A synthetic bioisoster of trimethadione and phenytoin elicits anticonvulsant effect, protects the brain oxidative damage produced by seizures and exerts antidepressant action in mice

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Abstract

Epilepsy is recognized as one of the most common and serious neurological disorder affecting 1–2% of the world's population. The present study demonstrates that systemic administration of 3-butyl-5,5-dimethyl-1,2,3-oxathiazolidine-4-one-2,2-dioxide (DIOXIDE), a synthetic compound bioisoster of trimethadione and phenytoin (classical anticonvulsants), elicits a dose dependent anticonvulsant response in mice submitted to the subcutaneous pentylenetetrazole seizure test (scPTZ). Among various factors supposed to play role in epilepsy, oxidative stress and reactive species have strongly emerged. The protection exerted by DIOXIDE over the extent of brain oxidative damage produced by PTZ was determined, by measuring the levels of lipid peroxidation and reduced glutathione and the activity of Na^+/K^+-ATPase.

Psychiatric disorders represent frequent comorbidities in persons with epilepsy. In this report, the potential anxiolytic and antidepressant activities of DIOXIDE were evaluated in several widely used models for assessing anxiolytic and antidepressant activities in rodents. Although DIOXIDE did not evidence anxiolytic activity at the doses tested, it revealed a significant antidepressant-like effect. Preliminary studies of its mechanism of action, by means of its capacity to act via the $GABA_A$ receptor (using the [^3H]flunitrazepam binding assay in vitro and the picrotoxin test in vivo) and the Na^+ channel (using the alkaloid veratrine, a voltage-Na^+ channel agonist) demonstrated that the anticonvulsant effect is not likely related to the

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GABAergic pathway and the antidepressant-like effect could be due to its Na^+ channel blocking properties.

The results for DIOXIDE suggested it as a new anticonvulsant–antioxidant and antidepressant compound that deserves further development.

1. Introduction

Epilepsy is a common chronic neurological disorder characterized by recurrent unprovoked seizures (Guo et al., 2011). About 50 million people worldwide have epilepsy, 90% of which are in developing countries.

Psychiatric disorders, particularly depression, represent frequent comorbidities in persons with epilepsy and many patients require treatment with antidepressants (Kühn et al., 2003). Nevertheless, the use of antidepressant in these patients has been controversial, as there is some evidence suggesting that antidepressants can facilitate the severity of seizures, particularly at high doses (Cramer et al., 2004).

The actual pathogenesis of epilepsy remains uncertain. It is known that oxidative injury may play a role in the initiation and progression of epilepsy, and therapies aimed at reducing oxidative stress may ameliorate tissue damage and favorably alter the clinical course of the disease (Costello and Delanty, 2004; Devi et al., 2008). Oxidative stress occurs when the normal balance between oxidative events and antioxidant defenses is disrupted either by loss of reducing agents/antioxidant enzymes or by increased production of oxidizing species (Halliwell, 2009; Sayre et al., 2008). A growing body of evidence has suggested that reactive oxygen species (ROS) generation may underlie the convulsant and neurotoxic effects of pentylenetetrazole (PTZ), the drug of choice to induce seizure in mice (Obay et al., 2008; Oliveira et al., 2006). In fact, several studies have demonstrated an increase in reactive species formation in central nervous system (CNS) of animals exposed to PTZ-induced convulsions model, and the treatment with antioxidants seems to attenuate convulsions and/or ROS-induced damage (Patsoukis et al., 2004). Therefore, antioxidant therapies aimed at reducing oxidative stress have received considerable attention in the treatment of epilepsy (Acharya et al., 2008; Azam et al., 2012).

The current clinically available antiepileptic drugs (AEDs) are associated with a variety of side-effects and chronic toxicity (Samren et al., 1997). In this regard, great efforts have been made in search of new AEDs with enhanced efficacy and minimal side-effects. However, the resistant tolerance to therapy observed in many patients treated with conventional AEDs, as well as wide global distribution of epilepsy, makes the design of novel compounds and the study of its mechanisms of action very important (Brunbech and Sabers, 2002).

AEDs comprise a heterogeneous group of agents with diverse mechanisms of action. Many have multiple effects on neuronal activities and overall mechanisms of action are a source of debate. Briefly, antiepileptic drugs aim to modify the dynamic balance between inhibition and excitation in neuronal circuits by targeting membrane ion channels, transmitter receptors and metabolic pathways. They can be generally assigned into three major groups; those that block voltage gated ion channels (Na^+ and Ca^{2+}), and those that either enhance GABAergic inhibition (by altering gamma amino butyric acid (GABA) synthesis/breakdown or potentiating $GABA_A$-receptor activity) or reduce glutamatergic excitation (blocking glutamate receptors or reducing glutamate release) (Landmark, 2007; Rogawski and Löscher, 2004).

As part of our search for potential anticonvulsant agents based on bioisosteric functional group information related to trimethadione and phenytoin, we have previously described the synthesis and anticonvulsant properties of a series of novel heterocycles N-derivative-1,2,3-oxathiazolidine-4-one-2, 2-dioxides and of their intermediates of synthesis, α-hydroxyamides (Pastore et al., 2013). Among them, 3-butyl-5,5-dimethyl-1,2,3-oxathiazolidine-4-one-2,2-dioxide (DIOXIDE) (Figure 1), resulted the most active compound. Moreover, DIOXIDE showed a median effective dose (ED_{50}) of 0.06 mg/kg in the maximal electroshock seizure test (MES) in mice, almost 5000 times more potent than valproic acid (VA), a typical antiepileptic drug of broad spectrum in clinical practice (Pastore et al., 2013).

In order to further expand the anticonvulsant profile of DIOXIDE, pentylenetetrazole seizure test (scPTZ) in mice was performed. In addition, the protection exerted by this compound over the extent of brain oxidative damage produced by PTZ was determined, by measuring the levels of lipid peroxidation and reduced glutathione (GSH) and the activity of Na^+/K^+-ATPase. The potential antidepressant activity of DIOXIDE was also evaluated. Furthermore, a preliminary study of its mechanism of action, by means of its capacity to act via the $GABA_A$ receptor and/or the sodium channel was attempted.

2. Experimental procedures

2.1. Drugs

DIOXIDE was prepared in our laboratory (Pastore et al., 2013). The chemical purity of the compound (99%) was confirmed by elemental
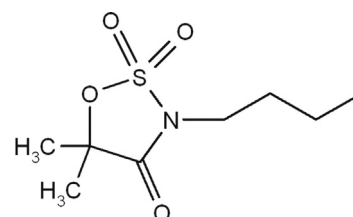
3-Butyl-5,5-dimethyl-1,2,3-oxathiazolidine-4-one-2,2-dioxide (DIOXIDE)

Figure 1 Molecular structure of the compound.

2.2. Animals and injection procedures

Adult male Swiss mice weighing 25–30 g were used in the pharmacological assays and adult male rats (200–300 g) Wistar strain for [³H]-flunitrazepam biochemical studies, both were obtained from the Central Animal House of the School of Pharmacy and Biochemistry, University of Buenos Aires. For behavioral assays mice were housed in groups of five in a controlled environment (20–23 °C), with free access to food and water and maintained on a 12 h/12 h day/night cycle, light on at 06:00 AM. Housing, handling, and experimental procedures complied with the recommendations set forth by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication no. 85-23, revised 1985) and the Institutional Committee for the Care and Use of Laboratory Animals, University of Buenos Aires, Argentina. Efforts were made to minimize animal suffering and to reduce the number of animals used. Mice were used only once. Pharmacological tests were performed between 10:00 AM and 2:00 PM.

DIOXIDE was administered in 30% polyethylene glycol (400) (PEG) in a volume of 0.15 mL/30 g of body weight. The rodents were intraperitoneally (i.p.) injected, except for PTZ and picrotoxin (PIC) that were subcutaneously (s.c.) administered. Veratrine (VER), PTZ and PIC were dissolved in saline (SAL). In each session, a control group receiving only vehicle (VEH) was tested in parallel with those animals receiving drug treatment. VEH control mice showed no significant differences in any of the tests assayed compared to mice treated with SAL (data not shown).

2.3. PTZ-induced convulsions

2.3.1. PTZ treatments

A preliminary anticonvulsant evaluation in the Maximal Electroshock Seizure test (MES) and subcutaneous Pentylenetetrazole Seizure test (scPTZ) was already performed following the procedures proposed by the National Institute of Health (NIH) via the Anticonvulsant Screening Project (ASP) (phase I) (Pastore et al., 2013). In the present work quantitative biological studies (phase II) were performed in scPTZ test. At this stage, the maximal time effect (MTE) of DIOXIDE (30 mg/kg) was determined. Then, the anticonvulsant activity, expressed as median effective dose, ED_{50}, was determined at the MTE using groups of six mice with different doses of DIOXIDE (Litchfield and Wilcoxon, 1949).

The scPTZ test involves a chemical induction to generate convulsions related to myoclonic seizures and entailed the s.c. administration of 85 mg/kg of PTZ in SAL in the posterior midline of the mice. Afterwards, the animals were placed in 15 cm × 30 cm chambers to record seizure during 30 min of observation. Protection was defined as the failure to observe even a threshold seizure (single episode of clonic spasms of at least 5 s duration). Compounds that are active in scPTZ may act raising seizure threshold (Malawska et al., 2004; Rogawski and Löscher, 2004).

2.3.2. Measurement of oxidative stress parameters

In order to further study glutathione reduced (GSH) contents, lipid peroxidation levels and Na⁺/K⁺-ATPase activity, mice were divided into 6 groups randomly assigned ($n=6$–12 in each group) as follows (indicated as pretreatment-treatment): (1) VEH-SAL, (2) VEH-PTZ, (3) VA (100 mg/kg)-SAL, (4) DIOXIDE (30 mg/kg)-SAL, (5) VA (100 mg/kg)-PTZ and (6) DIOXIDE (30 mg/kg)-PTZ. After the treatments (at the MTE previously determined) mice were placed in the chambers and observed during 30 min. The survivors of all groups were killed by decapitation and their brains dissected on ice to remove complete brain for biochemical determinations.

2.3.2.1. Tissue preparation. Subsequent to the seizure episode induced by PTZ, mice were decapitated and the cerebral tissue of the whole brain was rapidly removed, placed on ice and weighed. Tissues were immediately homogenized in cold 50 mM Tris-HCl, pH 7.4 (1:5 w/v). The homogenate was centrifuged for 10 min at 2000 rpm to yield a pellet that was discarded and a low-speed supernatant (S1) was obtained and used to determine GSH contents, lipid peroxidation levels and Na⁺/K⁺-ATPase activity. The amount of protein was estimated according to Bradford's method (Bradford, 1976).

2.3.2.2. Lipid peroxidation assays. Lipid peroxidation was estimated by the measurement of malondialdehyde (MDA) levels. MDA is an end product of lipid peroxidation, and its level was determined spectrophotometrically, in a Shimadzu 160A spectrophotometer, by the thiobarbituric acid reactive substances (TBARS) assay, as previously described by Yagi (1976). For this purpose, butylated hydroxytoluene (BHT, 50 μl 4%) and trichloroacetic acid (TCA, 500 μl, 20% p/v) were added to homogenate S1 (500 μl). The mixture was centrifuged for 10 min. Then, 500 μl of thiobarbituric acid (TBA, 0.8%) was added to the supernatant and incubated at 100 °C for 1 h. MDA reacts with TBA to form a colored complex which is measured at 532 nm. Results were expressed as pmol TBARS/mg protein.

2.3.2.3. Determination of reduced glutathione (GSH). GSH levels were evaluated to estimate endogenous defenses against oxidative stress. The method was based on Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid), DTNB) reaction with free thiol groups. Production levels of GSH were determined in S1 as described by Sedlak and Lindsay (1968). Briefly, S1 was deprotonized with 50%TCA and was allowed to stand at 4 °C for 2 h. The content was centrifuged at 3000 rpm for 10 min. This supernatant (400 μl) was added to 800 μl of 0.4 M Tris-buffer (pH: 8.9) containing 0.02 M EDTA (pH: 8.9) followed by the addition of 20 μl of 0.01 M DTNB. Finally the pH mixture was adjusted to pH:8-9 with 0.2 M Tris buffer (pH: 8.2). Absorbance was read at 412 nm, in a Shimadzu 160A spectrophotometer, and results expressed as μg GSH/g tissue.

2.3.2.4. Na⁺, K⁺ ATPase activity. Na⁺, K⁺ ATPase activity was determined in S1 in a reaction mixture that contained 5 mM MgCl, 80 mM NaCl, 20 mM KCl and 40 mMTris-HCl, pH 7.4, in a final volume of 500 μl. The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM. The samples were incubated at 37 °C for 10 min. Control samples were carried out under the same conditions with the addition of 1 mM ouabain. Na⁺, K⁺-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos, 1984). Released inorganic phosphate (Pi) was spectrofluorimetrically measured at 650 nm as described by Fiske and Subbarow (1925) and Na⁺, K⁺ ATPase activity was expressed as relative (%) to total activity (nmolPi/mg protein/min) in each sample.

2.4. [³H]-flunitrazepam binding assay

A radioligand binding assay was used to evaluate the putative action of the compounds on the benzodiazepine binding site of the GABA_A receptor complex. The binding of [³H]-flunitrazepam (81.8 Ci/mmol; obtained from PerkinElmer Life and Analytical Sciences, Boston, MA, USA) to the benzodiazepine binding site was performed in washed crude synaptosomal membranes from rat cerebral cortex according to Wasowski et al. (2012).

2.5. Subcutaneous PIC test

The GABA_A chloride-channel blocker PIC is a potent inducer of chemoconvulsant seizures (Velisek, 2006). DIOXIDE was given 2 h prior to the administration of PIC (2.5 mg/kg). Animals were

observed for 45 min. Absence of a 3 s clonic episode was used as the endpoint indicating protection.

2.6. Behavioral assays

2.6.1. Elevated plus-maze test
The elevated plus-maze is a validated model to evaluate anxiety in rodents (Lister, 1987). The set-up consisted of a maze of two open arms, $25 \, cm \times 5 \, cm$, crossed by two closed arms of the same dimensions, with free access to all arms from the crossing point. The closed arms had walls 15 cm high all around. The maze was suspended 50 cm from the room floor. DIOXIDE (10 mg/kg and 30 mg/kg) and diazepam (1 mg/kg, the reference compound) were administered (i.p.) 30 min before the experiment. Mice were placed on the central part of the cross facing an open arm. The number of entries and time spent in open arms were counted during 5 min under red dim light. An arm entry was defined as all four paws having crossed the dividing line between an arm and the central area. The total exploratory activity (number of entries in both arms) was also determined (Lister, 1987).

2.6.2. Locomotor activity test
Because all behaviors measured in the tests performed depend on locomotor activity that is probably the confounding issue; the spontaneous locomotor activity was recorded in separate experiments. The spontaneous locomotion was measured in a box made of Plexiglass, with a floor of $30 \, cm \times 15 \, cm$ and 15 cm high walls as previously described (Marder and Paladini, 2002). The mice were i. p. injected 30 min before performing this test. The locomotor activity was expressed as total light beam counts per 5 min.

2.6.3. Forced swimming test
The forced swimming test (FST) employed was similar to that described previously (Porsolt et al., 1977). This test is one of the most widely used tools for evaluation of antidepressant drugs with good reliability and predictive validity. Mice, 30 min after their i.p. injection of VEH, DIOXIDE or IMP, were individually dropped in glass cylinders (height: 25 cm; diameter: 10 cm; containing 10 cm of water at $24 \pm 1 \, °C$) for 6 min. The immobility time was recorded during the last 4 min of the test. Each mouse was judged to be immobile when it ceased struggling and remained floating in the water, making only the necessary movements to keep its head above water. The test was performed 30 min after the i.p. injection of DIOXIDE, imipramine (IMP, control drug) or VEH.

2.6.4. Tail suspension test (TST)
The tail suspension test was carried out according to the methodology proposed by Stéru et al. (1985). This test is frequently used in laboratory practice to identify compounds with antidepressant-like activity. Mice were individually suspended by the tail to a metal hook (distance from floor: 18 cm) using adhesive tape (distance from tip of tail: 2 cm) for 6 min. Typically, mice demonstrate several escape oriented behaviors interspersed with temporally increasing bouts of immobility. The duration of immobility was recorded during the final 4 min interval of the test. Mice were considered immobile only when they hung passively and completely motionless. The test is based on the fact that animals subject to the short-term, inescapable stress of being suspended by their tails will develop an immobile posture. The test was performed 30 min after the i.p. injection of DIOXIDE, IMP or VEH.

2.7. Effect of VER pre-treatment on the antidepressant effect of DIOXIDE in the forced swimming test

The possible role of Na^+ channels in the mechanism of action of DIOXIDE as antidepressant was investigated by using VER, a selective activator of Na^+ channels.

The dose of 0.125 mg/kg of VER was chosen as it did not induced, by itself, any effects in spontaneous locomotor activity and in the FST, based on a previous study (Prica et al., 2008). VER (0.125 mg/kg) was administered, in mice, in association with two active doses of DIOXIDE. The subactive dose of VER (or SAL) was administered 45 min before testing. DIOXIDE (or VEH) was administered 30 min before testing in the FST. The animals were randomly allocated in six experimental groups as follows (indicated as pretreatment-treatment): (1) SAL-VEH, (2) SAL-DIOXIDE (10 mg/kg), (3) SAL-DIOXIDE (30 mg/kg), (4) VER-VEH, (5) VER-DIOXIDE (10 mg/kg), (6) VER-DIOXIDE (30 mg/kg) (number of mice per group=6-11).

2.8. Statistical analyses

The effects of the compounds in mice were analyzed by one-way analysis of variance (ANOVA) and post-hoc comparisons between treatments were made using Dunnett's Multiple Comparison Test. The association study with VER was analyzed by two-way ANOVA (pre-treatment $vs.$ treatment) and post-hoc comparison was made using Bonferroni post test. ED_{50} value with its 95% confidence limits was calculated using GraphPad Prism 5.00 software after nonlinear curve fitting. A P value <0.05 was considered statistically significant. All data were expressed as mean $\pm$S.E.M. and analyzed with GraphPad Prism 5.00 software.

3. Results

3.1. Maximal time effect (MTE) and dose-response curve of DIOXIDE on convulsions induced by PTZ

The MTE of DIOXIDE at 30 mg/kg on scPTZ test in mice was determined at intervals of 0.5, 2, 4 and 6 h (Figure 2A) showing a value of 2 h. The dose–response curve of DIOXIDE at doses of 3, 10, 20, 30 and 40 mg/kg determined at the MTE is shown in Figure 2B. The ED_{50} (95% confidence intervals) was 23.3 mg/kg (18.5–29.3 mg/kg). Figure 2C shows the percentage of dead mice at the doses chosen to determine the ED_{50}.

3.2. Measurement of oxidative stress parameters

The effect of DIOXIDE and VA administrations on lipid peroxidation (TBARS content), glutathione levels (GSH content) and Na^+, K^+ ATPase activity in mice whole brains after PTZ injections is shown in Figure 3. The s.c. administration of 85 mg/kg of PTZ induced an increase in lipid peroxidation [$F(5,25)=5.04$, $P=0.0038$, Figure 3A], a decrease in glutathione levels [$F(5,21)=7.095$, $P=0.0011$, Figure 3B] and a reduced Na^+, K^+ ATPase activity [$F(5,17)=14.30$, $P=0.0001$, Figure 3C] in the whole brains. The pretreatment with DIOXIDE (30 mg/kg) or VA protected against these deleterious effects induced by PTZ.
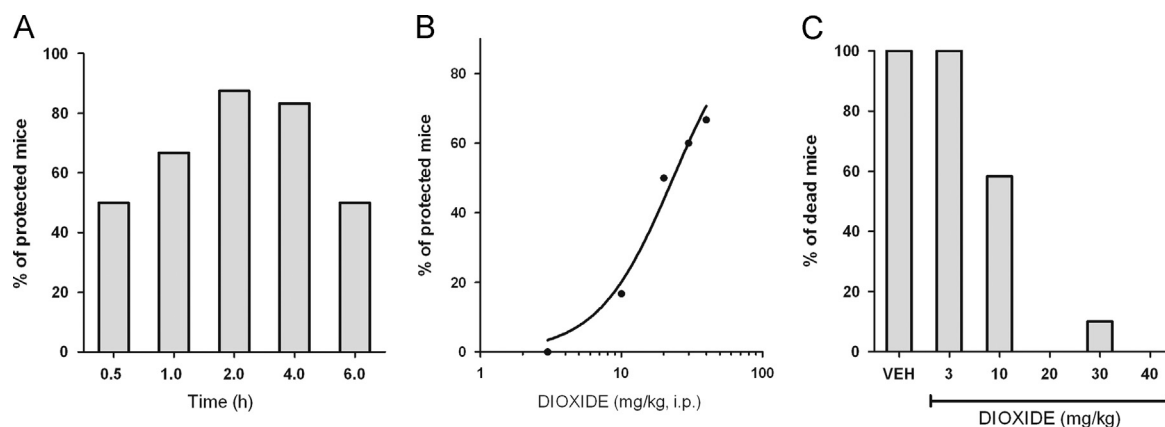
Figure 2 Performance of mice after the i.p. injection of 3-butyl-5,5-dimethyl-1,2,3-oxathiazolidine-4-one-2,2-dioxide (DIOXIDE) in the subcutaneous pentylenetetrazole seizure test (scPTZ). Data was calculated as number of mice protected from seizures or dead × 100/number of mice tested. (A) Maximal time effect (MTE) for DIOXIDE 30 mg/kg. (B) Dose-response curve determined at MTE (2 h). The median effective dose (ED_{50}) (95% confidence intervals) was 23.3 mg/kg (18.5-29.3 mg/kg). (C) % of dead mice for DIOXIDE (3, 10, 20, 30 and 40 mg/kg).
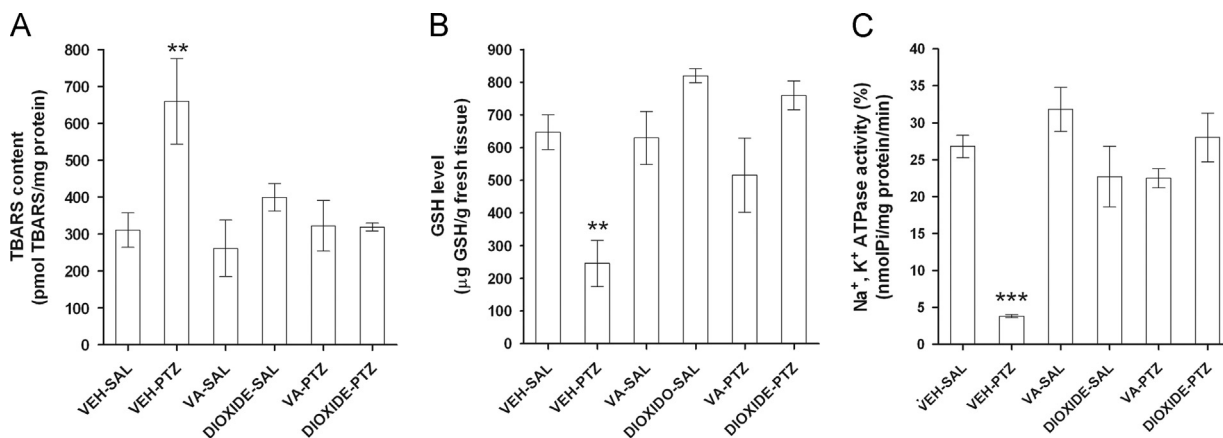

Figure 3 Effect of 3-butyl-5,5-dimethyl-1,2,3-oxathiazolidine-4-one-2,2-dioxide (DIOXIDE, 30 mg/kg) and valproic acid (VA, 100 mg/kg) administrations after PTZ injection (85 mg/kg, s.c.) on (A) lipid peroxidation (TBARS content); (B) reduced glutathione levels (GSH content); (C) Na^+, K^+ ATPase activity in mice whole brains. The data are presented as the mean $\pm$ S.E.M for $n=3$-5 determinations per group. **$P<0.01$, ***$P<0.001$ compared to the control group.

3.3. Study of possible mechanisms of action involved in the activity of DIOXIDE

3.3.1. [³H]-flunitrazepam binding assay
In the [³H]flunitrazepam binding assay DIOXIDE showed no potency in displacing the radioligand up to a concentration of 300 μM (data not shown).

3.3.2. Subcutaneous PIC test
DIOXIDE (30 mg/kg) was administrated 2 h before the injection of PIC. No protection was observed in any of the mice evaluated (data not shown).

3.4. Behavioral assays

3.4.1. Elevated plus-maze test and locomotor activity test
The effects of DIOXIDE and diazepam (the reference compound) in the plus maze and locomotor activity tests are shown in Table 1. ANOVA of the results obtained yielded

statistically significant differences in the percentage of open arms entries [$F(3,54)=16.99$, $P<0.0001$], percentage of time spent in open arms [$F(3,54)=20.88$, $P<0.0001$] and total entries [$F(3,54)=8.075$, $P=0.0002$] but not for the locomotor activity counts [$F(3,42)=1.941$, $P=0.1389$]. Comparisons between the vehicle control group and experimental groups (Dunnett's procedure) indicated that diazepam (1 mg/kg) increased the percentage of open arm entries, the percentage of time spent in open arms and the total arm entries ($P<0.0001$). DIOXIDE, i.p. injected at 10 mg/kg and 30 mg/kg, failed to show any effect in these assays.

3.4.2. Forced swimming test (FST)
The antidepressant-like effect of DIOXIDE is shown in Figure 4. ANOVA of the results obtained yielded statistically significant differences in the immobility time measured in the forced swimming test [$F(4,73)=11.02$, $P<0.0001$, Figure 4]. The comparison between the VEH control group and experimental groups by Dunnett's test indicated that DIOXIDE at 3 mg/kg ($P<0.05$), 10 mg/kg and 30 mg/kg

Table 1 Effect of 3-butyl-5,5-dimethyl-1,2,3-oxathiazolidine-4-one-2,2- dioxide in the plus-maze and locomotor activity tests in mice.

Test		VEH	DIOXIDE		Diazepam
			10 mg/kg	30 mg/kg	1 mg/kg
Plus-maze (mean±S.E.M.)	Number of total arms entries	18.1±0.7	17.6±1.6	19.5±1.2	26.2±2.0***
	Percentage of open arms entries	13.4±1.5	14.8±3.2	11.2±1.5	30.9±2.5***
	Percentage of time in open arms	10.5±1.4	7.5±1.5	9.1±1.5	28.2±2.9***
Locomotor activity (mean±S.E.M.)		675±15	741±60	677±35	763±31

Performance of mice in the plus maze and locomotor activity tests of vehicle (VEH), 3-butyl-5,5-dimethyl-1,2,3-oxathiazolidine-4-one-2,2-dioxide (DIOXIDE) 10 mg/kg and 30 mg/kg or diazepam 1 mg/kg. Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's test.
   ***$P<0.001$ compared with the control group ($n=6$-21 per group).
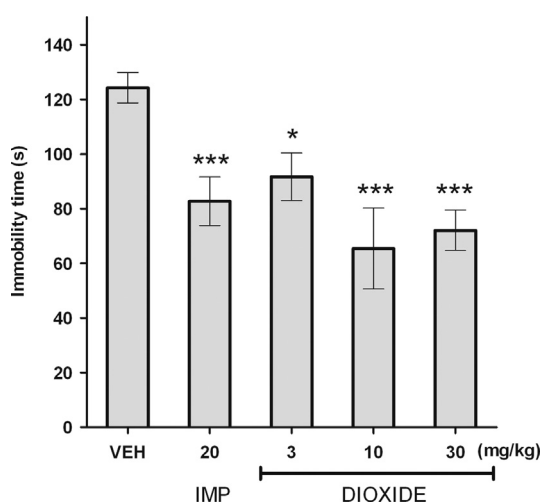

Figure 4 Effects of acute administration of 3-butyl-5,5-dimethyl-1,2,3-oxathiazolidine-4-one-2,2-dioxide (DIOXIDE 3, 10 and 30 mg/kg) and imipramine (IMP, 20 mg/kg) in the forced swimming test. Results are expressed as mean±S.E.M. of the immobility time (in s) in comparison to control animals (injected with VEH). Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's test. *$P<0.05$, ***$P<0.001$ compared with the control group ($n=7$-35 per group).

($P<0.001$) and IMP at 20 mg/kg ($P<0.05$), significantly reduced the immobility time of mice.

3.4.3. Tail suspension test (TST)
The antidepressant-like effect of DIOXIDE is shown in Figure 5. ANOVA of the results obtained yielded statistically significant differences in the immobility time measured in the tail suspension test [$F_{(4,58)}=15.71$, $P<0.0001$, Figure 5]. The comparison between the VEH control group and experimental groups by the Dunnett's test indicated that DIOXIDE at 10 mg/kg ($P<0.05$) and 30 mg/kg ($P<0.01$) and IMP at 20 mg/kg ($P<0.001$), significantly reduced the immobility time of mice.
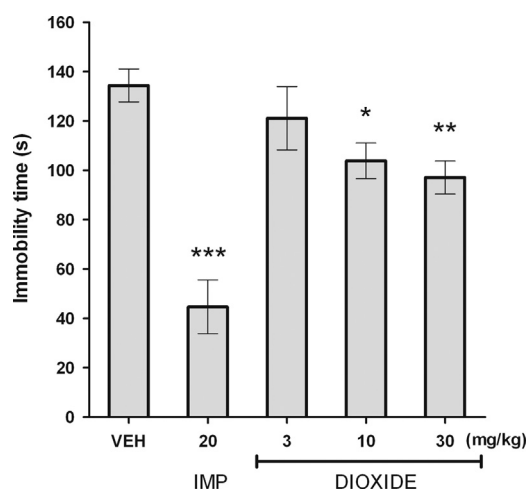

Figure 5 Effects of acute administration of 3-butyl-5,5-dimethyl-1,2,3-oxathiazolidine-4-one-2,2-dioxide (DIOXIDE, 3, 10 and 30 mg/kg) and imipramine (IMP, 20 mg/kg) in the tail suspension test. Results are expressed as mean ±S.E.M. of the immobility time (in s) in comparison to control animal (injected with VEH). Statistical analysis was performed by a one-way ANOVA followed by a Dunnett's test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to the control group ($n=7$-17 per group).

3.4.4. Effect of VER pre-treatment on the antidepressant effect of DIOXIDE in the forced swimming test
The study conducted in order to assess the effects of VER on the antidepressant-like effect of DIOXIDE in the FST is shown in Figure 6. It was already reported that VER tested alone in this assay and up to a dose of 2 mg/kg did not modify the immobility time of mice, and that VER 0.125 mg/kg have no effect on the locomotor activity of mice (Prica et al., 2008). As shown in Figure 6, VER (0.125 mg/kg, i.p.) did not evoke any response by itself. The two-way ANOVA (pre-treatment × treatment) performed revealed significant effects of pre-treatment [SAL or VER: $F_{(1,44)}=21.22$; $P<0.0001$], treatment [SAL or DIOXIDE: $F_{(2,44)}=16.73$; $P<0.0001$] and a significant interaction between these two factors [$F_{(2,54)}=5.605$; $P=0.0068$]. DIOXIDE given alone induced a significant antidepressant-like effect for the doses of 10 ($P<0.01$) and 30 mg/kg ($P<0.001$). The co-administration of VER
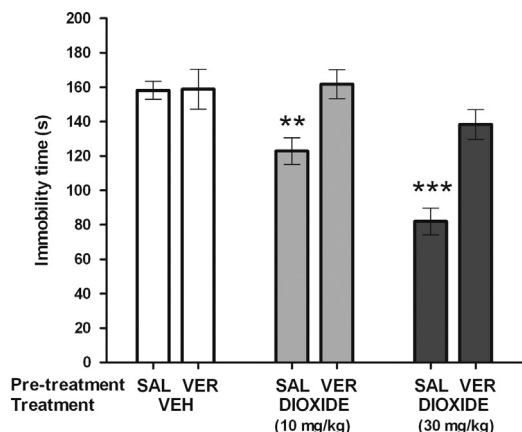
Figure 6 Effects of 3-butyl-5,5-dimethyl-1,2,3-oxathiazolidine-4-one-2,2- dioxide (DIOXIDE, 10 and 30 mg/kg, i.p. 30 min before the test) in the forced swimming test in mice pretreated with veratrine (VER, 0.125 mg/kg, i.p. 45 min before the test). Results are expressed as mean $\pm$S.E.M. of the immobility time (in s) in comparison to control group. Statistical analysis was performed by two way ANOVA followed by Bonferroni test. $**P<0.01$, $***P<0.001$ compared with the control group saline-VEH (SAL-VEH) (n=6-11 per group).

(0.125 mg/kg) significantly reversed the antidepressant-like effect of DIOXIDE.

4. Discussion

The present study demonstrates that systemic administration of DIOXIDE elicits a dose dependent anticonvulsant response in mice submitted to scPTZ, with an ED_{50} of 23.3 mg/kg (18.5-29.3 mg/kg), six times more active than VA, which shows an ED_{50} of 148.6 mg/kg in this assay (Phillips and Knaus, 1993). Valproic acid effect involves a variety of mechanisms, including increased GABAergic transmission, reduced release and/or effects of excitatory amino acids, blockade of voltage-gated sodium channels and modulation of dopaminergic and serotoninergic transmission. The broad spectrum of antiepileptic efficacy of valproate is reflected in preclinical *in vivo* and *in vitro* models. This drug is active either in the MES test (ED_{50}=283 mg/kg) as well as in the scPTZ assay and it is the drug of choice to use as a reference compound. However, phenitoyn (a sodium voltage channel blocker) and trimethadione (a voltage dependent T-type calcium channel inhibitor) are only effective in the MES test (ED_{50}=5.5 and 627 mg/kg, respectively), but not in the scPTZ test, due to their distinctive mechanisms of action. DIOXIDE had evidenced an ED_{50} of 0.06 mg/kg in the MES test, being almost 90 times more active than phenitoyn, 4700 times more active than VA and 10,000 times more active than trimethadione (Pastore et al., 2013). In view of the high anticonvulsant activity of DIOXIDE, it was selected for further extended pharmacological *in vivo* and *in vitro* studies.

PTZ is known to interact with a variety of channels and neurotransmitter receptors and after single or repeated administrations of PTZ several neurotransmitter systems, such as GABAergic (Olsen, 1981), adenosinergic (Pagonopoulou and Angelatou, 1998), and glutamatergic pathways (Ekonomou and

Angelatou, 1999) are affected. Various CNS disorders have been associated with ROS generation (Uttara et al., 2009) and there is emerging evidence that focuses on the role of oxidative stress and mitochondrial dysfunction both as a consequence and a cause of epileptic seizures (Patel, 2002). Previous studies demonstrated that acute PTZ-induced epileptic seizures lead to an increase in oxidative stress, indicated by an augmented lipid peroxidation, a decrease level of GSH and a reduced Na^+, K^+ ATPase activity (Bashkatova et al., 2003; Shin et al., 2011). In the present work, it is shown that the pretreatment of mice with DIOXIDE 30 mg/kg significantly prevented such PTZ deleterious effects.

GSH is the primary defense against oxidative damage to mitochondrial membranes by ensuring the reduction of hydroxyperoxide on phospholipids and other lipidic peroxides (Albrecht et al., 2011). It is a tripeptide that exerts a major cytoprotective function as a reductant and a cofactor for certain antioxidant enzymes (Atmaca and Fry; 1996). On the other hand, TBARS are products form as a result of free radical induced lipid peroxidation and their content is used as an index of oxidative stress. DIOXIDE alone did not modify GSH and TBARS levels and was effective in maintaining their levels after PTZ injection.

Na^+/K^+-ATPase is a membrane bound enzyme known to play a pivotal role in cellular ionic gradient maintenance, responsible for the active transport of sodium and potassium ions in the nervous system and necessary for neuronal excitability and regulation of neuronal cell volume. This enzyme is present in high concentration in brain cellular membranes, consuming about 40-50% of the ATP generated in this tissue and is particularly sensitive to reactive species (Petrushanko et al., 2006). Several studies have suggested that ROS inhibit the activity of Na^+/K^+ ATPase by oxidation of SH groups and alteration of the membrane fluidity (Barriviera et al., 2005; Muriel et al., 2003). Since seizures alter membrane lipid composition that can alter membrane fluidity and permeability, Na^+, K^+-ATPase is considered a marker of neuronal membrane. In the present study we also investigated the changes caused by PTZ induced seizures on this enzyme activity in membranes from mice brain. Results showed that DIOXIDE protects against the decrease in Na^+/K^+ ATPase activity induced by PTZ.

In brief, DIOXIDE administered before PTZ treatment countered the effects of PTZ and protected brain from oxidative stress by decreasing the MDA formation and restoring the Na^+/K^+ ATPase activity as well as GSH content. The antioxidant profile of this compound was comparable to VA.

As one of the proposed mechanism of action of PTZ is as a GABA$_A$ receptor antagonist (Olsen, 1981), the possible role of this receptor on the anticonvulsant activity of DIOXIDE was studied using the [³H]flunitrazepam binding assay *in vitro* and the PIC test *in vivo*. The results presented in this report revealed that DIOXIDE incubation did not alter the binding of [³H]flunitrazepam to GABA$_A$ receptors present in washed crude synaptosomal membranes from rat cerebral cortex. Furthermore, DIOXIDE was not able to prevent convulsions induced by PIC in mice. These data suggest that the anticonvulsant effect exerted by DIOXIDE is not likely related to the GABAergic pathway.

A chronic disease such as epilepsy can be an important stress factor, and inability to deal with the condition can

bring psychological difficulties and emotional discomfort. Seizures can affect a person's ability to work, drive, and perform other activities independently. In addition, studies have shown that many people with epilepsy experience symptoms of depression and/or anxiety. Indeed, some authors estimate the lifetime prevalence of depression in association with epilepsy to be as high as 55%. Despite this there has been remarkably little research into the mechanism of depression and anxiety in epilepsy, and even less of its treatment.

Depression lowers quality of life significantly and can directly increase seizure frequency through the mechanism of sleep deprivation; failure to recognize depression or inadequate treatment can lead to suicide. Some antidepressants, also, can facilitate the severity of seizures, particularly at high doses (Cramer et al., 2004). As with depression, anxiety can be seizure related. Anxiety is often a dominant symptom of the adjustment disorder which most patients go through when first diagnosed with epilepsy (Jackson and Turkington, 2005).

As many patients with epilepsy require treatment with antidepressants, the continued search for new, safer, and more effective drugs with both anticonvulsant and antidepressant activities is therefore imperative, and is a challenge in medicinal chemistry.

In this report the potential anxiolytic and antidepressant activities of DIOXIDE were evaluated in several traditional and validated behavioral models of anxiety and depression, such as the elevated plus maze and the FST and TST, respectively. The FST and TST measure the time during which mice, placed in an inescapable situation, remain immobile instead of struggling. Although DIOXIDE did not evidence anxiolytic activity at the doses tested, it revealed a significant antidepressant-like effect in both FST and TST, without producing alteration on locomotor activity, at doses quite similar to IMP, the reference compound.

It was reported that the administration of lamotrigine, topiramate and phenytoin, three well known antiepileptic drugs, induced a decrease in the immobility time on the FST which can be considered as an antidepressant-like activity. The antidepressant-like effect of these anticonvulsants was completely reversed by VER suggesting that this action might be due to their Na^+ channels blocking properties (Bourin et al., 2009; Prica et al., 2008). The alkaloid VER is a voltage-Na^+ channel agonist that induced an increase in the release of glutamate, the major CNS excitatory neurotransmitter, in cortical and cerebellum brain slices (Bourin et al., 2009). Thus, inhibition of glutamate release is considered as a potentially important mechanism for anticonvulsant and neuroprotective drug effects. It is well known that several anticonvulsants that demonstrated clinical efficacy in the treatment of partial and secondary generalized tonic-clonic seizures are suggested to exert their therapeutic effect, at least in part, via modulation of Na^+ channels (Kuo, 1998; Meldrum, 1996).

Voltage-gated Na^+ channels have a fundamental role in most electrically excitable cells since they conduct the inward currents that occur during the rising phase of action potentials (Hodgkin and Huxley (1952)).

The present study clearly demonstrated that the antidepressant-like effect of DIOXIDE was reversed when an active dose of this compound (30 mg/kg) in the FST is associated with a subactive dose of VER (0.125 mg/kg). This suggests that the antidepressant-like effect of DIOXIDE on the FST could be due to its Na^+ channel blocking properties. Consistent with this, it was found that the association studies between VER and the antidepressant drugs paroxetine, IMP and desipramine have no effect on the antidepressant-like effect of these serotonin-selective reuptake inhibitors and serotonin–norepinephrine reuptake inhibitors (Bourin et al., 2009; Prica et al., 2008). Moreover, the MES test is associated with a Na^+ channel mechanism (Fischer et al., 1992), where DIOXIDE has a striking effect, with an ED_{50} of 0.06 mg/kg. However, DIOXIDE also protects against damages produced by PTZ and this action seems not to be related to the GABAergic pathway, so another mechanism of action, in addition to its Na^+ channel blocking properties, could be involved. Further studies to obtain deeper insights into the molecular targets of action of DIOXIDE are required.

To summarize, the results of the present study demonstrate that the systemic administration of DIOXIDE in mice protected against seizures *in vivo* and neurochemical alterations *ex vivo* (increases in oxidative stress, measured by the extent of lipid peroxidation and GSH content, and Na^+, K^+-ATPase activity in the whole brain) induced by PTZ. DIOXIDE, also, exerts antidepressant like effects, with no alteration on locomotor activity, which seems to be dependent on the concentration of glutamate release, since this action was reversed by VER, a Na^+-channel opener.

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Contributors

V. Pastore carried out most of the experiments, undertook the statistical analyses and wrote the first draft of the paper. C. Wasowski carried out most of the experiments, managed the literature searches and contributed to the writing of the manuscript. L. Bruno-Blanch and V. Pastore designed and synthesized the compound used in this work. J. Higgs collaborated with the behavioral assays and I. C. Mangialavori with the measurement of the oxidative stress parameters. M. Marder designed this study, wrote the manuscript and contributed to the funding of the study. All the authors contributed significantly and have approved the final manuscript.

Conflict of interest

All the authors declare that they have no conflicts of interest.

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