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Neuroprotective Effect of Isobenzofuranones on Hydrogen Peroxide-Mediated Redox Imbalance in Primary Cultures of Hippocampal Neurons

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HIGHLIGHTS

- Primary neuronal cultures as a model on studies of cytotoxicity.
- Isobenzofuranones described neuroprotective activity.
- Isobenzofuranones control reactive oxygen species in cultured neurons.

Abstract: In live organisms, there is a balance between the production of reactive oxygen species (ROS) and their neutralization. The increased level of these species leads to a condition called redox imbalance. The aim of this study was to evaluate the protective action of isobenzofuranones in primary cultures of hippocampal neurons subjected to redox imbalance. To accomplish this, MTT and LIVE/DEAD assays were initially performed. In the cultures pretreated with isobenzofuranones 1 and 2, there was a higher number of live cells when compared to that in the untreated ones. Regarding redox imbalance, there was a significant increase in the intracellular levels of ROS. The cultures pretreated with isobenzofuranones showed a reduction in ROS levels. Lipid peroxidation caused by oxidative damage was significantly reduced in the cultures pretreated with isobenzofuranones 1 and 2. Taken together, these data show the ability of isobenzofuranones 1 and 2 to significantly minimize cytotoxicity, cell death, intracellular levels of ROS and lipid peroxidation induced by redox imbalance. These results suggest that isobenzofuranones 1 and 2 represent a possible alternative therapy for the neurodegenerative disturbances that are triggered by ROS production increases.

Keywords: reactive oxygen species, isobenzofuranones, phthalide, hippocampal neurons, redox imbalance.

INTRODUCTION

Under physiological conditions, there is a balance between the levels of reactive oxygen species (ROS) and their neutralization by antioxidant systems. When this balance is lost, there is an increase in ROS in the organism. The increased level of these species leads to a condition called redox imbalance [1], which can result in irreversible oxidative damage to proteins, lipids and DNA [2-5]. In the literature, there is evidence that redox imbalance is linked to the pathogenesis of several neurodegenerative diseases, such as Alzheimer´s and Parkinson´s diseases [6,7], ischaemic brain damage [8], and amyotrophic lateral sclerosis [9]. The human body has its own natural antioxidant defence system to protect it from the harmful effects of ROS [10]. Synthetic antioxidant compounds act by strengthening and/or helping the natural defence system of the body [11-13]. Isobenzofuranones, also known as phthalides, are chemical compounds with a structure involving a benzene ring fused to a *y*-lactone ring [14]. These substances have attracted the attention of several researchers due, in part, to the range of their biological effects, including antioxidant activity [15]. In China, an isobenzofuranone derivative, 1-3-n-butylphthalide (NBP), was originally extracted as a pure component of *Apium graveolens* Linn seeds and has been synthesized and developed as a neuroprotective agent for cerebral ischaemia [16,17]. In addition, studies on 3-butyl-6-bromo-1(3H)-isobenzofuranone (Br-NBP) have demonstrated a reduction in the volume of cerebral infarction and the amelioration of neurological behavioural deficits in rats with transient middle cerebral artery occlusion [18]. These compounds can be of synthetic or natural origins, which makes them part of a growing group of substances with therapeutic properties that have been used in the search for new medicines [19-21]. Based on the importance of the effects of antioxidant on redox imbalance and considering the importance of this process in the progression of neurodegenerative diseases, the present study aimed to evaluate the protective action of isobenzofuranones in primary cultures of hippocampal neurons exposed to redox imbalance. Within this context, the compounds 3-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-enyl) isobenzofuran-1(3*H*)-one (isobenzofuranone **1**) and 2-(-oxo-1,3-dihydroisobenzofuran-1-il)1*H*-indene-1,3(2*H*)-dione (isobenzofuranone **2**) (scheme 1) were evaluated. Previous *in silico* analyses have revealed that these isobenzofuranones exhibit physicochemical and pharmaceutical properties, which allows their *in vitro* activity to be evaluated [22]. To evaluate isobenzofuranones **1** and **2**, primary cultures of hippocampal neurons were used, as these cells are more susceptible to redox imbalance and are robustly involved in memory and learning processes, which are directly affected by neurodegenerative diseases [23,24].

MATERIAL AND METHODS

SYNTHESIS OF COMPOUNDS 1 AND 2

Isobenzofuranones **1** and **2** (Figure 1) were synthesized as previously described [22]. Briefly, condensation reactions, which were promoted by DBU, between phthalaldehydic acid and the appropriate diketones produced isobenzofuranones **1** and **2**. These isobenzofuranones were purified by silica-gel column chromatography and fully characterized by spectroscopy (IR and NMR) and mass spectrometry.

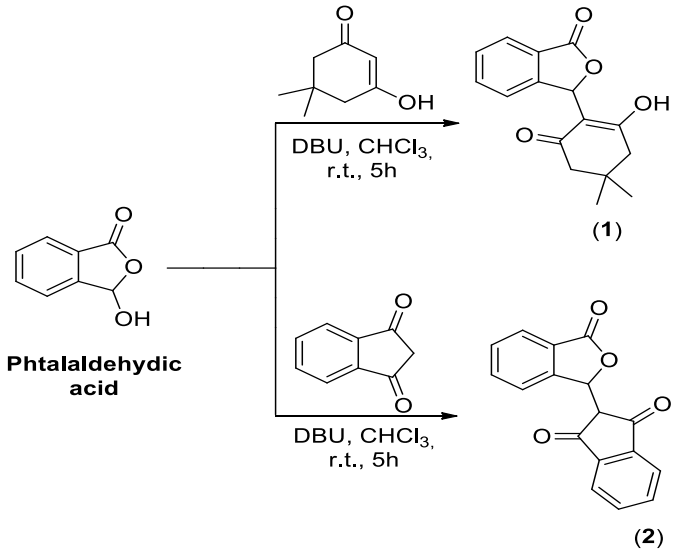

Figure 1. Synthesis of isobenzofuranones **1** and **2**.

PRIMARY HIPPOCAMPAL CELL CULTURE

Male and female C57BL/6 mice were kept in the Animal Science Center of the Federal University of Ouro Preto. All procedures performed on the animals were approved by the Animal Ethics Committee (protocol 45/2015). Primary cultures of hippocampal neurons were obtained from mouse embryos as described previously [25,26]. Briefly, brains were dissected from embryonic day 17 (E17) embryos. We considered embryos to be at E1 when the dam was found to be sperm-positive. The hippocampi were separated from the embryonic brains and placed in cold Hanks' balanced salt solution (Thermo Scientific) in a sterile microtube. The hippocampi were first incubated with trypsin (0.25%; Sigma-Aldrich) for 20 minutes at 37°C. After that, the trypsin was neutralized with plating medium (Eagle's minimum essential medium (Thermo Scientific) containing 10% FBS (Invitrogen) and antibiotics (100 mg/mL streptomycin and 100 units/mL penicillin). Afterwards, the hippocampi were dissociated mechanically in plating medium. The cells were then plated at a density of 10^5 or 10^6 per well on dishes pre-coated with 10 mg/mL poly-L-lysine (Sigma) and kept at 37°C and 5% CO_2. The plating medium was replaced by maintenance medium (neurobasal medium, B-27, L-glutamine (Thermo Scientific), 100 mg/mL streptomycin and 100 units/mL penicillin) 4 h after the cells were plated. The experiments were carried out 6 to 10 days after the neurons were seeded onto the dishes at an appropriate density according to each experimental scale.

Treatment of the primary hippocampal neurons

For each evaluated compound (isobenzofuranones **1** and **2**, Figure 1), a 2 mM stock solution in DMSO (20%) was prepared. The stock solutions were diluted to obtain concentrations of 50, 100 and 150 µM. To control for the cytotoxicity of DMSO, its final concentration was kept at 0.05% for all treatments. The neurons were seeded onto 96-well dishes coated with poly-L-lysine (1.0×10^5 cells/well). After 8 days, the cells were treated with 50, 100 and 150 µM of isobenzofuranone and incubated for 2 h in a humidified chamber at 37°C with 5% CO_2. After the treatment with isobenzofuranones **1** and **2**, redox imbalance was induced for 3 h with hydrogen peroxide (100 µM) [27,28].

Evaluation of cytotoxicity

The metabolic activity of hippocampal neurons was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich) assay (adapted from Sozio and coauthors [11]). Cell viability was based on the ability of viable cells to reduce MTT to the coloured formazan salt. The neurons were incubated for 2 h with isobenzofuranones **1** and **2** at concentrations of 50, 100 and 150 µM in 96-well dishes coated with poly-L-lysine (1.0×10^5 cells/well). After that, redox imbalance was induced with 100 µM of hydrogen peroxide (H_2O_2) for 3 h. Then, the medium was replaced with 0.5 mg/mL MTT, and the dish was incubated for 4 h at 37 °C. After this time, the dish was incubated with 10% SDS solution for 16 h at 37 °C to solubilize the formazan salt. Finally, the absorbance was measured at 570 nm with a VICTOR microplate reader (Multidetector VICTOR™ X3, Perkin Elmer).

Cell viability

For cell viability, the LIVE/DEAD (Thermo Scientific) assay was used (adapted from Beraldo and coauthors [29]). The LIVE/DEAD assay is a cell viability assay that uses two spectra of fluorescence and simultaneously identifies live and dead cells with calcein-AM and ethidium-1 (EthD-1) homodimers. Calcein-AM can permeate the cell membrane; however, after being cleaved by esterase, which is present in the cytoplasm of live cells, it is no longer capable of passing through the membrane and it remains stuck in the cell, where it produces an intense and uniform green fluorescence. Fluorescence was observed at 495 and 635 nm. Mouse primary hippocampal neurons were seeded onto poly-L-lysine-coated 4-well dishes at a density of 1.0×10^6 cells/well and cultured for 10 days. On day 11, the neurons were treated for 2 h with isobenzofuranones **1** and **2** and then incubated with H_2O_2 (100 µM) for 3 h. Afterwards, 20 µL of the solution prepared from the LIVE/DEAD kit was added to each well for 45 minutes. Live and dead cells were counted based on fluorescence images taken with a Zeiss LSM780 confocal microscope using a 20X/0.5 objective and a wavelength of 488 nm. The cell death rate was calculated as the percentage of dead cells relative to the total amount of cells.

ROS intracellular measurement

Intracellular ROS production was assessed by a fluorescence assay that employed 2,7-dichlorofluorescein diacetate (H2DCFDA). The methodology was adapted from a work previously reported by Halliwell and Whiteman30. H2DCFDA readily diffuses into cells, and intracellular esterases promote the cleavage of

the acetate groups to produce H2DCF, which remains inside the cells. Intracellular ROS oxidize H2DCF to form the highly fluorescent 2,7-dichlorofluorescein (2,7-DCF). After treatment with isobenzofuranones 1 and 2 and the induction of redox imbalance, the medium was replaced by an H2DCFDA (2 μM) solution for 45 minutes. After that, the wells were washed three times with colourless calcium (Ca^{2+}) and magnesium (Mg^{2+}) free Hanks' balanced salt solution. Fluorescence was monitored every 30 minutes for 3 h after the addition of 100 μM H_2O_2 using a VICTOR (multidetector VICTOR™ X3, PerkinElmer) microplate reader at wavelengths of 495 and 530 nm.

Lipid peroxidation

To analyse the membrane damage caused by redox imbalance induction, we adapted the protocol reported by Angelova and collaborators[31]. Mouse primary hippocampal neurons were seeded onto poly-L-lysine-coated 96-well dishes at a density of 1.0 x 10^5 cells/well and cultured for 6 days. On day 7, the cells were treated with isobenzofuranones **1** and **2** for 2 h. After this time, the medium was replaced by a solution containing $BODIPY_{581/591}$ C11 (2 μM) and incubated for 30 minutes. Then, the wells were washed three times with colourless Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution. Before reading the results, redox imbalance was induced by the addition of 100 μM H_2O_2. Readings were performed every 30 minutes for 3 h, with the first reading at time 0', using a VICTOR (multidetector VICTOR™ X3, PerkinElmer) microplate reader at wavelengths of 495 and 530 nm.

STATISTICAL ANALYSIS

The results were analyzed by analysis of variance (ANOVA) followed by Tukey's test. The differences were considered significant when the p-values were ≤0.01. The numeric values are presented as the average ±standard error of the mean (SEM).

RESULTS AND DISCUSSION

Cytotoxicity and cell viability

The cytotoxicity of isobenzofuranones **1** and **2** was evaluated by analyses of hippocampal neuron metabolism in response to isobenzofuranones and the induction of redox imbalance separately and in combination. As shown in Figure 1, there was a significant reduction in the metabolism of the untreated cultures compared to the cultures pretreated with isobenzofuranones **1** and **2** (Figure 1).

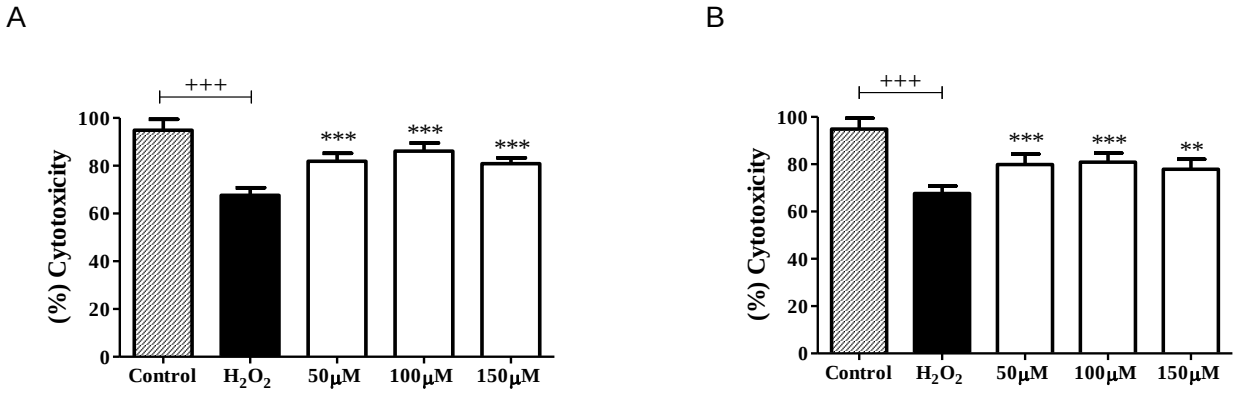

Figure 1. Cells were pretreated with isobenzofuranones **1** (A) and **2** (B) at concentrations of 50 μM, 100 μM and 150 μM for 2 h. Next, 100 μM H_2O_2 was, added for 3 h. The data represent the mean ± SEM. (+++) $p < 0.001$ between the control group and the H_2O_2-untreated group. (***) $p < 0.001$ and (**) $p < 0.01$ between the H_2O_2-untreated group and the H_2O_2-treated groups.

To confirm the neuroprotective effect of isobenzofuranones **1** and **2** on the cell death induced by H_2O_2, the LIVE/DEAD assay was carried out. In the cultures pretreated with isobenzofuranones **1** and **2,** there were a greater number of live cells compared to that in the untreated cultures (Figure 2 and SM1).

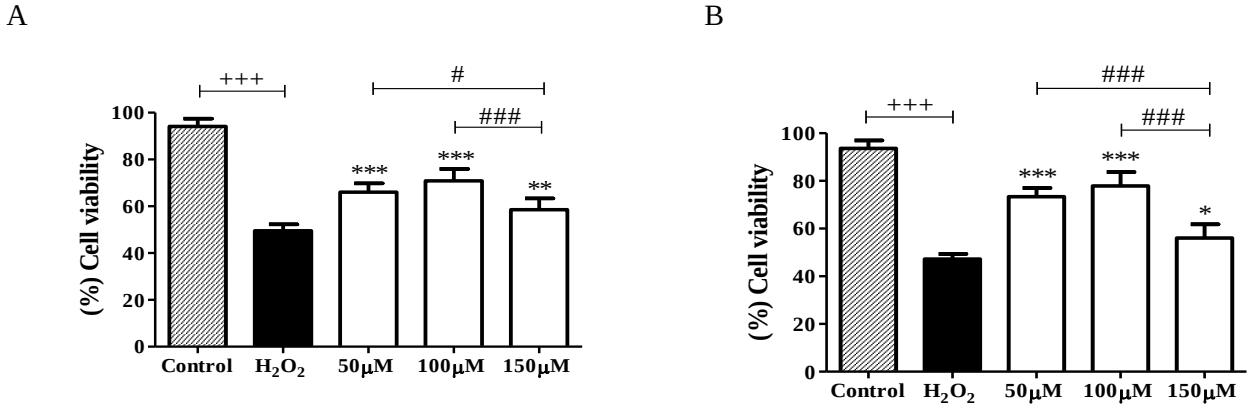

Figure 2. Cells were pretreated with isobenzofuranones **1** (A) and **2** (B) at concentrations of 50 μM, 100 μM and 150 μM for 2 h. Next, 100 μM H_2O_2 was added for 3 h. The graphics represent the percentage of live cells from the captured images. The data represent the mean ± SEM. (+++) $p < 0.001$ compared to the control group and the H_2O_2-untreated group. (***) $p < 0.001$, (**) $p < 0.01$ and (*) $p < 0.05$ between the H_2O_2-untreated group and the H_2O_2-treated group. (###) $p < 0.001$ (#) $p < 0.05$ between the groups treated with different concentrations.

Although isobenzofuranones attenuated the decrease in cell metabolism and, consequently, the neuronal death induced by redox imbalance, previous studies have demonstrated the cytotoxic effects of isobenzofuranones in the U937 (lymphoma) and K562 (myeloid leukaemia) cell lines[22] as well as in the HL-60 leukaemia, SF295 glioblastoma and MDA-MB435 melanoma cancer cell lines [32]. In the present investigation, it was shown that isobenzofuranones **1** and **2** were not toxic to cells at low concentrations. This can be explained by the hormesis effect, which describes a biphasic effect of isobenzofuranones characterized by a beneficial effect at lower concentrations and a cytotoxic effect at higher doses.

ROS Intracellular Measurement

Hydrogen peroxide is an endogenous source of hydroxyl free radicals that contributes to the background level of cellular oxidative stress [33]. After the induction of redox imbalance with H_2O_2, there was a significant increase in intracellular ROS levels during the 3 h of analysis. (Figure 3 and SM 2). However, in the primary cell cultures pretreated with isobenzofuranones, a significant reduction in ROS levels was observed (Figure 3 and SM 2). These results corroborate the findings of Xu and Zao [34], who reported that PC12 cells treated with the compound 3-butyl-6-fluoro-1(3H)-isobenzofuranone and subjected to redox imbalance showed a reduction in ROS levels. Strobel and coauthors [35] observed that isobenzofuranones extracted from the fungus *Pestalotiopsis microspora* were able to reduce the hydroxyl radical (OH·) levels that were chemically induced by hydrogen peroxide in the samples tested. Thus, considering that, in the present investigation, redox imbalance was induced by H_2O_2, it is plausible that the neuroprotective effect of isobenzofuranones **1** and **2** is related to their ability to scavenge free radical species.

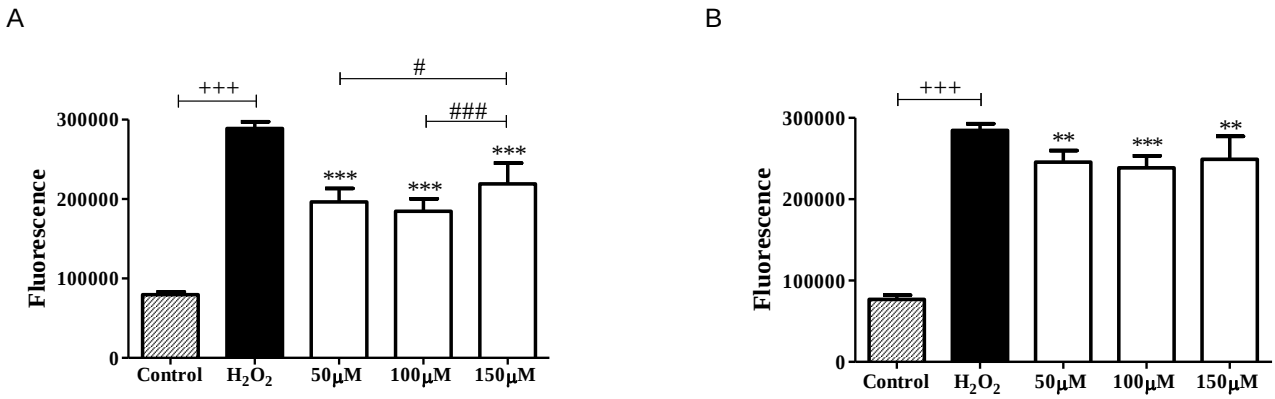

Figure 3. Cells were pretreated with isobenzofuranones **1** (A) and **2** (B) at concentrations of 50 μM, 100 μM and 150 μM for 2 h. Next, 100 μM of H_2O_2 was added for 3 h. Immediately afterwards, the fluorescence of 2,7-dichlorofluorescein (H_2DCFDA) was measured. The data represent the mean ± SEM. (+++) $p < 0.001$ between the control group and the H_2O_2-untreated group. (***) $p < 0.001$ and (**) $p < 0.01$ between the H_2O_2-untreated group and the H_2O_2-treated group. (###) $p < 0.001$ and (#) $p < 0.05$ between the groups treated with different concentrations.

Lipid Peroxidation

Lipid peroxidation caused by oxidative damage was significantly reduced in the cultures pretreated with isobenzofuranones **1** and **2** at 50 µM, 100 µM and 150 µM (Figure 4). It was also possible to observe a significant difference between the groups treated with compound **2** at concentrations of 100 µM and 150 µM (Figure 4B). The same did not occur with the groups treated with compound **1** (Figure 4A).

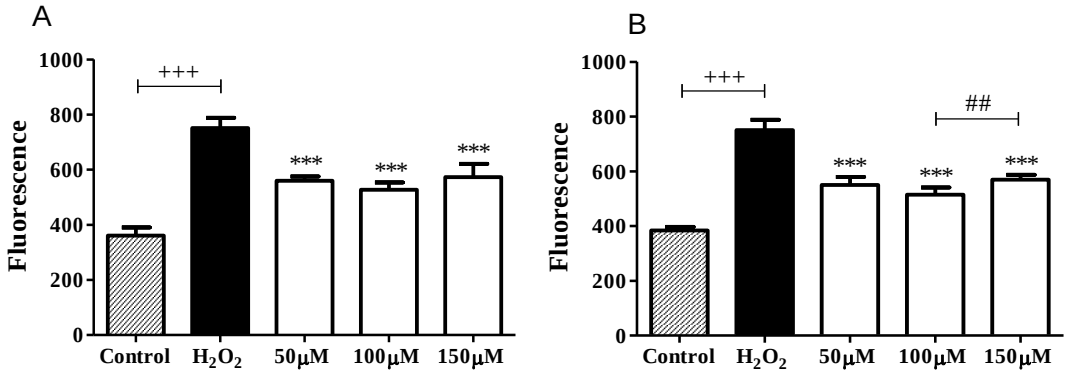

Figure 4. Cells were pretreated with isobenzofuranones **1** (A) and **2** (B) at concentrations of 50 µM, 100 µM and 150 µM for 2 h. Next, 100 µM H_2O_2 was added for 3 h. Then, the spectral emission levels of $BODIPY_{581/591}$ C11 were quantified. The data represent the mean ± SEM. (+++) $p < 0.001$ between the control group and the group H_2O_2-untreated group. (***) $p < 0.001$ between the H_2O_2-untreated group and the H_2O_2-treated groups. (##) $p < 0.01$ between the groups treated with different concentrations.

Similar results to those achieved when PC12 cells were treated with 3-butyl-6-fluoro-1(3H)-isobenzofuranone and 3-butyl-6-bromo-1(3H)-isobenzofuranone [30,34] were observed. Taken together, these data suggest that, because their concentrations can be increased without causing cytotoxicity, compounds 1 and 2 are able to decrease lipid peroxidation caused by ROS and act in a more efficient way to protect against membrane damage during redox imbalance in primary cultures of neurons.

CONCLUSION

The present study showed the ability of isobenzofuranones **1** and **2** to significantly decrease cytotoxicity, cell death, intracellular ROS levels and lipid peroxidation induced by redox imbalance. The findings reported herein are significant since this is the first study to show the protective activity of isobenzofuranones on redox imbalance induced in primary cultures of mouse hippocampal neurons. In addition, the performance of these compounds under physiological conditions was described to improve our understanding. Taken together, the data suggest that isobenzofuranones **1** and **2** represent possible alternative therapies for neurodegenerative disturbances that are triggered by ROS production increases, but further study is required to confirm this assumption.

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Conflicts of Interest: The authors declare no conflict of interest.

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