Our laboratory has characterized the presence of NTPDase and ecto-5'-nucleotidase activities in the zebrafish brain membranes (Rico et al., 2003; Senger et al., 2004). Zebrafish is a small freshwater teleost fish widely used as a model to study vertebrate developmental, neurobiological, and toxicological mechanisms (Fetcho and Liu, 1998; Hill et al., 2005). This species may be an ideal vertebrate model system for numerous human diseases, where genetic and biological mechanisms of the diseases may be studied (Dodd et al., 2000; Gerlai et al., 2000; Ivetic et al., 2000). Zebrafish genes present a high degree of conservation and share a 70–80% homology with human genes (Dooley and Zon, 2000).

Studies have shown that antipsychotics, such as chlorpromazine, are able to inhibit Na<sup>+</sup>, K<sup>+</sup>-ATPase, and Ca<sup>2+</sup>-ATPase activity in rat brain (Mazumder et al., 1990). Furthermore, fluphenazine and HAL (both of which can induce severe EPS in humans) develop movement defects in larval zebrafish. In contrast, OLZ, which produces mild to little EPS in humans, leads to minimal movement defects in larval zebrafish (Giacomini et al., 2006). Clozapine also produced a rapid and profound effect on locomotor activity (hypactivity) in larval zebrafish (Boehmler et al., 2007). In addition, it has been shown that serum adenosine deaminase activity, which converts adenosine into inosine, is increased in schizophrenic patients treated either with typical antipsychotics or clozapine (Brunstein et al., 2007).

Considering that zebrafish may be an ideal vertebrate model system for numerous human diseases and that purinergic system is present in the zebrafish brain, the aim of this study is to evaluate the *in vitro* effects of different concentrations of a typical antipsychotic (HAL) and two atypical (OLZ and Sulp) antipsychotic drugs on ectonucleotidase activities from the zebrafish brain membranes.

## 2. Material and methods

### 2.1. Animals

Adult zebrafish of both sexes were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimated for at least two weeks in a 50-l thermostated aquarium filled with unchlorinated and constantly aerated water. Fish were kept on a 12 h light/dark cycle (with lights turning on at 7:00 am), and at a temperature of 25 ± 2 °C. They were used according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication N° 85–23, revised 1996), being healthy and free of any signs of disease. The Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) approved the protocol under the number 014/08–CEUA.

### 2.2. Chemicals

SULP, HAL, OLZ, Trizma Base, EDTA, EGTA, sodium citrate, Coomassie blue G, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, calcium, and magnesium chloride were purchased from Sigma (St. Louis, MO, USA). All other reagents used were from analytical grade.

### 2.3. *In vitro* treatments

Antipsychotics, HAL, SULP, and OLZ, were added to reaction medium before the preincubation with the enzyme, and were maintained throughout the enzyme assays. Antipsychotics were tested at final concentrations of 1, 10, 50, 100, and 250 µM.

### 2.4. Membrane preparation

The preparation of brain membranes was performed as described previously by Barnes et al. (1993). Zebrafish were euthanized by decapitation, their brains were removed from the cranial skull by the dissection technique. For each sample (membrane preparation), a pool of five zebrafish brains was used, which were briefly homogenized in 60 volumes (v/w) of chilled Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor driven Teflon-glass homogenizer. The samples were centrifuged at 1000 g for 10 min and the pellet was discarded. The supernatant was centrifuged for 25 min at 40000g. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris-citrate buffer, and centrifuged for 20 min at 40000g. This fresh-thaw-wash procedure was used to ensure the lysis of the brain membranes. The final pellet was resuspended and used in the enzyme assays. All samples were maintained at 2–4 °C throughout preparation.

### 2.5. Enzyme assays

NTPDase and 5'-nucleotidase assays were performed as described previously (Rico et al., 2003; Senger et al., 2004). Zebrafish brain membranes (3 µg protein for NTPDase and 5 µg protein for 5'-nucleotidase) were added to the reaction mixture containing 50 mM Tris-HCl (pH 8.0) and 5 mM CaCl<sub>2</sub> (for the NTPDase activity) or 50 mM Tris-HCl (pH 7.2) and 5 mM MgCl<sub>2</sub> (for the 5'-nucleotidase activity) at a final volume of 200 µl. The samples were preincubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min by the addition of 200 µl trichloroacetic acid at a final concentration of 5%. The samples were chilled on ice for 10 min and 1 ml of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added in order to determine the inorganic phosphate released (Pi) (Chan et al., 1986). After 20 min, the quantification of inorganic phosphate (Pi) released was determined spectrophotometrically at 630 nm. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity was expressed as nanomoles of Pi released per minute per milligram of protein. All enzyme assays were run at least in triplicate.

### 2.6. Protein determination

Protein was measured using Coomassie blue as the color reagent (Bradford, 1976) and bovine serum albumin was used as standard.

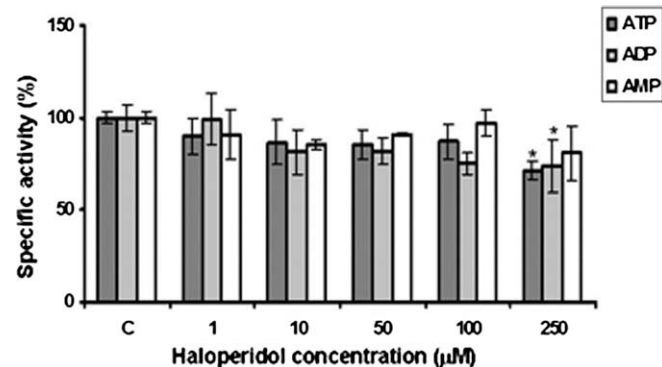
## 2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), being expressed as means  $\pm$  S.D. of three different experiments ( $n = 3$ ). A Tukey multiple test range as post-hoc was performed at  $\alpha = 0.05$ .

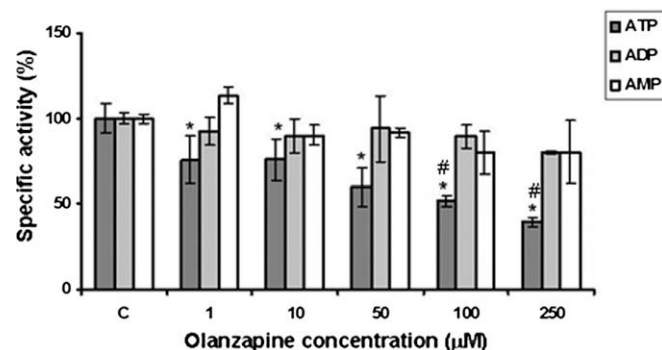
## 3. Results

We tested the *in vitro* effect of HAL, OLZ, and SULP (at concentrations varying from 1 to 250  $\mu$ M) on NTPDase and 5'-nucleotidase activities from zebrafish brain membranes. HAL promoted a significant inhibition on ATP and ADP hydrolysis (28.9% and 26.5%, respectively) only at 250  $\mu$ M, when compared to the control group (Fig. 1). In contrast, OLZ significantly inhibited ATP hydrolysis at all concentrations tested (23.8–60.7%) in a dose-dependent manner (Fig. 2). There were no significant changes in ADP hydrolysis in the presence of OLZ at all concentrations tested.

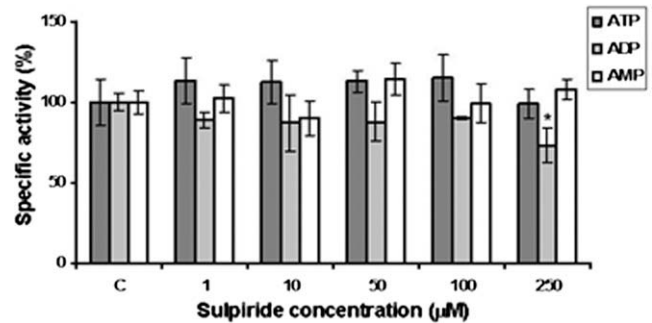
SULP did not alter ATP hydrolysis whereas ADP hydrolysis was inhibited at 250  $\mu$ M (25.6%) (Fig. 3). All drugs tested, HAL, OLZ, and SULP, did not promote any significant changes on 5'-nucleotidase activity in zebrafish brain membrane, when compared to the control group (Figs. 1–3, respectively).



**Fig. 1.** *In vitro* effect of varying concentrations of haloperidol (1–250  $\mu$ M) on ATP, ADP, and AMP hydrolysis in zebrafish brain membranes. Bars represent the mean  $\pm$  S.D. of at least three different experiments, each one performed in triplicate. The symbol (\*) indicates difference when compared to the control group. Data were analyzed by one-way ANOVA followed by Tukey test as post-hoc test, considering  $P \leq 0.05$  as significant.



**Fig. 2.** *In vitro* effect of varying concentrations of olanzapine (1–250  $\mu$ M) on ATP, ADP, and AMP hydrolysis in zebrafish brain membranes. Bars represent the mean  $\pm$  S.D. of at least three different experiments, each one performed in triplicate. The symbol (\*) indicates difference when compared to the control group. The symbol (#) indicates difference when compared to the 1 and 10  $\mu$ M OLZ. Data were analyzed by one-way ANOVA followed by Tukey test as post-hoc test, considering  $P \leq 0.05$  as significant.



**Fig. 3.** *In vitro* effect of sulpiride on NTPDase and ecto-5'-nucleotidase activity in zebrafish brain membranes. ATP, ADP, and AMP hydrolysis were evaluated in different concentrations (1–250  $\mu$ M). Bars represent the mean  $\pm$  S.D. of at least three different experiments, each one performed in triplicate. The symbol (\*) indicates difference when compared to the control group. Data were analyzed by one-way ANOVA followed by Tukey test as post-hoc test, considering  $P \leq 0.05$  as significant.

## 4. Discussion

The findings of the present study have demonstrated that NTPDase, but not 5'-nucleotidase, was altered by the action of antipsychotic drugs in the zebrafish brain membranes. Our results have shown that OLZ promoted the most intense effects on NTPDase activity, inhibiting ATP hydrolysis at all concentrations tested. However, HAL and SULP promoted a decrease on ATP hydrolysis only in the highest concentration tested. The ectonucleotidases may play a relevant role in several pathophysiological situations, such as schizophrenia, since these enzymes contribute to maintenance of extracellular ATP, ADP, AMP, and adenosine levels (Agteresch et al., 1999; Lara and Souza, 2000; Lara et al., 2006; Burnstock, 2008).

The discovery of antagonistic interactions between adenosine  $A_{2A}$  receptors and dopamine D2 receptors in the central nervous system suggests that the adenosine may be involved in the pathogenesis of psychiatric and neurological disorders (Martini et al., 2006). Different studies have demonstrated the effect of antipsychotic drugs on ATPase activities. Chlorpromazine *in vitro* inhibited  $Na^+$ ,  $K^+$ -ATPase activity in rat brain microsomal membranes in a time- and dose-dependent manner (Mazumder et al., 1990). Furthermore, *in vivo* treatment with this drug has shown that  $Na^+$ ,  $K^+$ -ATPase, and  $Ca^{2+}$ -ATPase activities were reduced with increasing concentrations or time exposures in microsomal membranes of different organs (Mazumder et al., 1990). Our results are consistent with these studies, demonstrating that the ATP hydrolysis in the zebrafish brain membranes is inhibited by antipsychotic drugs.

Antipsychotic drugs have shown high affinity for biomembranes due to their amphipathic and amphiphilic properties. This implies that antipsychotic drugs can interact with membrane lipid organization. Antipsychotic intercalation in the membrane can modify the membrane lipid dynamic, possibly inducing a subsequent modification of the receptor response (Tessier et al., 2008). Drug interaction with the biomembrane influences the bilayer structure, consequently, modulating processes that range from membrane-bound enzyme activity and receptor binding to membrane permeability and transport (Carfagna and Muhoberac, 1993). The intercalation of antipsychotic drug molecules into the plasma membrane may thus modulate the efficacy and tolerability profile of compounds able to exert their therapeutic effect through their binding with synapse receptors, in particular dopamine D2 receptors. This suggests that pharmacological activity of antipsychotic medications may result from a combination of drug-receptor and drug-membrane interactions

(Jutila et al., 2001). These results suggest additional mechanisms by which antipsychotics may exert some of their pharmacological activities. NTPDase1, 2, 3, and 8 are firmly anchored to the membrane via two transmembrane domains that, in the instance of NTPDase1, are important for maintaining catalytic activity and substrate specificity (Grinthal and Guidotti, 2006). Papanikolaou et al. (2005) have shown that removing cholesterol from membranes of NTPDase1-expressing cells reduces ATPase activity to the same extent as solubilization does. However, these authors have suggested that some aspects of cholesterol other than its effect on membrane fluidity are required for native transmembrane helix properties of the enzyme. If the balance between stability and mobility is a key feature of the interplay between the transmembrane domains and the active site of NTPDase1, anything that changes the bilayer stability might change NTPDase activity by altering this balance (Grinthal and Guidotti, 2006). Thus, changes in the membrane bilayer environment promoted by the interaction with haloperidol, olanzapine, and sulpiride may be able to promote the inhibitory effect observed on NTPDase activity. Thus, it is possible to suggest that changes induced by antipsychotics on membrane structure could affect NTPDase activities, and consequently, could modulate ATP and adenosine levels in the synaptic cleft. In contrast, ecto-5'-nucleotidase was not altered by antipsychotic drugs at the doses tested. Ecto-5'-nucleotidase is attached via a GPI (glycosylphosphatidylinositol) anchor to the extracellular membrane (Sträter, 2006). The different effects induced by antipsychotic drugs on NTPDase and ecto-5'-nucleotidase activities can be related to the differences in membrane anchorage of these enzymes. Previous studies from our laboratory have shown that antidepressant drugs can also promote similar effects on NTPDase activity (Pedrazza et al., 2007), which reinforce the idea that changes in the membrane bilayer induced by these drugs can modulate the extracellular nucleotide hydrolysis.

In summary, this study has shown that HAL, OLZ, and SULP can alter the ectonucleotidase activities, suggesting that the extracellular adenosine levels can be modulated by antipsychotic drugs.

### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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