**THE THIOL-MODIFIER EFFECTS OF ORGANOSELENIUM COMPOUNDS AND THEIR
CYTOPROTECTIVE ACTIONS IN NEURONAL CELLS**

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Abstract

Most pharmacological studies concerning the beneficial effects of organoselenium compounds have focused on their ability to mimic glutathione peroxidase (GPx). However, mechanisms other than GPx-like activity might be involved in their biological effects. This study was aimed to investigate and compare the protective effects of two well known [(PhSe)₂ and PhSeZnCl] and two newly developed (MRK Picolyl and MRK Ester) organoselenium compounds against oxidative challenge in cultured neuronal HT22 cells. The thiol peroxidase and oxidase activities were performed using the glutathione reductase (GR)-coupled assay. In order to evaluate protective effects of the organoselenium compounds against oxidative challenge in neuronal HT22 cells, experiments based on glutamate-induced oxytosis and SIN-1-mediated peroxynitrite generation were performed. The thiol peroxidase activities of the studied organoselenium compounds were smaller than native GPx enzyme. Besides, (PhSe)₂ and PhSeZnCl showed higher thiol peroxidase and lower thiol oxidase activities compared to the new compounds. MRK Picolyl and MRK Ester, which showed lower thiol peroxidase activity, showed higher thiol oxidase activity. Both pre- or co-treatment with (PhSe)₂, PhSeZnCl, MRK Picolyl and MRK Ester protected HT22 cells against glutamate-induced cytotoxicity. (PhSe)₂ and MRK Picolyl significantly prevented peroxynitrite-induced dihydrorhodamine oxidation, but this effect was observed only when HT22 were pre-treated with these compounds. The treatment with (PhSe)₂ increased the protein expression of antioxidant defences (Prx3, CAT and GCLC) in HT22 cells. Our results suggest that the biological effects elicited by these compounds are not directly related to their GPx-mimetic and thiol oxidase activities, but might be linked to the up-regulation of endogenous antioxidant defences through their thiol-modifier effects.

Keywords: Oxidative damage, Antioxidant, Glutathione peroxidase, Organoselenium compounds, Thiol-modifier effect, neuronal cells.

Introduction

Oxidative damage is a crucial event in neurodegenerative diseases, including Alzheimer's, Parkinson's and Huntington's diseases [1]. Neuronal cells are particularly susceptible to oxidative damage because of their high oxygen consumption rate, which promotes the production of reactive species by the mitochondrial electron transport chain [2]. Moreover, neuronal cells contain relatively low levels of antioxidant enzymes and high levels of polyunsaturated fatty acids content, favouring lipid peroxidation [3].

Antioxidant enzymes such as glutathione peroxidase (GPx), catalase (CAT) and peroxiredoxin (Prx) have essential roles in the detoxification of reactive species. The GPx catalytic system depends on reduced glutathione (GSH) to metabolize lipo- or hydroperoxides and regenerate the native enzyme [4] [5]. Pharmacological strategies to counteract oxidative damage and preserve the neuronal cell homeostasis represent emerging and promising approaches to treat oxidative-related neuropathological conditions. Notably, organoselenium compounds have been reported to show antioxidant properties and beneficial effects on Alzheimer's and Parkinson's disease models [6, 7]. The antioxidant effects of organoselenium compounds have been mostly attributed to their GPx-like (thiol peroxidase) activity [8, 9].

Several organoselenium compounds, including diselenides, can react with thiol groups forming a selenol-containing molecule, with an active centre similar to that found in GPxs [8]. This selenol group may decompose H_2O_2 (and organic hydroperoxides) or peroxynitrite (ONOO^-) with varying catalytic efficiency depending on the characteristics of each compound [10, 11]. Various organoselenium compounds have been synthesized aiming to mimic the thiol peroxidase activity of GPx [8, 12]. However, the catalytic efficiency of organoselenium compounds is significantly lower when compared to that of native GPx [12]. The biological and pharmacological properties of some organoselenium compounds seem to be more complex and go far beyond GPx mimetic activity [13]. From a molecular point of view, some organoselenium compounds can react with unspecific thiol groups (thiol oxidase activity), resulting in cellular adaptive responses [10, 12, 14, 15]. Studies have shown that specific organoselenium compounds activate cellular signalling pathways such as Nrf2, increasing the expression of antioxidant defences [10, 15, 16]. The organoselenium compound Ebselen was able to reduce the oxidative damage and to increase the glutathione levels and heme-oxygenase (HO-1) protein expression in HT22 neuronal

cells [17]. Another organoselenium compound, diphenyl diselenide (PhSe)₂, was also effective in preventing oxidative damage and cell death in HT22 cells exposed to *tert*-Butyl hydroperoxide (t-BuOOH) in a mechanism that seems to be dependent on the endogenous GPx1 activity [16]. In cultured endothelial cells, (PhSe)₂ treatment stimulated the Nrf2 translocation to the nucleus and promoted the protein expression of GPx1 [10].

As already mentioned, recent evidence indicate that some biological effects of specific organoselenium compounds may depend on their direct oxidant effects toward endogenous thiols (thiol oxidase activity), thus leading to the modulation of endogenous antioxidant systems [10, 15]. However, it is not known whether the thiol peroxidase and thiol oxidase activities of organoselenium compounds are necessarily linked to their protective/beneficial effects. In this study, we compared the thiol peroxidase and thiol oxidase activities of four organoselenium compounds - two well known [(PhSe)₂ and PhSeZnCl] and two newly developed (MRK Picolyl and MRK Ester) organic selenides - against oxidative challenge in cultured neuronal HT22 cells.

Materials and Methods

Reagents

(PhSe)₂, GPx (isoform 1) enzyme from bovine erythrocytes (code G6137), β-Nicotinamide adenine dinucleotide phosphate sodium salt reduced (NADPH), dimethyl sulfoxide (DMSO), glutathione reductase from baker's yeast, reduced glutathione, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), dihydrorhodamine (DHR), tiophenol and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) and fetal bovine serum (FBS) were obtained from Gibco Life Technologies (Carlsbad, CA). All other chemicals were of the highest grade available commercially. Phenyl selenium zinc chloride (PhSeZnCl) was synthesized according to literature [18]. Synthetic and analytical procedures related to MRK Picolyl and MRK Ester (Figure 1) are described in the Supplementary material.

Thiol peroxidase and thiol oxidase activity of organoselenium compounds in polar medium

Thiol peroxidase and oxidase activities of the organoselenium compounds were performed using the glutathione reductase (GR)-coupled assay, measuring the consumption of NADPH at 340 nm [19]. Glutathione reductase (0.38 U/ml) was used to reduce back the glutathione disulfide formed from GSH (1 mM) in the H₂O₂ (0.2 mM) reduction reaction of GPx (0.075 – 0.15 U/ml) or organoselenium compounds (1-20 μM) (thiol peroxidase), with reduced NADPH (0.2 mM) as a source of electrons. In the protocol defined as thiol oxidase, the reaction medium did not receive H₂O₂; the capability of either GPx or the organoselenium compounds in directly oxidizing GSH in the absence of peroxide was measured. The molar extinction of NADPH was measured at 340 nm using on a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices). The results were expressed as μmol NADPH consumed per min.

Thiol peroxidase activity of organoselenium compounds in nonpolar medium

The thiol peroxidase activity of the organoselenium compounds in nonpolar medium was evaluated according to the Iwaoka and Tomoda method [20]. The organoselenium compounds (100 μM), thiophenol (PhSH) (2 mM) and methanol were mixed and incubated at 25 °C for 120 s. Thereafter, the catalytic thiol peroxidase reaction ($\text{H}_2\text{O}_2 + 2\text{PhSH} \rightarrow 2\text{H}_2\text{O} + \text{PhSSPh}$) was initiated by the addition of H₂O₂ (30 mM). The reduction rates of H₂O₂ were monitored through the UV absorption increase at 305 nm due to diphenyl disulfide (PhSSPh) formed, using a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices). Results are expressed as slope/min/organoselenium concentration.

Cell culture and treatments

The HT22 cells, an immortalized mouse hippocampal neuronal cell line, were a gift from Dr. David Schubert (Salk Institute, La Jolla, CA, USA). HT22 were cultured in growth medium (DMEM) supplemented with 5% of fetal bovine serum (FBS; Gibco/Invitrogen), containing 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mM Hepes, 25 mM glucose, 44 mM NaHCO₃ and incubated at 37 °C in a humidified atmosphere of 5% CO₂. Cells suspensions were seeded in dish plates (100 × 20 mm), 96, 24 or 6-well plaques at different cell densities, depending on the experimental procedure. Cells were subcultured at confluences of 90% and treated with (1-20 μM) of organoselenium compounds for 24 h (pre-treatment). In other set of experiment, the cell treatment with the organoselenium compounds occurred simultaneously (co-treatment) with the oxidants glutamate (5 mM) or SIN-1 (25 μM).

Cell viability assays

HT22 cells were plated into 96-well plates at a cell density of 1×10^3 cells/well and cultivated at 24 h. To establish the non-toxic concentration of organoselenium compounds, the cells were treated with 1, 5, 10 and 20 μM of organoselenium compounds or 0.05% DMSO (vehicle) for 24 h. To evaluate the protective effect of organoselenium compounds against glutamate cytotoxicity, HT22 were treated with 1 μM of organoselenium compounds for 24 h (pre-treatment); afterward, the medium was replaced by a fresh medium and then exposure to glutamate (5 mM) for additional 24 h. In parallel experiments, the cell treatment occurred simultaneously (co-treatment) with glutamate. The concentration and timeline of glutamate toxicity for HT22 cells has already been determined by previous studies of literature [21]. The cell viability was measured by MTT reduction and propidium iodide (PI) uptake assays.

The reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl-tetrazolium bromide (MTT) to the formazan product by mitochondrial dehydrogenases in viable cells was conducted as described by Mosmann [22]. PI, which is excluded by living cells but rapidly enters cells with damaged membranes and binds to DNA, rendering them brightly fluorescent, was measured according to Riccardi and Nicoletti [23]. Results of MTT assays were expressed as percentage of untreated cells and the results of PI uptake assays were expressed as percentage of 2% Triton X-100-treated cells that represent the 100% of death. All experiments were performed in triplicate and read on a SpectraMax Paradigm spectrofluorometer (Molecular Devices).

Dihydrorhodamine (DHR) oxidation

The fluorescent probe DHR reacts with peroxynitrite-derived free radicals but not with $\text{O}_2^{\cdot-}$ or $\cdot\text{NO}$ directly [24, 25]. HT22 cells were grown in 24-well culture (1×10^4 cells/well) plates and treated with 1 μM $(\text{PhSe})_2$ or vehicle for 24 h (pre-treatment) or simultaneously (co-treatment) with SIN-1 as explained above. The protocol was performed in Dulbecco's phosphate buffered solution (dPBS) consisting of 137 mM NaCl, 8.1 mM Na_2HPO_4 , 0.9 mM CaCl_2 , 0.5 mM MgCl_2 , 2.7 mM KCl, and 1.45 mM KH_2PO_4 (pH 7.4), supplemented with 5 mM glucose and 1 mM L-arginine, containing 10 μM DHR. Immediately after the cell exposure to SIN-1 (25 μM) the detection of rhodamine 123, an oxidation product of DHR, was followed online in a fluorescence plate reader at 37 °C for 60 min with excitation at 485 nm and an emission at 525 nm and 590 nm.

Protein extraction and Western blotting

HT22 were grown in 6-well culture (5×10^4 cells/well) plates and treated with (PhSe)₂ or vehicle for 3, 6, 12, 24 and 48 h. After treatments, cells were washed with phosphate-buffered saline (PBS) and lysed in 150 µl RIPA lysis buffer containing 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, 1% NP40 and 25 mM Tris-HCl pH 7.6, in the presence of protease (Complete, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Cells were harvested by scraping, samples were clarified by centrifugation at 13,000 rpm for 15 min at 4 °C and protein concentration was measured with the BCA assay (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (40 – 50 µg) from the total extract were separated by electrophoresis using an acrylamide/bisacrylamide (10 or 12%) gel and transferred to PVDF Hybond-P membranes (Amersham/GE healthcare) at 20V for 1 h in a semi-dry Trans-Blot SD cell system (Bio-Rad, Hercules, CA). After blocking the membranes in 5% skimmed milk in TBS-T (10 mM), they were incubated with the appropriate primary antibodies, anti-MnSOD (1:3000, ADI-SOD-110, Enzo Life Sciences), anti-Prx3 (1:1000; LabFrontier, Daehyun-dong Seodaemun-gu Seoul, Korea), anti-Catalase (1:3000; Chemicon International, Temecula, CA), anti-GCLC (1:1000; generous gift of Dr. Santiago Lamas laboratory), anti-GCLM (1:1000; Santa Cruz Biotechnology, CA, USA), and anti-HO-1 (1:1000; Santa Cruz Biotechnology, CA, USA). Secondary antibodies against rabbit and mouse were from LI-COR Biosciences (Lincoln, NE). Densitometry of at least 3 Western blots for each experiment was done using β-Tubulin (1:80000; Sigma, St. Louis, M) as housekeeping control for the total levels of protein loaded. Image J software (NIH) was used to measure and analyze protein band intensity. These analyses were represented in bar graphs showing the mean ± SEM of protein respective to vehicle conditions.

Statistics

Statistical analysis and graphics were made using the GraphPad PRISM® software version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). Normal (Gaussian) distribution was evaluated with the Shapiro-Wilk normality test. Significant differences were evaluated by linear regression, one-way or two-way of variance analysis (ANOVA), depending on the experimental design. Multiple comparisons were performed using the Dunnett's or Tukey *post-hoc* test. Results are expressed as mean ±

SEM. $p < 0.05$ was considered statistically significant. $n \geq 4$ in all experiments. The number of independent experiments, for each articular experiment, is indicated in the corresponding figure legends.

Results

Thiol peroxidase and oxidase activity of purified GPx and organoselenium compounds in polar and nonpolar medium.

The rate of the thiol peroxidase activity displayed by the native GPx enzyme and the organoselenium compounds is depicted in Fig. 2A. Using fixed concentrations of H_2O_2 (0.2 mM) and GSH (1 mM) and varying concentrations of the purified GPx (2.9 – 5.9 nM) or the organoselenium compounds (1 – 20 μM), the purified enzyme showed a significantly higher catalytic efficiency compared to the organoselenium compounds. The rate of peroxidase activity displayed by the purified GPx, present at the nanomolar (nM) range, was achieved only when the organoselenium compounds were present at concentrations approximately 5.000 fold higher, at the micromolar (μM) range. We founded a significant linear concentration-dependent response for native GPx ($r = 0.9605$, $p = 0.0094$), for $(PhSe)_2$ ($r = 0.9879$, $p = 0.0121$) and for $PhSeZnCl$ ($r = 0.9629$, $p = 0.0371$), while experiments with the compounds MRK Picolyl and MRK Ester did not show significant linear concentration-dependent response.

In order to investigate the thiol oxidase activity (the direct interaction with thiol groups), the same protocol was used (Fig. 2A), however, in the absence of H_2O_2 (Fig. 2B). At concentrations similar to those used in Fig. 2A (nM range), the measurement of thiol oxidase activity showed no significant concentration-dependent responses for the native GPx ($r = 0.5102$, $p = 0.3798$) and $(PhSe)_2$ ($r = 0.8098$, $p = 0.1902$), while $PhSeZnCl$, MRK Picolyl and MRK Ester displayed significant concentration-responses for thiol oxidase activity ($r = 0.9981$, $p = 0.0019$; $r = 0.9746$, $p = 0.0254$ and $r = 0.9716$, $p = 0.0284$ respectively).

We also evaluated the thiol peroxidase activity of the organoselenium compounds in a nonpolar medium, as proposed by Iwaoka and Tomoda (Iwaoka; Tomoda 1994). Interestingly all organoselenium compounds here evaluated exhibited thiol peroxidase activity in nonpolar medium (Fig. 2C).

Organoselenium compounds protect HT22 cells against glutamate-mediated oxidative toxicity

Firstly, to assess non-toxic concentrations of organoselenium compounds, a concentration-response study was conducted by treating HT22 cells for 24 h with concentrations of such compounds ranging from 1 to 20 μ M. Cell viability was evaluated by the MTT assay. As shown in Fig. 3A, the compound MRK Picolyl caused a significant decline in MTT reduction starting at 5 μ M, while the cytotoxicity of PhSeZnCl started at 10 μ M. (PhSe)₂ and MRK Ester caused significant cellular cytotoxicity only at 20 μ M. There was no significant decrease in cell viability over a 24 h exposure to organoselenium compounds here tested at 1 μ M (Fig. 3A). Based these results, the concentration at 1 μ M, which did not significantly decrease cell viability, was chosen to evaluate the potential protective effect of the organoselenium compounds against glutamate-induced oxidative toxicity (Fig. 3B and 3C). Glutamate caused a marked decrease in MTT reduction and a significant increase in PI uptake in HT22 cells after 24 h of exposure. Our data show all the tested organoselenium compounds were able to significantly reduce glutamate-induced cell death in both protocols used (pre or co-treatment; Fig. 3B-E). However, the protective effects of organoselenium compounds were more pronounced in the co-treatment protocol (Fig. 3C and E).

Effect of organoselenium compounds on SIN-1-mediated DHR oxidation

The exposure of HT22 cells to SIN-1 (25 μ M) led to intracellular DHR oxidation (Fig. 4A), due to the production of peroxynitrite-derived radicals. Pre-treatments (24h) with 1 μ M of (PhSe)₂ and MRK Picolyl (Fig. 4B) significantly decreased the generation of rhodamine 123 (a product of DHR oxidation) in SIN-1-treated cells. This indicates that these compounds can prevent DHR oxidation resulting from SIN-1 exposure. When co-treated with SIN-1, the organoselenium compounds displayed no significant effects against DHR oxidation (Fig. 4C).

(PhSe)₂ modulates antioxidant enzymes in HT22 cells

The lack of association between the protective/beneficial effects of the organoselenium compounds with their thiol peroxidase/oxidase activities (Fig. 2-4) suggested additional mechanisms involved in the observed protection. Thus, we evaluated the ability of the prototype (PhSe)₂ in modulating cellular antioxidant enzymes. Fig. 5 shows a significant increase in Prx3 (at 6 h), CAT (24 h), GCLC (6 and 24 h) protein expression after (PhSe)₂ treatment. No significant changes in the protein expression of MnSOD, glutamate-cysteine ligase, modifier subunit (GCLM) and HO-1 were observed after (PhSe)₂ treatment.

Discussion

Several synthetic organoselenium compounds, designed mainly to mimic GPx, have been developed over the last three decades; among them, several have exhibited beneficial pharmacological properties in experimental models of human pathologies, including neurodegenerative diseases [6, 7, 16, 26]. Although the GPx-like activity displayed by some synthetic organoselenium compounds represents a key factor for their beneficial biological effects [27], recent literature data suggests that some of these compounds can up-regulate the expression of antioxidant and pro-survival proteins, thus adding up new mechanisms concerning their pharmacological actions. In this study, we evaluated the thiol-peroxidase/oxidase activities of some synthetic organoselenium compounds and compared with the native GPx. Although our data showed lower thiol-peroxidase activity of the selenium compounds compared to the purified GPx, significant protective effects of the studied organoselenium compounds against oxidative challenge in neuronal HT22 cells were observed. Such protection, whose magnitude was dependent upon the molecular structure and treatment schedule (pre- or co-treatment), could be in part attributed to their thiol modifier effects.

Among the analyzed compounds, (PhSe)₂, the prototype organoselenium compound, displayed a linear concentration-dependent response for thiol peroxidase activity and the lowest thiol oxidase activity here observed. In accordance with the present results, previous studies have proposed kinetic parameters of thiol peroxidase activity for (PhSe)₂ [12]. In general, diselenides enter the GPx cycle via its reduction by thiol containing molecules, such as GSH. This leads to the formation of selenol intermediate, which reduces peroxides with concomitant formation of selenenic acid. Finally, GSH regenerated back the selenol group [13].

Our data showed that PhSeZnCl, MRK Picolyl and MRK Ester displayed a significant thiol-oxidase activity, reacting efficiently with nonspecific thiols group, like those from GSH. It is relevant to consider that the ability of these compounds to oxidize the thiol groups of proteins (the thiol-modifier effect) might be a proper way to explain their pharmacological effects. Even without displaying thiol-peroxidase activity, these compounds were effective in preventing cell death induced by oxidants. These data suggested that these organoselenium compounds can to modulate the redox homeostasis by a hormetic effect, as previously reported for organic diselenides [28].

The organoselenium compounds here evaluated showed different patterns of toxicity in HT22 cells. The MRK Picolyl showed high cytotoxicity, decreasing cell viability at 5 µM, followed by

PhSeZnCl (10 μ M), while (PhSe)₂ and MRK Ester showed a cytotoxic effect on HT22 neuronal cells only at 20 μ M. It is essential to mention that only (PhSe)₂ and PhSeZnCl, at high concentrations (above 10 μ M), achieve a thiol peroxidase activity comparable to the efficiency of GPx enzyme (in the degradation of H₂O₂). However, at 10 μ M, most of organoselenium compounds exhibited cytotoxic to HT22 neuronal cells. This fact suggests other mechanisms, besides GPx mimetic activity are involved in the actions of organoselenium compounds.

To evaluate the hormetic hypothesis to explain the protective action of organoselenium compounds, we submitted HT22 cells to an oxidative stress-induced cell death pathway, called oxytosis [29]. Two protocols were performed, where (i) cells were pre-treated for 24 h with the organoselenium compounds and then exposed to glutamate, or (ii) simultaneously treated with the compounds and glutamate. In both protocols, the organoselenium compounds were able to reduce the cell death induced by glutamate. In particular, organoselenium compounds were slightly more effective when simultaneously exposed to glutamate. In this sense, it is important to state that the cell viability assay was evaluated at 24 h after glutamate exposure, therefore sufficient time for organoselenium compounds trigger cellular signalling pathways related to modulating antioxidant defences.

The hormetic effect of organoselenium was also verified by their effect as a scavenger of ONOO⁻ on HT22 cells. When HT22 cells were simultaneously exposed to SIN-1, a chemical donor of ONOO⁻, and then to organoselenium compounds, no ONOO⁻ scavenger effect was observed. However, when cells were previously treated with the organoselenium compounds, HT22 cells were able to detoxify the ONOO⁻ generated by SIN-1 flux. This result highlights the ability of organoselenium compounds to induce cellular defences, which renders the cells more capable of dealing with the toxic effect induced by the oxidative challenge (SIN-1-derived peroxynitrite). We have previously reported this kind of effect in endothelial cells [10, 15] supporting the notion that organoselenium compounds could induce chemical changes in biological molecules leading an improved cellular antioxidant response.

In the biological milieu (either intra- or extracellularly), organoselenium compounds react with thiols, becoming weak nucleophiles able to oxidize not only GSH, but also redox-sensitive thiols [13]. The transcriptional factor, nuclear erythroid 2-related factor (Nrf2), works as a master regulator of intracellular antioxidant response modulating the transcription of several of antioxidant genes. Under normal conditions, Nrf2 is negatively regulated by Keap1. The oxidation of thiol bounds between Keap1 and Nrf2 leads to Nrf2 activation, stimulating the transcription of antioxidants elements [30, 31]. In this

context, we proposed to verify the ability of the prototype, (PhSe)₂, to modulate antioxidant enzymes in HT22 cells, since in a previous study we demonstrated the effect of (PhSe)₂ in activating the Nrf2 [10, 15]. Our results showed that (PhSe)₂ was able to increase the protein levels of Prx3, catalase and GCLC, but did not alter HO-1 protein levels in HT22 cells after 24 h of treatment. Supporting these data, recently we founded that (PhSe)₂ increased the mRNA levels of GPx1, GPx4, GCLC, HO-1 and catalase in HT22 cells. The modulation in the cellular antioxidant environment turns the cells treated with (PhSe)₂ capable to resist to the oxidative damage induced by tert-butyl hydroperoxide [16].

In summary, the results presented herein reinforce the hypothesis that the biological effects of organoselenium compounds go far beyond their thiol-peroxidase activities. Such activities were not directly associated with the magnitude of their beneficial effects against oxidative challenges (glutamate and SIN-1). The thiol oxidase activities of the studied compounds, which are directly related to their thiol-modifier abilities, should also be taken into account when considering their biological (either beneficial or toxic) effects. The GPx-like activities of these compounds were much lower than that of the native GPx enzyme, achieving comparable efficiency only at very high concentrations, which are toxic to cells. Our results build up the idea that the studied organoselenium compounds - particularly (PhSe)₂ - can act as redox-modulating molecules, increasing the expression of antioxidant and pro-survival proteins (Prx3, Catalase and GCLC) in a redox-mediated manner. These findings encourage the investigation of the beneficial effects of these compounds under *in vivo* experimental conditions, especially those where hormesis and the upregulation of the endogenous adaptive stress responses are relevant.

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Figures

Fig. 1 Organoselenium compounds MRK-Picolyl, MRK-Ester, (PhSe)₂ and PhSeZnCl.

Fig. 2 Thiol peroxidase and oxidase activity of organoselenium compounds. (A) Thiol peroxidase activity of purified GPx enzyme from bovine erythrocytes and of organoselenium compounds in polar medium. (B) Thiol oxidase activity of purified GPx enzyme from bovine erythrocytes and of organoselenium compounds in polar medium. (A-B) Results are represented as mean ± SEM (n = 5), linear regression was

determined (concentration-dependent response). (C) Thiol peroxidase activity of organoselenium compounds (100 μ M) in nonpolar medium (thiophenol method). Results are represented as mean $\pm$ SEM (n = 5). Data were analyzed by one-way ANOVA followed by the Tukey's *post hoc* test. Different letters indicate significant differences among the groups ($p < 0.05$).

Fig. 3 Effect of organoselenium compounds in glutamate-exposed HT22 cells. (A) HT22 cells were exposed to different organoselenium concentrations (1 – 20 μ M) for 24 h. (B and D) HT22 cells were pre-treated with 1 μ M of organoselenium compounds for 24h followed by exposure to glutamate (5 mM) for additional 24 h. (C-E) HT22 cells were co-treated with 1 μ M of organoselenium compounds at the same time of glutamate (5 mM) exposure. The cell viability was evaluated after 24 h. Results of MTT (B-C) assay were expressed as the percentage of MTT reduction with respect to vehicle (0.05% DMSO) group. Results of PI (D-E) assays are expressed as percent of PI uptake, where the 100% of cell death value represent cells treated with 2% Triton X-100 during 15 min. Data are represented as mean $\pm$ SEM; n = 5. # $p < 0.05$ indicate statistical difference between glutamate and vehicle or organoselenium groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ indicate statistical difference between glutamate and organoselenium group by one-way ANOVA followed by the Dunnett's *post hoc* test.

Fig. 4 Effect of pre and co-treatment with organoselenium compounds in HT22 cells against SIN-1-mediated DHR oxidation. (A) Effect of SIN-1 (25 μ M) or vehicle (HCl 3 mM + DMSO 0.05%) in HT22 cells. (B) HT22 cells were pre-treated for 24h with organoselenium compounds and exposed to SIN-1 (25 μ M) for 40 min. (C) Cells were simultaneously treated with organoselenium compounds and exposed to SIN-1 (25 μ M for 40 min). The cells were loaded with DHR (10 μ M) and rhodamine 123 (RH123) formation was detected, at 37°C, in a fluorescence plate reader during 60 min at ex = 485 nm and em = 590 nm, results are expressed as relative slope per minute. Data are represented as mean $\pm$ SEM (n = 5), * $p < 0.05$ indicate statistical difference from SIN-1 group (dashed line) by one-way ANOVA followed by the Dunnett's *post hoc* test.

Fig. 5 Effect of (PhSe)₂ in modulating the cellular antioxidant enzymes. HT22 cells were treated with (PhSe)₂ (1 μ M) at different time points (3 – 24 h). (A) MnSOD, (B) Prx3, (C) Catalase, (D) GCLC, (E) GCLM and (F) HO-1 expression were determinate by Western blotting and Tubulin expression was used

477 as loading control, V = vehicle, D = (PhSe)₂. Data were represented as mean ± SEM (n = 5). **p* < 0.05,
478 indicate statistical difference from vehicle group (dashed line) by two-way ANOVA, followed by the
479 Tukey's *post hoc* test.

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