Triterpene 3β, 6β, 16β trihidroxilup-20(29)-ene protects against excitability and oxidative damage induced by pentylenetetrazol: The role of Na\(^+\),K\(^+\)-ATPase activity

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**Abstract**

Administration of the compound triterpene 3\(\beta\), 6\(\beta\), 16\(\beta\) trihidroxilup-20(29)-ene (TTHL) resulted in antioxidantive activity in several pain models in mice. Because pain and epilepsy have common mechanisms, and several anticonvulsants are clinically used to treat painful disorders, we investigated the anticonvulsant potential of TTHL. Behavioral and electrographic recordings revealed that pretreatment with TTHL (30 mg/kg; i.g.) increased the latencies to the first clonic seizure to the tonic-clonic and reduced the duration of the generalized seizures induced by the GABA\(_{\alpha}\) receptor antagonist PTZ (80 g; i.p.). The TTHL pretreatment also protected against PTZ-induced deleterious effects, as characterized by protein carbonylation, lipid peroxidation, \(^3\)H glutamate uptake and the inhibition of Na\(^+\),K\(^+\)-ATPase (subunits \(\alpha_1\) and \(\alpha_2\)). Although TTHL did not exhibit DPPH, ABTS radical scavenging activity per se and does not alter the binding of \(^1\)Hflunitrazepam to the benzodiazepinic site of the GABA\(_{\alpha}\) receptor, this compound was effective in preventing behavioral and EEG seizures, as well as the inhibition of Na\(^+\),K\(^+\)-ATPase induced by ouabain. These results suggest that the protection against PTZ-induced seizures elicited by TTHL is due to Na\(^+\),K\(^+\)-ATPase activity maintenance. In fact, experiments in homogenates of the cerebral cortex revealed that PTZ (10 mM) reduced Na\(^+\),K\(^+\)-ATPase activity and that previous incubation with TTHL (10 \(\mu\)M) protected against this inhibition. Collectively, these data indicate that the protection exerted by TTHL in this model of convulsion is not related to antioxidant activity or GABAergic activity. However, these results demonstrated that the effective protection of Na\(^+\),K\(^+\)-ATPase elicited by this compound protects against the damage due to neuronal excitability and oxidation that is induced by PTZ.

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1. Introduction

Epilepsy is a neurological disorder characterized by behavioral and electroencephalographic (EEG) changes in a group of neurons in the central nervous system (CNS) (Dichter et al., 2007). This neurological disorder consists of a large group of neurological
diseases with incidence of 0.5–1% in the general population (Andrade and Minassian, 2007). Our understanding of the pathophysiology of epilepsy has advanced dramatically in last 30 years, particularly in terms of its cellular physiology and genetic bases (Meldrum and Rogawski, 2007). Conversely, although single-drug therapies provide optimal seizure control in approximately 80% of all patients, seizure activity remains uncontrolled in a significant number of individuals, regardless of the type of therapy (Dichter et al., 2007).

From this point of view, several studies have suggested that a cascade of biological events, including overstimulation of the glutamatergic system, understimulation of the GABAergic system, and oxidative stress on selected targets as Na+/K+-ATPase, underlies the development and propagation of epilepsy (Patsoukis et al., 2005; Souza et al., 2009). This finding is particularly important, considering that this ion-motive pump plays a key role in regulating and controlling nerve excitability (Vasilets and Schwarz, 1993). Based on the hypothesis that these selected targets are involved in epilepsy, an alternative approach to treatment of this disorder would be to use in order to prevent or slow the progression of seizures.

There are several mechanisms shared by epilepsy and pain induction (de Oliveira et al., 2011; Freitas, 2009; King et al., 2011). Of note, some anticonvulsants are used clinically to treat painful disorders (Feuerbach et al., 2009; Mares and Rokyta, 2009). In this context, Longhi-Balbinito et al. (2009) have demonstrated that triptene 3β, 6β, 16β-trihydroxilup-20(29)-ene (TTHL) produced significant and specific antinociceptive action against glutamate-, NMDA- and trans-ACPD-induced nociception without causing any detectable locomotor impairment. These experimental findings suggest that the antinociceptive effect of TTHL is closely related to an effect on the glutamatergic system. Because overstimulation of glutamatergic system is a common mechanism for pain and epilepsy, we decided to investigate the effect of TTHL on PTZ-induced behavioral, electrographic and neurochemical alterations in mice.

2. Materials and methods

2.1. Animals

The experiments were conducted using Swiss mice (25–35 g) maintained in a controlled environment (12:12 h light–dark cycle, 24 ± 1 °C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water. All of the experimental protocols were developed with the goals of keeping the number of animals used to a minimum and maintaining these animals’ wellbeing. All of the experiments were conducted in accordance with national and international standards (i.e., the Brazilian School of Animal Experimentation (COBEA) policy and the U.S. Public Health Service’s Policy on Humane Care and Use of Laboratory Animals) and with the approval of the Ethics Committee to the Federal University of Santa Maria (113/2010).

2.2. Placement of cannula and surgical procedures for electrocorticographic recording

To determine whether TTHL protects against convulsions induced by ouabain and PTZ, a subset of animals was anesthetized with ketamine (100 mg/kg; i.p.) and xylazine (30 mg/kg; i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula and a set of electrodes were implanted for the purpose of EEG recording. The guide cannula was glued to a multipin socket and inserted into the right ventricle through a previously opened skull orbit. Two screw electrodes were placed over the right (ipsilateral) and left (contralateral) parietal cortices (coordinates in mm: AP = −4.5, 1.25) along with a ground lead positioned over the nasal sinus. The electrodes were connected to a multipin socket and fixed to the skull with dental acrylic cement.

All of the intracerebroventricular (i.c.v.) injections were performed with a needle (30 gauge) protruding 1 mm below a guide cannula. All drugs were injected during a period of 1 min with a Hamilton syringe, and an additional minute was allowed to elapse before the removal of needle to avoid backflow of drug through the cannula. The procedures for EEG recording were carried out as previously described (Cavalheiro et al., 1992). Briefly, the animals were allowed to habituate to a Plexiglas cage (25 cm × 25 cm × 60 cm) for at least 30 min before the EEG recordings. Animals were subsequently connected to the lead socket that resides inside a Faraday’s cage. The EEG recordings were executed using a digital encephalograph (Neuromap EQ5250, Neurotec LTDA, Irajubá, MG, Brazil). EEG signals were amplified, filtered (0.1–70.0 Hz, band pass), digitized (sampling rate 256 Hz) and stored on a PC for off-line analysis.

2.3. Reagents, drug administration protocol and seizure evaluation

All of the reagents were purchased from Sigma (St. Louis, USA). The drug TTHL was isolated from the flowers of Combretum leprosum by the Department of Organic Chemistry (Universidade Federal de Rondônia, Brazil) and characterized by spectral analyses (RMN-H) and RMN-13C and by comparison with the spectrum literature data (Facundo et al., 1993); the drug showed a degree of purity greater than 98%. All reagents were dissolved in saline solution (0.9%) with the exception of triptene, which was dissolved in saline plus DMSO/Tween 80. The final concentration of DMSO and Tween 80 did not exceed 10% and did not cause any effect per se (Fig. 1).

To evaluate the effect of TTHL on electroencephalographic, oxidative and neurochemical alterations in the cerebral cortex of mice induced by PTZ, the animals were treated with TTHL (1, 3, 10 or 30 mg/kg) or its vehicle by intragastric gavage (i.g.) (Longhi-Balbinito et al., 2012) 60 min before the systemic administration of PTZ (80 mg/kg; i.p.) (Zandieh et al., 2010). In the present study, we also evaluated the participation of Na+/K+-ATPase in electroencephalographic alterations exerted by TTHL in this model of excitotoxicity. For this purpose, a subset of animals was treated with TTHL (30 mg/kg) or its vehicle (i.g.) 60 min before administration of ouabain (1 µg/site; i.c.v.) (Bagetta et al., 1995; Doggett, 1975).

The presence of seizures was monitored in all animals by electroencephalographic recordings. A 10-min baseline recording was obtained to establish an adequate control period. After this baseline recording, the animals were observed for the appearance of generalized tonic–clonic convulsive episodes for 20 min (Ferraro et al., 1999); tonic–clonic convulsive episodes were defined by generalized whole-body clonus involving all four limbs and the tail, rearing, wild running and jumping, followed by sudden loss of upright posture and autonomic signs, such as hypersalivation and defecation. During the 20-min observation period, the latency for generalized tonic–clonic convulsions was measured. The EEG recordings were visually examined for seizure activity, as defined by the occurrence of the following alterations in the recording leads (McColl et al., 2003); isolated sharp waves (>1.5 s baseline); multiple sharp waves (>2× baseline) in brief spindle episodes (>1 s and ≤5 s); multiple sharp waves (>2× baseline) in long spindle episodes (>5 s); spikes (>2× baseline) plus slow waves; multispikes (>2× baseline, ≥3 spikes/complex) plus slow waves; and major seizure (repetitive spikes plus slow waves obliterating background rhythm, ≥5 s).

2.4. Ex vivo experiments

2.4.1. Tissue processing for neurochemical analyses

Immediately after the behavioral and electroencephalographical evaluation, animals were sacrificed by decapitation. The brain of each animal was later exposed by the removal of the parietal bone. The cerebral cortex was rapidly dissected on an inverted ice-cold Petri dish and homogenized in cold 30 mM Tris–HCl buffer (pH 7.4). This homogenate was used for determination of thoribarbituric acid-reactive substances (TBARS), glutamate uptake, carbonyl content and Na+-K+-ATPase activity.

2.4.2. Determination of Na+-K+-ATPase activity

Na+-K+-ATPase and its α isomers were assayed as previously described (Nishi et al., 1999; Rambo et al., 2009). Briefly, the assay medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM MgCl2 and 50 µg of protein in the presence or absence of ouabain (1 mM); the final volume of

Fig. 1. Molecular structure of triterpene 3β, 6β, 16β-trihydroxilup-20(29)-ene.
this solution was 350 μL. The reaction was started adding adenosine triphosphate to a final concentration of 5 mM. After 30 min at 37 °C, the reaction was stopped by adding 70 μL of 50% (w/v) trichloroacetic acid. Saturating substrate concentrations for these compounds were used, and the reaction was linear with protein and time. The appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate [Lauren et al., 2007] released was quantified by a previously described colorimetric method (Fiske and Subbarow, 1927), using KH2PO4 as reference standard. The specific Na⁺/K⁺-ATPase activity (nmol Pi/mg protein/min) was calculated by subtracting the ouabain-inhibitable activity from the overall activity in the absence of ouabain.

In the same set of experiments, we investigated whether some Na⁺/K⁺-ATPase α isoforms are selectively modulated by TTHL or PTZ. For this purpose, we used a previously described pharmacological approach that takes advantage of the isoform-specific sensitivity to ouabain (Nishi et al., 1999). The cerebral cortex contained 30 mM Tris–HCl buffer, pH 7.4; 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM MgCl₂, and 50 μg of protein in the presence of 3 μM (enough to inhibit Na⁺/K⁺-ATPase isoforms containing α2 and α3 subunits) or 4 μM ouabain (sufficient to inhibit α1 isoforms).

2.4. Measurement of thiobarbituric acid-reactive substances (TBARS)

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2.4.5. Determination of glutamate uptake

For the assay of [3H] glutamate uptake, a synaptosomal preparation was obtained from isotonic Percoll/sucrose discontinuous gradients at 4 °C as previously described (Dunkley et al., 1986). Briefly, homogenates from the cerebral cortex (10% [w/v]) were made in homogenization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 320 mM sucrose, 50 mM glucose, and 1.2 mM CaCl₂; the homogenate was then centrifuged twice at 16,000 × g for 10 min at 4 °C until analyzed.

The level of radioactivity in the synaptosomal pellet was measured in a Packerd 464 L. The specific activity from the overall activity in the absence of ouabain.

2.5. In vitro experiments

2.5.1. DPPH, ABTS radical and anion superoxide radical scavenging activity

The atom density or the electron donation capacity of the respective extracts was computed from the bleaching property of the purple-colored methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The stable DPPH radical scavenging activity was determined by Boish method (1958). The samples and reference were dissolved in ethanol (75%) and mixed with DPPH solution (final concentration 0.15 mM). The remaining DPPH amount was measured at 520 nm using a Shimadzu UV–visible double beam spectrophotometer (15A). The results were compared to that of n-acetylcysteine (NAC), which was employed as the reference. The antioxidant capacity was calculated as compared to a blank tube containing DPPH solution and ethanol (100% oxidation). The experiments were run in triplicate, and the results were conveyed as average values with S.E.M. (standard errors of the mean).

The ABTS radical (2,2′-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid]) scavenging ability was measured using slight modifications of an existing protocol (Fernandes et al., 2008). The ABTS radical cation was generated by mixing the ABTS stock solution (7 mM) with potassium persulfate (140 mM) and allowing the reaction to occur in the dark at room temperature for 16 h before use. Prior to the assay, the ABTS stock solution was diluted with a KH2PO4/K3HPO4 (100 μM, pH 7.4) buffer solution (ratio 1:88). The ABTS solution (1 mL) was added to glass test tubes containing different concentrations of TTHL (1–100 μM). The tubes were incubated for 30 min at room temperature. The scavenging capacity was measured at 734 nm against the corresponding blank. The superoxide radical scavenging activity was performed according a previously described method (Zhao et al., 2006). The determination is performed with the pyrogallol/xanthine oxidase (HPX/XOD) system, which is based on the enzymatic generation of the O₂− species from the oxidation of pyrogallol to xanthine and uric acid. The produced free radical reduces the absorbing probe nitroblue tetrazolium (NBT) to formazan at pH 7.4, indicated by a color change when monitored at 560 nm.

2.5.2. Na⁺/K⁺-ATPase activity

It was presumed that the behavioral effects of PTZ and TTHL are due to its capacity to alter Na⁺/K⁺-ATPase activity by directly interacting with the enzyme; therefore, this activity was measured directly. A subset of animals were killed by decapitation and had their brains rapidly removed and placed in an inverted ice-cold Petri dish. The cerebral cortex was homogenized in cold 10 mM Tris–HCl buffer (pH 7.4), and the experiments were performed at a final volume of 500 μL. The homogenates were pre-incubated for 30 min at 37 °C with increasing concentrations of TTHL (1 and 10 μM). After 30 min of incubation, the homogenates were incubated with PTZ (10 μM) during 20 min. The concentration of PTZ used in the current study was chosen based previous studies that have demonstrated a direct interaction of the PTZ with Na⁺/K⁺-ATPase (Dubberke et al., 1998). After the incubation period, the Na⁺/K⁺-ATPase activity was determined as previously described (Nishi et al., 1999; Rambo et al., 2009).

2.5.3. [3H]flunitrazepam binding

The cerebral cortex from each animal was thawed and homogenized in 10 ml of homogenization buffer A (10 mM Tris–HCl, 300 mM sucrose, and 2 mM EDTA, pH 7.4) per gram of tissue. This homogenate was centrifuged at 1000 × g for 10 min at 4 °C. The resulting supernatant was centrifuged at 16,000 × g for 20 min at 4 °C. The resulting pellet was then re-suspended in 1 ml of homogenization buffer and frozen at −70 °C until analyzed.

The binding of [3H]flunitrazepam to the benzodiazepinic site of GABAA receptors was determined by first washing the cell membrane preparation in the following manner: individual aliquots were diluted with five volumes of wash buffer B (50 mM Tris–HCl and 2 mM EDTA, pH 7.4); the aliquots were mixed and centrifuged at 16,000 × g for 10 min at 4 °C, and the samples were incubated for 30 min at 37 °C. This washing procedure was repeated twice, and the final pellet was re-suspended in binding assay buffer C (20 mM HEPES and 1 mM EDTA, pH 7.4). The protein concentration of each sample was determined by a spectrophotometric protein dye-binding assay based on a previously described method that uses bovine serum albumin as the standard (Bradford, 1976). The incubation was carried out in duplicate in polycarbonate tubes (total volume 500 μL containing 50 mM Tris–HCl (pH 7.4), 0.5 mg of protein membrane and TTHL (0.1, 1 and 10 μM). Diazepam (10 μM) was used as a positive control. The incubation was started by adding 1 nM [3H]flunitrazepam (85.8 Ci/mmol) and was run in ice for 60 min. The reaction was stopped by vacuum filtration, and each filter was washed with 15 ml of cold 10 mM Tris–HCl buffer. The filters were placed individually in polycarbonate tubes, and 1 ml of scintillation liquid was added. The radioactivity of each sample was determined using a Packard Tri-Carb 2100TR liquid scintillation counter. The non-specific binding of each sample was determined by adding 100 μM non-radioactive flunitrazepam to the medium in parallel assays. The specific binding of each sample was considered as the difference between total binding and non-specific binding. The results were expressed as percentage inhibition of TTHL specific binding. [3H]flunitrazepam. Protein content was measured colorimetrically by a previously described method (Bradford, 1976) that used bovine serum albumin (1 mg/ml) as the standard.

2.5.4. [3H]glutamate binding

For membrane preparation, the cerebral cortex from three mice were homogenized (1:20, w/v) in homogenization buffer (10 mM Tris–acetate, 320 mM sucrose, 1 mM MgCl₂). The homogenate was centrifuged at 4 °C 1000 × g for 15 min, and the supernatant (S1) was collected from this initial centrifugation. The initial pellet (P1)
was homogenized again and subjected to an identical centrifugation, and the resulting supernatant (S1) was combined with S1. The combined supernatant was centrifuged at 4 °C at 16,000 × g for 15 min, and the resulting pellet (P2) was re-suspended (1:20, w/v) in 10 mM Tris–acetate (pH 7.4) without sucrose and placed on ice for 30 min. After this incubation, the membrane solution was centrifuged at 4 °C at 16,000 × g for 15 min, the supernatant was discarded and the pellet was re-suspended in the same buffer. This last wash sequence was repeated more than three times, and the resulting membranes were frozen at −20 °C for 48 h. After this period, the membranes were defrosted, re-suspended (1:20, w/v) in 10 mM Tris–acetate and incubated at 37 °C for 30 min. Next, the membranes were centrifuged and washed in the same conditions as described above four additional times, and they were frozen at −20 °C. On the assay day, the membranes were centrifuged and washed four additional times. After the last centrifugation, the pellets were re-suspended in 10 mM Tris–acetate (pH 7.4), and the protein content was determined using a previously described method (Bradford, 1976).

The glutamate binding experiments were carried out in the presence of sodium to target glutamate binding to the transporters that depend on sodium for their activity as previously described (Dalcin et al., 2007). The incubations were carried out in triplicate in polycarbonate tubes (total volume 500 μL) containing 50 mM Tris–acetate/120 mM NaCl, pH 7.4, 50 μg of protein membrane and TTHL (1–10 μM). The level of [3H] glutamate binding was evaluated in the presence of 10 μM l-trans-pyrrolidine-2,4-dicarboxylate (PDC), a substrate inhibitor of glutamate transporters. The experimental controls did not contain TTHL. The incubation step was started by adding 40 nM [3H] glutamate (0.3 μCi), and the process was run at 30 °C for 30 min. The reaction was stopped by vacuum filtration, and each filter was washed with 20 ml of cold 10 mM Tris–acetate buffer. The filters were placed individually in polycarbonate tubes, and 1 ml of scintillation liquid was added to each tube. The radioactivity of each sample was determined using a Packard Tri-Carb 2100TR liquid scintillation counter. The non-specific binding of each sample (10–20% of the total binding) was determined by adding 40 μM non-radioactive glutamate to the medium in parallel assays. The specific binding of each sample was considered as the difference between total binding and non-specific binding. The results were expressed as percentage of specific binding.

2.6. Statistical analysis

Statistical analysis was carried out by one- or two-way analysis of variance (ANOVA), and only F-values at a significance level of P < 0.05 are presented. Post-hoc analyses were carried out when appropriate using the Student–Newman–Keuls test. All data are expressed as the mean ± S.E.M.

3. Results

3.1. Dose response curve of TTHL on convulsions induced by PTZ

Fig. 2 shows the effect of the acute administration of TTHL (1, 3, 10 and 30 mg/kg) on PTZ-induced behavioral seizures, as characterized by the latencies to the first tonic–clonic and the generalized convulsion and the duration of the generalized convulsions. Electroencephalographic recordings confirmed that the behavioral seizures were elicited by PTZ (80 mg/kg; i.p) (Fig. 2C); these seizures were characterized by the occurrence of multispeaks plus slow waves and major seizure activity. The multispeaks plus slow waves correlated with myoclonic jerks, which are characteristic of clonic convulsions. The generalized convulsions appeared in the EEG recordings as the major seizure activity and were characterized by 2–3 Hz high-amplitude activity. After the ictal discharge, postictal EEG suppression and slow waves were observed, and this activity correlated with behavioral catalepsy. In the present study we showed that pretreatment with TTHL (30 mg/kg i.g.) increased the latency to first clonic seizure [H (5) = 10.96, P < 0.001, Fig. 2G] and the latency to the tonic-clonic [H (5) = 14:46, P > 0.05 Fig. 2H] but reduced the duration of generalized seizures [H (5) = 17:33, P < 0.01 Fig. 2I] induced by PTZ (80 mg/kg; i.p). The EEG recordings confirmed that systemic administration of TTHL (30 mg/kg) protected against PTZ-induced behavioral seizures (Fig. 2F).

However, a lower dose of TTHL (1–10 mg/kg) was not capable of altering both behavior and EEG seizures induced by PTZ (Fig. 2G–I and data not shown). Thus, we decided to select only the dosage of TTHL (30 mg/kg) that had anticonvulsant effects for the investigation of...
the action of TTHL on neurochemical alterations following the injection of PTZ.

3.2. Involvement in TTHL in \( ^{3} \)Hflunitrazepam binding, specific \( ^{3} \)H glutamate binding and antioxidant activity in vitro

To determine whether the prevention exerted by TTHL in this model of convulsion may result from an interaction with GABA\(_{A}\) receptors, we performed a \( ^{3} \)Hflunitrazepam binding assay in membranes from the cerebral cortex of mice. A statistical analysis revealed that the incubation of TTHL (1–10 \( \mu \)M) did not alter the binding of \( ^{3} \)Hflunitrazepam to the benzodiazepinic site of GABA\(_{A}\) receptors or to \( ^{3} \)H glutamate (Fig. 3C–D). A statistical analysis also revealed that TTHL (1–100 \( \mu \)M) did not exhibit ABTS and DPPH radical scavenging activity (Fig. 3A–B). The effect of TTHL (1–100 \( \mu \)M) against enzymatically generated \( O_{2}^{-} \) radicals was also evaluated, but no antioxidant activity was observed for concentrations of TTHL ranging from 1 to 100 \( \mu \)M (data not shown).

3.3. Involvement in TTHL lipid peroxidation and protein carbonylation in ex vivo

In the present study, we showed that the injection of a consultant dose of PTZ (80 mg/kg) induced an increase in lipid peroxidation \( [F(3,22) = 5.38, P < 0.05, \text{Fig. 4A}] \) and protein carbonylation \( [F(3,24) = 4.37, P < 0.05, \text{Fig. 4B}] \) in the cerebral cortex. In addition, our data showed that pretreatment with TTHL (30 mg/kg i.g.) protected against these deleterious effects induced by PTZ. These experimental data suggest that anticonvulstant effect exerted by TTHL protects against increases in protein carbonyl and TBARS after PTZ injection.

3.4. TTHL effect on glutamate uptake and \( \text{Na}^{+}.\text{K}^{+}-\text{ATPase} \) activity in ex vivo

Considering that some selected targets, such as \( \text{Na}^{+}.\text{K}^{+}-\text{ATPase} \) and glutamate transporters, might lead to the enhancement of neuronal excitability and the appearance of convulsions (Rambo et al., 2009), we decided to investigate whether TTHL pretreatment protects against PTZ-induced deleterious effects; the level of this protection is characterized here by glutamate uptake and Na\(^{+}\).K\(^{+}\)-ATPase activity inhibition. A statistical analysis showed that pretreatment with TTHL (30 mg/kg; i.g.) protected against inhibition of the total activity of Na\(^{+}\).K\(^{+}\)-ATPase induced by PTZ \( [F(3,19) = 5.06, P < 0.05, \text{Fig. 5A}] \), as well as that of the Na\(^{+}\).K\(^{+}\)-ATPase subunits \( x_{1} \) \( [F(3,19) = 5.91, P < 0.05, \text{Fig. 5B}] \) and \( x_{2}/x_{3} \) \( [F(3,19) = 3.72, P < 0.05, \text{Fig. 5C}] \). A statistical analysis also revealed that the administration of TTHL (30 mg/kg; i.g.) had no effect on \( ^{3} \)H glutamate uptake per se \( [F(1,19) = 1.54, P > 0.05] \), but the administration of TTHL was effective against PTZ-induced \( ^{3} \)H glutamate uptake inhibition \( [F(3,19) = 14.02, P < 0.05, \text{Fig. 6}] \). These experimental results suggest that the inhibition of the Na\(^{+}\).K\(^{+}\)-pump and inhibition of \( ^{3} \)H glutamate uptake contribute to the ictogenic activity of PTZ. In addition, the anticonvulstant effect exerted by pretreatment with TTHL involves the Na\(^{+}\).K\(^{+}\)-ATPase activity maintenance.

3.5. TTHL effect on ouabain-induced convulsions and \( \text{Na}^{+}.\text{K}^{+}-\text{ATPase} \) activity inhibition ex vivo

To determine whether the prevention exerted by TTHL in this model of convulsion may result from an Na\(^{+}\).K\(^{+}\)-ATPase maintenance, we investigated the effect of TTHL on seizures induced by ouabain, which is a Na\(^{+}\).K\(^{+}\)-ATPase inhibitor. As depicted in the

Fig. 3. The effect of TTHL (100 \( \mu \)M) or N-acetylcysteine (10 \( \mu \)M) on the ABTS scavenger assay (A) (\( n = 4 \)/triplicate). The effect of TTHL (1–100 \( \mu \)M) or N-acetylcysteine (10 \( \mu \)M) on the DPPH scavenger assay (B) (\( n = 4 \)/triplicate). The effect of TTHL (1 or 10 \( \mu \)M) or diazepam (DZP, 10 \( \mu \)M) on \( ^{3} \)H-flunitrazepam binding in mice cerebral cortex membranes (C) (\( n = 4 \)/triplicate); The effect of TTHL (1 or 10 \( \mu \)M) or trans-2,3-trans-pyrrolidine-2,3-dicarboxylic acid (PDC, 10 \( \mu \)M) on sodium-dependent \( ^{3} \)H-glutamate binding in mice cerebral cortex membranes (\( n = 4 \)/duplicate). The data are presented as the mean ± S.E.M. *P < 0.05 or **P < 0.01 compared with the control group (One-way ANOVA followed by Student Newman–Keuls’ test).
representative EEG recordings (Fig. 7C), an ouabain injection at a fully convulsive dose (1 µg/i.c.v.) caused the appearance of clonus of the left forelimb and/or left hind limb and head; these behaviors were accompanied by rotational behavior. The seizures were defined by episodes consisting of the simultaneous occurrence of at least two of the following alterations in all four recording leads: high-frequency complexes, polyspike complexes, or high-voltage synchronized spike activity. A statistical analysis also revealed that pretreatment with TTHL (30 mg/kg, i.g.) increased the latency to the tonic–clonic \([H (5) = 9.64, P < 0.05; \text{Fig. 7G}]\) and reduced the duration of generalized seizures \([H (5) = 17:33, P < 0.05; \text{Fig. 7H}]\) (1 µg/i.c.v.). The EEG recordings confirmed that behavioral seizures were elicited by injection of ouabain (Fig. 7C) and that the behavioral seizures were prevented by the systemic administration of TTHL (Fig. 7F). In addition, the pretreatment with TTHL (30 mg/kg; i.g.) protected against inhibition of the total activity of Na\(^{+},K^{+}\)-ATPase induced by ouabain (1 µg/i.c.v.) \([F(3, 19) = 5.06, P < 0.05, \text{Fig. 8A}]\), as well as that of the subunits \(\alpha_2/\alpha_3\) \([F(3,19) = 3.72, P < 0.05, \text{Fig. 8C}]\).

### 3.6. TTHL effect on Na\(^{+},K^{+}\)-ATPase activity in vitro

To determine whether PTZ decreases Na\(^{+},K^{+}\)-ATPase activity by directly interacting with the enzyme, PTZ (10 mM) and TTHL (1, 10 µM) were added directly to the reaction medium containing the cerebral cortex homogenates. A statistical analysis revealed that the inhibitory effect of PTZ (10 mM) on Na\(^{+},K^{+}\)-ATPase activity in homogenates is not isoform-specific, as it occurred in the presence of 4 mM ouabain (inhibiting all isoforms) \([F(3, 40) = 9.44, P < 0.05, \text{Fig. 8A}]\).
The data are presented as median and interquartile range for vehicle- and TTHL-treated animals, respectively. The arrows indicate the ouabain injection; the arrowheads indicate the EEG patterns associated with clonic seizures. The calibration bars are 500 µV and 15 s. The effect of TTHL administration (30 mg/kg, p.o.) on the convulsive behavior induced by ouabain (1 µg/5 µL, i.c.v) is shown in Figures G (latency for generalized tonic-clonic seizure) and H (time spent in generalized tonic-clonic seizure). The data are presented as median and interquartile range for n = 8–9 animals per group. “p < 0.05 compared with the control group (Mann–Whitney test).

Fig. 7. Several representative electroencephalographic recordings from animals treated with the vehicle (A–C) or TTHL (30 mg/kg, p.o.) (D–F). The basal period is showed in recording A and D. Recordings B and E show the period after vehicle or TTHL treatment, respectively. Recordings C and F show the period after ouabain injection (1 µg/5 µL, i.c.v) in vehicle- and TTHL-treated animals, respectively. The arrows indicate the ouabain injection; the arrowheads indicate the EEG patterns associated with clonic seizures. The calibration bars are 500 µV and 15 s. The effect of TTHL administration (30 mg/kg, p.o.) on the convulsive behavior induced by ouabain (1 µg/5 µL, i.c.v) is shown in Figures G (latency for generalized tonic-clonic seizure) and H (time spent in generalized tonic-clonic seizure). The data are presented as median and interquartile range for n = 8–9 animals per group. “p < 0.05 compared with the control group (Mann–Whitney test).

Fig. 9A] and 3 µM ouabain (inhibiting Na⁺,K⁺-ATPase isoforms containing α2/α3) [F(3, 40) = 6.70; P < 0.05, Fig. 9B]. A statistical analysis also showed that previous incubation with TTHL (10 µM) protected against Na⁺,K⁺-ATPase inhibition induced by PTZ (10 mM) [F(3, 40) = 4.11; P < 0.05, Fig. 9C]. This result reinforced the idea that the effective protection exerted by TTHL in this model of seizure involves the maintenance of Na⁺,K⁺-ATPase activity.

4. Discussion

According to the World Health Organization, approximately 50 million people in the world have epilepsy. Despite progress in understanding the pathogenesis of the disorder, there remain a significant number of patients with poorly controlled seizures (at least 20% of patients) regardless of the type of therapy. An alternative approach to treatment of this disorder would be to use drugs to prevent or slow the progression of seizures. The results presented in the present report show, for the first time, the anticonvulsant properties of TTHL. The systemic administration of TTHL in mice protected against behavioral and electrographic seizures in vivo and neurochemical alterations ex vivo (increases in oxidative stress and inhibition of glutamate uptake and Na⁺,K⁺-ATPase activity in the cerebral cortex) induced by PTZ, an GABA_A receptor antagonist. However, the incubation of TTHL in vitro did neither scavenged free radicals nor altered flunitrazepam and sodium-dependent glutamate binding, indicating that the TTHL anticonvulsant effect was unrelated with its direct antioxidant action or interaction with GABA_A receptor and glutamate transporters. Conversely, the TTHL pretreatment was effective in preventing behavioral and electrographic seizures and Na⁺,K⁺-ATPase inhibition induced by ouabain, an inhibitor of Na⁺,K⁺-ATPase. Moreover, the results presented in this report show that PTZ exert an isoform-specific inhibitory effect on Na⁺,K⁺-ATPase activity in homogenates, an action prevented by the incubation with TTHL. These experimental findings imply Na⁺,K⁺-ATPase as a target for TTHL, which may underlie its protection against ouabain and PTZ-induced seizures and neurochemical alterations.

In the current study, we have confirmed and extended previous findings that PTZ, a chemical convulsant that affects several neurotransmitter systems, elicits behavioral and electrographic seizures, increases oxidative stress and inhibits glutamate uptake and Na⁺,K⁺-ATPase activity in vivo (Rambo et al., 2009; Souza et al., 2009). Moreover, the treatment with TTHL by oral route prevented PTZ-induced seizures and neurochemical alterations. Knowing that the antagonism of the GABA_A receptor has been considered the main mechanism for biological actions of PTZ (Zandieh et al., 2010), we determined whether the prevention exerted by TTHL in this model of convulsion may result in part from a direct interaction with the GABA_A receptor using the [³H]flunitrazepam binding assay in vitro. Of note, the binding of [³H]flunitrazepam to the benzodiazepinic site of the GABA_A receptor is sensitive to GABA_A receptor agonists that interact with several sites in the receptor (Lauren et al., 2007). The results presented in this report revealed that TTHL incubation did not alter the binding of [³H]flunitrazepam to the GABA_A receptor in cerebral cortex membranes, suggesting that the anticonvulsant effect exerted by TTHL is not closely related to GABAergic pathway.

Various central nervous system disorders have been associated with the reactive oxygen species (ROS) generation, such as seizures (Rambo et al., 2009; Souza et al., 2009). ROS can damage essential cellular constituents such as lipids and proteins, which can be measured by identification of their by products malondialdehyde and protein carbonyl, respectively (Souza et al., 2009). We observed that PTZ-induced cerebral cortex increase of TBARS (including malondialdehyde) and production and protein carbonylation ex vivo, indicating that oxidative stress occurs as a consequence of seizures, thereby contributing to seizure-induced brain damage. In this context, a number of natural products have been demonstrated to have direct antioxidant and anticonvulsant effects (Jiang et al., 1993; Lian et al., 2008). Moreover, there has been an increasing interest in the biochemical effects of medicinal plants with antioxidant properties, as they could be candidates for the prevention of oxidative damage associated with epilepsy (Noda et al., 1997). We showed that TTHL pretreatment protected against the
electrographic seizures and the oxidative damage elicited by PTZ ex vivo. However, our data revealed that TTHL did not present any direct action against the DPPH, ABTS and anion superoxide radicals. The enzymatically generated O$_2^-$ radical is a free radical more representative of the reactivity of the radical species generated under physiological conditions. Considering that this anion superoxide radical has a higher oxidation potential ($+0.94$ V) (Halliwell and Gutteridge, 2007) compared to the relative stable DPPH free radical, it also corroborated the results obtained by the DPPH and ABTS methods, in which we did not observe an antioxidant activity of TTHL. These results suggest that the protection of PTZ-induced convulsion and oxidative damage elicited by TTHL was not due to its direct action on DPPH, ABTS and anion superoxide radicals generation, and that other mechanisms might be used to explain the currently reported effects.

A large body of literature has accumulated that indicates that in human and experimental epilepsy, Na$^+$.K$^+$.ATPase activity is altered (Rambo et al., 2009; Souza et al., 2009). Moreover, the Na$^+$.K$^+$.ATPase inhibitor ouabain causes electrographically recorded seizures in mice (Jamme et al., 1995). It is also remarkable that decreased Na$^+$.K$^+$.ATPase activity has been found in the postmortem epileptic human brain (Grisar et al., 1992). In addition, a mutation in the Na$^+$.K$^+$.ATPase alpha subunit gene has been associated with epilepsy in humans (Jurkat-Rott and Lehmann-Horn, 2004). Accordingly, the results presented in this report reveal that PTZ treatment in vivo inhibited total and subunits $\alpha_1$ and $\alpha_2/\alpha_3$ Na$^+$.K$^+$.ATPase activity, an effect prevented by the pretreatment with TTHL. Knowing that there is a strong correlation between Na$^+$.K$^+$.ATPase activity inhibition and the duration of PTZ-induced convulsions (Fighera et al., 2006) and that oxidative damage may be a consequence of seizures, the maintenance of the Na$^+$.K$^+$.ATPase activity produced by the treatment with TTHL may be related not only with its anticonvulsant effect, but also with its protection against oxidative damage. The behavioral and EEG recordings reinforce this assumption because an injection of the Na$^+$.K$^+$.ATPase inhibitor ouabain caused the appearance of seizures.
and reduction of total and \( \alpha_2/\alpha_3 \) subunit Na\(^+\),K\(^+\)-ATPase activity, both effects protected by TTHL pretreatment.

Na\(^+\),K\(^+\)-ATPase is an ubiquitous plasma membrane protein that plays a key role in the maintenance of intracellular electrolyte homeostasis in virtually all tissues (Skoou and Esmann, 1992). In the central nervous system (CNS), Na\(^+\),K\(^+\)-ATPase activity significantly accounts for the maintenance of the electrochemical gradient across the plasma membrane underlying resting and action potentials and modulation of neurotransmitter release and uptake (Stahl and Harris, 1986). In line with this view, recent studies have demonstrated that glutamate transporters and Na\(^+\),K\(^+\)-ATPase are part of the same macromolecular complexes and operate as a functional unit to regulate glutamatergic neurotransmission in astrocytes and neurons (Rose et al., 2009; Zhang et al., 2009.). As a consequence, a decrease of Na\(^+\),K\(^+\)-ATPase activity directly affects neurotransmitter signaling, neural activity and animal behavior. Accordingly, the Na\(^+\),K\(^+\)-ATPase inhibition causes seizures in mice (Jammé et al., 1995) and glutamate release by reversal of Na\(^+\)-dependent transporter in the rat spinal cord (Li and Stys, 2001). Because glutamate is the principal excitatory neurotransmitter, it inevitably plays a role in the initiation and spreading of seizures (Danbolt, 2001). Our data show that inhibition of PTZ decreases Na\(^+\),K\(^+\)-ATPase activity and that TTHL pretreatment prevented this effect. This result is particularly important considering that the activity of glutamate transporters can be impaired by several indirect mechanisms, including ROS formation and the reduction of Na\(^+\),K\(^+\)-ATPase activity (Rambo et al., 2009; Souza et al., 2009). Although the exact mechanism through which PTZ reduces glutamate uptake is still unknown, it is tempting to propose that a reduction in Na\(^+\),K\(^+\)-ATPase decreases glutamate uptake because it depends on Na\(^+\) gradients across cell membrane (Trotti et al., 1997).

Finally, our results showed that PTZ (10 mM) inhibited the Na\(^+\),K\(^+\)-ATPase activity without isoform specificity when added directly to the reaction medium containing the cortex homogenates. These data agree with several studies that have demonstrated that millimolar concentrations of PTZ affect a variety of ion-selective channels and neurotransmitter receptors (Bloms et al., 1992; Walden et al., 1984) and inhibit the Na\(^+\),K\(^+\)-ATPase with Ki values approximately 10–20 mM (Dubberke et al., 1998). In addition, we showed that previous incubation with TTHL (10 \( \mu \)M) protected against \( \alpha_2/\alpha_3 \) Na\(^+\),K\(^+\)-ATPase inhibition induced by PTZ. The selective protection exerted by TTHL in vitro demonstrates differing roles of \( \alpha_1 \) and \( \alpha_2/\alpha_3 \) isoforms in neuronal excitability. For example, in excitable cells, the catalytic \( \alpha_1 \)-subunit of Na\(^+\),K\(^+\)-ATPase is relatively ROS- and ouabain-resistant. Conversely, \( \alpha_2/\alpha_3 \) Na\(^+\),K\(^+\)-ATPase are ouabain-sensitive and ROS-vulnerable (Karpova et al., 2010). In addition, the results presented in this report suggest that the \( \alpha_2/\alpha_3 \) Na\(^+\),K\(^+\)-ATPase maintenance elicited by TTHL protects against neuronal excitability and oxidative damage induced by PTZ. In agreement with this view, our data reveal that pretreatment with TTHL also protects against the inhibition of the total activity of Na\(^+\),K\(^+\)-ATPase and that of subunits \( \alpha_2/\alpha_3 \) induced by convulsive doses of ouabain.

In summary, the present results provide convincing evidence that the systemic administration of TTHL, a pentacyclic triterpene isolated from C. leprosum, protects against seizures and neurochemical alterations induced by convulsant agent PTZ. Furthermore, the results presented in this report revealed that the action elicited by TTHL is not due to its direct antioxidant action or interaction with GABA\(_A\) receptor, but the mechanism involves the Na\(^+\),K\(^+\)-ATPase activity maintenance. Therefore, TTHL could be a new therapeutic approach for preventing seizures and some of their deleterious effects.

References
Cavalheiro, E.A., Fernandes, M.J., Turski, L., Mazzacoratti, M.G., 1992. Neurochemical mechanisms, including ROS formation and the reduction of Na\(^+\),K\(^+\)-ATPase activity, directly affect neurotransmitter signaling, neural activity and animal behavior. According to this view, the Na\(^+\),K\(^+\)-ATPase inhibition causes seizures in mice (Jammé et al., 1995) and glutamate release by reversal of Na\(^+\)-dependent transporter in the rat spinal cord (Li and Stys, 2001). Because glutamate is the principal excitatory neurotransmitter, it inevitably plays a role in the initiation and spreading of seizures (Danbolt, 2001). Our data show that inhibition of PTZ decreases Na\(^+\),K\(^+\)-ATPase activity and that TTHL pretreatment prevented this effect. This result is particularly important considering that the activity of glutamate transporters can be impaired by several indirect mechanisms, including ROS formation and the reduction of Na\(^+\),K\(^+\)-ATPase activity (Rambo et al., 2009; Souza et al., 2009). Although the exact mechanism through which PTZ reduces glutamate uptake is still unknown, it is tempting to propose that a reduction in Na\(^+\),K\(^+\)-ATPase decreases glutamate uptake because it depends on Na\(^+\) gradients across cell membrane (Trotti et al., 1997).

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