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In vitro effects of antiepileptic drugs on acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain

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ABSTRACT

Carbamazepine (CBZ), phenytoin (PHT), and gabapentine (GBP) are classical antiepileptic drugs (AEDs) that act through a variety of mechanisms. We have tested the *in vitro* effects of CBZ, PHT, and GBP at different concentrations on ectonucleotidase and acetylcholinesterase activities in zebrafish brain. CBZ inhibited ATP hydrolysis at 1000 μM (32%) whereas acetylcholine hydrolysis decreased at 500 μM (25.2%) and 1000 μM (38.7%). PHT increased AMP hydrolysis both at 500 μM (65%) and 1000 μM (64.8%). GBP did not promote any significant changes on ectonucleotidase and acetylcholinesterase activities. These results have shown that CBZ can reduce NTPDase (nucleoside triphosphate diphosphohydrolase) and PHT enhance ecto 5′-nucleotidase activities. Therefore, it is possible to suggest that the AEDs induced-effects on ectonucleotidases are related to enzyme anchorage form. Our findings have also shown that high CBZ concentrations inhibit acetylcholinesterase activity, which can induce an increase of acetylcholine levels. Taken together, these results showed a complex interaction among AEDs, purinergic, and cholinergic systems, providing a better understanding of the AEDs pharmacodynamics.

1. Introduction

Epilepsy, a neurological disorder characterized by the occurrence of spontaneous recurrent seizures, is one of the most common pathologies of the central nervous system (CNS), affecting individuals of all ages (Badawy et al., 2009; Banerjee et al., 2009). Epilepsy damages the brain as a whole showing significant influence on dynamic and functional properties, mental development, cognition, and behavior (Hamed, 2009). Classical AEDs such as GBP, PHT, and CBZ work through a variety of mechanisms, often acting to suppress ion channels, promote gabaergic neurotransmission, and/or decrease glutamatergic neurotransmission. AEDs may be used for the treatment of several types of epilepsy and GBP, PHT and CBZ are treatments for partial and generalized tonic-clonic seizures (Rogawski and Löscher, 2004). Studies have suggested that AEDs can interfere in the purinergic (Borowicz et al., 1997, 2002) and cholinergic systems in CNS (Boccia et al., 2001; D'Antuono et al., 2007).

The purinergic system employs extracellular nucleotides as signaling molecules. ATP is a neurotransmitter co-released with other signaling molecules, such as glutamate, GABA, and acetylcholine in different subpopulations of neurons in CNS. ATP acts through activation of G-protein-coupled P2Y receptors and P2X ionotropic receptors, linked to Ca^{2+} channels (Burnstock, 2004, 2009; Pankratov et al., 2009). Extracellular nucleotides can be hydrolyzed by a variety of soluble or cell-surface-located enzymes named ectonucleotidases (Zimmermann et al., 1998). Nucleoside 5′-tri- and diphosphates may be hydrolyzed by members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase), ectonucleoside pyrophosphatase/phosphodiesterase (E-NPP) and alkaline

Abbreviations: AChE, acetylcholinesterase; AEDs, antiepileptic drugs; BuChE, butyrylcholinesterase; CBZ, carbamazepine; CNS, central nervous system; GBP, gabapentine; NTPDase, nucleoside triphosphate diphosphohydrolase; PHT, phenytoin.

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phosphatase protein families. AMP may be hydrolyzed by the ecto-5′-nucleotidase family and alkaline phosphatases to produce the nucleoside adenosine (for review see Abbracchio et al. (2009)). In this way, ectonucleotidases control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors and, consequently, the extent and duration of receptor activation (Chen and Guidotti, 2001). Therefore, this is an enzymatic pathway with double function of removing one signaling molecule, ATP, and generating a second one, adenosine, a well-known neuromodulator (Zimmermann, 2006; Abbracchio et al., 2009; Burnstock and Verkhratsky, 2009). Adenosine acts through four known subtypes of P1 metabotropic receptors (A_1, A_{2A}, A_{2B} and A_3) and reduces excessive neuronal activity through inhibitory A_1 receptors, acting as an endogenous anticonvulsant (Fredholm et al., 2001; Sebastião and Ribeiro, 2009). Adenosine modulates the acetylcholine release through A_1 receptor-mediated inhibition or by A_{2A} receptor-mediated facilitation of release (Cunha, 2001).

In cholinergic neurotransmission, acetylcholine is synthesized by cholineacetyltransferase in the presynaptic neuron, then released into the synaptic cleft and bound to muscarinic (metabotropic) and nicotinic (ionotropic) acetylcholine receptors. After its release, acetylcholine is degraded by the cholinesterases that cleave acetylcholine into choline and acetate. Two different types of cholinesterases are able to hydrolyze acetylcholine:acetylcholinesterase (AChE) (E.C.3.1.1.7) and butyrylcholinesterase (BuChE) (E.C.3.1.1.8) (Soreq and Seidman, 2001). Cholinergic mechanisms, in particular those related to the activation of muscarinic receptors, regulate brain excitability and can promote and maintain synchronous epileptiform discharges (Liu et al., 1994; Nagao et al., 1996; Dickson and Alonso, 1997). Moreover, cholinergic agents, such as pilocarpine, produce limbic seizures that are followed by histopathological changes resembling that encountered in temporal lobe epileptic patients (Turski et al., 1989; Mello et al., 1993).

PHT and CBZ blocked the pilocarpine-induced increase on ATP, ADP, and AMP hydrolysis in synaptosomes from hippocampus and cerebral cortex in rats (Cognato et al., 2007). Moreover, CBZ inhibited *in vitro* ATP hydrolysis from synaptosomal plasma membranes (Horvat et al., 2006). GBP and other AEDs, when administered together with adenosine receptor antagonists, showed decreased effect in mice models of seizure (Zuchora et al., 2004). In rats, the supraeffective dose of CBZ (100 mg/kg) inhibited the activities of acetylcholinesterase (9.5%) and butyrylcholinesterase (24.7%) whereas the effective dose of CBZ (25 mg/kg) did not alter acetylcholine degradation (Mizuno et al., 2000). Moreover, Sudha et al. (1995) showed that PHT decreased acetylcholinesterase activity in the hippocampus (50 and 75 mg/kg) and in the striatum (75 mg/kg).

Zebrafish is a small freshwater teleost fish that has been used to study learning and memory process, development, pharmacology, toxicology, behavior, and teratology (Kosmehl et al., 2008; Bencan et al., 2009; Gerlai et al., 2009; Ingham, 2009; Yang et al., 2009). This specie has been used as a tool for the study of seizure and to screen potential novel AEDs (Baraban et al., 2005; Baraban, 2007; Berghmans et al., 2007). NTPDase and ecto-5′-nucleotidase activities have been characterized in zebrafish in our laboratory (Rico et al., 2003; Senger et al., 2004). Acetylcholinesterase is encoded by a single gene and butyrylcholinesterase was not found in zebrafish genome (Clemente et al., 2004). Other drugs used for neurological disorder treatments were tested *in vitro* (haloperidol, olanzapine, and sulpiride) and inhibited NTPDase and acetylcholinesterase activities whereas did not change ecto-5′-nucleotidase activity in zebrafish brain (Seibt et al., 2009a,b). However, haloperidol significantly increased the acetylcholinesterase activity after *in vivo* treatments (Seibt et al., 2009b).

Considering that zebrafish may be a model organism to study human diseases and drug mechanisms, and purinergic and cholinergic systems have been described in this specie, the aim of this study was to evaluate the *in vitro* effects of different concentrations of the antiepileptic drugs GBP, PHT, and CBZ on ectonucleotidase and acetylcholinesterase activities of zebrafish brain.

2. Materials and methods

2.1. Animals

Adult wild-type zebrafish (*Danio rerio*) of both sexes were obtained from a commercial supplier (Red Fish, RS, Brazil) and acclimated for 2 weeks before the experiments in a 50 l thermostated aquarium filled with continuously aerated and unchlorinated water. The fish were conditioned at 26 ± 2 °C under a 14–10 h light/dark cycle photoperiod. The animals were maintained healthy and free of any signs of disease and fed twice a day with commercial food for fish. The use and maintenance of zebrafish were according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. The protocol was approved by the Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the number 085/06-CEP.

2.2. Chemicals

PHT, GBP, CBZ, acetylthiocholine, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), Trizma Base, EDTA, EGTA, sodium citrate, Coomassie blue, bovine serum albumin, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, calcium, and magnesium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were from analytical grade.

2.3. In vitro treatments

PHT, GBP, and CBZ were tested at 10, 50, 100, 500, and 1000 μM. PHT and GBP were diluted in deionized water. CBZ was diluted in 2% ethanol. Control treatments with equal volume of vehicle were performed to exclude the ethanol effect on the enzyme activities. Antiepileptic drugs were added to the reaction medium before the preincubation with the enzyme and were maintained during the enzyme assays.

2.4. Determination of ectonucleotidase activities

The brain membranes for the ectonucleotidase assays were prepared as described previously by Barnes et al. (1993). For each membrane preparation, a pool of five whole brains was obtained and briefly homogenized in 60 vol. (v/w) of chilled Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4) in a glass-Teflon homogenizer. This homogenate was centrifuged at 1000*g* for 10 min and the pellet was discarded in order to remove cell debris. The supernatant was centrifuged for 25 min at 40,000*g*. The resultant pellet was frozen in liquid nitrogen and thawed in order to ensure the lysis of the brain membranes. The pellet was resuspended in Tris–citrate buffer and centrifuged for 20 min at 40,000*g*. The final pellet was resuspended and used in the enzyme assays. The samples were maintained at 2–4 °C throughout preparation.

NTPDase and 5′-nucleotidase assays were performed as described previously (Rico et al., 2003; Senger et al., 2004). Brain membranes of zebrafish (3 μg protein for NTPDase and 5 μg protein for 5′-nucleotidase) were added to the reaction medium containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the 5′-nucleotidase activity) at a total 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. After 30 min the reaction was stopped by the addition of 200 μl 10% trichloroacetic acid and the samples were kept on ice during 10 min. In order to determine the inorganic phosphate released (Pi) 1 ml of a colorimetric reagent composed of 2.3% polyvinyl alcohol, 5.7% ammonium molybdate, and 0.08% malachite green was added to the samples for 20 min (Chan et al., 1986). The quantification of inorganic phosphate (Pi) released was determined spectrophotometrically at 630 nm and the specific activity was expressed as nanomole of Pi released per minute per milligram of protein. In order to correct non-enzymatic hydrolysis of the substrates we used controls with the addition of the enzyme preparation after the addition of trichloroacetic acid. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. All enzyme assays were performed in at least four different experiments, each one performed in triplicate.

2.5. Determination of acetylcholinesterase activity

Zebrafish were euthanized and their whole brains were removed by dissection. The brains (five whole brains for each sample) were homogenized on ice in 60 vol. (v/w) of 50 mM Tris–HCl, pH 8.0, in a glass-Teflon homogenizer. Acetylcholinesterase activity was measured as the method described previously (Ellman et al., 1961) determining the rate of hydrolysis of acetylthiocholine (ACSCh, 0.8 mM) in 2 ml assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB. Samples containing protein (10 μg) and the reaction medium described above were preincubated during 10 min at 25 °C followed by starting of reaction with addition of substrate. The hydrolysis of substrate was monitored by the formation of thiolate dianion of DTNB at 412 nm every 30 s for 2–3 min. The linearity of absorbance towards time and protein concentration was previously determined. Acetylcholinesterase activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. All enzyme assays were performed in at least four different experiments, each one performed in triplicate.

2.6. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976) and bovine serum albumin was used as standard.

2.7. Statistical analysis

Results are expressed as means ± S.D. Data were analyzed by one-way ANOVA followed by Duncan post-hoc test, considering $P < 0.05$ as significant. SPSS 16.0 was used for statistical analysis.

3. Results

The *in vitro* effect of GBP, PHT, and CBZ (at concentrations ranging from 10 to 1000 μM) was tested on acetylcholinesterase, NTPDase, and ecto-5′-nucleotidase activities in zebrafish brain. CBZ significantly decreased (32%; $P < 0.05$) ATP hydrolysis at 1000 μM (285 ± 63.06 nmol Pi min^{-1} mg^{-1} of protein) when compared to the ethanol group (419.54 ± 111.17 nmol Pi min^{-1} mg^{-1} of protein) (Fig. 1) whereas this drug did not alter both ADP and AMP hydrolysis (data not shown). The results demonstrated that CBZ inhibited (25.2% and 38.7%, respectively; $P < 0.05$) acetylcholinesterase activity at 500 μM (24.13 ± 5.35 μmol SCh h^{-1} mg^{-1} of protein) and at 1000 μM (19.75 ± 3.76 μmol SCh h^{-1} mg^{-1} of protein) when compared to the ethanol group (32.25 ± 6.49 μmol SCh h^{-1} mg^{-1} of protein; Fig. 2).

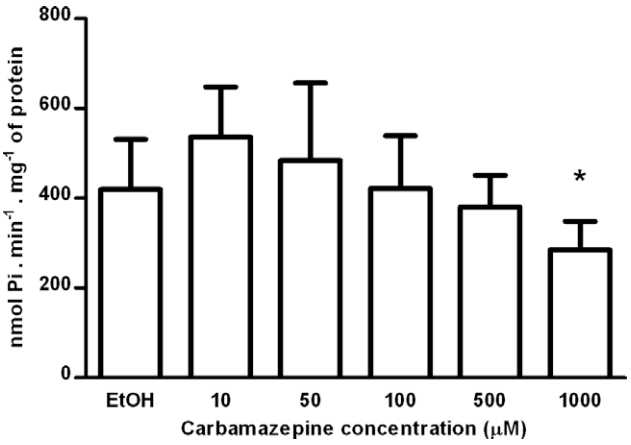

Fig. 1. *In vitro* effect of carbamazepine on ATP hydrolysis evaluated in different concentrations (10–1000 μM). Bars represent the mean ± S.D. The symbol (*) indicates significantly difference when compared to the ethanol group. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein.

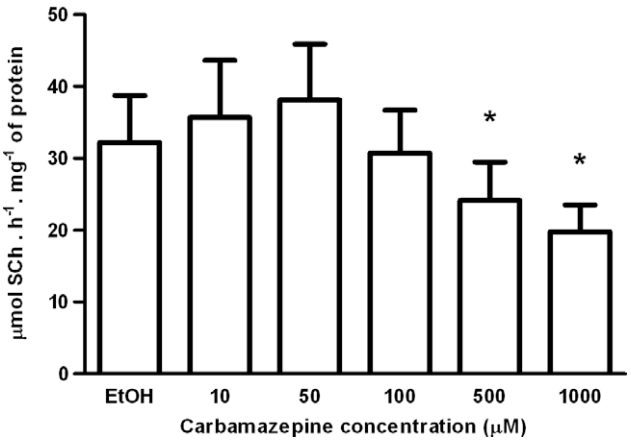

Fig. 2. *In vitro* effect of carbamazepine on acetylcholine hydrolysis evaluated in different concentrations (10–1000 μM). Bars represent the mean ± S.D. The symbol (*) indicates significantly difference when compared to the ethanol group. The specific enzyme activity is reported as micromole of thiocholine released per hour per milligram of protein.

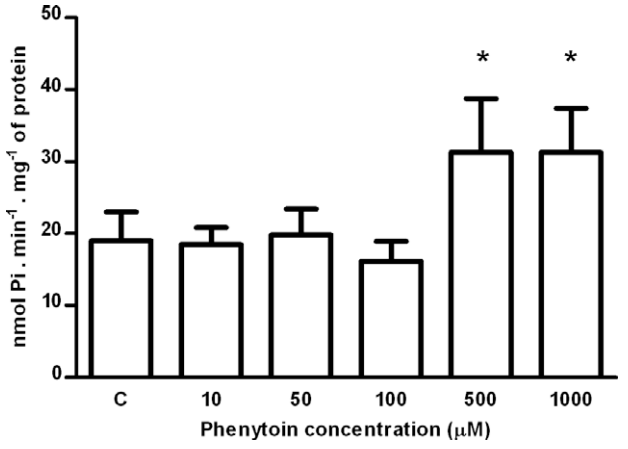

Fig. 3. *In vitro* effect of phenytoin on AMP hydrolysis evaluated in different concentrations (10–1000 μM). Bars represent the mean ± S.D. The symbol (*) indicates significantly difference when compared to the control group. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein.

PHT significantly increased (65%; $P < 0.05$ and 64.8%; $P < 0.05$) AMP hydrolysis at 500 μM (31.31 ± 7.42 nmol Pi min^{-1} mg^{-1} of protein) and 1000 μM (31.28 ± 6.14 nmol Pi min^{-1} mg^{-1} of protein) when compared to the control group (18.98 ± 3.98 nmol Pi min^{-1} mg^{-1} of protein; Fig. 3). However, PHT did not change ATP, ADP, and acetylcholine hydrolysis (data not shown). Similarly, GBP did not alter ATP, ADP, AMP, and acetylcholine hydrolysis (data not shown).

4. Discussion

The interactions of AEDs and ectonucleotidases have been investigated and these drugs have demonstrated different effects in several studies. Cognato et al. (2007) observed the *in vitro* and *in vivo* effects of PHT, valproate, and CBZ on ectonucleotidase activities in synaptosomes from hippocampus and cerebral cortex of rats. These results have shown that these drugs did not alter the ectonucleotidase activities both *in vitro* and *in vivo* experiments in hippocampus and cerebral cortex. However, the authors observed that previous treatment with CBZ and PHT can modulate plastic events associated to the nucleotidase activities preventing the pilocarpine-induced increase on ATP, ADP, and AMP hydrolysis (Cognato et al., 2007). Horvat et al. (2006) observed ecto-ATPase inhibition in rat brain synaptosomal plasma membranes treated with CBZ. This effect may represent a potential neuromodulatory action of this drug given that by inhibiting ecto-ATPase activities, CBZ may increase the extracellular ATP content and decrease the production of adenosine.

Ectonucleotidases modulate activation of P2 or P1 receptors by controlling extracellular concentrations of ATP and adenosine. NTPDase family, which hydrolyzes both nucleoside triphosphates and diphosphates, comprises cell-surface-enzymes with an extracellular active site, anchored to the membrane via two transmembrane domains that are essential to catalytic activity and substrate specificity (Grinthal and Guidotti, 2006; Robson et al., 2006). The ectonucleotidase chain is also constituted by ecto-5′-nucleotidase, which is able to promote the hydrolysis of nucleoside monophosphates to adenosine. Unlike NTPDases, ecto-5′-nucleotidase is attached to the extracellular membrane by a glycosyl phosphatidylinositol (GPI) anchor (Sträter, 2006).

Changes in membrane constituents can affect membrane-bound enzymes activity. Cholesterol depletion from membranes of NTPDase1-expressing cells reduces ATPase activity to the same extent as solubilization does (Papanikolaou et al., 2005). Intra and intermolecular oxidative cross-linking decreases ATPase activity (Chiang and Knowles, 2008). These results showed that changes at the balance between stability and mobility of the transmembrane domains can alter NTPDase activity (Grinthal and Guidotti, 2006).

Our findings demonstrated that CBZ decreased NTPDase whereas did not change ecto-5′-nucleotidase activities. A study with human erythrocyte have shown that CBZ perturbed outer moiety lipids inducing a disordering effect on the polar head groups and acyl chains of the membrane lipid showing that CBZ can lead interactions in membrane bilayer (Suwalsky et al., 2006). Therefore, it is possible to suggest that the effects on NTPDase activities induced by CBZ may be due to changes promoted in the bilayer membrane. Previous studies from our laboratory have shown that other drug classes, such as antidepressant (Pedrazza et al., 2007) and antipsychotic drugs (Seibt et al., 2009a) also promoted different effects on NTPDase when compared with ecto-5′-nucleotidase activities, probably by modifying plasma membranes.

PHT did not change NTPDase while significant increase of ecto-5′-nucleotidase activity was observed. Ecto-5′-nucleotidase has several functions, including generation of adenosine leading to the activation of adenosine receptors (Hunsucker et al., 2005). This enzymatic effect may represent further ways by PHT modulate the neuronal activity. Adenosine is known to be very effective in the suppression of seizures. Binding of adenosine to the high affinity A_1 receptor reduces excitability of the cells by modulation of Ca^{2+} and K^+ fluxes and inhibits the release of various neurotransmitters, such as glutamate, dopamine, serotonin, and acetylcholine (Boison, 2005, 2008).

Our results showed that CBZ and PHT can increase ATP and adenosine levels, respectively. It is possible to suggest that these effects are involved, at least partially, in the antiepileptic mechanisms of these AEDs. Although it has been shown that adenosine inhibits neurotransmission (Dunwiddie and Masino, 2001; Fredholm et al., 2005; Boison, 2008), some studies indicate that ATP can also inhibit synaptic transmission (Yoshioka and Nakata, 2004; Nakata et al., 2005). The colocalization of A_1 and $P2Y_1$ receptors in several regions in the brain suggests a potential heterodimerization and functional interaction mechanism between these receptors. $A_1/P2Y_1$ heterodimerization forms an ATP-sensitive adenosine receptor and ATP can work as an A_1 agonist to inhibit neurotransmission (Yoshioka and Nakata, 2004; Nakata et al., 2005). Furthermore, a cross-talk of A_1–$P2Y_1$ receptors might be a mechanism by which low and high concentrations of adenosine or purines could regulate glutamate release (Tonazzini et al., 2007).

Acetylcholine is a neurotransmitter involved in essential brain functions, including memory and learning (Shaked et al., 2008). Acetylcholinesterase, the key enzyme that hydrolyzes and inactivates acetylcholine, modulates also non-cholinergic functions, such as glutamatergic and dopaminergic systems (Soreq and Seidman, 2001; Zimmermann and Soreq, 2006; Shaked et al., 2008). Several studies have shown the cholinergic system is also involved in the mechanisms related to epilepsy and acetylcholinesterase inhibitors lead to an increase in acetylcholine levels inducing seizures (Friedman et al., 2007; Pernot et al., 2009). Different studies have demonstrated the effect of AEDs on acetylcholinesterase activity and acetylcholine levels. Previous studies showed that cortical injury and enhanced neural excitability are associated with modifications in the isoform of acetylcholinesterase. The variation in distribution patterns suggested a possible isoform shift of acetylcholinesterase enzyme in epileptic brains from membrane adhered AChE-S protein, located in cellular layers, to the soluble AChE-R monomers, which may diffuse to extrasynaptic regions. The results suggested an increased production of AChE-R monomers, which may access the synaptic microenvironment and potentially protect the epileptic tissue from cholinergic hyperexcitation (Zimmermann et al., 2008). In another study with rats, chronic treatment with PHT (ranging from 5 to 75 mg/kg) did not modify significantly the acetylcholinesterase activity in the motor cortex, pyriform cortex, and olfactory bulb when compared to control group. On the other hand, PHT decreased acetylcholinesterase activity in the hippocampus (50 and 75 mg/kg) and also in the striatum (75 mg/kg) (Sudha et al., 1995). Likewise, in rats, acute treatment with effective dose of CBZ (25 mg/kg) enhances acetylcholine synthesis without affecting the degradation, increasing acetylcholine levels. The supraeffective tested dose of CBZ (100 mg/kg) decreased the activity of acetylcholinesterase and butyrylcholinesterase and also inhibited acetylcholine synthesis, reducing the levels of this neurotransmitter. Therefore, effective dose of CBZ increased acetylcholine levels, which were reduced by increasing of CBZ dose (Mizuno et al., 2000). It also has been reported that CBZ had biphasic effects on acetylcholine release and synthesis (Zhu et al., 2002).

Previous studies have shown the IC_{50} of CBZ for acetylcholinesterase and butyrylcholinesterase activities were more than 300 μM (Mizuno et al., 2000). The authors suggest that the inhibitory ef-

fects of supraeffective CBZ concentrations on cholinesterase activities may not play a role in acetylcholine transmission (Mizuno et al., 2000). In contrast, both toxic concentration of acetylcholine receptor agonists, antagonists and cholinesterase inhibitor have been shown to have proconvulsant activities, whereas low doses of cholinesterase inhibitor reduced seizure activities (Bhattacharya et al., 1991; Cruickshank et al., 1994). This evidence supports the hypothesis that drastic elevation of cholinergic function induces seizure activity. The therapeutic ranges of serum concentration of CBZ are from 15 to 45 μM (Loiseau and Duche, 1995). Our results showed an inhibitory effect on acetylcholinesterase only at high CBZ concentrations (500 and 1000 μM). Therefore, the inhibitory effect on acetylcholinesterase at these high doses of CBZ could promote an increase of acetylcholine levels, which could induce a seizure activity or a neurotoxic effect promoted by CBZ high concentrations. Here, the CBZ biphasic profile also was observed in purinergic system. Our results demonstrated a trend to increase ATP hydrolysis in the presence of low doses of CBZ (10 and 50 μM) and a decreased hydrolysis of this neurotransmitter in high CBZ dose (1000 μM). Such results demonstrate that CBZ induces a complex set of effects characterized by a biphasic profile. Additional studies are required to verify the *in vivo* effect of the high CBZ doses in the seizure activity and purinergic and cholinergic transmission.

These findings indicate that AEDs could show different pharmacokinetic profile depending on relevant end points, such as route of administration, dose of the drug tested and animal model used in the study. However, there are some limitations about the side effects and resistance developed of AEDs. The discovery and screening of new molecular targets and AEDs may increase the treatment spectrum, which will be reached with pharmacology studies focusing to this issue (Stefan and Feuerstein, 2007; Bialer and White, 2010; Luna-Tortós et al., 2010). In summary, this study highlight that enzymes related to purinergic and cholinergic systems can be modulated by antiepileptic drugs in zebrafish. Furthermore, these findings can contribute to a better understanding about the pharmacology of classical AEDs and their interaction with purinergic and cholinergic neurotransmission.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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