Evaluation of the Neurotoxic/Neuroprotective Role of Organoselenides Using Differentiated Human Neuroblastoma SH-SY5Y Cell Line Challenged with 6-Hydroxydopamine

ARTICLE *in* NEUROTOXICITY RESEARCH · JANUARY 2012

Impact Factor: 3.54 · DOI: 10.1007/s12640-012-9311-1 · Source: PubMed

11 AUTHORS, INCLUDING:

Guilherme Antônio Behr
Universidade Federal do Rio Grande do Sul
32 PUBLICATIONS **435** CITATIONS
SEE PROFILE

Valeska Oliveira
Universidade Federal do Rio Grande do Sul
2 PUBLICATIONS **23** CITATIONS
SEE PROFILE

Mohammad Ibrahim
Universidade Federal de Santa Maria
21 PUBLICATIONS **143** CITATIONS
SEE PROFILE

Joao Batista Teixeira da Rocha
Universidade Federal de Santa Maria
575 PUBLICATIONS **13,026** CITATIONS
SEE PROFILE

Neurotox Res
DOI 10.1007/s12640-012-9311-1

Evaluation of the Neurotoxic/Neuroprotective Role of Organoselenides Using Differentiated Human Neuroblastoma SH-SY5Y Cell Line Challenged with 6-Hydroxydopamine

Fernanda Martins Lopes · Giovana Ferreira Londero · Liana Marengo de Medeiros · Leonardo Lisbôa da Motta · Guilherme Antônio Behr · Valeska Aguiar de Oliveira · Mohammad Ibrahim · José Cláudio Fonseca Moreira · Lisiane de Oliveira Porciúncula · João Batista Teixeira da Rocha · Fábio Klamt

Received: 14 December 2011 / Revised: 6 January 2012 / Accepted: 9 January 2012

Abstract It is well established that oxidative stress plays a major role in several neurodegenerative conditions, like Parkinson disease (PD). Hence, there is an enormous effort for the development of new antioxidants compounds with therapeutic potential for the management of PD, such as synthetic organoselenides molecules. In this study, we selected between nine different synthetic organoselenides the most eligible ones for further neuroprotection assays, using the differentiated human neuroblastoma SH-SY5Y cell line as in vitro model. Neuronal differentiation of exponentially growing human neuroblastoma SH-SY5Y cells was triggered by cultivating cells with DMEM/F12 medium with 1% of fetal bovine serum (FBS) with the combination of 10 μM retinoic acid for 7 days. Differentiated cells were further incubated with different concentrations of nine organoselenides (0.1, 0.3, 3, 10, and 30 μM) for 24 h and cell viability, neurites densities and the immunocontent of neuronal markers were evaluated. Peroxyl radical scavenging potential of each compound was determined with TRAP assay. Three organoselenides tested presented low cytotoxicity and high antioxidant properties. Pre-treatment of cells with those compounds for 24 h lead to a significantly neuroprotection against 6-hydroxydopamine (6-OHDA) toxicity, which were directly related to their antioxidant properties. Neuroprotective activity of all three organoselenides was compared to diphenyl diselenide $(PhSe)_2$, the simplest of the diaryl diselenides tested. Our results demonstrate that differentiated human SH-SY5Y cells are suitable cellular model to evaluate neuroprotective/neurotoxic role of compounds, and support further evaluation of selected organoselenium molecules as potential pharmacological and therapeutic drugs in the treatment of PD.

Keywords SH-SY5Y cells · Organoselenides · Oxidative stress · 6-Hydroxydopamine · Neuroprotection

F. M. Lopes · G. F. Londero · L. M. de Medeiros · L. L. da Motta · G. A. Behr · V. A. de Oliveira · J. C. F. Moreira · L. de Oliveira Porciúncula · F. Klamt (✉)
Departamento de Bioquímica, Laboratório 24, ICBS/ Universidade Federal do Rio Grande do Sul (UFRGS), Rua Ramiro Barcelos, 2600, Porto Alegre, RS 90035-003, Brazil
e-mail: 00025267@ufrgs.br

F. M. Lopes · G. F. Londero · L. M. de Medeiros · L. L. da Motta · G. A. Behr · V. A. de Oliveira · J. C. F. Moreira · L. de Oliveira Porciúncula · J. B. T. da Rocha · F. Klamt
Rede Gaúcha de Estresse Oxidativo e Sinalização Celular (FAPERGS), Porto Alegre, RS, Brazil

F. M. Lopes · G. F. Londero · L. M. de Medeiros · L. L. da Motta · V. A. de Oliveira · F. Klamt
Institutos Nacionais de Ciência e Tecnologia—Translacional em Medicina (MCT/CNPq INCT-TM), Porto Alegre, RS, Brazil

M. Ibrahim · J. B. T. da Rocha
Departamento de Química, CCNE/UFSM, Santa Maria, RS, Brazil

M. Ibrahim
Department of Chemistry, Abdul Wali Khan University Mardan (AWKUM) KPK, Mardan, Pakistan

Introduction

Many lines of evidence indicate that the central nervous system (CNS) is prone to oxidative damage (Halliwell 2006). Some attributes of this tissue can contribute to this situation: modest antioxidant defenses (Halliwell 2001), high amount of polyunsaturated fatty acids (Floyd and

Published online: 20 January 2012

Hensley 2002), auto-oxidization of several neurotransmitters (Spencer et al. 1998), and high oxygen consumption (Halliwell 1992). The process of neurodegeneration also involves oxidative damage (Halliwell 2006). It is well known that many neurodegenerative disease share common pathophysiologic mechanisms: oxidative stress (Halliwell 2001); protein aggregation (Stefanis and Keller 2006); and mitochondrial dysfunction (Zeevalk et al. 2005).

One example of these disorders is Parkinson disease (PD). The motor symptoms of Parkinson's disease result from the death of dopamine (DA)-generating neurons of *substancia nigra pars compacta*, which are exposed to chronic oxidative stress generated by DA metabolism. High amount of iron present in this region also contributes to hydroxyl radical ($^{\bullet}$OH) generation by Fenton Chemistry (Barnham et al. 2004; Drechsel and Patel 2008). Moreover, the neurotoxin 6-hydroxydopamine (6-OHDA), an analog of DA, is widely used to mimic PD through the generation of a massive oxidative damage in cells (Lehmensiek et al. 2006).

Since the overproduction of reactive species (RS) is a milestone in PD, investigations on antioxidant molecules with neuroprotective potential are at the forefront of PD therapeutic management (Posser et al. 2008). Agents currently under investigation as potential treatments include anti-apoptotics (omigapil, CEP-1347), compound that improve mitochondrial activity (coenzyme Q10), antiglutamatergics, monoamine oxidase inhibitors (selegiline, rasagiline), calcium channel blockers (isradipine), and growth factors (GDNF) (Pedersen and Schmidt 2000; Mandel et al. 2003; McCarthy et al. 2004; Naoi and Maruyama 2010; Ilijic et al. 2011; Safi et al. 2011). One example of these candidates are organochalcogenides, especially Se-containing organic compounds (Bhabak and Mugesh 2007). Since the discovery that these compounds (in the form of selenocysteine) are a critical component of glutathione peroxidase (GPx) (Flohe et al. 1973; Rotruck et al. 1973) and thioredoxin reductase (Arnér and Holmgre 2000; Santos et al. 2009), the chemistry at the active site of GPx has been extensively investigated to make synthetic Se-containing compounds (Rossato et al. 2002; Luchese et al. 2007; Posser et al. 2006, 2008; Ibrahim et al. 2011).

One example of this compound is ebselen. Ebselen is a synthetic organoselenide that mimics the activity of GPx both in vitro and in vivo (Müller et al. 1984; Bhabak and Mugesh 2007). Due to its antioxidant function, it can neutralize the free radical damage and also has neuroprotective effects against brain injuries involving glutamatergic system (Porciúncula et al. 2003), ischemia (Xu et al. 2006), and spinal cord injury (Kalayci et al. 2005). Hence, the success obtained with ebselen inspired several research groups to synthesize other low-molecular-weight compounds with high availability, both qualities that improve its therapeutic potential (Sies 1993; Schewe 1995; Mugesh et al. 2001; Geoghegan et al. 2006; Talas et al. 2008; Ozdemir et al. 2010). Even though other ebselen analog, diphenyl diselenide $(PhSe)_2$, can inhibit glutamate uptake in rat hippocampus (Ardais et al. 2010) and also confers neuroprotection in hippocampus slices through antioxidant mechanisms (Posser et al. 2008), the organoselenides can be very toxic to many tissues, such as liver (Meotti et al. 2003), blood (Santos et al. 2009), and brain (Souza et al. 2010). Paradoxically, the abovementioned toxicity can be mediate due to pro-oxidative conditions, such as GSH depletion (Farina et al. 2004). Hence, due to its contradictory data, more studies are necessary to elucidate its mechanisms.

There are many studies evaluating the cytoprotective/cytotoxic features of organochalcogenides, however, there is little information regarding their effects over the CNS and neuronal cells models, such as differentiated human neuroblastoma SH-SY5Y cells (Posser et al. 2011). This cell line has been widely used as an in vitro experimental model and present several advantages for neuroscience studies, such as: (i) is derived from humans; (ii) as a cell line, is homogenous (which increases data reproducibility); (iii) has mitogenic potential, which decreases the time of the culture process compared to primary and organotypic culture; (iv) it can be differentiated into dopaminergic neurons through neurotrophins, for instance, retinoic acid (RA); and (v) once differentiated, it acquires important neuronal attributes (e.g., high expression of tyrosine hydroxylase—TH, dopamine transporter—DAT, neuron specific enolase—NSE, neuronal nuclear protein—NeuN, Synaptobrevin and others, and a typical neuronal morphology with abundant neurites outgrow). Hence, the RA-differentiated SH-SY5Y cells are considered a more suitable in vitro model to study not only the pathophysiological mechanism of CNS diseases, but also to evaluate neuroprotective/neurotoxicity of compounds (Lopes et al. 2010).

The aim of this study was to screen between different organoselenides, investigating their antioxidant potential and neurototoxicity, for subsequent selection the most promising compounds for neuroprotection assays. For this purpose, we used differentiated human neuroblastoma SH-SY5Y cells challenged with 6-OHDA. Our data demonstrate that at least two different organoselenides tested—compound IV (1,1′-(2,2′-diselanediylbis(2,1-phenylene)) diethanamine) and V (2,2′-(1*Z*,1′*E*)-(1,1′-(2,2′-diselanediylbis(2,1-phenylene))bis(ethane-1,1-diyl))bis(azan-1-yl-1-ylidene)bis(methan-1-yl-1-ylidene)diphenol) (Fig. 1) present neuroprotective potential and are promising candidates for further investigations for PD therapeutic management.

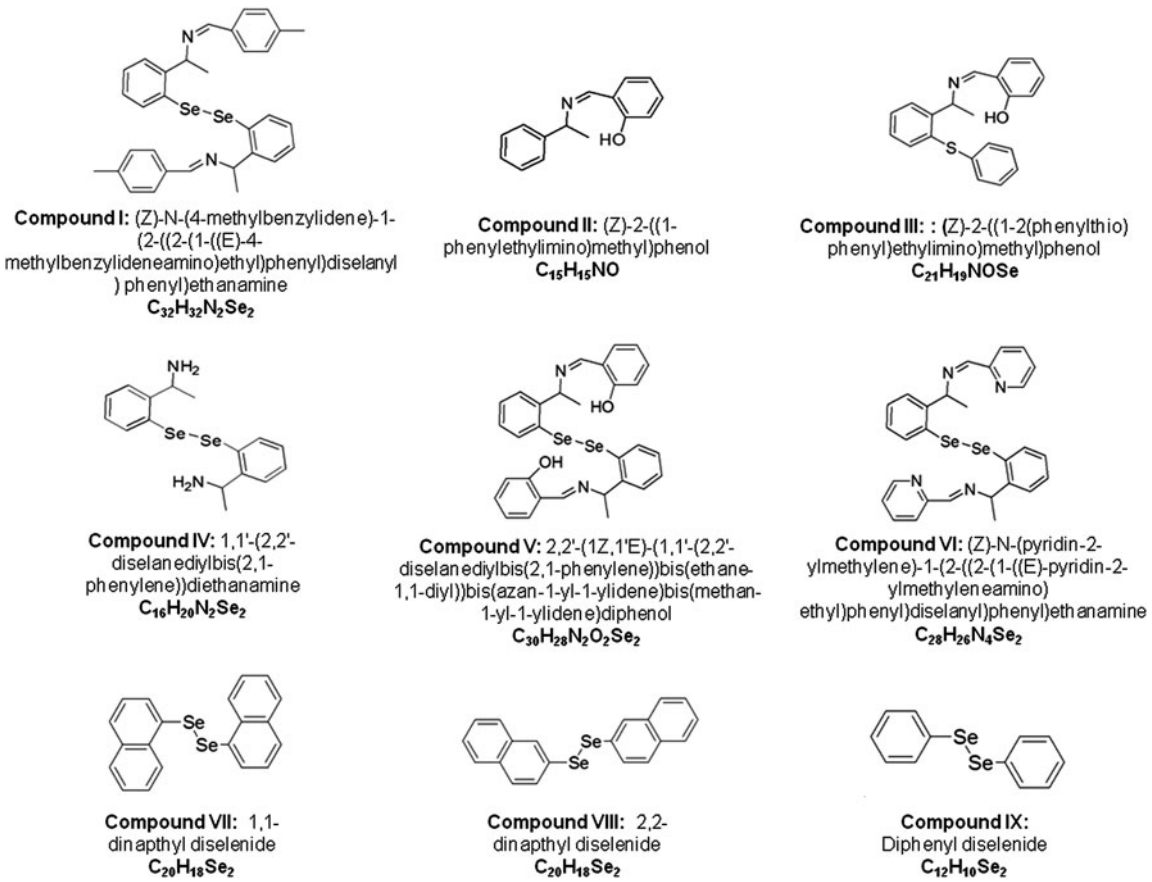

Fig. 1 Molecular structures of the organoselenides tested. Basic structures of compounds were derived from ebselen (not shown) and diphenyl diselenide $(PhSe)_2$, the simplest diaryl diselenides tested. All the compounds names were substitute for roman numerals (I–IX)

Experimental Procedures

Chemicals

Materials used in cell culture were acquired from Gibco®/Invitrogen (São Paulo, SP, Brazil). Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The structures of the nine organoselenides tested in this study are shown in Fig. 1. Analysis of the ^{1}H-NMR and ^{13}C-NMR spectra showed that all the compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structures. The purity of the compounds were assessed by high resonance mass spectroscopy (HRMS) and was higher than 99.9%. To simplify the understanding of this work, the compound name was replaced by roman numerals. All of them were diluted in ultrapure dimethyl sulfoxide (DMSO).

Cell Culture, Differentiation, and Treatments

Exponentially growing human neuroblastoma SH-SY5Y cell line, obtained from ATCC (Manassas, VA, USA), was maintained at 37°C in a humidified atmosphere of 5% CO_2. The cells were grown in a mixture of 1:1 of Ham's F12 and Dulbecco's Modified Eagle Minimum (DMEM) supplemented with 10% of fetal bovine serum (FBS), 2 mM of glutamine, 0.28 μg/μL of gentamicin, and 250 μg of amphotericin B. Medium was changed each 3 days and cells were sub-cultured once they reached ~80% confluence. After 24 h of plating, cell differentiation was triggered by lowering the FBS in medium to 1% with the addition of 10 μM of RA during 7 days (Lopes et al. 2010). In the seventh day of RA-induced differentiation, the SH-SY5Y cells were treated with different concentrations of the nine compounds (0.1; 0.3; 3; 10; 30 μM) for 24 h at 37°C. For viability assay, cells were seeded in 96-wells plate at density of 2×10^4 cells/well. For the evaluation of the redox parameters, cells were seeded into flasks of 75 cm^3 at a density of 3×10^6 cells/well.

Total Radical-Trapping Antioxidant Potential

To evaluate the non-enzymatic antioxidant capacity of samples, we used the total radical-trapping antioxidant potential (TRAP) assay, which is based on the measurement of luminescence generated by luminol oxidation by

AAPH (2, 20-azobis 2-amidinoprepane) decomposition, in glycine buffer (pH 8.6). After system stabilization (buffer plus luminol and AAPH), sample was added (organoselenides or extract of cells pre-incubated with selected concentration of compounds) and the luminescence decreases proportionately to its antioxidant potential. The luminescence was monitored in a Wallace 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer (Perkin Elmer). For data analysis, a time per chemiluminescence curve was obtained and the relative "area under the curve" (AUC) in the recovery phase was used, as previously established (Lissi et al. 1995; Dresch et al. 2009).

Cellular Viability

Cell viability was evaluated by the quantification of 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to a blue formazan product by cellular dehydrogenases. At the end of the treatment, cells were incubated with 0.5 mg/mL of MTT during 1 h at 37°C. Then, medium was discarded and DMSO was added to solubilize the formazan crystals. The absorbance was determined at 560 and 630 nm in a SoftMax Pro Microplate Reader (Molecular Devices®, USA).

Neurites Density and Immunocontent of Neuronal Markers

To further evaluate the implication of the organoselenides in neuronal parameters, we evaluated the stellate morphology and neurites densities in treated cells. Cellular treatments were performed in six-well plates (density: 2×10^5 cell/well) for 24 h. First, 10 microscopic fields (200× magnification) were randomly selected from three independent experiments ($n = 3$), photographed using an Olympus IX70 inverted microscope and analyzed with NIS-elements software. Neurite density was assessed by counting the number of "nodes" per cell. Primary nodes were considered branches from the cell body. The results were expressed in percentage of untreated cells (mean ± SD value).

For western blot analysis of neuronal markers, after 24 h treatment, cells were washed with PBS and resuspended in a Tris-buffer (pH 7.0) with protease inhibitor (Roche®), lysed, and total protein extracts (30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane. Thereafter, nonspecific binding was blocked with 5% of BSA in TTBS for 1 h at room temperature. Membranes were then incubated overnight at 4°C with rabbit anti-tyrosine hydroxylase—TH (1:3,000) from Abcam®, rat anti-dopamine transporter—DAT (1:1,000) from Santa Cruz®, and mouse anti-synaptobrevin (1:1,000) from Sigma® (Billerica, MO, USA). After washing, the membrane was incubated with peroxidase-conjugated secondary antibodies (1:5,000) from Dako® (Glostrup, DK) for 2 h at room temperature. Bands were visualized with Super Signal West Pico Chemiluminescence Substrate from PIERCE® (Rockford, IL, USA). Membranes were then striped and reprobed with rabbit anti-β-actin antibody (1:2,000) from Sigma Chemical Co. (St. Louis, MO, USA) followed by goat anti-rabbit peroxidase-conjugated secondary antibody (1:5,000) from Dako® (Glostrup, DK). Quantification of band was done with ImageJ 1.36b software (National Institutes of Health).

Antioxidant Enzymes Activities and –SH levels

Differentiated SH-SY5Y cells were treated with selected compounds during 24 h. The medium was removed and the cells were washed with PBS to avoid the contamination of non-incorporated compound. After that, cells were frozen at −80°C and thawed twice in 10 mM PBS. Cells extracts were centrifuged and the supernatant was collected and Elman's sulfhydryl group (–SH) levels were determined. Briefly, samples were dilute in 10 mM of boric acid with 0.2 mM EDTA (pH 8.5). 10 mM of DTNB was added and –SH levels were determined by reacting samples with 5-thio-2-nitrobenzoic acid (Nbs) and measuring absorbance at 412 nm ($\varepsilon_{412\ nm} = 27{,}200\ M^{-1}\ cm^{-1}$). Results are expressed an nmol –SH/mg protein (Ellman 1959). GPx activity (E.C. 1.11.1.9) was assayed measuring NADPH oxidation at 340 nm in the presence of GSH, *tert*-butyl hydroperoxide and glutathione reductase. GPx unit was defined as nmol NADPH oxidized/min (Wendel1981). Catalase (CAT) (E.C. 1.11.1.6) activity was determined by monitoring the rate of H_2O_2 consumption at 240 nm (Aebi 1984). Superoxide dismutase (SOD) (E.C. 1.15.1.1) activity was assayed by measuring the inhibition of adrenaline auto-oxidation at 480 nm. SOD unit was defined as the sample amount that inhibits 50% of adrenaline auto-oxidation (Misra and Fridovich 1972). Glutathione *S*-transferase (GST) (E.C. 2.5.1.13) activity is determined measuring the formation of the conjugate of GSH and chloro-dinitro benzene (CDNB) (Pabst et al. 1974).

Neuroprotection and RS Generation

Cells were incubated with selected compounds for 24 h, washed with PBS and challenged with 6-OHDA ($LD_{50} = 15$ µM) to evaluate the neuroprotection features of the organoselenides through MTT assay. Moreover, to evaluate if the compounds were able to decrease the RS generated by 6-OHDA, we use the probe DCF-DA (2′, 7′-dichlorodihydrofluorescein diacetate) (Wang and Joseph 1999; Halliwell and Whiteman 2004). After treatment, the

medium was removed and 10 μM DCF-DA was added. Cells were treated with 6-OHDA (LD_{50} = 15 μM) and the fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm.

Protein Quantification

The proteins contents were measured by the Lowry assay (Lowry et al. 1951).

Statistical Analysis

Data are expressed as percentage of untreated cells (control) (mean ± SD) from at least three independent experiments. For statistical analysis, data were analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls test. Differences were considered significant at $P < 0.05$.

Results

Screening of Organoselenides Compounds for Neuroprotective Evaluations

This study was designed to screen between different synthetic organoselenides compounds to select the most eligible ones for further neuroprotection evaluation. The organoselenides evaluated here were synthesized using the basic structure of $(PhSe)_2$, the simplest diaryl diselenides tested (Fig. 1, compound IX). Since it is well known that oxidative stress is related to pathophysiologic mechanisms of many neurodegenerative diseases, including PD, our screening was based on two distinct parameters: find the concentration of compounds that presents high in vitro antioxidant potential with concomitant low cytotoxicity against the differentiated human neuroblastoma SH-SY5Y cells. TRAP assay showed that the majority of the organoselenides tested were not able to scavenge the peroxyl radical generated by AAPH decomposition (Fig. 2). In the concentrations evaluated in this study (0.1, 0.3, 3, 10, and 30 μM) only compound III (concentration ranging from 10 to 30 μM), IV, V, and VI (concentration ranging from 3 to 30 μM) presented antioxidant activity against the peroxyl radical.

MTT assay was used to evaluate the basal cytotoxicity of the nine compounds over the differentiated human neuroblastoma SH-SY5Y cells. Figure 3 shows that compound III, IV, V, and VI, considered as antioxidant based on the in vitro TRAP assay, presented also high cytotoxicity in treated cells. In all concentrations tested, compound I, II, VII, and VIII did not show any significant signs of cytotoxicity. Hence, based on TRAP and MTT assays, we selected the concentrations of three compounds that presented high antioxidant potential and low cytotoxicity: compound III at 10 μM, IV at 3 μM, and V at 3 μM. Even though the compound IX $(PhSe)_2$ presented no antioxidant potential in the concentrations tested, we select this compound based on previous findings about its neuroprotective features (Porciúncula et al. 2003; Posser et al. 2008; Ardais et al. 2010).

Effect of Selected Organoselenides Compounds Over Cellular Morphology and Neuronal Markers

The RA-induced differentiated SH-SY5Y cells have several morphological and biochemical parameters of a dopaminergic cell (Lopes et al. 2010). One important feature of this cell model is the stellate (neuron-like) morphology and the abundance of neurites, which are important parameters to be evaluated in neuroscience studies (Radio and Mundy 2008). Here, we treated the SH-SY5Y cells with the previously selected concentration of compounds and further evaluated their effect over neurites density and the immunocontent of several neuronal markers (Fig. 4). Even though the concentration selected of all compounds was considered sub-lethal by MTT assay, our data showed a significant change in cell morphology with concomitant decrease in neurites density caused by compound III and IX (Fig. 4a, b). Densitometric analysis showed no change in the immunocontent of the neuronal markers tested (Synaptobrevin, DAT, and TH) (Fig. 4c).

Effect of Selected Organoselenides Compounds Over Cellular Redox Parameters

Based on the widely documented peroxidase-like activity of organoselenides, we first determined the TRAP capacity and Elman's reduced thiol levels of cells incubated with compound III at 10 μM, IV at 3 μM, and V at 3 μM for 24 h, in order to evaluate the effect of these three compounds in cellular redox status. All these experiments were compared to DMSO to determine the solvent effect. The results shown that control and vehicle groups were not different from each other and all the data were expressed in percentage to control cells.

TRAP assay showed that all the selected compounds were able to increase radical scavenging capacity when compared to vehicle ($P < 0.05$) (Fig. 5a). These increases in TRAP capacity were followed by an increase in reduced thiol levels found in treated cells (Fig. 5b). Incubation with compounds III, IV, and V increased the GPx activity (Fig. 5e), and SOD activity was increased in SH-SY5Y cells treated with compounds IV and V (Fig. 5d). No

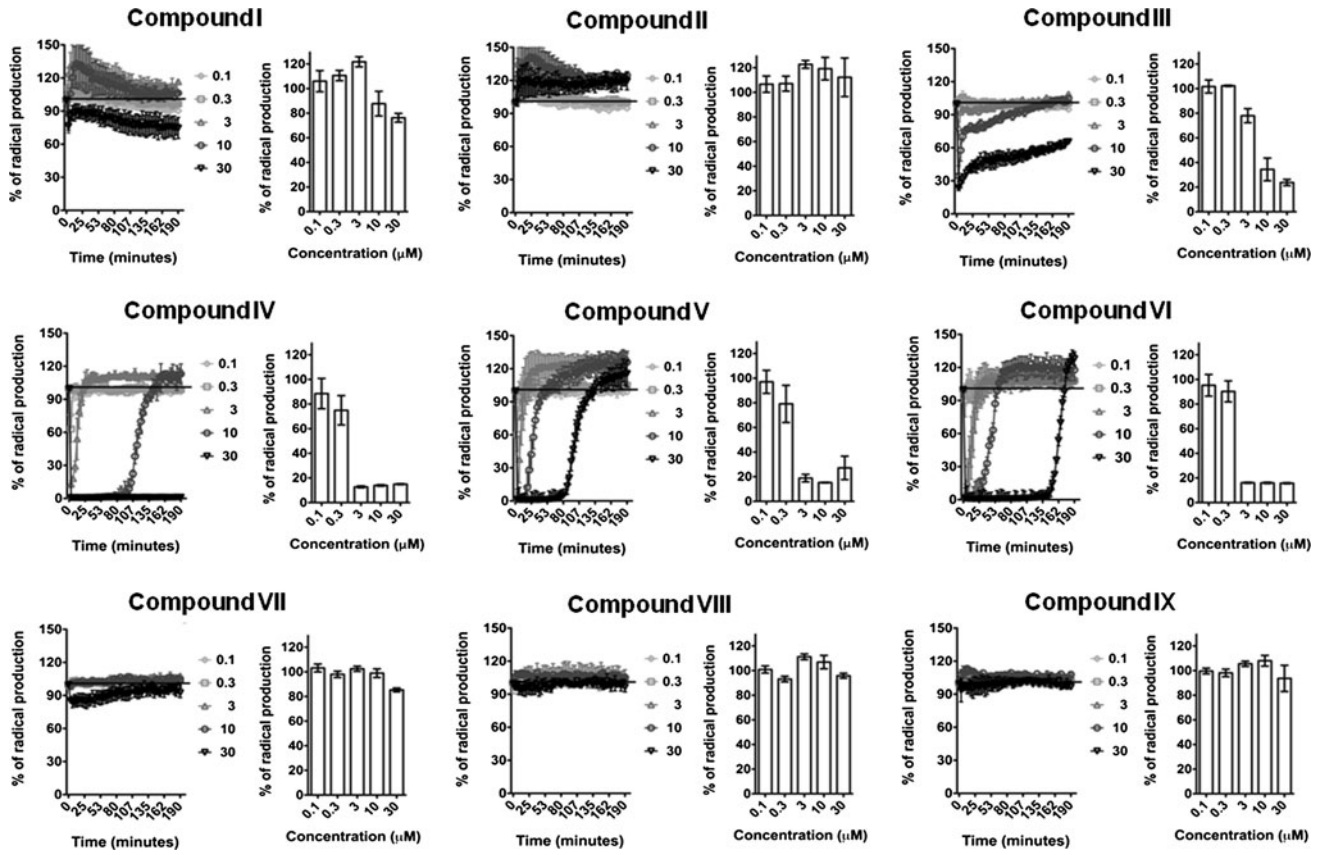

Fig. 2 In vitro total radical-trapping antioxidant potential (TRAP) of the organoselenides. *Left figures* represents TRAP traces, evidencing the system measurements (AAPH + buffer + luminol) (*black line*), which represent 100% of radical production, and the effect of the addition of compounds at the concentrations of 0.1, 0.3, 3, 10, and 30 μM. *Right figures* represent the "AUC" values and expressed as % of radical production, as described in "Experimental Procedures" section. Data are presented as mean ± SD of four independent experiments carried out in triplicates ($n = 4$)

differences were observed in CAT and GST activities (Fig. 5c, f).

Evaluation of Neuroprotection by Selected Organoselenides Against 6-Hydroxydopamine Toxicity

To evaluate neuroprotection, we pre-incubate the differentiated SH-SY5Y cells with compounds III, IV, V, and IX during 24 h. After cells were washed and challenged with 6-OHDA ($LD_{50} = 15$ μM). All the compounds were able to inhibit the cell death (Fig. 6a) and to decrease the generation of RS (Fig. 6b) caused by 6-OHDA treatment.

Discussion

The ongoing management of neurodegenerative diseases is only palliative; basically aimed to decrease the symptoms of these debilitating illnesses. Finding a drug that can reduce disease progression and thus, delay the onset of disability is a critical goal (Shobha et al. 2006; Meissner et al. 2011). However, to develop targeted therapeutics, it is fundamental to understand the molecular mechanisms of these diseases. Even though the pathophysiology of neurodegenerative diseases is unknown, for instance, PD, there are many lines of evidence supporting the major involvement of oxidative stress (Schapira 2008). In this context, the search for antioxidant molecules as therapeutic adjuvant can be an alternative for PD (Jaisin et al. 2011).

Organoselenides have been documented as promising pharmacological agents against a number of diseases associated with oxidative stress, mainly related to hepatotoxicity (Ibrahim et al. 2010). However, it is not well elucidated their role in neuroprotection. These molecules present several features that support their potential role in the management of neurodegenerative disease, such as their low-molecular weight in combination with high antioxidant potential, high availability and hydrophobicity, all qualities that improve its therapeutic potential. For instance, one clinical trial showed that early treatment with ebselen improved the patient outcome in acute ischemic stroke (Yamaguchi et al. 1998). Further studies demonstrated a

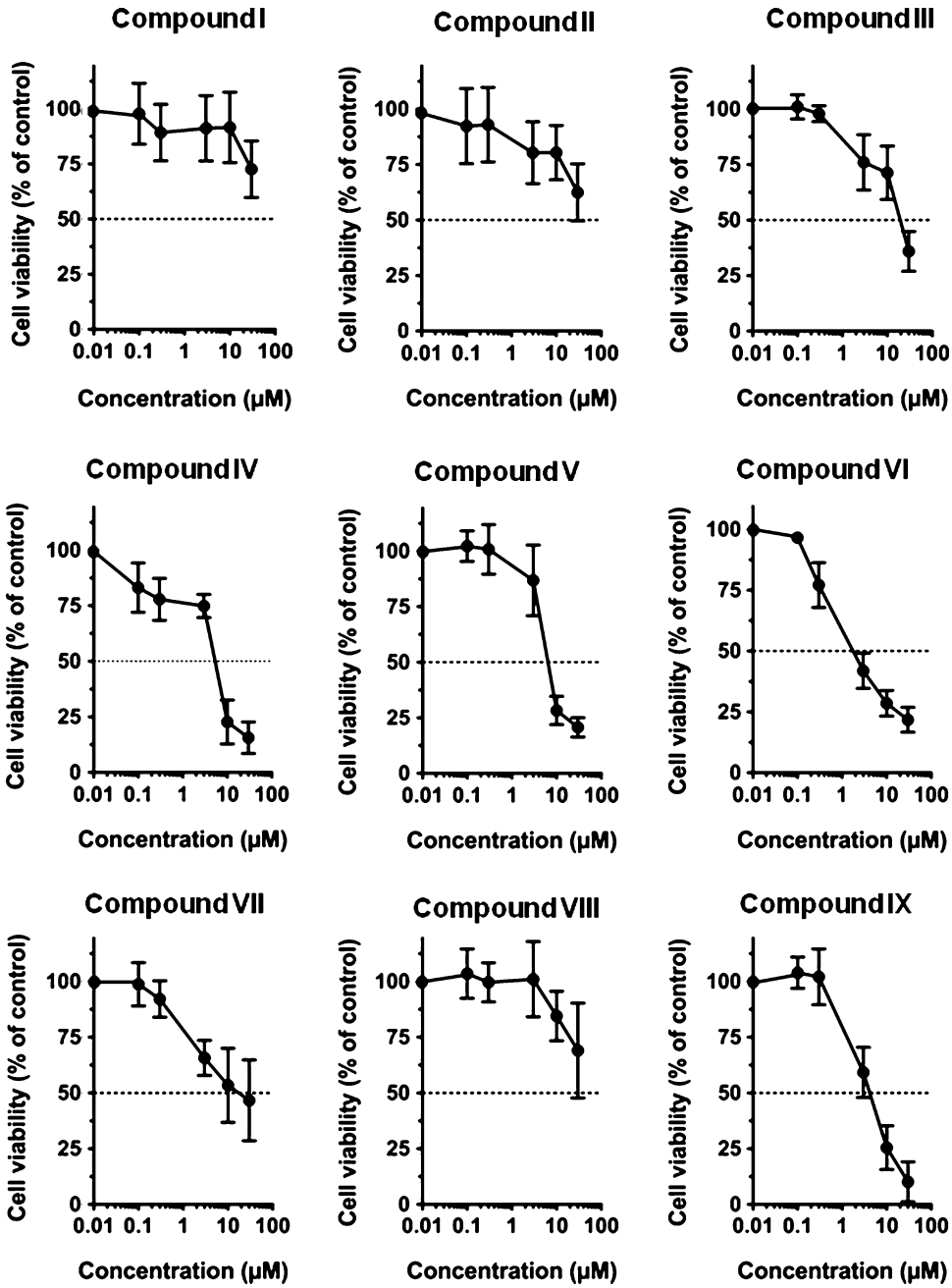

Fig. 3 Cytotoxicity curves of the organoselenides compounds. Cells were treated with each compound at the concentrations of 0.1, 0.3, 3, 10, and 30 μM during 24 h and cell viability was evaluated by MTT assay. *Dashed line* represents LD_{50} value of compound. Data are presented as mean ± SD for four independent experiments carried out in quadruplicates ($n = 4$)

neuroprotective effect of organoselenides in hippocampal slices submitted to oxygen-glucose deprivation (Porciúncula et al. 2003) and hydrogen peroxide administration (Posser et al. 2008). However, there is no data exploring the neurotoxic or neuroprotective role of organoselenides in cell systems, such as differentiated human neuroblastoma SH-SY5Y cells.

We aimed here to screen between different synthetic organoselenides compounds to select the most eligible ones for neuroprotection evaluation. Taking into account the results obtained in this study, we found a significant correlation between the antioxidant potential of synthetic organoselenides compounds and cellular toxicity in RA-differentiated human neuroblastoma SH-SY5Y cells. The concept of antioxidant supplementations being beneficial to human health should be taking with caution. Antioxidant is any substance that, when present in low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substances so that, they keep the RS in low levels (Halliwell 2006). However, oxidants are not only villains in biological systems and they can act in normal physiology of the cells, such as in the activity of cytochrome P450, the immune response and in normal signal transduction pathways

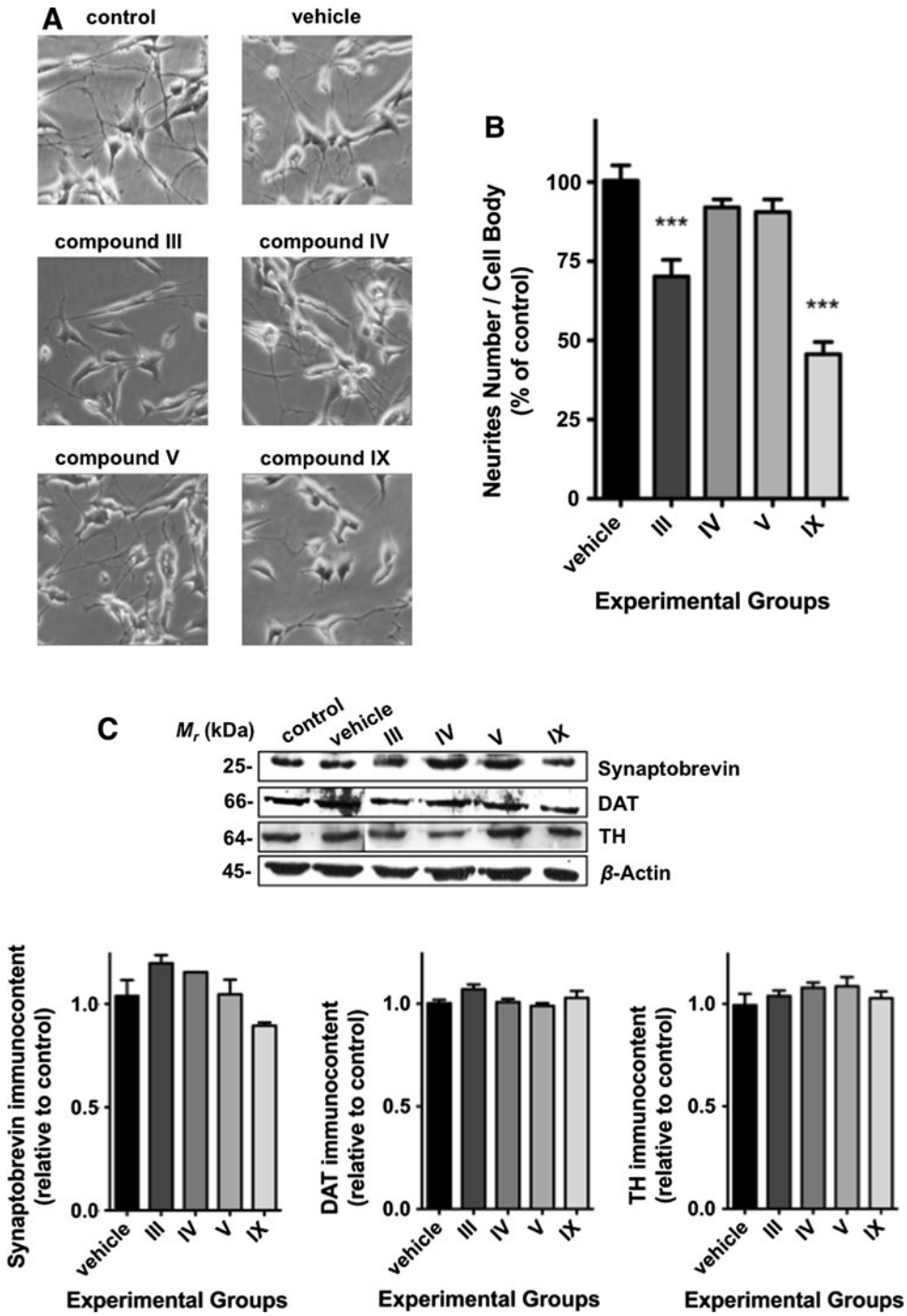

Fig. 4 The effect of sub-lethal doses of selected organoselenides over the cellular morphology and neuronal markers of differentiated human neuroblastoma SH-SY5Y cells. **a** Representative phase contrast images of cells treated by 24 h with 10 μM of compound III, 3 μM of compound IV, 3 μM of compound V, 0.3 μM of compound IX or vehicle, evidencing the stellate morphology with abundant neurites outgrow of cells (200× magnification). Note the change in cell morphology and the lost of neurites caused by compound III and IX. **b** Representative quantification of the neurites density per cell body in treated cells. Data are presented as mean ± SD for three independent experiments carried out in quadruplicates ($n = 3$). ***$P < 0.001$ (one-way analysis of variance). **c** Western blot analysis of neuronal markers (synaptobrevin, tyrosine hydroxylase—TH, dopamine transporter—DAT) of differentiated SH-SY5Y cells treated with selected sub-lethal doses of compounds. β-Actin was used as loading control. Representative blots of three independent experiments ($n = 3$)

(Stone and Yang 2006). Thus, as found with the organoselenides studied here, any antioxidant compound may alter the intracellular redox balance toward a more reduced state, causing as much cytotoxicity response as oxidative stress (Gutteridge and Halliwell 2010).

Our study also established several advantages of the use of differentiated human neuroblastoma SH-SY5Y cells as an in vitro experimental model for neurotoxicity/neuroprotection drug screening evaluations. Even though the differentiated SH-SY5Y cells posses many desired features of an in vitro cell model for neuroscience research (e.g., neuronal morphology with abundant of neurites outgrow and the expression of several neuronal markers) (Pahlman et al. 1984; Lopes et al. 2010), there is still some controversy about the necessity of cellular differentiation (Xie et al. 2010). As an example, in a recent report Cheung et al. (2009) found no change in the expression of dopaminergic markers during SH-SY5Y cellular differentiation by RA.

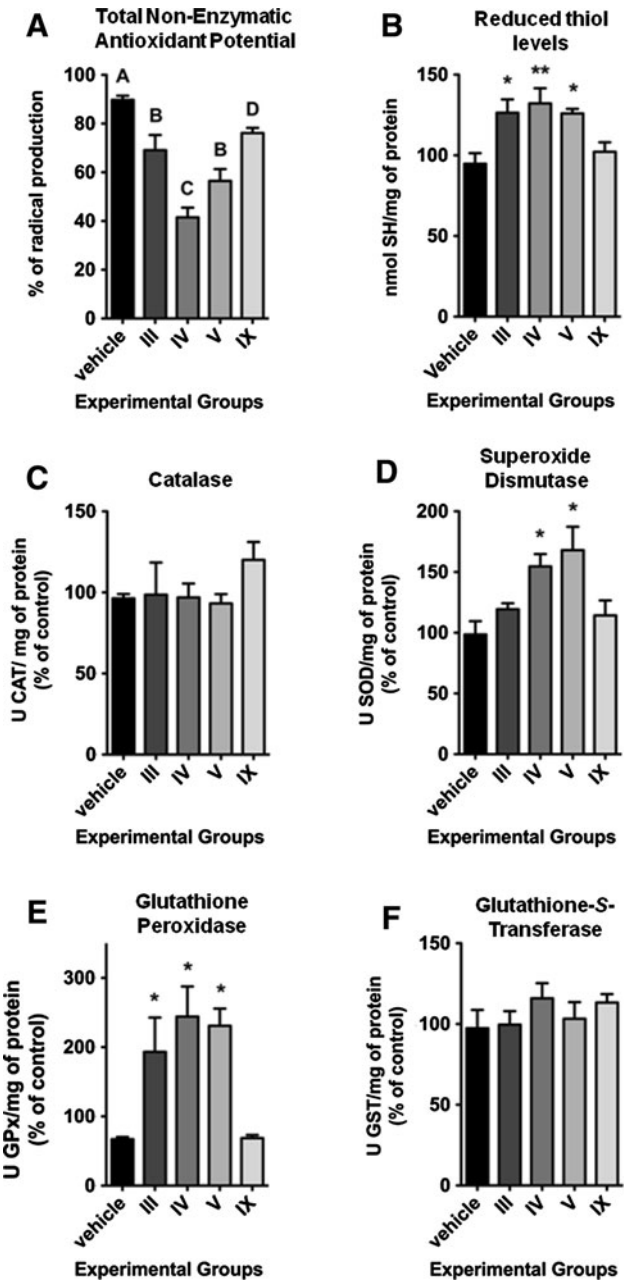

Fig. 5 The effect of sub-lethal doses of selected organoselenides over the redox parameters in differentiated human neuroblastoma SH-SY5Y cells. SH-SY5Y cells were treated by 24 h with 10 μM of compound III, 3 μM of compound IV, 3 μM of compound V, 0.3 μM of compound IX or vehicle, washed and the redox parameters were determined as described in "Experimental Procedures" section. All the results were compared to control cells and expressed as % of control. **a** Total radical-trapping antioxidant potential (TRAP) of treated cells. Data represent the "AUC" values and expressed as % of radical production, as described in "Experimental Procedures" section. Statistical differences are expressed by *letters*. $P < 0.05$ (one-way analysis of variance). **b** Elmann's reduced thiol levels (basal levels ranging from 24 to 48 nmol –SH/mg of protein). **c** Catalase activities in treated cells (basal levels ranging from 1.18 to 1.73 U CAT/mg of protein). **d** SOD activities in treated cells (basal levels ranging from 9.50 to 16.23 U SOD/mg of protein). **e** GPx activities in treated cells (basal levels ranging from 2.84 to 4 U GPx/mg of protein). **f** Glutathione-*S*-transferase activities in treated cells (basal levels ranging from 21.11 to 28.35 U GST/mg of protein). Data are presented as mean ± SD for four independent experiments carried out in quadruplicates ($n = 4$). $^{*}P < 0.05$; $^{**}P < 0.01$ (one-way analysis of variance)

Furthermore, they found a reduced sensitivity to the neurotoxins MPP^{+} and 6-OHDA in RA-differentiated cells, which was attributed to an increased activity of survival signals such as Akt pathway. Thus, with the lack of change in dopaminergic properties and decreased susceptibility to neurotoxins during RA differentiation, they claimed that undifferentiated SH-SY5Y cell might be a more appropriate model in experimental PD research (Cheung et al. 2009). These conclusions have been criticized (Luchtman and Song 2010), and contrast with our previous report, in which we found an increase in the expression of several neuronal markers and an increased sensitivity to 6-OHDA in RA-differentiated SH-SY5Y cells (Lopes et al. 2010). These contradictories results could be explained by the differences in differentiation protocols used, for instance, serum origin and amount (Dr. Raymond Chang, personal communication). The study of neurotoxicity induced by chemicals represents a major challenge due to the physiological and morphological complexity of nervous system (Radio and Mundy 2008; Bal-Price et al. 2010). Most of in vitro models uses cell lines derived from CNS tumors and do not exhibit the same phenotype as the progenitor cells (Banker and Goslin 1998). Moreover, most of the screening strategies use simple cell viability assays (such as MTT, sulforhodamine B, trypan blue exclusion, and others) as outcome of the studies. Using the differentiated SH-SY5Y cells, we found that sub-lethal doses of two compounds (III and IX) significantly decrease the neurites densities of treated cells. The term neurite refers to axons and dendrites extended by neuronal cell lines, and neurites densities are important morphological parameters in neuroscience studies (Carmeliet 2003). These results should be taken into account in futures neurotoxic/neuroprotective evaluations of organoselenides or other potential molecules.

The synthesis of different organoselenides, such as ebselen and $(PhSe)_2$, was based on the structure of the selenocysteine in the active site of GPx enzyme (Rotruck et al. 1973; Brigelius-Flohé 1999; Sarma and Mugesh 2005). Since they all present a peroxidase-like activity in vitro, is not surprising that incubation of cells with selected compounds leads to an increased in TRAP and reduced thiol levels, in combination with increased GPx activity. The increase in the cellular antioxidant defense in response to organoselenides treatment is the basis of the neuroprotection against 6-OHDA toxicity. 6-OHDA is an analog of DA with similar structural characteristics and affinity with DA transporter. Once inside the neuron, it accumulates in the

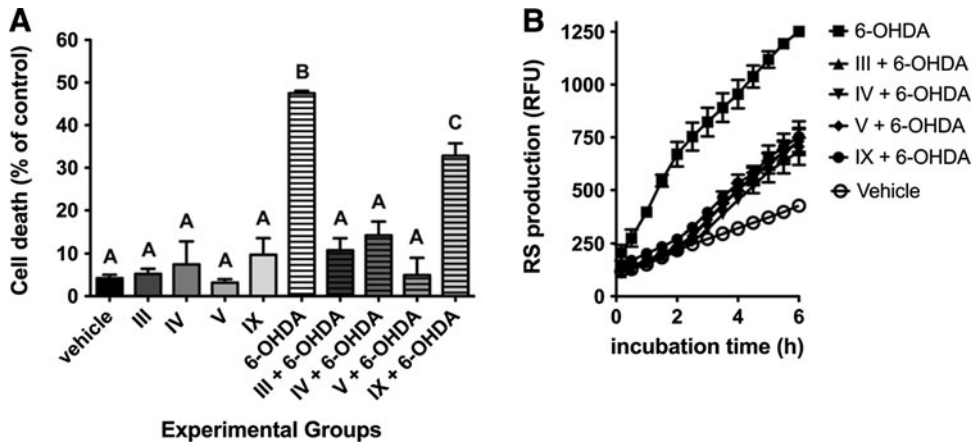

Fig. 6 Evaluation of neuroprotection of sub-lethal doses of compounds III, IV, V, and IX against 6-OHDA toxicity. SH-SY5Y cells were pre-incubated by 24 h with 10 μM of compound III, 3 μM of compound IV, 3 μM of compound V, 0.3 μM of compound IX or vehicle, washed with PBS, and further treated with 6-OHDA (LD_{50} = 15 μM) for 24 h. **a** Cell viability was determined by MTT assay, as described in "Experimental Procedures" section. **b** RS production was evaluated by DCF assay. Data are presented as mean ± SD of four independent experiments carried out in quadruplicates ($n = 4$). Statistical differences are expressed by *letters*. $P < 0.05$ (one-way analysis of variance)

cell causing cellular alterations, such as oxidative damage (Lehmensiek et al. 2006). This neurotoxin is a potent complex I inhibitor and may lead to the production of superoxide radicals and H_2O_2 (Glinka et al. 1997). 6-OHDA can suffer auto-oxidation and consequently can generate a massive RS production, such as quinones, hydrogen peroxide, superoxide, and hydroxyl radicals (Blandini et al. 2010; Bove and Perier 2011). Moreover, 6-OHDA leads to a reduction in endogenous cellular antioxidants activities such as SOD and GPx (Ahmad et al. 2011). The neuroprotection against 6-OHDA in differentiated SH-SY5Y cells should be related to a high radical scavenge capacity of the incorporated organoselenides. High peroxidase-like activity and thiol levels found in treated cells are important (and desired) features of organoselenides, since many lines of evidence indicate that the CNS posses modest antioxidant defenses and is highly susceptibility to oxidative damage (Halliwell 2006; Jaisin et al. 2011). These features of organoselenides suggest their use as therapeutic adjuvant for PD and demand futures investigations.

In conclusion, our data demonstrated that RA-differentiated human neuroblastoma SH-SY5Y cells treated with sub-lethal doses of both compounds IV and V present an increase in cellular antioxidant status without alteration of neurites densities and neuronal markers. Moreover, these treatments also confer a complete protection against 6-OHDA neurotoxicity and we can infer that these organoselenides are the most promising ones as potential drugs in the treatment of PD.

Acknowledgments This study was supported by the Brazilians funds FINEP/IBN-Net (01060842-00), PRONEX/FAPERGS (1000274), and MCT/CNPq INCT-TM (573671/2008-7). F.K. received a fellowship from MCT/CNPq (303613/2008-4).

References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126

Ahmad S, Khan MB, Hoda MN, Bhatia K, Haque R, Fazili IS, Jamal A, Khan JS, Katare DP (2011) Neuroprotective effect of sesame seed oil in 6-hydroxydopamine induced neurotoxicity in mice model: cellular, biochemical and neurochemical evidence. Neurochem Res. doi:10.1007/s11064-011-0638-4

Ardais AP, Viola GG, Costa MS, Nunes F, Behr GA, Klamt F, Moreira JC, Souza DO, Rocha JB, Porciúncula LO (2010) Acute treatment with diphenyl diselenide inhibits glutamate uptake into rat hippocampal slices and modifies glutamate transporters, SNAP-25, and GFAP immunocontent. Toxicol Sci 113:434–443

Arnér ES, Holmgre A (2000) Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem 267:6102–6109

Bal-Price AK, Hogberg HT, Buzanska L, Coecke S (2010) Relevance of in vitro neurotoxicity testing for regulatory requirements: challenges to be considered. Neurotoxicol Teratol 32:36–41

Banker G, Goslin K (1998) Type of neurvous cells, their advantages and limitations. The MIT Press, Cambridge

Barnham KJ, Masters CL, Bush AI (2004) Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov 3:205–214

Bhabak KP, Mugesh G (2007) Synthesis, characterization, and antioxidant activity of some ebselen analogues. Chemistry 13:4594–4601

Blandini F, Cova L, Armentero MT, Zennaro E, Levandis G, Bossolasco P, Calzarossa C, Mellone M, Giuseppe B, Deliliers GL, Polli E, Nappi G, Silani V (2010) Transplantation of undifferentiated human mesenchymal stem cells protects against 6-hydroxydopamine neurotoxicity in the rat. Cell Transplant 19:203–217

Bové J, Perier C (2011) Neurotoxin-based models of Parkinson's disease. Neuroscience. doi:10.1016/j.neuroscience.2011.10.057

Brigelius-Flohé R (1999) Tissue-specific functions of individual glutathione peroxidases. Free Radic Biol Med 27:951–965

Carmeliet P (2003) Blood vessels and nerves: common signals, pathways and diseases. Nat Rev Genet 4:710–720

Cheung YT, Lau WK, Yu MS, Lai CS, Yeung SC, So KF, Chang RC (2009) Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology 30:127–135

Drechsel DA, Patel M (2008) Role of reactive oxygen species in the neurotoxicity of environmental agents implicated in Parkinson's disease. Free Radic Biol Med 44:1873–1886

Dresch MT, Rossato SB, Kappel VD, Biegelmeyer R, Hoff ML, Mayorga P, Zuanazzi JA, Henriques AT, Moreira JC (2009) Optimization and validation of an alternative method to evaluate total reactive antioxidant potential. Anal Biochem 385:107–114

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77

Farina M, Soares FA, Zeni G, Souza DO, Rocha JB (2004) Additive pro-oxidative effects of methylmercury and ebselen in liver from suckling rat pups. Toxicol Lett 146:227–235

Flohe L, Günzler WA, Schock HH (1973) Glutathione peroxidase: a selenoenzyme. FEBS Lett 32:132–134

Floyd RA, Hensley K (2002) Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases. Neurobiol Aging 23:795–807

Geoghegan M, McAuley D, Eaton S, Powell-Tuck J (2006) Selenium in critical illness. Curr Opin Crit Care 12:136–141

Glinka Y, Gassen M, Youdim MBH (1997) Mechanism of 6-hydroxydopamine neurotoxicity. J Neural Transm 104:55–66

Gutteridge JM, Halliwell B (2010) Antioxidants: molecules, medicines, and myths. Biochem Biophys Res Commun 393:561–564

Halliwell B (1992) Reactive oxygen species and the central nervous system. J Neurochem 59:1609–1623

Halliwell B (2001) Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 18:685–716

Halliwell B (2006) Oxidative stress and neurodegeneration: where are we now? J Neurochem 97:1634–1658

Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 142:231–255

Ibrahim M, Prigol M, Hassan W, Nogueira CW, Rocha JB (2010) Protective effect of binaphthyl diselenide, a synthetic organoselenium compound, on 2-nitropropane-induced hepatotoxicity in rats. Cell Biochem Funct 28:258–265

Ibrahim M, Luchese C, Pinton S, Roman SS, Hassan W, Nogueira CW, Rocha JB (2011) Involvement of catalase in the protective effect of binaphthyl diselenide against renal damage induced by glycerol. Exp Toxicol Pathol 63:331–335

Ilijic E, Guzman JN, Surmeier DJ (2011) The L-type channel antagonist isradipine is neuroprotective in a mouse model of Parkinson's disease. Neurobiol Dis 43:364–371

Jaisin Y, Thampithak A, Meesarapee B, Ratanachamnong P, Suksamrarn A, Phivthong-Ngam L, Phumala-Morales N, Chongthammakun S, Govitrapong P, Sanvarinda Y (2011) Curcumin I protects the dopaminergic cell line SH-SY5Y from 6-hydroxydopamine-induced neurotoxicity through attenuation of p53-mediated apoptosis. Neurosci Lett 489:192–196

Kalayci M, Coskun O, Cagavi F, Kanter M, Armutcu F, Gul S, Acikgoz B (2005) Neuroprotective effects of ebselen on experimental spinal cord injury in rats. Neurochem Res 30:403–410

Lehmensiek V, Tan EM, Liebau S, Lenk T, Zettlmeisl H, Schwarz J, Storch A (2006) Dopamine transporter-mediated cytotoxicity of 6-hydroxydopamine in vitro depends on expression of mutant a-synucleins related to Parkinson's disease. Neurochem Int 48:329–340

Lissi E, Salim-Hanna M, Pascual C, del Castillo MD (1995) Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. Free Radic Biol Med 18:153–158

Lopes FM, Schröder R, da Frota ML Jr, Zanotto-Filho A, Müller CB, Pires AS, Meurer RT, Colpo GD, Gelain DP, Kapczinski F, Moreira JC, Fernandes MdaC, Klamt F (2010) Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain Res 1337:85–94

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Luchese C, Zeni G, Rocha JB, Nogueira CW, Santos FW (2007) Cadmium inhibits delta-aminolevulinate dehydratase from rat lung in vitro: interaction with chelating and antioxidant agents. Chem Biol Interact 165:127–137

Luchtman DW, Song C (2010) Why SH-SY5Y cells should be differentiated. Neurotoxicology 31:164–165

Mandel S, Grunblatt E, Riederer P, Gerlach M, Levites Y, Youdim MB (2003) Neuroprotective strategies in Parkinson's disease: an update on progress. CNS Drugs 17:729–762

McCarthy S, Somayajulu M, Sikorska M, Borowy-Borowski H, Pandey S (2004) Paraquat induces oxidative stress and neuronal cell death; neuroprotection by water-solubleCoenzyme Q10. Toxicol Appl Phamacol 201:21–31

Meissner WG, Frasier M, Gasser T, Goetz CG, Lozano A, Piccini P, Obeso JA, Rascol O, Schapira A, Voon V, Weiner DM, Tison F, Bezard E (2011) Priorities in Parkinson's disease research. Nat Rev Drug Discov 10:377–393

Meotti FC, Borges VC, Zeni G, Rocha JB, Nogueira CW (2003) Potential renal and hepatic toxicity of diphenyl diselenide, diphenyl ditelluride and ebselen for rats and mice. Toxicol Lett 143:9–16

Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247:3170–3175

Mugesh G, Panda A, Singh HB, Punekar NS, Butcher RJ (2001) Glutathione peroxidase-like antioxidant activity of diaryl diselenides: a mechanistic study. J Am Chem Soc 123:839–850

Müller A, Cadenas E, Graf P, Sies H (1984) A novel biologically active seleno-organic compound—I. Glutathione peroxidase-like activity in vitro and antioxidant capacity of PZ 51 (ebselen). Biochem Pharmacol 33:3235–3239

Naoi M, Maruyama W (2010) Monoamine oxidase inhibitors as neuroprotective agents in age-dependent neurodegenerative disorders. Curr Pharm Des 16:2799–2817

Ozdemir I, Talas ZS, Gok Y, Ates B, Yilmaz I (2010) Changes in tyrosine hydroxylase activity, adrenomedullin and total RNA levels by treatment of organoselenium compounds in rat hypothalamus exposed to DMBA. Regul Pept 159:137–141

Pabst MJ, Habig WH, Jakoby WB (1974) Glutathione S-transferase. A novel kinetic mechanism in which the major reaction pathway depends on substrate concentration. J Biol Chem 249:140–147

Pahlman S, Rusala AI, Abrahamsson L, Mattsson ME, Esscher T (1984) Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. Cell Differ 14:135–144

Pedersen V, Schmidt WJ (2000) The neuroprotectant properties of glutamate antagonists and antiglutamatergic drugs. Neurotoxic Res 2:179–204

Porciúncula LO, Rocha JBT, Cimarosti H, Vinadé L, Ghisleni G, Salbego CG, Souza DO (2003) Neuroprotective effect of ebselen on rat hippocampal slices submitted to oxygen-glucose deprivation: correlation with immunocontent of inducible nitric oxide synthase. Neurosci Lett 346:101–104

Posser T, Moretto MB, Dafre AL, Farina M, da Rocha JB, Nogueira CW, Zeni G, Ferreira J, Dos S, Leal RB, Franco JL (2006) Antioxidant effect of diphenyl diselenide against sodium nitroprusside (SNP) induced lipid peroxidation in human platelets and erythrocyte membranes: an in vitro evaluation. Chem Biol Interact 164:126–135

Posser T, Franco JL, dos Santos DA, Rigon AP, Farina M, Dafré AL, Teixeira Rocha JB, Leal RB (2008) Diphenyl diselenide confers neuroprotection against hydrogen peroxide toxicity in hippocampal slices. Brain Res 1199:138–147

Posser T, de Paula MT, Franco JL, Leal RB, da Rocha JB (2011) Diphenyl diselenide induces apoptotic cell death and modulates ERK1/2 phosphorylation in human neuroblastoma SH-SY5Y-cells. Arch Toxicol 85:645–651

Radio NM, Mundy WR (2008) Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. Neurotoxicology 29:106–118

Rossato JI, Ketzer LA, Centurião FB, Silva SJ, Lüdtke DS, Zeni G, Braga AL, Rubin MA, Rocha JB (2002) Antioxidant properties of new chalcogenides against lipid peroxidation in rat brain. Neurochem Res 27:297–303

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science 179:588–590

Safi R, Gardaneh M, Panahi Y, Maghsoudi N, Zaefizadeh M, Gharib E (2011) Optimized quantities of GDNF overexpressed by engineered astrocytes are critical for protection of neuroblastoma cells against 6-OHDA toxicity. J Mol Neurosci. doi: 10.1007/s12031-011-9654-8

Santos DB, Schiar VP, Paixão MW, Meinerz DF, Nogueira CW, Aschner M, Rocha JB, Barbosa NB (2009) Hemolytic and genotoxic evaluation of organochalcogens in human blood cells in vitro. Toxicol In Vitro 23:1195–1204

Sarma BK, Mugesh G (2005) Glutathione peroxidase (GPx)-like antioxidant activity of the organoselenium drug ebselen: unexpected complications with thiol exchange reactions. J Am Chem Soc 127:11477–11745

Schapira AH (2008) Mitochondrial dysfunction in neurodegenerative disease. Neurochem Res 12:2502–2509

Schewe T (1995) Molecular actions of ebselen—an antiinflammatory antioxidant. Gen Pharmacol 26:1153–1169

Shobha SR, Hofmann LA, Shakil A (2006) Parkinson's disease: diagnosis and treatment. Am Fam Physician 74:2046–2054

Sies H (1993) Ebselen, a selenoorganic compound as glutathione peroxidase mimic. Free Radic Biol Med 14:313–323

Souza AC, Stangherlin EC, Ardais AP, Nogueira CW (2010) Diphenyl diselenide and diphenyl ditelluride: neurotoxic effect in brain of young rats, in vitro. Mol Cell Biochem 340:179–205

Spencer JP, Jenner P, Daniel SE, Lees AJ, Marsden DC, Halliwell B (1998) Conjugates of catecholamines with cysteine and GSH in Parkinson's disease: possible mechanisms of formation involving reactive oxygen species. J Neurochem 71:2112–2122

Stefanis L, Keller JN (2006) The proteasome in neurodegeneration. Springer, New York

Stone JR, Yang S (2006) Hydrogen peroxide: a signaling messenger. Antioxid Redox Signal 8:243–270

Talas ZS, Ozdemir I, Yilmaz I, Gok Y, Orun I (2008) The investigation of the antioxidative properties of the novel synthetic organoselenium compounds in some rat tissues. Exp Biol Med 233:575–579

Wang H, Joseph JA (1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 27:612–616

Wendel A (1981) Glutathione peroxidase. Methods Enzymol 77:325–333

Xie HR, Hu LS, Li GY (2010) SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. Chin Med J (Engl) 123:1086–1092

Xu JH, Hu HT, Liu Y, Qian YH, Liu ZH, Tan QR, Zhang ZJ (2006) Neuroprotective effects of ebselen are associated with the regulation of Bcl-2 and Bax proteins in cultured mouse cortical neurons. Neurosci Lett 399:210–214

Yamaguchi T, Sano K, Takakura K, Saito I, Shinohara Y, Asano T, Yasuhara H (1998) Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Ebselen Study Group. Stroke 29:12–17

Zeevalk GD, Bernard LP, Song C, Gluck M, Ehrhart J (2005) Mitochondrial inhibition and oxidative stress: reciprocating players in neurodegeneration. Antioxid Redox Signal 7:1117–1139