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Bis(phenylimidazoselenazolyl) diselenide as an antioxidant compound: An *in vitro* and *in vivo* study

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ABSTRACT

The organoselenium compounds have been reported for many biological properties, especially as potent antioxidants. The compound bis(phenylimidazoselenazolyl) diselenide (BPIS) is a novel diaryl diselenide derivative, which shows antinociceptive and anti-inflammatory properties in mice, but whose antioxidant activity has not been studied. The present study aimed to investigate the antioxidant and toxicological potential of BPIS in brain of rats *in vitro*, and the effect of BPIS against the oxidative damage induced by sodium nitroprusside (SNP) in mouse brain. BPIS, at low molecular range, reduced lipid peroxidation (LP) and protein carbonyl (PC) content in rat brain homogenates (IC_{50} values of 1.35 and 0.74 μM, respectively). BPIS also presented dehydroascorbate reductase-like and glutathione-S-transferase-like, as well as DPPH and NO-scavenging activities. Related to togicological assays, BPIS inhibited δ-ALA-D and Na^+, K^+-ATPase activities in rat brain homogenates and [^{3}H]glutamate uptake in synaptosomes *in vitro*, but these effects were observed at higher concentrations than it had antioxidant effect (IC_{50} values of 16.41, 26.44 and 3.29 μM, respectively). *In vivo*, brains of mice treated with SNP (0.335 μmol per site; i.c.v.) showed an increase in LP and PC and a reduction in non protein thiol content, however, it was not observed significant alterations in antioxidant enzyme activities. BPIS (10 mg/kg; p.o.) protected against these alterations caused by SNP. In conclusion, the results demonstrated the antioxidant action of BPIS in *in vitro* assays. Furthermore, BPIS protected against oxidative damage caused by SNP in mouse brain, strengthening the potential antioxidant effect of this compound.

1. Introduction

Reactive oxygen species (ROS), electrophilic molecules which are physiologically produced can react with lipids, proteins and nucleic acids, resulting in oxidative damage to these macromolecules, when produced in high concentrations [4]. ROS can also react with nitric oxide (NO), forming the reactive nitrogen species (RNS), which are equally deleterious [42]. Both species are generally detoxified by cellular enzymatic and non-enzymatic antioxidant compounds, maintaining the natural redox state of the cell [42].

However, in situations when there is an imbalance between the production of ROS/RNS and the natural antioxidants; this event is called oxidative stress, which can lead to cell damage. Studies have reported the involvement of oxidative stress in the pathophysiology of diseases and neurodegenerative processes like aging [4], inflammation [46] and cancer [17].

Sodium Nitroprusside (SNP; Fig. 1A) is considered a NO donor, generally used as an inductor of apoptosis and oxidative stress *in vitro* and *in vivo* [26,23]. SNP interacts with oxyhemoglobin in the blood to produce methemoglobin while releasing cyanide (CN^-) and NO spontaneously [24]. Then, SNP could induce reduction of enzyme mitochondrial activity as well as production of ROS and RNS, triggering to oxidative stress and subsequently cellular damage [5,24].

The interest in the treatment of diseases related to oxidative stress with antioxidants has increased in the last years [31]. Intending to prevent the oxidative stress-related damage, natural and synthetic antioxidants are tested in *in vitro* and *in vivo* models of toxicity [27,20].

In this context, a special attention can be given to the organoselenium compounds and their derivatives. This class of compounds has been reported as potent antioxidants, and this property seems to be related to their ability to mimick enzymes as glutathione peroxidase (GPx), dehydroascorbate reductase (DHAR) and glutathione-S-transferase (GST), as well as act as substrate for the

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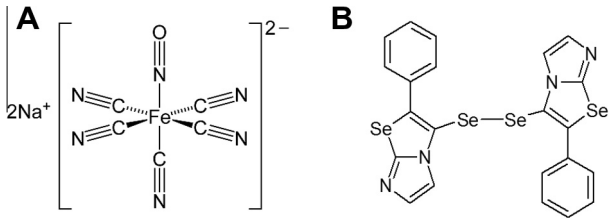

Fig. 1. Chemical structure of (A) SNP and (B) BPIS.

enzyme thioredoxin reductase (TrxR) [32,9]. Considering that slight modifications in molecular structures could partially or completely alter the effect of a drug, novel organoselenium compounds have been synthesized. The compound bis(phenylimidazoselenazolyl) diselenide (BPIS; Fig. 1B) is a novel diaryl diselenide derivative, which has been already proven to have antinociceptive and anti-inflammatory properties in mice [7] but its antioxidant activity has not been studied.

Whereas many of the organoselenium compounds properties are related to their antioxidant activity, the present study aimed to investigate the antioxidant potential of BPIS *in vitro*. Toxicological assays, such as the effect of BPIS on the activity of sulfhydryl enzymes and glutamate uptake, which are end points related to organoselenium compounds toxicity, were also performed. Based on the *in vitro* data, we also evaluated the *in vivo* effect of BPIS on the model of SNP-induced cerebral oxidative stress.

2. Material and methods

2.1. Chemicals

BPIS was prepared and characterized according to Roehrs et al. [40]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra shed analytical and spectroscopic data in full agreement with its assigned structure [40]. The chemical purity of BPIS (99.9%) was determined by GC/MS. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt, δ-aminolevulinic acid (δ-ALA), ascorbic acid, adenosine 5′-triphosphate (ATP) disodium salt hydrate, 1-chloro-2,4-dinitrobenzene (CNDB), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 2′,7′-dichlorofluorescin diacetate (DCF-DA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), [^{3}H]-L-glutamic acid, glutathione (GSH), SNP, thiobarbituric acid (TBA) and vanadium (III) chloride were purchased from the Sigma Chemical Co. (St Louis, Missouri, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers. BPIS was dissolved in dimethylsulfoxide (DMSO) and canola oil for *in vitro* and *in vivo* experiments, respectively.

2.2. Animal

Male adult albino Wistar rats (200–300 g) and male adult C57BL/6 mice (25–35 g) from our own breeding colony were used for *in vitro* and *in* vivo experiments, respectively. Animals were kept on a separate animal room, in a 12 h light/dark cycle, at a room temperature of 22 ± 2 °C, with free access to food (Guabi, RS, Brazil) and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil (#066/2014).

2.3. Tissue preparation

Animals were killed and the brain tissue was rapidly dissected, weighed and placed on ice. Tissues were immediately homogenized in cold 50 mM Tris–HCl, pH 7.4 (1/5, w/v). Protein carbonyl content was assayed using the fresh brain homogenate without centrifugation. For *in vitro* lipid peroxidation, δ-aminolevulinate dehydratase (δ-ALA-D) and Na^{+}, K^{+}-ATPase assays, the homogenate was centrifuged at 2400*g* for 10 min to yield a pellet that was discarded and a low-speed supernatant (S_1), the latter was used to determine the effect of different concentrations of BPIS on the previously mentioned assays. Differently, for the [^{3}H]glutamate uptake assay, the rats were decapitated and the whole brain was removed and used to prepare synaptosomes [45].

2.4. In vitro experiments

The *in vitro* experiments were carried out in order to investigate the antioxidant and potential toxicological effect of BPIS.

2.4.1. Lipid peroxidation induced by Fe (II)/EDTA

Fe(II)/EDTA were used as classical inductors of lipid peroxidation. An aliquot of 200 μL of S_1 was added to the reaction mixture containing: 30 μL of 500 μM EDTA solution (in water), 30 μL of 1.44 mM $FeCl_2$ solution and 10 μL of BPIS at different concentrations (final concentrations of 0.1 to 5 μM) and water to complete a final volume of 300 μL. The $FeCl_2$ solution was prepared in water, maintained in a dark tube on the ice and immediately used. Afterward the mixture was pre-incubated at 37 °C for 1 h. The reaction product was determined using 500 μL TBA (0.8%), 200 μL sodium dodecyl sulfate (SDS, 8.1%) and 500 μL acetic acid (pH 3.4) with subsequent incubation at 95 °C for 1 h. TBA reactive species (TBARS) were spectrophotometrically determined at 532 nm as previously described [33], using malondialdehyde (MDA, an end product of the peroxidation of lipids) as an external standard. Results were expressed as nmol MDA/g tissue. Ascorbic acid (final concentration 1–1000 μM) was used as positive control.

2.4.2. Protein carbonyl determination

Carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone [39]. Homogenate was diluted with Tris–HCl buffer, pH 7.4 in a proportion of 1:8 (homogenate:Tris–HCl). Aliquots of 940 μL of homogenate dilutions were incubated at 37 °C for 2 h in the presence of 10 μL of BPIS at different concentrations (final concentrations of 0.1–1 μM) and 50 μL of 20 mM SNP. SNP was used to stimulate the protein carbonyl production and was prepared in water, maintained in a dark tube on the ice and immediately used. In two tubes, it was added 200 μL of 10 mM DNPH in 2.0 M HCl. In the third tube, only 200 μL of 2.0 M HCl solution (blank) was added. All tubes were incubated for 1 h at room temperature, in dark and shaken using a vortex mixer every 15 min. After that, 0.5 mL of denaturizing buffer (sodium phosphate buffer, pH 6.8, containing 3% SDS), 1.5 mL of ethanol and 1.5 mL of hexane were added to all tubes. The tubes were shaken with a vortex mixer for 40 s and centrifuged for 15 min at 2400*g*. The pellet obtained was separated, washed two times with 1 mL of ethanol: ethyl acetate (1:1, volume/volume), and dried at room temperature for 2 min. The pellet was immediately dissolved in 1 mL of denaturizing buffer solution with mixing. Absorbance was measured at 370 nm. Results were expressed as carbonyl content (nmol carbonyl content/mg protein). Trolox (final concentration 1–100 μM) was used as positive control.

2.4.3. Dehydroascorbate (DHA) reductase-like assay

The DHA reductase-like activity of BPIS was assayed as described previously with minor modifications [50,49]. In brief, 10 μL of BPIS at different concentrations (final concentrations of 0.1–25 μM) were incubated (2 min) with 955 μL of 100 mM sodium phosphate buffer, pH 6.9, at 25 °C in the presence or

absence of 10 μL of 100–300 mM GSH (final concentrations of 1–3 mM, diluted in water) in a final volume of 1 mL. The mixture was incubated at 25 °C for 2 min. The DHA reductase assay was initiated by adding 25 μL of 20 mM DHA to a final volume of 1.0 mL. DHA solution was prepared on the day of experiments. For this end, ascorbic acid was added to a solution containing 10 mM sodium phosphate dibasic and 0.5 mM EDTA to achieve a final concentration of 20 mM ascorbic acid. The pH of mixture was adjusted to 5.5 with NaOH. After that, 10 μL of bromine to each 2 mL of ascorbic acid solution pH 5.5 were added and mixed at room temperature for 30 s. Afterward, the solution was bubbled in argon for 10 min. The DHA solution obtained was stored protected from light in ice for up to 4 h. Ascorbic acid regeneration from DHA was recorded at 265 nm. A blank without BPIS was run, and the difference gave the BPIS DHA reductase activity in nmol/min using the molar extinction coefficient of ascorbic acid of 14,700 $cm^{-1}M^{-1}$. Ebselen (final concentration 1–25 μM) was used as positive control.

2.4.4. Glutathione S-transferase (GST)-like assay

The reaction of GSH with CDNB is typically the preferred system used to measure the catalysis imparted by naturally occurring GSTs [14]. An aliquot of 10 μL of BPIS at different concentrations (final concentrations of 0.1–25 μM) was incubated with 20 μL of 50 mM GSH and 950 μL of 100 mM sodium phosphate buffer, pH 6.9 at 25 °C for 3 min. The reaction was initiated by adding 20 μL of 25 mM CDNB to achieve a final volume of 1.0 mL and recorded for 3 min at 340 nm. This assay was also performed without GSH in order to discard possible direct reaction of CDNB with BPIS. CDNB was used as substrate. A blank without BPIS was included and the difference was expressed as Δabs (delta absorbance)/min. Ebselen (final concentration 1–25 μM) was used as positive control.

2.4.5. Scavenging activity of ABTS radical

The determination of the ABTS radical scavenging activity was performed according to the method previously described with some modifications [38]. Initially, the ABTS radical was generated by reacting 7 mM ABTS solution in water with 140 mM potassium persulfate in the dark for 12–16 h. In the day of the assay, the preformed ABTS radical solution was diluted in potassium phosphate buffer in a proportion of 1:88 (1 mL ABTS radical + 87 mL 10 mM potassium phosphate buffer, pH 7.0). Briefly, 1 mL of ABTS radical solution was added to tubes containing 10 μL of BPIS at different concentrations (final concentrations of 0.1–100 μM). The mixture was incubated at 25 °C for 30 min in dark. The decrease in absorbance was measured at 734 nm. Ascorbic acid (1–25 μM) was used as a positive control. Results were expressed as percentage of the control. Ascorbic acid (final concentration 0.1–100 μM) was used as positive control.

2.4.6. Scavenging activity of DPPH radical

Radical-scavenging activity was determined by the reaction of the stable DPPH radical with the compound in accordance with the method previously described [8]. An aliquot of 10 μL of BPIS at different concentrations (final concentrations of 0.1–100 μM) was mixed with 1 mL of methanolic solution containing DPPH radical, resulting in a final concentration of 85 μM DPPH. The mixture was left to stand for 30 min at room temperature in the dark and the absorbance was measured at 517 nm. Results are expressed as percentage of the control. Ascorbic acid (final concentration 0.1–100 μM) was used as positive control.

2.4.7. Scavenging activity of NO and related species (NO_x)

The procedure is based on the principle that SNP in aqueous solution at physiological pH spontaneously generates NO. For the experiment, SNP (10 mM) was mixed with different concentrations of BPIS (final concentrations of 1–100 μM) and incubated at room temperature for 150 min. The same reaction mixture, without the compounds but with an equivalent amount of water, served as the control. After the incubation period, 0.5 mL of Griess reagent (1% sulfanilamide, 2% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The NO generated interacts with oxygen to produce nitrite ions that can be estimated using this reagent. Then, NO scavengers could reduce the production of nitrite ions. The absorbance of the chromophore formed was read at 540 nm [28]. The oxime butane-2,3-dionethiosemicarbazone (OXIME; final concentration 25 μM) was used as positive control [37].

2.4.8. δ-ALA-D activity

δ-ALA-D activity was assayed according to the method described by Sassa [41], with some modifications. S_1 (200 μL) was pre-incubated for 10 min at 37 °C in the presence of BPIS at different concentrations (final concentrations of 1–25 μM) or DMSO in the control tube. The enzymatic reaction was initiated by adding the substrate (δ-ALA) to a final concentration of 2.2 mM in a medium containing 45 mM phosphate buffer, pH 6.8 and incubated for 3 h at 37 °C. The incubation was stopped by adding 10% trichloroacetic acid solution (TCA) with 10 mM $HgCl_2$. The reaction product (porphobilinogen) was measured at 555 nm using modified Ehrlich's reagent. The values are expressed as nmol PBG/mg protein/h.

2.4.9. Na^+, K^+-ATPase activity

The reaction mixture for Na^+, K^+-ATPase activity assay contained 3 mM $MgCl_2$, 125 mM NaCl, 20 mM KCl, and 50 mM Tris/HCl, pH 7.4, in a final volume of 500 μL. S_1 (50 μL) was pre-incubated at 37 °C for 10 min in the presence of BPIS (final concentrations of 1–100 μM) or DMSO. The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM and incubated at 37 °C for 30 min. For obtaining the ouabain-sensitive activity, other samples were carried out under the same conditions with the addition of 0.1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow [13]. The values are expressed as nmol Pi/mg protein/h.

2.4.10. [^{3}H]glutamate uptake by synaptosomes

[^{3}H]glutamate uptake was assayed according to [45]. The synaptosomal preparation was washed twice by suspending in three volumes of 0.3 M sucrose, in 15 mM Tris/acetate buffer (pH 7.4), and centrifuging at 35,000*g* for 15 min. The final pellet was suspended in 0.3 M sucrose, 15 mM Tris/acetate buffer (pH 7.4), and incubated in Tris/HCl buffer (composition in mM Tris/HCl 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 12, $CaCl_2$ 1.0) pH 7.4 (adjusted with HCl), in the presence of BPIS (final concentrations of 1.75–5 μM) or DMSO for 10 min at 37 °C. The [^{3}H]glutamate uptake was initiated by adding to the medium [^{3}H]glutamate (final concentration 100 mM) for 1 min at 37 °C. The reaction was stopped by centrifugation (16,000*g*, 1 min, 4 °C), and the pellets were washed three times in Tris/HCl buffer by centrifugation at 16,000*g* for 1 min (at 4 °C). Radioactivity present in pellet was measured in a scintillation counter. Specific [^{3}H]glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium described above, and the uptake obtained with a similar incubation medium in which NaCl was replaced by choline chloride.

2.5. In vivo experiments

Considering that the *in vitro* experiments pointed out the antioxidant properties of BPIS, the compound was used to

investigate its antioxidant effect against damage induced by SNP in the brain of C57BL/6 mice.

2.5.1. Protocol of Exposure

Mice were divided into 4 groups of 7–9 animals each. Animals belonging to groups I and III received oral application of canola oil (10 mL/kg of body weight). Mice of groups II and IV received oral administration of BPIS (10 mg/kg). Thirty minutes after the treatment, mice of groups III and IV received SNP (0.335 μmol per site/2 μL) intracerebroventricular (i.c.v.). Groups I and II received saline solution (i.c.v.). The dosage of SNP was based on our previous study [36] and the BPIS dose was selected based on our previous study that demonstrated the antinociceptive action of BPIS through a series of acute models [7]. I.c.v. injections were given as described previously [22], with the bregma fissure as a reference point.

2.5.2. Tissue preparation

One hour after SNP or saline administration all mice were kiled and the brains of animals were removed and homogenized as described in Section 2.3. The low-speed supernatants (S_1) were separated and used for *in vivo* assays. For protein carbonyl content, it was used the homogenate without centrifugation. In addition for NO_x levels, the brains were homogenized with $ZnSO_4$ (200 mM) and acetonitrile (96%), centrifuged at 16,000*g* at 4 °C for 30 min, and the supernatant was collected.

2.5.3. Lipid peroxidation

Lipid peroxidation was carried out with an aliquot of S_1 (200 μL) as described in Section 2.4.1, excepting for the absence of the preincubation step.

2.5.4. Reactive species (RS) determination

RS levels were determined by a spectrofluorimetric method, using DCHF-DA assay [25]. S_1 (10 μL) was incubated with 10 μL of DCHF-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) is measured for the detection of intracellular RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 30 min after the addition of DCHF-DA to the medium. RS levels were expressed as arbitrary units (AU) of fluorescence/g tissue.

2.5.5. Non-protein thiol (NPSH) content

NPSH levels were determined by the method previously described [10]. To determine NPSH, S_1 was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded and free –SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB. The color reaction was measured at 412 nm. NPSH levels were expressed as optic density (DO)/g tissue.

2.5.6. Protein carbonyl determination

Protein carbonyl content was carried out with an aliquot of 940 μL of a homogenate diluted with Tris–HCl buffer, pH 7.4 in a proportion of 1:8, as described above Section 2.4.2, excepting for the absence of the preincubation step.

2.5.7. NO_x levels

The brains were dissected on an inverted ice-cold Petri dish and homogenized with $ZnSO_4$ (200 mM) and acetonitrile (96%), centrifuged at 16,000*g* at 4 °C for 30 min, and the supernatant was collected for assay of the nitrite plus nitrate content [28]. NO_x content was estimated in a medium containing 900 μL of the previously described Griess Reagent. After incubating at 37 °C for 60 min, nitrite levels were determined spectrophotometrically at 540 nm, based on NO_x/g tissue.

2.5.8. Catalase (CAT) activity

Enzymatic reaction was initiated by adding an aliquot of 20 μL of the S_1 and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.5. The enzymatic activity was measured at 240 nm and expressed as Unit (U)/mg protein (1 U decomposes 1 μmol of H_2O_2 per minute at pH 7 at 25 °C) [1].

2.5.9. Superoxide dismutase (SOD) activity

S_1 was diluted 1:10 (v/v) for determination of SOD activity in the test day. Aliquots of supernatant were added in a Na_2CO_3 buffer 50 mM pH 10.3. Enzymatic reaction was started by adding of epinephrine. The color reaction was spectrophotometrically measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C [29]. The enzymatic activity was expressed as U/mg protein.

2.5.10. Glutathione-S-trasferase (GST) activity

GST activity was assayed spectrophotometrically at 340 nm by the method of Habig et al. [14]. The reaction mixture contained an aliquot of S_1, 0.1 M potassium phosphate buffer pH 7.4, 100 mM GSH and 100 mM CDNB, which was used as substrate. The enzymatic activity was expressed as nmol CDNB conjugate/min/mg ptn.

2.5.11. Glutathione reductase (GR) activity

GR activity in S_1 was determined as described by Calberg and Mannervik [6]. In this assay, GSSG is reduced by GR at the expense of NADPH consumption, which was followed at 340 nm. GR activity is proportional to NADPH decay. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.5.12. Glutathione peroxidase (GPx) activity

GPx activity in S_1 was assayed spectrophotometrically by the method of Wendel [51], through the GSH/NADPH/glutathione reductase system, by the dismutation of H_2O_2 at 340 nm. S_1 was added in GSH/NADPH/glutathione reductase system and the enzymatic reaction was initiated by adding H_2O_2. In this assay, the enzyme activity was indirectly measured by means of NADPH decay. H_2O_2 is reduced and generates GSSG from GSH. GSSG is regenerated back to GSH by glutathione reductase present in the assay media at the expenses of NADPH. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.6. Protein quantification

Protein concentration was measured by the method of Bradford [3], using bovine serum albumin (1 mg/mL) as the standard.

2.7. Statistical analysis

The results are presented as mean ± SEM. For *in vitro* experiments, the data were performed using one-way analysis of variance followed by the Newman–Keul's multiple range test when appropriate. The IC_{50} values were calculated considering responses between 20 and 80% and reported as geometric means accompanied by their 95% confidence limits, using the program GraphPad InSTAT. Maximal inhibition (I_{MAX}) values were calculated at the most effective dose used. For *in vivo* experiments, data were analyzed using two-way analysis of variance (ANOVA) followed by the Newman–Keul's multiple range test when appropriate. p values less than 0.05 ($p < 0.05$) were considered as indicative of significance.

3. Results

3.1. In vitro experiments

3.1.1. Effect of BPIS on Fe (II)/EDTA-induced TBARS in rat brain homogenate

BPIS significantly reduced the lipid peroxidation induced in rat brain homogenate at concentrations equal or greater than 1 μM [$F_{(7,16)} = 36.03$; $p < 0.001$] (Fig. 2A). The IC_{50} value was 1.35 (1.29–1.42) μM and I_{MAX} 89 ± 1%. The positive control ascorbic acid was only effective in the concentration of 1000 μM with I_{MAX} 38 ± 6% (data not shown).

3.1.2. Effect of BPIS on protein carbonyl production induced by SNP in rat brain homogenate

As seen in Fig. 2B, BPIS decreased the protein carbonyl levels induced by SNP in rat brain homogenate at concentrations equal or greater than 0.5 μM [$F_{(6,14)} = 39.58$; $p < 0.001$]. The IC_{50} value was 0.74 (0.70–0.77) μM and I_{MAX} 65 ± 5%. The positive control trolox was effective at concentrations equal or greater than 50 μM with I_{MAX} 32 ± 2% (data not shown).

3.1.3. DHA-Reductase-like activity of BPIS

BPIS, at concentration equal or greater than 5 μM demonstrated DHA-Reductase-like activity, being effective to reduce DHA to

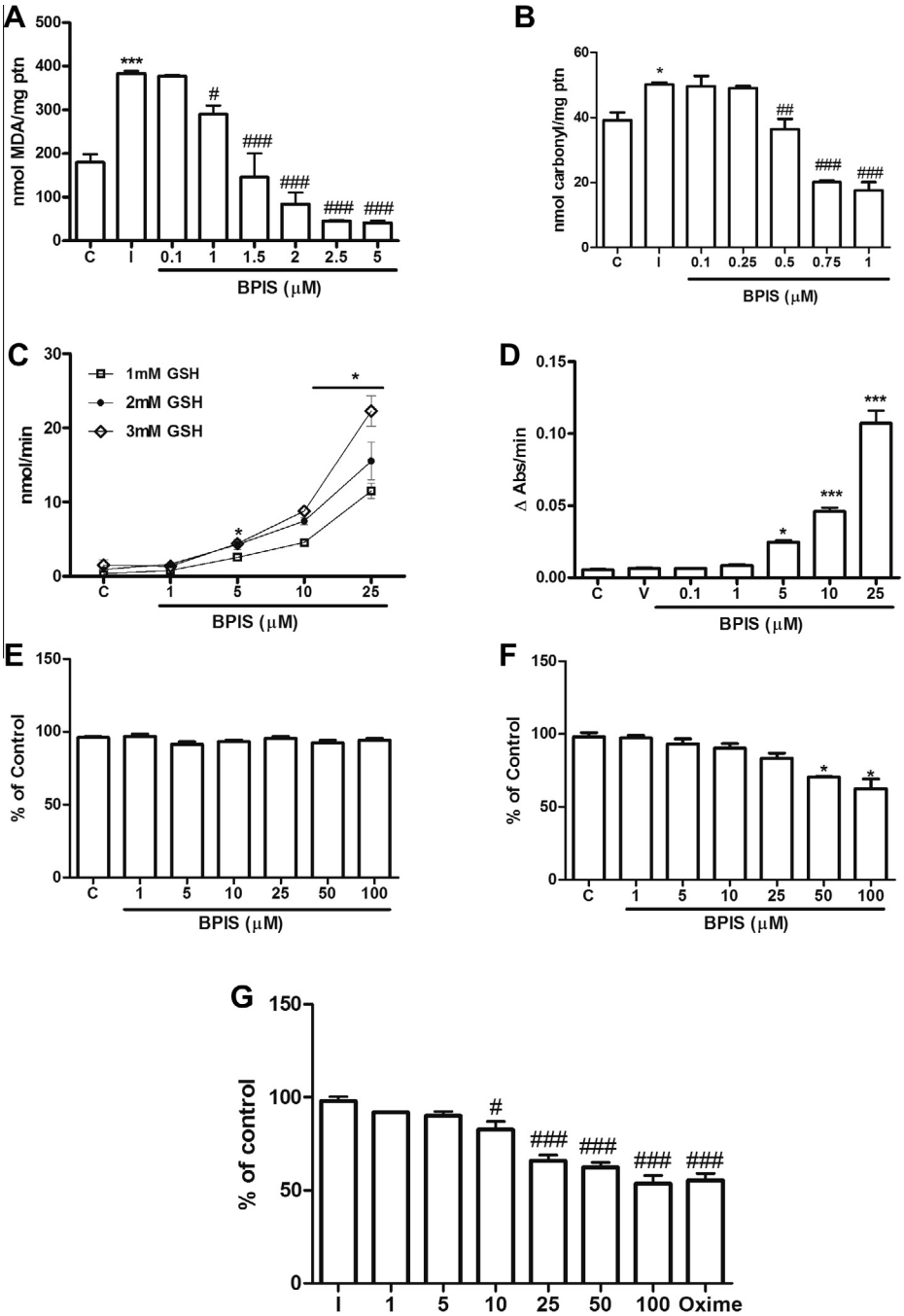

Fig. 2. Effect of BPIS on (A) lipid peroxidation induced by Fe(II)/EDTA and (B) protein carbonyl production induced by SNP in rat brain homogenates *in vitro*; (C) DHA-Reductase-like and (D) GST-like activities of BPIS; (E) ABTS, DPPH and NO_x-scavenging activities of BPIS. Data are reported as mean ± SEM for 3–4 experiments performed in duplicate, in different days, using different animals. (*) denotes $p < 0.05$ as compared to the control; (***) denotes $p < 0.001$ as compared to the control; (#) denotes $p < 0.05$ as compared to the induced; (##) denotes $p < 0.01$ as compared to the induced; (###) denotes $p < 0.001$ as compared to the induced (one way ANOVA/Newman–Keuls).

ascorbic acid. The rate of reduction was proportional to the concentration of BPIS, but similar to the different concentrations of GSH (Fig. 2C): in the presence of 1 mM of GSH [$F_{(6,14)} = 86.45$; $p < 0.001$]; 2 mM of GSH [$F_{(6,14)} = 29.24$; $p < 0.001$] and 3 mM of GSH [$F_{(6,14)} = 73.18$; $p < 0.001$]. The assay was also performed without GSH, and no ascorbic acid formation was observed, discarding any direct reaction of BPIS directly to DHA (data not shown).

The positive control ebselen did not show DHA-Reductase-like activity in the presence of 1 mM of GSH. In the presence of 2 mM of GSH, ebselen showed DHA-Reductase-like activity at concentration equal or greater than 10 μM. At last, in the presence of 3 mM of GSH, ebselen showed DHA-Reductase-like activity at concentration equal or greater than 5 μM (data not shown).

3.1.4. GST-like activity of BPIS

The data show that BPIS had GST-like activity in concentrations equal or greater than 5 μM [$F_{(5,12)} = 103.0$; $p < 0.01$] (Fig. 2D). The assay was also performed without GSH, and no product formation was observed, discarding any direct reaction of BPIS directly to CDNB (data not shown). The positive control ebselen only showed a significant GST-like activity at the concentration of 25 μM (data not shown).

3.1.5. ABTS and DPPH radical-scavenging activity

As seen in Fig. 2E, BPIS did not show ABTS radical scavenging activity at assayed concentrations [$F_{(6,14)} = 1.952$; $p = 0.1418$]. By contrast, BPIS showed DPPH radical scavenging activity at concentrations equal or greater than 50 μM [$F_{(6,14)} = 14.79$; $p < 0.001$] (Fig. 2F). For the DPPH assay, the IC_{50} value was not calculated considering that the I_{MAX} was 36 ± 7%.

The positive control ascorbic acid showed both ABTS and DPPH radical-scavenging activity at concentrations equal or greater than 5 μM (data not shown). For the ABTS assay the IC_{50} value was 8.63 μM and I_{MAX} was 96 ± 1% and for DPPH assay the IC_{50} value was 9.01 μM and I_{MAX} was 93 ± 2%.

3.1.6. NO_x-scavenging activity

BPIS diminuished the NO and related species formation induced by SNP at concentrations equal or greater than 10 μM [$F_{(7,16)} = 32.63$; $p < 0.001$] (Fig. 2G). The IC_{50} value was not calculated considering that the I_{MAX} was 45 ± 4%. OXIME was evaluated only in the concentration of 25 μM, it presented inhibition of 43 ± 4%.

3.1.7. Effect of BPIS on δ-ALA-D Activity in rat brain homogenate

BPIS significantly reduced the δ-ALA-D activity from rat brain homogenate at concentrations equal or greater than 10 μM [$F_{(4,10)} = 15.82$; $p < 0.001$] (Fig. 3A). The IC_{50} value was 16.41 μM (15.25–17.65) and I_{MAX} was 74 ± 14%.

3.1.8. Effect of BPIS on Na^+, K^+-ATPase activity in rat brain homogenate

As seen in Fig. 3B, BPIS showed a dual effect on the Na^+, K^+-ATPase activity in rat brain homogenates, significantly acting as an enzyme inductor at low concentrations (i.e. 1–10 μM) and as an inhibitor at high concentrations (i.e. equal or greater than 50 μM) [$F_{(6,14)} = 37.96$; $p < 0.001$]. The IC_{50} value was 26.44 (25.55–27.36) μM and I_{MAX} value was 86 ± 10%.

3.1.9. Effect of BPIS on [^{3}H]glutamate uptake by synaptosomes

BPIS significantly inhibited [^{3}H]glutamate uptake by synaptosomes at concentrations equal or greater than 3.75 μM [$F_{(4,10)} = 10.52$; $p < 0.01$] (Fig. 3C). The IC_{50} value was 3.294 μM and I_{MAX} was 74 ± 10%.

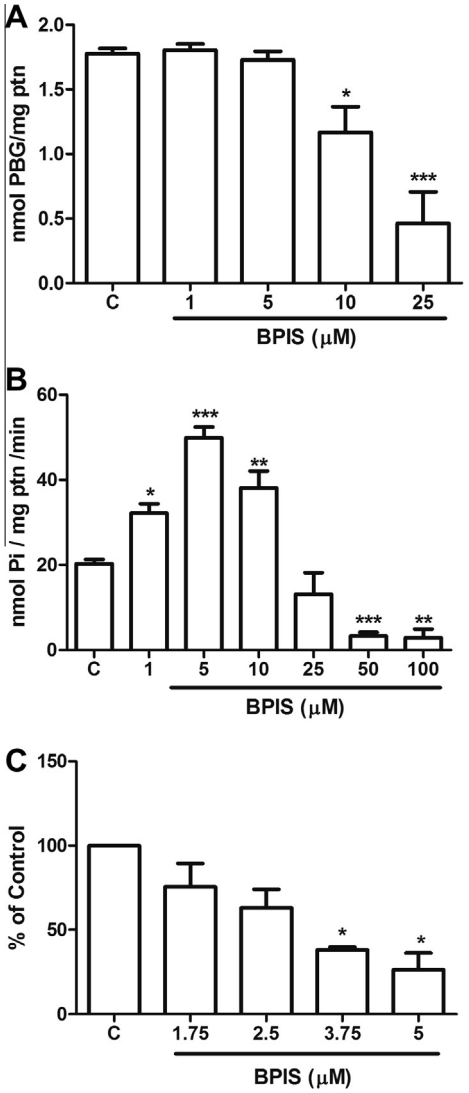

Fig. 3. Effect of BPIS on (A) δ-ALA-D, (B) Na^+, K^+-ATPase activities in rat brain homogenates and (C) [^{3}H]glutamate uptake by synaptosomes *in vitro*. Data are reported as mean ± SEM for 3–5 experiments performed in duplicate, in different days, using different animals. (*) denotes $p < 0.05$ as compared to the control; (**) denotes $p < 0.01$ as compared to the control; (***) denotes $p < 0.001$ as compared to the control (one way ANOVA/Newman–Keuls).

3.2. In vivo experiments

3.2.1. Lipid peroxidation

Two-way ANOVA of TBARS determination yielded a significant SNP × BPIS interaction [$F_{(1,26)} = 5.956$; $p < 0.05$]. Post-hoc comparisons demonstrated that SNP significantly induced lipid peroxidation in brains of mice and BPIS protected against the increase in these levels (Fig. 4A).

3.2.2. RS levels

According to two-way ANOVA, RS levels were not modified in rats in none of the experimental groups [$F_{(1,26)} = 0.011$; $p = 0.973$] (Fig. 4B).

3.2.3. NPSH levels

Regarding the NPSH levels, the two-way ANOVA showed a significant interaction between SNP and BPIS [$F_{(1,26)} = 4.291$; $p < 0.05$]. The BPIS pretreatment was effective in preventing the decrease in NPSH levels caused by SNP i.c.v. injection in brains of mice (Fig. 4C).

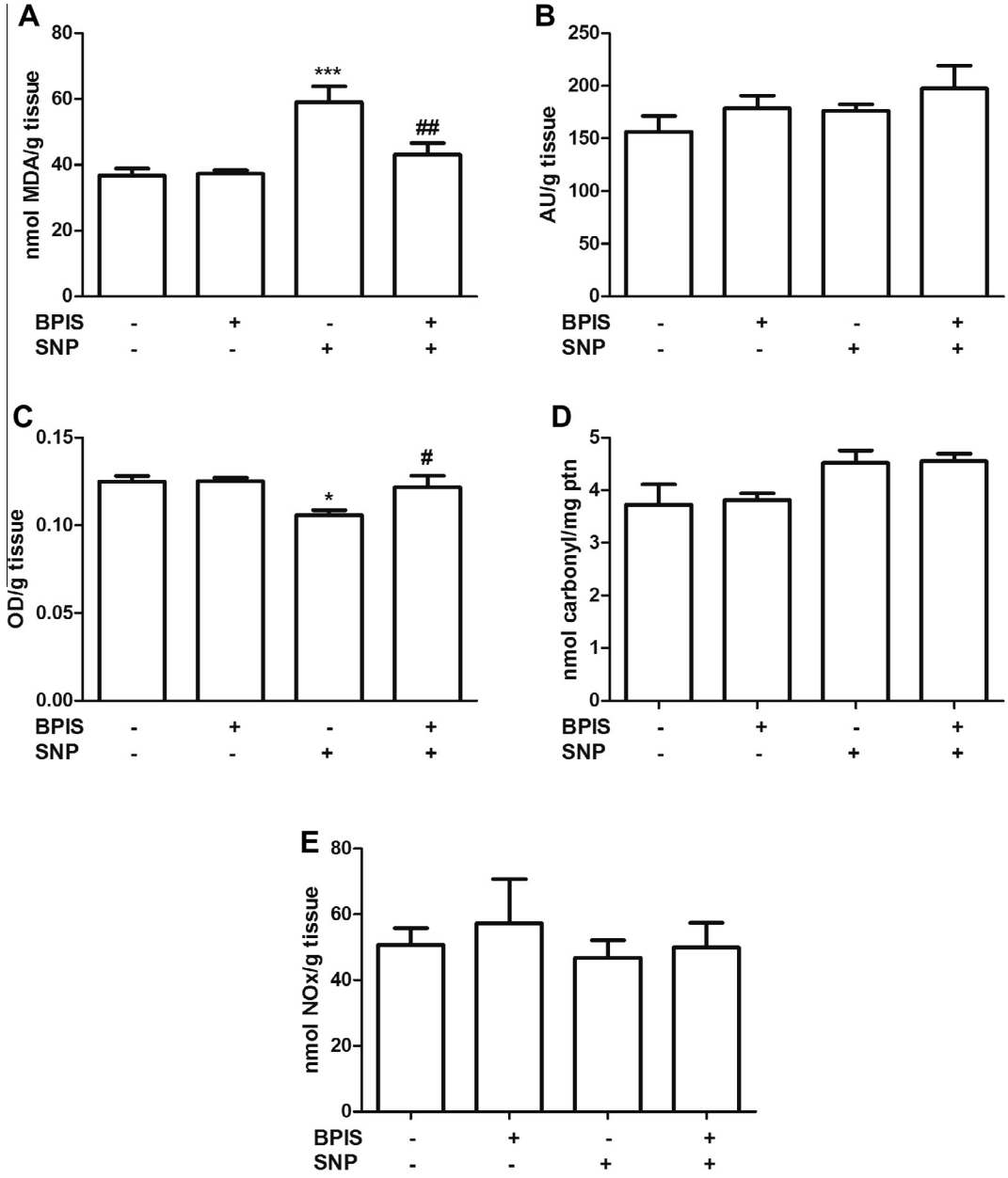

Fig. 4. Effect of BPIS (10 mg/kg) on (A) lipid peroxidation, (B) RS, (C) NPSH, (D) protein carbonyl and (E) NO_x levels in brains of mice treated with SNP. Data are reported as mean ± SEM of 7–9 animals per group. (*) denotes $p < 0.05$ as compared to the control group; (***) denotes $p < 0.001$ as compared to the control group; (#) denotes $p < 0.05$ as compared to the SNP group; (##) denotes $p < 0.01$ as compared to the SNP group (two-way ANOVA/Newman–Keuls).

3.2.4. Protein carbonyl content

In relation to carbonyl content, the two-way analysis revealed that there a main effect of SNP i.c.v. injection [$F_{(1,26)} = 10.579$; $p < 0.05$]; however, there was no interaction between SNP and BPIS treatments [$F_{(1,26)} = 0.018$; $p = 0.894$] (Fig. 4D).

3.2.5. NO_x levels

As seen in Fig. 4E, two-way ANOVA indicated that the administration of SNP and/or BPIS did not change NO_x levels in the brain of mice [$F_{(1,26)} = 0.049$; $p = 0.827$].

3.2.6. Antioxidant enzyme activities

Two-way analysis demonstrated that there was no significant interaction between SNP and BPIS treatments for CAT [$F_{(1,26)} = 0.068$; $p = 0.796$] (Fig. 5A), SOD [$F_{(1,26)} = 0.150$; $p = 0.702$] (Fig. 5B), GR [$F_{(1,26)} = 2.930$; $p = 0.099$] (Fig. 5C) and GST activities [$F_{(1,26)} = 2.464$; $p = 0.129$] (Fig. 5D).

Regarding GPx activity, two-way ANOVA revealed a signifcant main effect of BPIS treatment [$F_{(1,26)} = 5.106$; $p < 0.05$] (Fig. 5E). Although there was no significant interaction between SNP and BPIS treatments [$F_{(1,26)} = 3.53$; $p = 0.071$], GPx data from animals that received both BPIS and SNP was different from all the other groups.

4. Discussion

The results of the present study demonstrate that the compound BPIS had an *in vitro* and *in vivo* antioxidant activity. *In vitro,* BPIS protected against lipid peroxidation induced by Fe (II)/EDTA and protein carbonyl formation induced by SNP in rat brain homogenate. The mechanism of action by which BPIS shows antioxidant

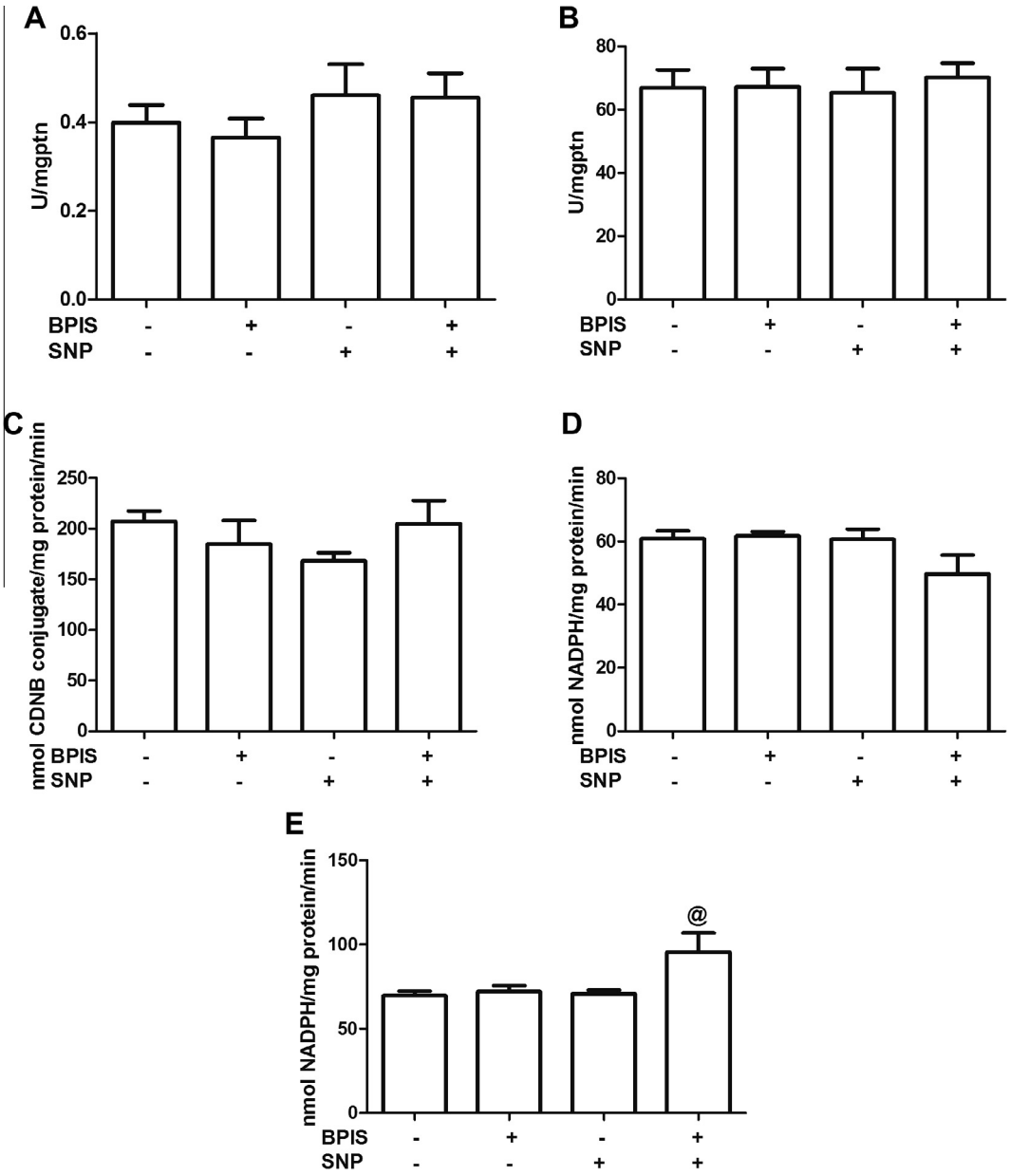

Fig. 5. Effect of BPIS (10 mg/kg) on (A) CAT, (B) SOD, (C) GST, (D) GR and (E) GPx activity in brains of mice treated with SNP. Data are reported as mean ± SD of seven animals per group. Data are reported as mean ± SEM of 7–9 animals per group. (@) denotes $p < 0.05$ as compared to all the other groups (two-way ANOVA/Newman–Keuls).

activity is related to its GST-like and DHA-Reductase-like activities as well as DPPH and NO-scavenging activity. It was also demonstrated, that BPIS can inhibit enzymes such as δ-ALA-d and Na^+, K^+-ATPase and the glutamate uptake, which could represent a toxicological potential, however, these effects were demonstrated to occur at higher concentrations than those of it showed antioxidant potential. In *in vivo* experiments, BPIS protected against alterations in parameters of oxidative stress induced by i.c.v. SNP administration, strengthening the BPIS potential as an antioxidant compound.

It is known that basal levels of ROS and RNS are physiologically produced by several mechanisms, including partial reduction of O_2 via mitochondrial electron transport chain, the oxidative deamination of biogenic amines and as part of the immune response by polymorphonuclear cells [4,11]. ROS and RNS are generally detoxified by enzymatic and non-enzymatic antioxidant defenses, maintaining the natural redox status of the cells [16]. However, when there is an imbalance between the production of RS and the natural antioxidant defenses, this situation is considered oxidative stress. Diverse molecules can be oxidized due to an augment in RS levels, among these macromolecules, we can mention protein, lipids and nucleic acids, generating cellular damage [34].

Lipid peroxidation and protein carbonylation can be observed in situations like aging, cancer and neurodegeneration [17,4,46]. The brain is extremely susceptible to oxidative stress, especially because many areas of this organ are rich in non-haem iron, which is catalytically involved in the generation of free radicals; as well as the brain contains high degree of polyunsaturated fatty acids, substrates particularly vulnerable to oxidation [15]. For this reason, we investigated the *in vitro* antioxidant effect of BPIS in brain homogenates, and the results demonstrated that BPIS acts as an antioxidant at very low concentrations: The results demonstrated IC_{50} values of 1.35 μM in the lipid peroxidation induced by Fe(II)/EDTA and 0.74 μM in the protein carbonyl production induced by SNP. Studies have demonstrated organoselenium compounds,

among them diaryl diselenides, as promising antioxidant agents against models of oxidative stress [27,36,2].

However, BPIS showed lower IC_{50} values when compared to other diselenides: for example, Prigol et al. [36] demonstrated that among many disubstituted diaryl diselenides tested, *p*-chlorodiphenyl diselenide $(p\text{-ClPhSe})_2$ showed the best antioxidant profile (IC_{50} 1.90 μM for SNP-induced lipid peroxidation, 2.7 μM for malonate-induced lipid peroxidation and 85 μM for SNP-induced protein carbonyl formation). Dithienyl diselenide $(ThSe)_2$, another parental compound, presented IC_{50} values of 15.33 μM for FE(II)/EDTA-induced lipid peroxidation and 11.89 μM for SNP-induced protein carbonyl formation.

Although the precise mechanism by which BPIS elicits antioxidant activity is still not completely understood, a large part of its action is the mimetic activity of the physiological enzymes DHA-Reductase and GST. DHA-Reductase is the enzyme responsible for the recycling of dehydroascorbate into ascorbic acid, one of the most important physiological antioxidants and GST is a detoxifying enzyme for xenobiotics, lipid peroxidation end products and other oxidative stress-related molecules [50,43]. BPIS acts similar to these enzymes, using GSH as a reducing agent in order to prevent oxidative stress, then contributing to ascorbic acid recycling and thiol-dependent xenobiotic detoxification.

In addition, BPIS showed scavenging activity against free radicals, represented by the ability to scavenge DPPH, as well as NO and related compounds. ABTS and DPPH radical-scavenging activity assays are widely used as antioxidant activity screening, differences in the scavenging activity can be due to DPPH reactions involve H-atom transfer and reactions with ABTS radicals involve electron-transfer processes [30]. BPIS also demonstrated NO_x scavenging activity, it is known that NO can undergo reactions with O_2, superoxide ion and reducing agents to produce RNS, such as nitroxyl (HNO), the oxides NO_2/N_2O_4, and N_2O_3, peroxynitrite, and S-nitrosothiols (RSNO), molecules that contribute to nitrosative and oxidative stress [19]. We have to mention that the concentrations in which BPIS showed scavenger activities were higher than those in which BPIS elicited antioxidant activity, but these could be contributing to the sum of antioxidant properties of this compound.

The inhibition of sulfhydryl enzymes, such as δ-ALA-d and Na^+, K^+-ATPase activities, and the blockade of glutamate uptake can represent important points to be investigate related to the toxicity of organoselenium compounds. The interaction with these enzymes or transporter is associated with the fact that most of organoselenium compounds are highly prone to attack thiol groups present in these proteins [32]. δ-ALA-D is an important enzyme related to haem biosynthesis, Na^+, K^+-ATPase is responsible for the active transport of sodium and potassium ions in the central nervous system and the glutamate uptake, carried out by excitatory amino-acid transporters, regulates concentrations of glutamate in the extracellular space [41,48,47]. The inhibition of these pathways generally leads to excitotoxicity, a situation reported in toxicity caused by high concentrations of organoselenium compounds [31]. However, we have to highlight that these parameters were mainly affected at higher concentrations than BPIS showed antioxidant effect. Other interesting fact is that in low concentrations, BPIS even stimulated the Na^+, K^+-ATPase activity; considering that oxidative regulation of this enzyme has important implications, reducing agents could increase the Na^+, K^+-ATPase activity or reverse the physiological inhibition caused by normal oxidative status [12].

Based on the *in vitro* results, we expanded the study in order to investigate the effect of an oral administration of BPIS on the model of oxidative damage induced by i.c.v. injection of SNP in mice. It was demonstrated that BPIS prevented against SNP-mediated alterations in oxidative stress parameters in brains of mice. BPIS avoided the increase in lipid peroxidation levels and the decrease in NPSH levels induced by the injection of SNP. SNP treatment did not affect all of the analyzed parameters, but the oxidation of protein, lipids and endogenous thiols is something to be highlighted, whereas that these are important parameters of oxidative stress [35].

Regarding that there is no significant alteration in the levels of RS or NO_x in the animals treated with SNP, it could be reacting more directly to lipids, thiols and proteins. We could not discard that other species related to SNP and NO metabolism could be elevated, whereas that the griess reagent mainly reacts with NO_2 and NO_3. The greater prevalence and reactivity of thiols over other biological nucleophiles could explain the propensity for S-nitrosothiol formation, what could explain the decrease in NPSH in the groups that received only SNP. Thiols in the presence of electron acceptors (redox-activated thiols) react with the nitrosonium moiety of NO to form S-nitrosothiol which may represent a storage pool for NO [19]. As already mentioned, SNP release cyanide and iron from its structure, what induce reduction of enzyme mitochondrial activity as well as production of ROS and RNS, triggering to oxidative stress and damage, represented by the oxidation of macromolecules [21,24]. We cannot affirm, regarding that we do not know the concentrations of BPIS in the brain tissue, when administered orally, but we can suppose that it could be acting detoxifying the SNP-induced damage by mechanisms similar to observed *in vitro*, such as GST- and DHA-Reductase-like, as wells as free radicals-scavenging activities, especially to NO-related molecules.

SNP did not affect the activity of the evaluated antioxidant enzymes (CAT, SOD, GR, GST and GPx), an important point to be investigated, but that seems not to be related to the toxic effects of SNP. Despite this, when the animals received both BPIS and SNP, the GPx activity was increased. It is known that some organoselenium compounds could stimulate the expression of GPx, and some organoselenium (e.g. selenocystein) can even be incorporated in selenoproteins as GPx [32,18]. However, the time following exposure to SNP and/or BPIS and tissue extraction is insufficient for induction of the enzyme expression, and it seems to merely reflects enzyme activation, this could also explain why some enzyme activities were not affected by the treatments. GPx is an antioxidant selenoenzyme that reduces variable hydroperoxides at the expense of glutathione and/or other reducing equivalents [18]. One interesting point is that both situations were necessary to increase GPx activity, just BPIS administration does not increase its activity, but also in association with a situation of oxidative damage.

Some of the limitations of this study include the dose of SNP which does not generate much harm, even if the results related to oxidative damage are similar to those observed in other studies [36,44]. In addition, the *in vitro* data cannot be extrapolated to the *in vivo* because the blood or brain concentrations of BPIS were not determined after oral administration of this compound to mice and in view of the fact that these experiments were carried out with species differentes. Despite these limitations, the present study provides valuable information for the *in vitro* and *in vivo* antioxidant potential of BPIS.

5. Conclusions

In conclusion, the results demonstrated the *in vitro* antioxidant action of BPIS on rat brain homogenates, at low concentrations, and this could be explained for its ability to mimic physiological antioxidant enzymes, as well as scavenge free radicals. BPIS administered to mice protected against alterations in parameters of oxidative stress caused by SNP in mouse brain, strengthening the potential antioxidant effect of this compound. Although the results

showed here indicate that BPIS might be a good candidate for future drug development in the prevention or treatment of diseases related to oxidative stress, more results would be required for better understanding the complete mechanism by which this compound acts and its physiological effects.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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