



Article

Discovery of New Antioxidant Molecules Enhancing the Nrf2-Mediated Pathway: Docking Studies and Biological Evaluation

Simona De Vita ¹, Elena González-Burgos ², Stefania Terracciano ¹, Maria Giovanna Chini ³, María Pilar Gómez-Serranillos ^{2,*} and Giuseppe Bifulco ^{1,*}

¹ Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 134, 84084 Fisciano, SA, Italy; sdevita@unisa.it (S.D.V.); sterracciano@unisa.it (S.T.)

² Department of Pharmacology, Pharmacognosy and Botanical, Faculty of Pharmacy, Universidad Complutense de Madrid, 28040 Madrid, Spain; elenagon@ucm.es

³ Department of Biosciences and Territory, University of Molise, Contrada Fonte Lappone, 86090 Pesche, IS, Italy; mariagiovanna.chini@unimol.it

* Correspondence: pserra@ucm.es (M.P.G.-S.); bifulco@unisa.it (G.B.); Tel.: +34-91-394-1767 (M.P.G.-S.); +39-089-969741 (G.B.)

Abstract

Oxidative stress has been reported to be implicated in the pathogenesis of many neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases. Enhancing antioxidant response, through the activation of the transcription factor Nrf2, may represent a potential strategy, based on in vitro models. To identify scaffolds potentially able to modulate the Nrf2-Keap1 interaction, docking experiments were carried out using a library of commercially available and in-house synthesized molecules. Compounds 1–4 were selected, and their direct and indirect antioxidant activity was evaluated in an acute oxidative stress model induced by Fenton's reaction in the human neuroblastoma SH-SY5Y cell line. Results showed that these compounds exerted the most pronounced protective effect under the tested conditions at the following concentrations: 10 μ M for 1, 25 μ M for 2, 10 μ M for 3, and 5 μ M for 4. Moreover, these molecules notably decreased intracellular ROS production and lipid peroxidation by-products and increased the GSH/GSSG ratio. Furthermore, these molecules promoted the protein expression of antioxidant enzymes downstream of the Nrf2 transcriptional pathway. Interestingly, compound 3 resulted in being the most active among the four.

Keywords: antioxidant; oxidative stress; Nrf2 activation; virtual screening; cytoprotection; SH-SY5Y



Academic Editor: Daniele Lana

Received: 29 December 2025

Revised: 9 February 2026

Accepted: 12 February 2026

Published: 15 February 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and

conditions of the [Creative Commons](#)

[Attribution \(CC BY\)](#) license.

1. Introduction

Reactive Oxygen Species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) are by-products naturally produced by cells [1] or derived from external stimuli. The hydroxyl radicals are the most damaging free radicals; they abstract hydrogen atoms and generate organic radicals, leading to lipid peroxidation and modifications of DNA and proteins [2]. Cells use different mechanisms to keep ROS levels under control, including prevention, repair, antioxidant actions, and physical defenses [3]. When ROS are produced in excess, endogenous antioxidant systems are unable to neutralize them completely, causing damage to biomolecules, leading to major cell issues [3,4]. High cellular ROS levels have been evidenced to play a pivotal role in several common

pathological states such as Alzheimer's disease [3,5–7] and Parkinson's disease [5,8–11], cancer [3,8], and diabetes [3,12]. Although oxidative stress is not considered the sole triggering factor of those pathologies, the inflammatory state derived from the action of ROS species is obviously a condition that contributes to the progression of the disease, and vice versa, the inflammatory response amplifies the production of ROS [7,13–15]. Therefore, upregulating the cellular antioxidant systems could represent an important strategy to attenuate ROS damage [3,6,8,16,17].

The nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is a basic leucine zipper (bZIP) protein that, directly or indirectly, regulates the expression of several antioxidant enzymes involved in different defense mechanisms including ROS direct metabolism (catalase and superoxide dismutase enzymes), regeneration of antioxidant molecules such as glutathione (glutathione reductase and glutathione peroxidase, NADPH regeneration, heme and/or iron metabolism [4,18–21]. Usually, Nrf2 is bound to its inhibitor, the protein Keap1, through ETGE and DLG motifs on the Neh2 domain of Nrf2 (Figure 1), which keeps the transcription factor in the cytoplasm and promotes its ubiquitination and subsequent degradation by the proteasome [22–24].

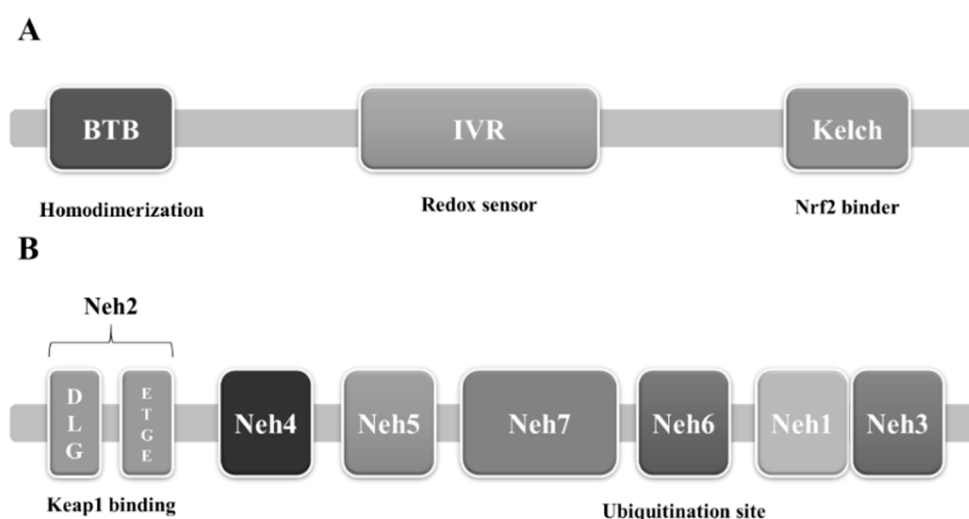


Figure 1. Domain structures of Keap1 (A) and Nrf2 (B).

Disrupting this binding prevents Nrf2 degradation, releases the transcription factor, and activates antioxidant gene expression. Therefore, promoting antioxidant response represents a promising strategy to ameliorate ROS levels in patients who suffer from any disorder related to oxidative stress [23,25,26].

In the last few years, many efforts have been made to discover new molecules capable of interfering with the binding between Nrf2 and Keap1 [27–32]. Such molecules are divided into two categories: cysteine-binding activators and Keap–Nrf2 protein–protein interactions (PPI) inhibitors [27,28]. The first group is based on molecules containing chemical structures that can bind cysteines on Keap1 (Cys273, Cys288, and Cys297), like dimethyl fumarate and chalcone derivatives, pyrazino[2,1-*a*]isoquinoline, 3-alkylamino-1H-indole acrylates, 2-hydroxybenzamide, and natural compounds (e.g., curcumin, vitamins, flavonoids) [27]. Owing to their ability to interact with generic thiols, these molecules may react with any cysteine present in proteins, potentially leading to off-target effects and context-dependent toxicity [27,29]. In light of these concerns regarding selectivity and long-term safety, disrupting the protein–protein interaction between Nrf2 and Keap1 represents an attractive and promising alternative strategy [30–32]. To do so, based on the recent crystallographic structures available, PPI disruptors should target the Kelch domain on Keap1 and, specifically, form hydrogen bonds or salt bridges with Ser363, Arg380,

Asn382, Arg415, Arg483, Ser508, and Ser555, and hydrophobic interactions with Tyr334, Tyr525, Tyr572, and Tyr577 [26,27] (Figure 2).

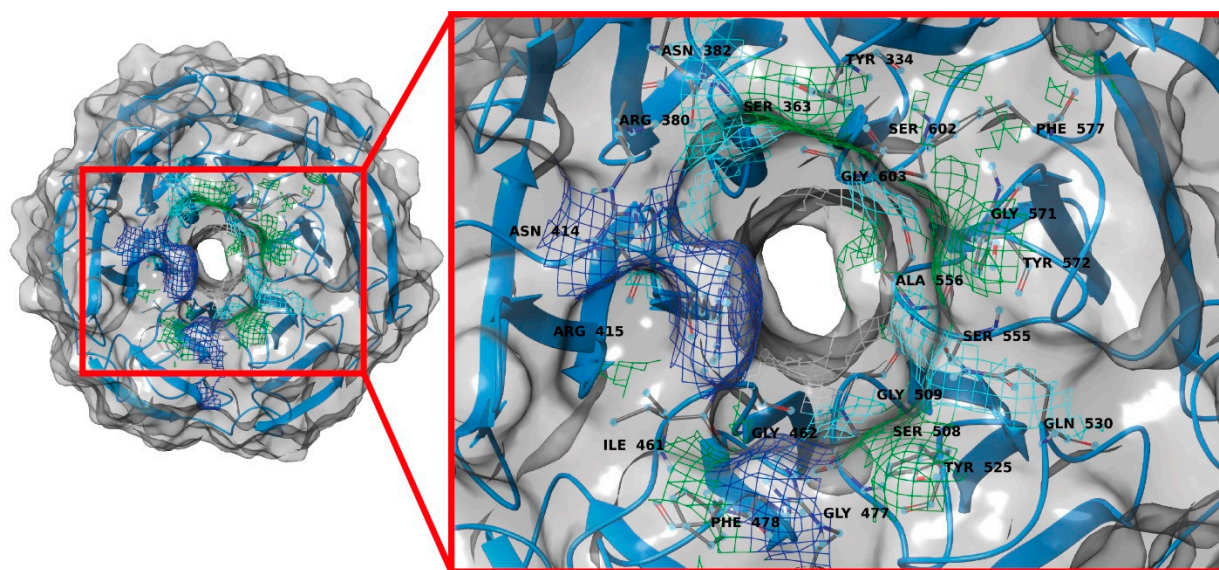


Figure 2. Key binding site residues of Keap1. A zoom-in view is reported in the red box.

Notably, a broad range of small-molecule Keap1-Nrf2 PPI inhibitors (peptide-based and nonpeptidic) has been reported to date, including compounds containing diverse chemical scaffolds like 1,4-diaminonaphthalenes, 1,4-diphenyl-1,2,3-triazoles, tetrahydroisoquinolines, indoline derivatives, pyrazolecarboxylic acids, phenylpropanoic acids, phenols, and organometallic complexes [31].

In this study, we present the outcomes of a combined computational and biological approach to identify new antioxidant lead compounds from a small library of commercially available and in-house compounds. The primary aim of this work was to search for small molecules capable of interfering with the protein–protein interaction (PPI) between the transcription factor Nrf2 and its negative regulator Keap1. By targeting this interaction, the selected compounds were intended to promote Nrf2 activation and downstream antioxidant responses without involving cysteine binding. The identified candidates were subsequently evaluated in cellular models of acute oxidative stress to assess their ability to mitigate oxidative damage, a pathological process frequently implicated in neurodegenerative disorders such as Alzheimer’s and Parkinson’s diseases.

2. Results

2.1. Molecular Docking

To obtain a general idea about putative new scaffolds that could act as PPI inhibitors of the Keap1-Nrf2 binding, we performed preliminary molecular docking experiments using a small mixed library containing commercially available and in-house synthesized compounds (see Section 4 “Materials and Methods”) against the Kelch domain of Keap1 using the software Glide (v. 7.4) [33–36].

As reported [26], the binding site of Nrf2 on the Kelch domain on Keap1 can be divided into five sub-pockets (P1 to P5), each one with peculiar characteristics (see Section 3 “Discussion”). After the molecular docking, the resulting poses generated for each ligand were ranked based on the estimated binding energy, setting a minimum energetic cutoff at -6.0 kcal/mol. Afterwards, the remaining complexes were visually inspected to highlight protein–ligand interactions involving important amino acids. Based on that qualitative evaluation, we identified **1** (N-(benzo[d][1,3]dioxol-4-ylmethyl)-4-(1,1-dioxido-

3-oxoisothiazolidin-2-yl)benzenesulfonamide), **2** (4-(1,1-dioxido-3-oxoisothiazolidin-2-yl)-2-methoxy-N-methyl-N-(2-(3-ylidine-4-yl)ethyl)benzenesulfonamide), **3** (N-(2-benzoylphenyl)-6-(2,4-dioxo-1,4-dihydroquinazolin-3(2H)-yl)hexanamide), and **4** ((S)-3-(5-(ethoxycarbonyl)-4-(6-(3-(methoxycarbonyl)phenyl)3-pyridin-2-yl)-6-methyl-2-oxo-3,4-dihydropyrimidin-1(2H)-yl)propanoic acid) as the most promising ones (Figure 3).

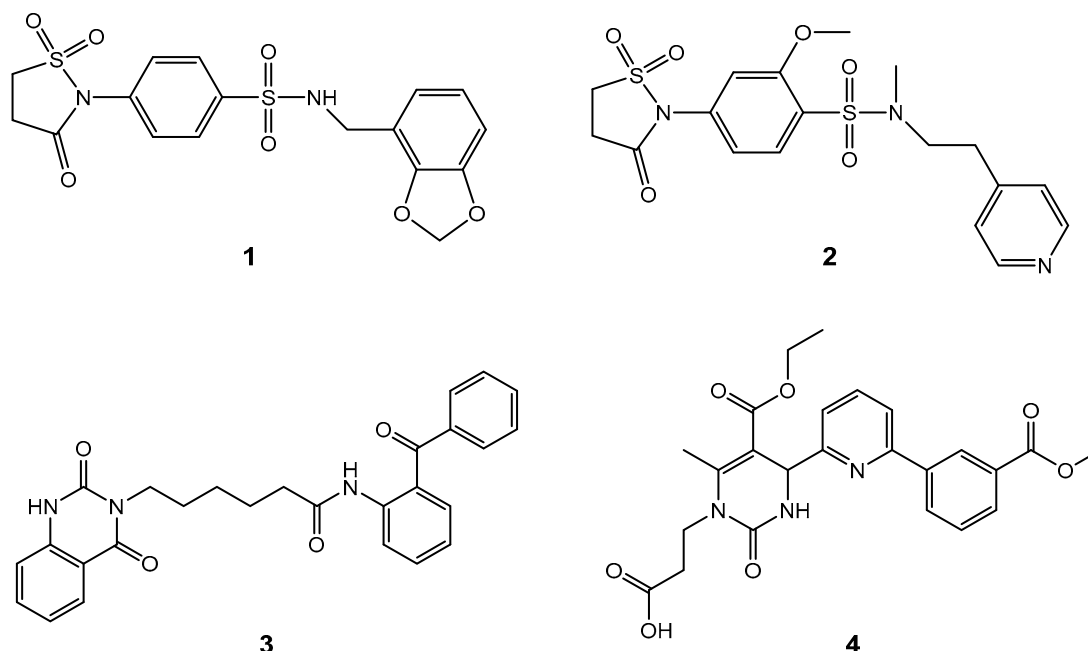


Figure 3. Chemical structure of compounds 1–4.

In the table below (Table 1), all the interactions made by the four molecules with the target are listed, and, as expected, hydrophobic contacts represent the most prevailing ones, along with interactions with Arg415, which was always present in the list of interacting residues.

Table 1. List of the interactions made by compounds 1–4 with the target. Interactions made with residues that do not belong to the five sub-pockets are in bold. If multiple interactions are made with the same residue, the total number is in brackets.

Molecule	H-Bond	π -Cation	π - π Stacking	Hydrophobic	Salt Bridge
1	Asn414, Arg415 ($\times 2$), Ser508, Tyr525, Gln530, Ser602.	Arg415.		Tyr334, Ser363, Gly364, Arg380, Asn382, Gly462, Arg483, Gly509, Ser555, Ala556, Tyr572, Gly574, Gly603.	
2	Asn414, Arg415 ($\times 2$), Ser508, Ser555, Ser602.	Arg415 ($\times 2$), Arg483.	Phe478.	Tyr334, Ser363, Gly364, Arg380, Asn382, His436, Ile461, Gly462, Gly509, Tyr525, Gln530, Ala556, Tyr572, Gly603.	
3	Arg415, Asn414, Ser602, Val604 .	Arg415 ($\times 2$).	Tyr334, Phe577.	Ser363, Gly364 , Leu365 , Ala366 , Gly367 , Arg380, Asn382, Ile416 , Gly417 , Gly462, Val463 , Gly464 , Val465 , Gly509, Ala510 , Gly511 , Val512 , Ser555, Ala556, Leu557 , Gly558 , Ile559 , Tyr572, Gly603, Gly605 , Val606 .	
4	Asn414, Arg415 ($\times 2$), Ser508, Ser602.	Arg415 ($\times 2$).	Tyr334.	Ser363, Gly364, Arg380, Asn382, Ile461, Gly462, Gly477, Phe478, Arg483, Gly509, Tyr525, Gln530, Ser555, Ala556, Tyr572, Phe577, Gly603.	Arg415.

Compounds 1–4 made a relevant number of interactions with the target protein, namely 21, 24, 34, and 25, respectively (Figure 4), fitting well in the binding pocket, as shown in Figure 5.

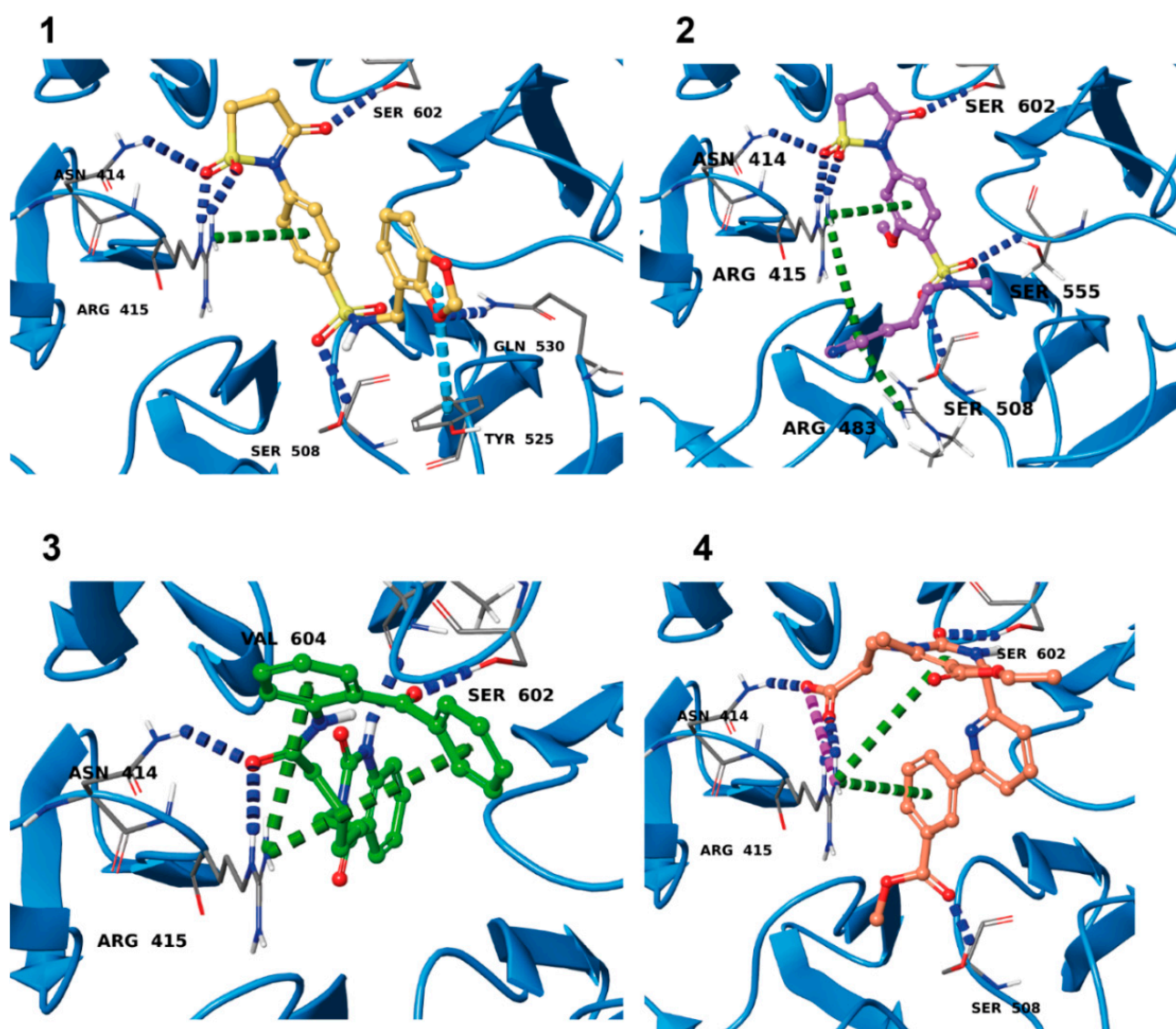


Figure 4. Molecular interactions of 1–4 with Keap1. Compounds are represented in ball-and-stick with custom carbons (yellow for 1, purple for 2, green for 3, and orange for 4). Blue dotted lines are hydrogen bonds, pink dotted lines are salt bridges, green dotted lines are π -cation interactions, and cyan dotted lines are π - π stackings. Interacting residues are labeled.

Based on molecular docking analyses, the simultaneous engagement of multiple subpockets (P1–P5; see Section 3 “Discussion”) together with interaction with the key residue Arg415 was observed among the most favorable binding poses. These features may represent recurrent interaction patterns in predicted Keap1 binders, suggesting that the combination of a cationic moiety and hydrophobic contacts within the P4–P5 region could contribute to effective binding *in silico*, rather than constituting a demonstrated requirement for functional Keap1-Nrf2 disruption.

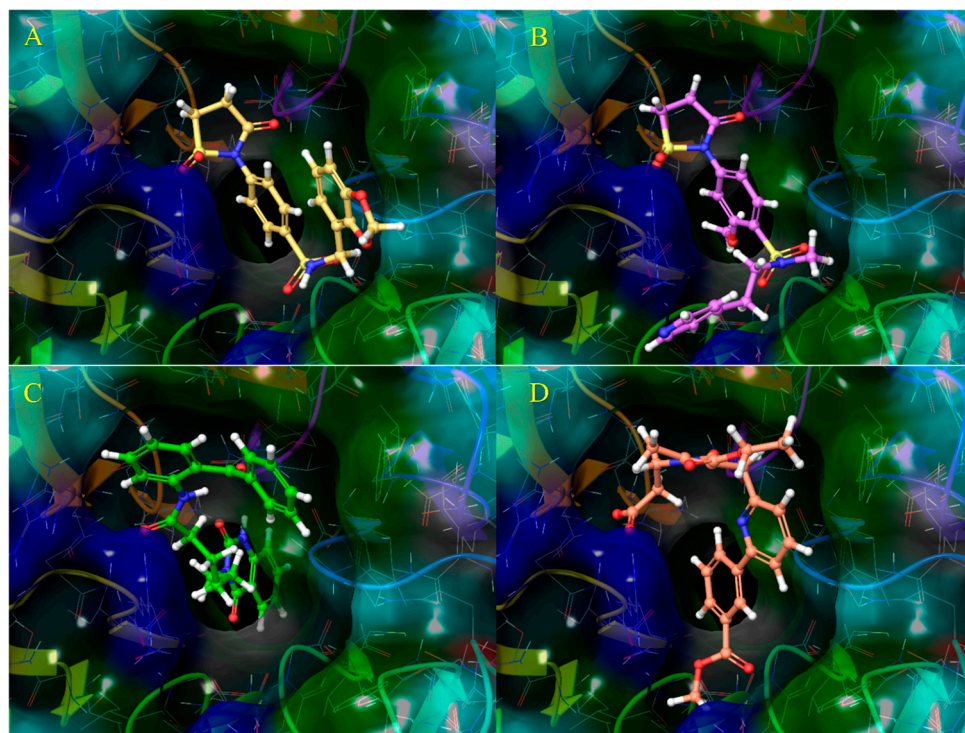


Figure 5. Three-dimensional view of **1** (A), **2** (B), **3** (C), and **4** (D) inside the binding pocket of Keap1. Compounds are represented in ball-and-stick with custom carbons (yellow for **1**, purple for **2**, green for **3**, and orange for **4**). The molecular surface is colored according to the following scheme: positively charged areas in blue, hydrophobic regions in green, and hydrophilic areas in cyan.

To further corroborate the predictions made by the virtual screening experiments, following what was recently reported [37], molecular docking studies were performed for compounds **1–4** using the crystal structure of the human Keap1 Kelch domain in complex with an Nrf2-derived peptide (PDB: 2FLU [38]). This model was selected to qualitatively evaluate whether the identified compounds could spatially interfere with the Keap1-Nrf2 interaction. Docking results indicated that the compounds are able to occupy the Nrf2 binding region within the Kelch domain and engage residues known to be critical for peptide recognition. In particular, Arg415 and Arg483, which play a key role in stabilizing the Keap1-Nrf2 interface, were preferentially involved in interactions with the docked compounds rather than with the peptide, suggesting a potential mechanism consistent with PPI inhibition (Figure S1).

In addition, several properties and descriptors were computed with QikProp [37] (Table S1). In particular, we focused our attention on pharmacokinetic properties, physically significant descriptors, pharmaceutically relevant properties for the prediction of absorption, distribution, metabolism, and excretion (ADME), and types of reactive functional groups (Table S1).

Overall, the predicted ADME profiles indicated that all compounds possess physicochemical properties consistent with a drug-like behavior. None of the compounds exhibited critical deviations from the recommended ranges, as reflected by low #stars values, and their molecular weights fall within an acceptable range for small-molecule development. Predicted brain/blood partition coefficients (QPPlogBB) suggest a moderate ability of all compounds to access the central nervous system, with values compatible with compounds acting in neuronal cellular models. Summing up the data obtained by these *in silico* experiments, we were confident in testing the antioxidant properties of the above-mentioned compounds.

2.2. Cytotoxicity Assay

The effect of the compounds 1–4 on SH-SY5Y cell viability was assessed to determine non-toxic concentrations using the MTT assay. The human neuroblastoma SH-SY5Y cell line is commonly used for studying neurotoxicity and neuroprotection, being especially relevant for experimental Alzheimer's and Parkinson's diseases [38]. SH-SY5Y cells were treated with a range of concentrations from 2.5 μ M up to 50 μ M for 24 h. As shown in Figure 6, compounds 2 and 3 did not affect SH-SY5Y cell viability at any assayed concentrations compared to control cells. Moreover, compound 2 showed a peculiar non-dose-dependent, biphasic response that generates a reverse U-shaped dose–response curve as reported in other neuroprotective studies [39].

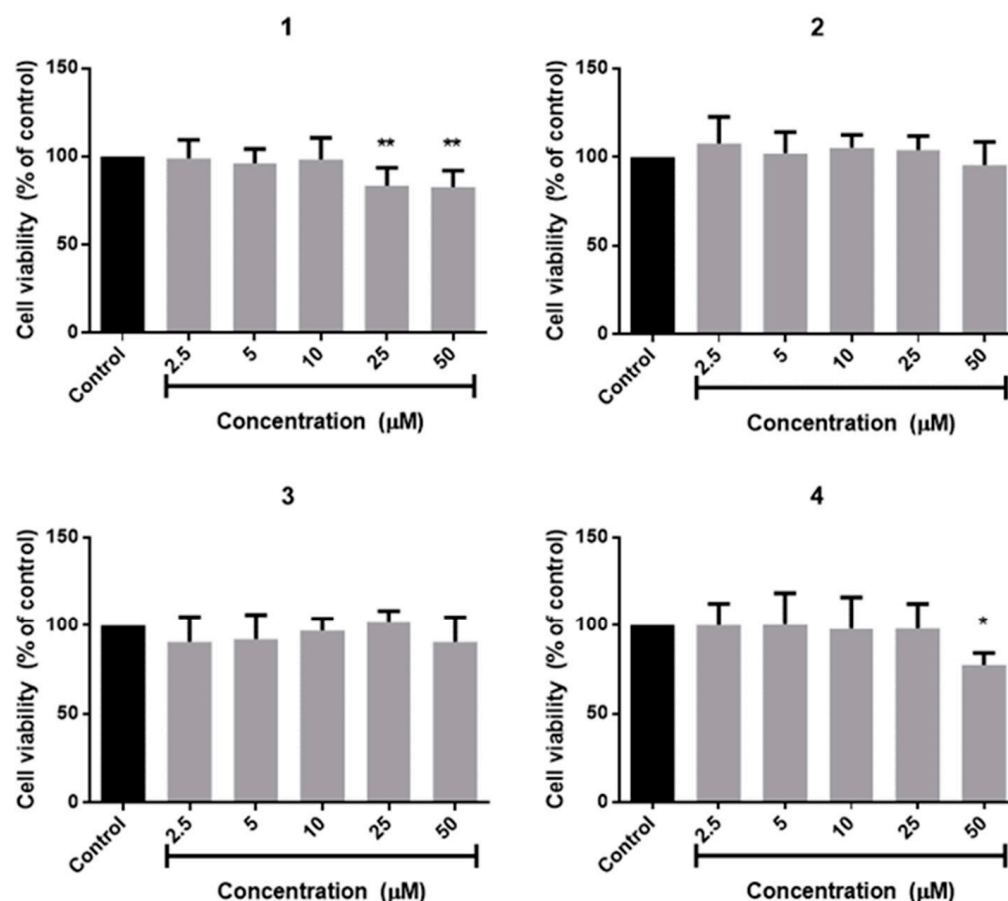


Figure 6. Cytotoxicity assay. SH-SY5Y cells were treated with compounds 1–4 from 2.5 μ M to 50 μ M for 24 h. The effect on cell viability was measured using an MTT assay. Data (mean $\pm$ SD of three independent experiments, grey bars) are expressed as a percentage of cell viability compared to control (100%, black bar). * $p < 0.05$ and ** $p < 0.005$.

Conversely, compound 1 caused significant cell death at 25 μ M and 50 μ M (around 20.0% of cell death compared to control; $p < 0.05$), and compound 4 showed a similar effect at 50 μ M (23.0% of cell death compared to control at 50 μ M; $p < 0.05$). Therefore, those cytotoxic concentrations were excluded from further investigations.

2.3. Protective Effects Against Oxidative Stress Induced by Fenton's Reaction

Then, we evaluated whether non-cytotoxic concentrations of the compounds 1–4 (Figure 3) could exert a neuroprotective effect in an induced experimental oxidative model. To generate ROS, we used Fenton's reagent (a mixture of a Fe^{2+} salt and hydrogen peroxide), which generates hydroxyl radicals ($\cdot\text{OH}$) [40]. SH-SY5Y cells were pre-treated for 6, 16, and

24 h at non-cytotoxic concentrations (from 2.5 μM to 10 μM for **1**, from 2.5 μM to 50 μM for **2** and **3**, and from 2.5 μM to 25 μM for **4**) before the exposure to Fenton's reagent (300 μM of FeSO_4 + 300 μM of H_2O_2) for 2 h. Cell viability, assessed by MTT, was used to evaluate the potential cytoprotective effects of the assayed compounds under oxidative stress conditions. As shown in Figure 7, we observed a time-dependent neuroprotective effect that starts being statistically significant after 16 h of treatment ($p < 0.05$), with an overall increase in cell viability of 24.0–31.0% compared to cells treated only with Fenton's reagent, except for **3** at 50 μM . In detail, **1** reached the best protective effect after 24 h of incubation at the concentration of 10 μM (cell viability 31.0% higher compared to Fenton's reagent); **2** had a similar effect at a concentration of 25 μM (cell viability 24.0% higher compared to Fenton's reagent); **3** showed the highest cytoprotective action after 24 h of treatment at a concentration of 10 μM (cell viability 26.0% higher compared to Fenton's reagent) and, finally, **4** showed the best protection after 24 h at a concentration of 5 μM (cell viability 26.3% higher compared to Fenton's reagent).

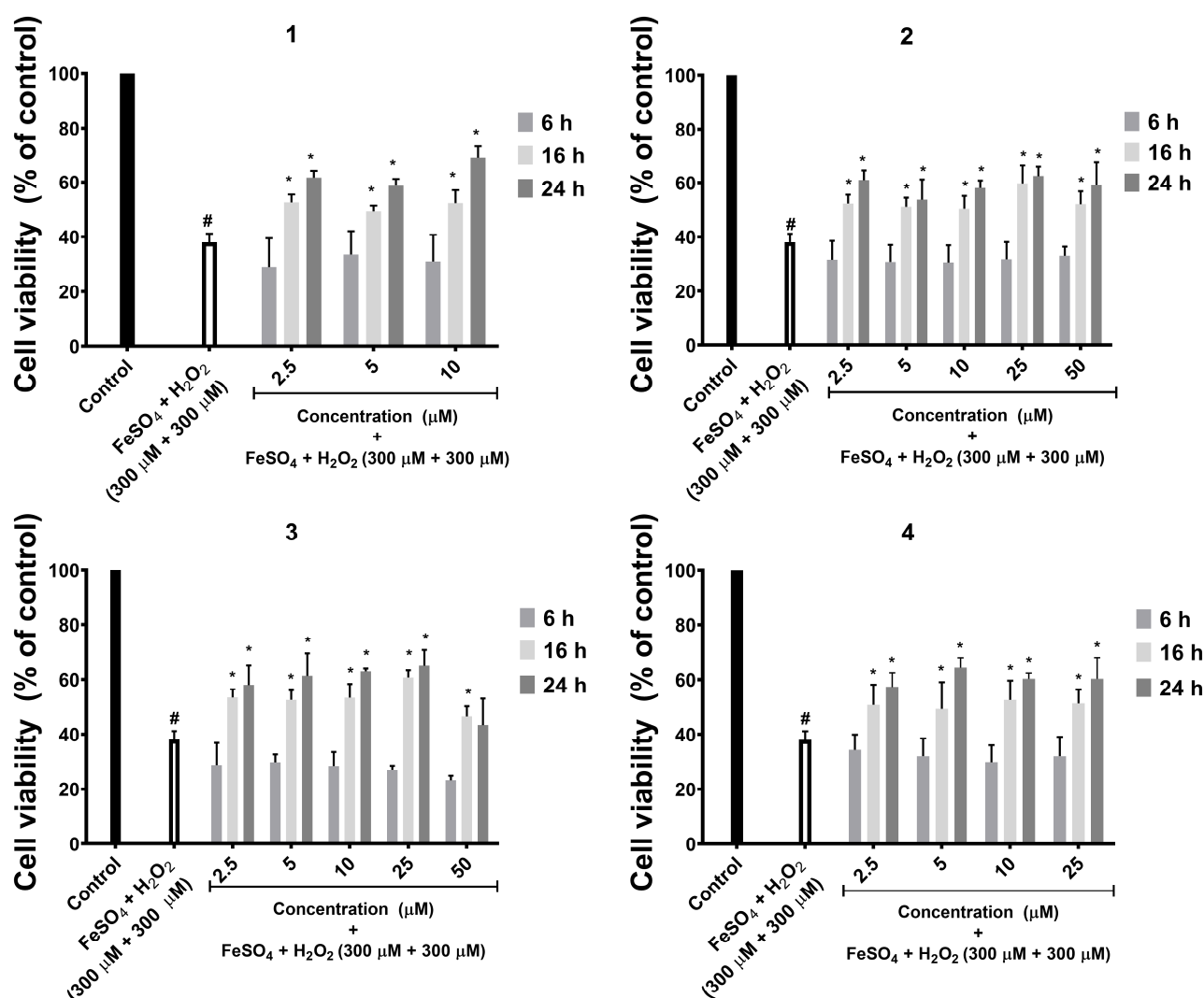


Figure 7. Cytoprotective assay. SH-SY5Y cells were pre-treated with the compounds 1–4 at non-cytotoxic concentrations for 6, 16, and 24 h before exposure to Fenton's reagent (300 μM of FeSO_4 + 300 μM of H_2O_2) for 2 h. The effect on cell viability was measured using an MTT assay. Data (mean $\pm$ SD of the three independent experiments) are expressed as a percentage of cell viability compared to the control (100%). * $p < 0.05$ versus Fenton's reagent and # $p < 0.05$ versus control.

Therefore, we selected the above-mentioned concentrations as the most protective ones for each compound and 24 h as the time of incubation for all the following experiments.

2.4. Effect on Intracellular ROS Production

Since oxidative stress is the result of an imbalance between ROS production and the endogenous antioxidant system, we measured the effect of these compounds on intracellular reactive species generation under oxidative stress induced by Fenton's reaction using the 2,7-dichlorofluorescein diacetate (DCFH-DA) assay. For this purpose, the most protective concentrations of each compound (10 μ M for 1, 25 μ M for 2, 10 μ M for 3, and 5 μ M for 4) were used. As shown in Figure 8, fluorescence significantly increased when cells were exposed only to Fenton's reagent (+33.8% compared to control cells; $p < 0.05$), proving high intracellular oxidant species levels. When cells were pre-treated with 1, 3, and 4, a significant reduction in DCFH-DA fluorescence was observed (−20.0%, −32.0%, and −29.0%, respectively), compared to those treated only with Fenton's reagent.

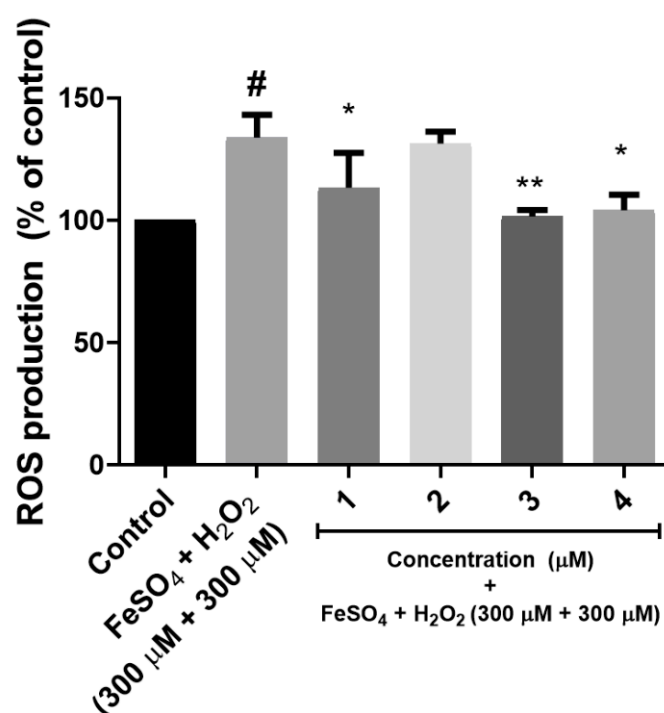


Figure 8. Effect of pre-treatment with 1 (10 μ M), 2 (25 μ M), 3 (10 μ M), and 4 (5 μ M) on ROS production induced by exposure to Fenton's reagent (FeSO₄ 300 μ M + H₂O₂ 300 μ M) for 2 h in SH-SY5Y cells. Data (mean $\pm$ SD of three independent experiments, greyscale bars) are expressed as a percentage of ROS production compared to control cells (100%, black bar). # $p < 0.01$ versus control, * $p < 0.05$, and ** $p < 0.01$ versus Fenton's reagent.

Therefore, compounds 3, 4, and 1 showed the greatest ability to reduce DCFH-DA-detectable intracellular reactive species under the experimental conditions used. On the other hand, 2 did not show significant protection against intracellular ROS overproduction induced by the Fenton reagent (just a 2.5% reduction compared to SH-SY5Y cells treated only with Fenton's reagent).

2.5. Effect on GSH/GSSG Ratio

When SH-SY5Y cells were treated only with Fenton's reagent, a marked decrease in the GSH/GSSG ratio (−72.0% compared to control cells; $p < 0.05$) was observed (Figure 9). Results highlight how 3 showed the most pronounced effect, presenting an increase of 15.0% in the GSH/GSSG ratio compared to control cells and a lower standard deviation.

Compounds **2** and **4** presented similar good results with an increase of 10.0% and 21.0% in GSH/GSSG ratio, respectively, compared to control cells, but with a higher standard deviation. Compound **1** showed significantly higher levels of GSH compared to cells treated only with Fenton's reagent (+51.0%), but the total ratio is still lower than control cells, although not significantly ($p > 0.005$).

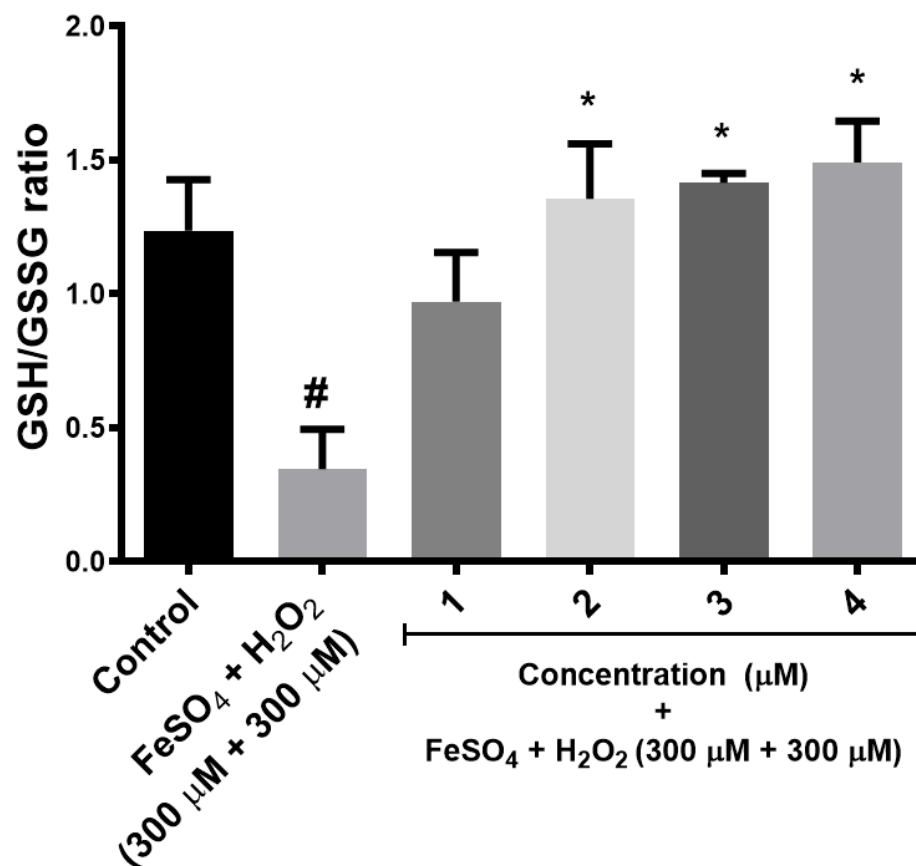


Figure 9. GSH/GSSG ratio in SH-SY5Y cells after pre-treatment with **1** (10 μM), **2** (25 μM), **3** (10 μM), and **4** (5 μM) and exposure to Fenton's reagent (FeSO₄ 300 μM + H₂O₂ 300 μM) for 2 h. Data are expressed as mean ± SD of three independent experiments (greyscale bars). # $p < 0.05$ versus control (black bar) and * $p < 0.005$ versus cells treated only with Fenton's reagent (light grey bar).

2.6. Effect on Lipid Peroxidation

The SH-SY5Y cells were pre-treated with protective concentrations of the compounds **1–4** and exposed to Fenton's reagent for 2 h. The effect of these compounds on lipid peroxidation, shown in Figure 10, was measured indirectly and is expressed as thiobarbituric acid reactive substances (TBARS) (% of control). In cells exposed only to Fenton's reagent, a notable increase in the TBARS concentration is detected (+141.7%), which shows that Fenton's reagent causes lipid peroxidation. In contrast, pre-treatment with all assayed compounds significantly inhibited lipid peroxidation, as shown in Figure 10.

Interestingly, all these preliminary data (reduction in ROS and TBARS concentration and restoration of the GSH/GSSG ratio) are in line with the molecular docking predictions, which suggested that compound **3** might favourably bind to the protein counterpart, due to its high number of predicted contacts.

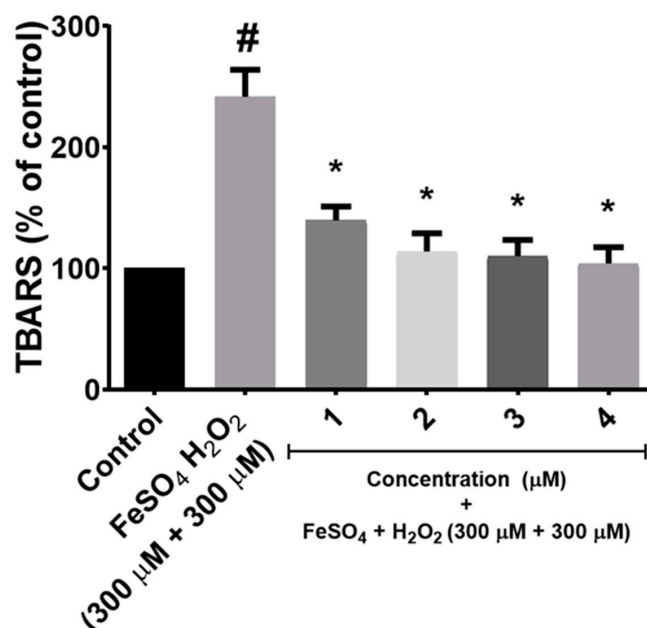


Figure 10. TBARS concentration in SH-SY5Y cells after pre-treatment with **1** (10 μM), **2** (25 μM), **3** (10 μM), and **4** (5 μM) and exposure to Fenton's reagent (FeSO₄ 300 μM + H₂O₂ 300 μM) for 2 h. Data are expressed as mean ± SD of three independent experiments (greyscale bars). # $p < 0.05$ versus control (black bar) and * $p < 0.005$ versus cells treated only Fenton's reagent (light grey bar).

2.7. Compounds 1–4 Increased the Expression of Antioxidant Enzymes

Antioxidant enzymes are the first line of defense against oxidative stress. Then, we quantified the effect of compounds on the protein expression of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (GPx) by Western blot. As shown in Figure 11, the protein expression for all antioxidant enzymes analyzed was significantly reduced in those cells treated with Fenton's reagent. Particularly, CAT was reduced by 51.3%, SOD by 39.0%, GR by 45.7%, and GPx by 75.0% ($p < 0.05$) compared to control cells. Pre-treatments of SH-SY5Y cells with compounds **1** (10 μM), **2** (25 μM), **3** (10 μM), and **4** (5 μM) resulted in a global up-regulation of the above-mentioned enzymes (Figure 11). Notably, compound **3** exerted the highest effect in increasing the expression of CAT (+87.8%), SOD (+36.5%), and GR (+65.3%). On the other hand, compound **4** was the most effective in upregulating GPx protein expression (+60.5%).

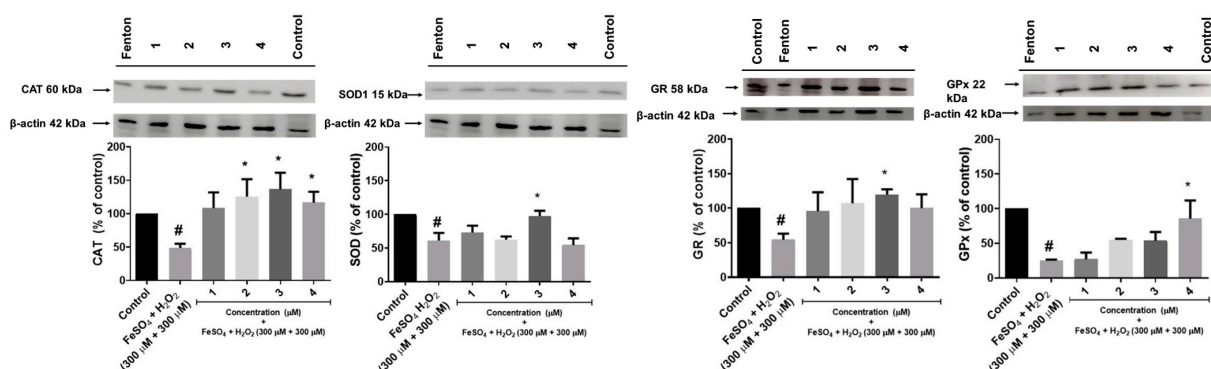


Figure 11. Effect of **1** (10 μM), **2** (25 μM), **3** (10 μM), and **4** (5 μM) on the expression of antioxidant enzymes (CAT, SOD, GR, and GPx) in the SH-SY5Y cell line. Cells were pre-treated with the above-mentioned compounds at non-toxic concentrations for 24 h, then we exposed the cells to Fenton's reagent (FeSO₄ 300 μM + H₂O₂ 300 μM) for 2 h. Protein expressions were measured by Western blot. All data shown represent the mean ± SD of at least three independent experiments (greyscale bars). # $p < 0.05$ versus control cells (black bar), * $p < 0.05$ versus Fenton's reagent (light grey bar).

2.8. Nuclear Translocation of Nrf2 After Treatment with Compounds 1–4

The expression of antioxidant enzymes is regulated by nuclear factor erythroid 2-related factor 2 (Nrf2); this factor plays a crucial role in cellular defense against oxidative stress. Nuclear extracts were analyzed using Western blot using β -actin as an internal control, as reported in other studies [41–45]. After pre-treatments with the four investigated molecules, Nrf2 levels in nuclear extracts were, except for compound 4, higher than both control cells and those treated only with Fenton's reagent ($p < 0.05$; Figure 12).

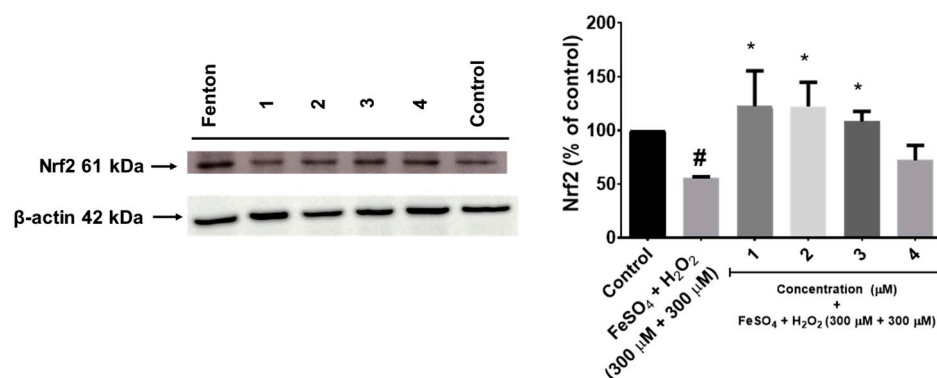


Figure 12. Nuclear localization of Nrf2 after pre-treatment with 1 (10 μ M), 2 (25 μ M), 3 (10 μ M), and 4 (5 μ M) for 24 h and exposure to Fenton's reagent (FeSO₄ 300 μ M + H₂O₂ 300 μ M) for 2 h. Protein expressions were measured by Western blot. All data shown represent the mean $\pm$ SD of at least three independent experiments (greyscale bars). # $p < 0.05$ versus control cells (black bar), * $p < 0.05$ versus Fenton's reagent (light grey bar).

Specifically, compound 1 showed an increase in nuclear Nrf2 level of 22.5% compared to control cells, compound 2 showed an increase of 22.3%, and compound 3 showed an increase of 8.5%. However, compound 4 increased the Nrf2 factor translocation but not significantly.

3. Discussion

The combined computational and biological data highlighted a coherent structure-activity relationship that may help rationalize the antioxidant and cytoprotective effects observed for compounds 1–4. All selected molecules share scaffolds capable of targeting the Kelch domain of Keap1, thereby potentially mitigating some of the drawbacks associated with covalent cysteine modification. Both safety and mechanistic considerations drove the choice of PPI disruptor Keap1 over cysteine-binding Nrf2 activators. Indeed, covalent thiol-targeting strategies have been reported to rely on inherently low selectivity and, depending on compound reactivity and exposure, may result in off-target interactions and associated toxicities [27,29–32].

The Kelch domain of Keap1 contains a well-characterized binding groove responsible for the recognition of the ETGE and DLG motifs of Nrf2. This groove is organized into five adjacent sub-pockets (P1–P5). In detail, these five sub-pockets are: P1) Arg415, Ile461, Gly462, Gly477, Phe478, Arg483, and Ser508 (positively charged region); P2) Ser363, Arg380, Asn382, and Asn414 (positively charged surface); P3) Gly509, Ser555, Ala556, Gly571, Ser602, and Gly603 (neutrally charged pocket); P4) Tyr525, Gln530, and Tyr572; P5) Tyr334, and Phe577 [26]. Docking analysis revealed that these compounds occupy the canonical Nrf2-binding groove, engaging multiple sub-pockets (P1–P5) through a combination of hydrogen bonding, π - π stacking, π -cation interactions, and hydrophobic contacts, which are considered essential for effective disruption of the Keap1–Nrf2 protein–protein interaction. The interaction patterns made are consistent with the disruption of the Keap1–Nrf2 contact

interface and were used prospectively to select compounds **1–4** for the biological validation. Importantly, docking results provide only theoretical estimates of binding and were used exclusively to guide compound prioritization for in vitro assays.

Reactive oxygen species (ROS) are naturally generated during normal cellular processes such as oxidative phosphorylation and are efficiently removed by cellular antioxidant defense systems. However, when an imbalance occurs between ROS production and endogenous antioxidant capacity, a condition known as oxidative stress, oxidative damage to biomolecules may ensue. Prolonged oxidative stress can impair cellular structures and functions and may ultimately lead to cell death [46,47]. In this study, the optimal protective concentrations and incubation times were identified, which conferred enhanced cytoprotection against damage induced by the Fenton's reaction. At these optimal concentrations and after a 24 h incubation period, compounds **1**, **3**, and **4** significantly reduced intracellular ROS production.

To further characterize the impact on cellular redox homeostasis, glutathione balance (GSH/GSSG ratio) and lipid peroxidation (TBARS) were measured as complementary indicators of cytosolic redox buffering capacity and membrane oxidative damage, respectively. GSH (γ -glutamyl-L-cysteinyl-glycine) is the primary non-enzymatic antioxidant in human cells, capable of reducing intracellular ROS through its reactive thiol groups [48,49]. The ratio of reduced glutathione (GSH) to its oxidized form (GSSG) is commonly used to assess cellular antioxidant capacity [50–52]. Elevated levels of GSSG during oxidative stress can induce mitochondrial dysfunction through glutathionylation of target proteins [53]. On the other hand, increases in the GSH/GSSG ratio or in GSH levels alone do not necessarily indicate a complete restoration of redox homeostasis and should be interpreted in the context of the overall cellular response to oxidative stress. Another major consequence of severe oxidative stress is lipid peroxidation. Polyunsaturated fatty acids are particularly susceptible to oxidation by ROS, producing various reactive products such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and F2-isoprostanes [54]. MDA, in particular, reacts with thiobarbituric acid to form a spectrophotometrically detectable adduct, which serves as an indirect marker of cellular oxidative damage [55,56]. Lipid peroxidation is initiated by hydroxyl radicals abstracting hydrogen atoms from lipids, generating lipid radicals that react with molecular oxygen to form lipid peroxyl radicals, a process often propagated by Fenton reaction-derived radicals [57].

Finally, the molecular mechanisms underlying the observed antioxidant and cytoprotective effects were investigated by assessing the expression of antioxidant enzymes and the nuclear translocation of Nrf2, thereby connecting the functional outcomes to activation of the Keap1-Nrf2 signaling pathway.

Among the four compounds, compound **3** consistently emerged as the most active in the in vitro assays and was also prioritized by molecular docking analyses. Structurally, it features a flexible hexanamide linker connecting a quinazolinone core to a benzophenone moiety, enabling an extended binding mode within the Keap1 pocket. Docking simulations suggested that this flexibility may allow simultaneous engagement of multiple sub-pockets, as suggested by the high number of protein-ligand contacts observed. Notably, the frequent interactions with Arg415 and Asn414, along with the extensive hydrophobic contacts involving Tyr334, Tyr572, and Phe577 that were computationally predicted, provide a plausible structural framework to rationalize the pronounced biological profile of compound **3**. This prediction is, indeed, a qualitative framework consistent with the pronounced effects of compound **3**, including a marked reduction in intracellular DCFH-DA-detectable reactive species, restoration of the GSH/GSSG ratio, inhibition of lipid peroxidation, and robust upregulation of antioxidant enzymes [57].

Compound **4**, despite inducing a lower extent of Nrf2 nuclear translocation, exhibited a pronounced antioxidant profile, particularly with respect to lipid peroxidation inhibition and upregulation of glutathione peroxidase (GPx). Docking analyses suggest that its dihydropyrimidinone scaffold, which incorporates both polar and hydrophobic functionalities, may favor interactions with aromatic residues within the P4 and P5 sub-pockets. Although the presence of a carboxylic acid moiety is typically associated with reduced passive membrane permeability, the marked biological activity observed for compound **4** at low micromolar concentrations indicates that cellular uptake is nevertheless sufficient.

Compounds **1** and **2**, both sulfonamide derivatives bearing an isothiazolidinone dioxide moiety, displayed intermediate biological activity. Molecular docking suggested that their shared scaffold may allow anchoring within positively charged regions of the binding site, particularly through interactions with Arg415 and Ser508. However, their relatively rigid conformations and smaller aromatic surface area compared with compound **3** may limit optimal engagement of hydrophobic sub-pockets, potentially accounting for their reduced efficacy in modulating intracellular ROS levels and downstream antioxidant responses. Notably, although compound **2** reduced cell death, it did not significantly decrease intracellular oxidant species levels, suggesting that its cytoprotective effect may be associated with indirect or compensatory cellular responses, such as modulation of antioxidant defenses, rather than with an immediate reduction in intracellular oxidative stress.

Although the Fenton reaction-based model represents an acute and supraphysiological source of oxidative stress, which does not fully recapitulate endogenous redox regulation, it may, in principle, allow the detection of direct antioxidant effects such as Fe^{2+} chelation or H_2O_2 scavenging. However, the available evidence indicates that this is not the primary mechanism of action of the compounds investigated. The protective effects, indeed, were strongly time dependent [58,59], with the compounds requiring sufficient incubation to mitigate oxidative damage through activation of intracellular antioxidant systems and reduction in ROS production, behavior inconsistent with the immediate chemical neutralization of Fe^{2+} or H_2O_2 . Moreover, the pronounced upregulation of antioxidant enzymes, restoration of the GSH/GSSG ratio, and nuclear translocation of Nrf2 collectively indicate activation of endogenous cellular defense mechanisms rather than direct radical scavenging. In addition, the chemical structures of the compounds lack classical iron-chelating motifs (e.g., catechol groups or deferoxamine-like functionalities), further supporting an indirect, signaling-mediated antioxidant effect.

Accordingly, the observed increase in antioxidant enzyme expression, together with enhanced Nrf2 nuclear localization, particularly for compounds **1–3**, supports the hypothesis that these molecules may interfere with the Keap1-Nrf2 protein–protein interaction, leading to activation of Nrf2-dependent antioxidant pathways, while not excluding the contribution of additional redox-sensitive mechanisms. With respect to lipid peroxidation and the observed reduction in TBARS levels, the upregulation of enzymes involved in lipid hydroperoxide detoxification, such as glutathione peroxidase (GPx), along with increased glutathione availability, is consistent with activation of the Keap1-Nrf2 pathway. Although minor contributions from local, membrane-associated effects cannot be entirely excluded, the time-dependent nature of the response and its correlation with Nrf2 accumulation and antioxidant enzyme induction are more consistent with the involvement of endogenous cellular defense pathways. Accordingly, these observations suggest that the observed effects are likely mediated primarily by activation of cellular antioxidant responses rather than by immediate membrane trapping or direct lipid scavenging, which were not directly assessed in this study.

Finally, although no direct protein–protein interaction disruption assays were performed, the biological data provide strong functional evidence for effective activation

of the Nrf2 antioxidant pathway. Treatment with compounds **1–4** induced significant Nrf2 nuclear translocation and subsequent upregulation of Nrf2-dependent antioxidant enzymes, providing robust functional support for the proposed mechanism of action. Overall, these findings identify compound **3** as a particularly promising lead scaffold for further optimization.

4. Materials and Methods

4.1. Chemistry

Compounds **1** and **2** were purchased from Otava Chemicals (Vaughan, Ontario, Canada). Otava product codes are reported in Table 2. For all the compounds, the company has undergone quality control to confirm their chemical structures.

Table 2. List of product codes corresponding to **1** and **2**.

Compound	Otava Code
1	7020470041
2	7020530643

4.1.1. General Methods

All chemicals were purchased from Merck Life Science S.r.l. (Milan, Italy) and used as received. All solvents used for the synthesis were of HPLC grade. NMR spectra (^1H and ^{13}C) were recorded on a Bruker Avance 400 MHz instrument (Bruker, Berlin, Germany) (^1H at 400 MHz and ^{13}C at 100 MHz); coupling constants (J) are reported in Hertz and chemical shifts in ppm (δ units), relative to Methanol- d_4 as an internal standard ($\delta_{\text{H}} = 3.31$ and $\delta_{\text{C}} = 49.0$ ppm). Spin multiplicities are given as s (singlet), d (doublet), t (triplet), and m (multiplet). ESI-MS spectra were recorded using a Micromass QTOF Premiere spectrometer (Waters Co., Milford, MA, USA) and carried out using water/acetonitrile (1:1) + 0.1% of acetic acid. Reactions were monitored by TLC using silica gel 60 F₂₅₄ plates (Merck) and visualized with cerium sulfate and under UV light ($\lambda = 254$ nm, 365 nm). Analytical and semi-preparative reversed-phase HPLC was performed on Agilent Technologies 1200 Series high-performance liquid chromatography (Agilent, Santa Clara, CA, USA). The HPLC method used the following conditions: Nucleodur C₈ reversed-phase column (NUCLEODUR® 100-5 C8 ec, 5 μm , 250 $\times$ 10.00 mm, flow rate = 4 mL/min; NUCLEODUR® 100-5 C8 ec, 5 μm , 250 $\times$ 4.6 mm, flow rate = 1 mL/min, Macherey-Nagel, Dueren, Germany); solvent A of 0.1% TFA in water, solvent B of 0.1% TFA in CH₃CN. The absorbance was detected at 240/280 nm. The purity of all tested compounds (>96%) was determined by HPLC analysis, and the full NMR and MS characterization excluded the presence of side products in the fraction.

4.1.2. Microwave Irradiation Experiments

All microwave irradiation reactions were carried out in a CEM-Discover® Focused Microwave synthesizer (CEM s.r.l., Cologno Al Serio, Italy), operating with continuous irradiation power from 0 to 300 W, utilizing the standard absorbance level of 300 W maximum power. The reactions were carried out in 10 mL sealed microwave glass vials. The temperature was monitored using the CEM-Discover built-in vertically focused IR temperature sensor. After the irradiation period, the reaction vessel was cooled to room temperature by air-jet cooling.

4.1.3. Synthesis of Compound **3**

To a solution of 6-aminohexanoic acid (1.1 mmol) in water (2.5 mL) was added triethylamine (1.1 mmol) and isatoic anhydride (1.2 mmol). The reaction mixture was stirred

for 2 h at 30–40 °C, cooled to room temperature, and evaporated under vacuum to form an oily residue, which was refluxed for 7 h in the presence of formic acid (3.5 mL). Then the mixture was cooled at room temperature and evaporated. The solid was dissolved in DCM and extracted with H₂O/DCM. The organic layers were combined, dried over anhydrous sodium sulfate, and filtered. The filtrate was concentrated by evaporation to yield the 6-(2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)-hexanoic acid, as a light pink powder (88%). The quinazolinone (0.4 mmol) was coupled with 2-aminobenzophenone (0.7 mmol) in the presence of HOBt (0.7 mmol) and DIC (0.5 mmol) in DMF (200 µL) under microwave irradiation for 30 min. A portion of the crude product was purified by semi-preparative reversed-phase HPLC (on NUCLEODUR® 100-5 C8 ec, 5 µm, 250 × 10.00 mm, flow rate = 4 mL) using the gradient conditions reported below. The final product was obtained with high purity > 96% detected by HPLC analysis and was fully characterized by ESI-MS and NMR spectra.

RP-HPLC t_R = 28.3 min, gradient condition: from 5% B to 100% B in 40 min, flow rate of 4 mL/min, λ = 240 nm. ¹H NMR (400 MHz, Methanol-d₄): δ = 1.34–1.41 (m, 2H), 1.54–1.62 (m, 2H), 1.76–1.85 (m, 2H), 2.23 (t, J = 7.3 Hz, 2H), 4.08 (t, J = 7.4 Hz, 2H), 6.76 (t, J = 8.3 Hz, 1H), 6.99 (d, J = 8.3 Hz, 1H), 7.29 (t, J = 7.8 Hz, 1H), 7.37–7.45 (m, 2H), 7.48–7.55 (m, 3H), 7.58–7.66 (m, 2H), 7.71–7.77 (m, 2H), 8.28 (d, J = 7.7 Hz, 1H); ¹³C NMR (100 MHz, Methanol-d₄): δ = 24.5, 25.6, 28.5, 36.0, 46.5, 114.7, 117.0, 127.8, 127.9, 128.5, 129.6, 130.7, 134.0, 134.1. ESI-MS, calculated for C₂₇H₂₅N₃O 455.51; found m/z = 478.17 [M + Na]⁺. In this case, the ESI-MS experiments revealed that a sodium adduct is formed.

4.1.4. Synthesis of Compound 4 by Microwave-Assisted Biginelli Reaction

A mixture of 6-(3-(methoxycarbonyl)phenyl)-2-pyridinecarboxaldehyde (1.0 mmol), 3-ureidopropionic acid (1.5 mmol), ethyl acetoacetate (1.0 mmol) in acetonitrile (1.5 mL) was placed in a 10 mL microwave glass vial equipped with a small magnetic stirring bar. Trimethylsilyl chloride (TMSCl) (1.0 mmol) was added, and the mixture was stirred under microwave irradiation at 120 °C for 20 min. Then, the reaction mixture was cooled to room temperature by air-jet cooling, cold water was added, and the vial was poured into crushed ice and then at 4 °C overnight. The resulting precipitate was filtered and washed with a cold mixture of ethanol/water (1:1) (3 × 3 mL), to give the desired product 4 as a pale-yellow solid (90% yield). HPLC purification was performed by semi-preparative reversed-phase HPLC (on NUCLEODUR® 100-5 C8 ec, 5 µm, 250 × 10.00 mm, flow rate = 4 mL) using the gradient conditions reported below. The final product was obtained with high purity > 98% detected by HPLC analysis and was fully characterized by ESI-MS and NMR spectra.

RP-HPLC t_R = 32.3 min, gradient condition: from 5% B to 100% B in 50 min, flow rate of 4 mL/min, λ = 280 nm. ¹H NMR (400 MHz, Methanol-d₄): δ = 1.16 (t, J = 7.1 Hz, 3H), 2.51 (s, 3H), 2.37–2.47 (m, 1H), 2.56–2.63 (m, 1H), 3.90 (s, 3H), 3.86–3.92 (m, 2H), 4.12 (q, J = 7.1 Hz, 2H), 5.59 (s, 1H), 7.24 (d, J = 7.6 Hz, 1H), 7.51 (t, J = 7.6 Hz, 1H), 7.68 (d, J = 7.6 Hz, 1H), 7.78 (t, J = 7.8 Hz, 1H), 8.06 (d, J = 7.8 Hz, 1H), 8.11 (d, J = 7.8 Hz, 1H), 8.52 (s, 1H); ¹³C NMR (100 MHz, Methanol-d₄): δ = 13.1, 15.1, 33.3, 38.3, 50.6, 51.3, 60.1, 104.0, 118.9, 119.3, 127.6, 128.6, 129.6, 130.5, 138.8, 139.3, 155.4, 157.3, 161.3, 166.4, 166.9, 171.9. ESI-MS, calculated for C₂₄H₂₅N₃O₇ 467.17; found m/z = 468.22 [M + H]⁺.

4.2. Molecular Docking

4.2.1. Input File Preparation

The X-ray structures of the Kelch domain of Keap1 bound to a small molecule and to the 16-mer Nrf2 peptide were downloaded from the Protein Data Bank (PDB 6TYM [30] and 2FLU [60]). The crystal structures were prepared using the Protein Preparation Wizard software (v. 72156) [61,62]. The process started by assigning the correct bond order and

protonation state, then missing atoms in the residues' side chains were added, the first and last residue were capped, the intramolecular H-bond network was optimized, and finally, the whole structure was minimized.

The small raw compound library, containing around 82,000 commercially available and synthesized molecules, was prepared using LigPrep [37]. In detail, protonation states at physiological pH (7.4 ± 1.0) are assigned (the carboxyl moiety of compound **4** was deprotonated), and the resulting chemical structure is minimized.

4.2.2. Docking Experiments

The *in silico* virtual screening workflow was performed using the software Glide (v. 7.4) [33–36]. In the primary and secondary phase (High Throughput Virtual Screening (HTVS) and Standard Precision (SP) mode), only one pose per ligand was kept for the following minimization. Afterward, only 50% of the compounds moved on to the next docking step, performed using the Extra Precision (XP) mode of Glide, generating at most 15 poses for each ligand and keeping all of them for the minimization. The resulting poses were first filtered by their predicted binding energy (i.e., only poses with a value below -6.0 kcal/mol were kept) and then visually inspected to select the best compounds.

4.3. Cell Culture and Pre-Treatment

The SH-SY5Y human neuroblastoma cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 0.5% gentamicin in a humidified atmosphere with 5% CO₂ and 37 °C. For experiments, cells were seeded into 96-well plates and 100 mm Petri dishes.

The compounds **1–4** were dissolved in DMSO and diluted with PBS to reach the necessary concentrations, with the final DMSO percentage $< 0.1\%$ in cells. SH-SY5Y cells were pre-treated with the above-mentioned compounds for 6, 16, and 24 h at concentrations ranging from 2.5 μ M to 50 μ M. Fenton's reagent as an oxidative stress inducer was added at 300 μ M of FeSO₄ and 300 μ M of H₂O₂ for 2 h. Each subsequent experiment was carried out in triplicate.

4.4. MTT Assay

MTT assay was performed following Mosmann [63]. Briefly, after cell treatments, SH-SY5Y cells were incubated for 1 h with a solution of MTT (2 mg/mL in PBS) [64,65]. Then, the solution was removed, and 100 μ L of DMSO was added to dissolve the crystals of formazan produced by the reduction in MTT. The viability of the cells was measured by absorbance at 550 nm using a microplate reader (Digiscan 340, Asys Hitech, Eugendorf, Austria) and calculated as a percentage of the control line (set at 100%).

4.5. Measurement of ROS Production

The 2,7-dichlorofluorescein diacetate (DCFH-DA), a fluorescent probe, was employed to measure the intracellular ROS production according to the protocol described by LeBel, Ischiropoulos, and Bondy [66]. After cell treatments, SH-SY5Y cells were incubated with DCFH-DA (0.01 M) dissolved in PBS-glucose for 30 min at 37 °C. After that, cells were washed twice with PBS-glucose, and fluorescence was measured after 2 h using a microplate fluorescence reader (FLx800, Bio-Tek Instruments, Winooski, VT, USA) at 480 nm excitation and 510 nm emission. All the operations were carried out in low ambient light, and the materials used were protected with foil paper.

4.6. TBARS Assay

Cells were treated as before, harvested, washed with PBS, and transferred into Eppendorf tubes to be stored at -80 °C. 1 μ L of each sample was used to measure the total

protein concentration using the bicinchoninic method [67,68]. For the assay, we prepared a solution of trichloroacetic acid (TCA), HCl 5N, and 2-thiobarbituric acid (TBA); 100 μ L of this solution was added to 50 μ L of pellets (defrosted at room temperature). The mixture was heated at 100 °C for 10 min. Then, samples were put on ice for 10 more minutes to stop the reaction. Eventually, they were centrifuged for 10 min at 4 °C at 3000 rpm, and 130 μ L of each sample was placed in a 96-well plate. We measured the absorbance at 530 nm using a microplate fluorescence reader (FLx800, Bio-Tek Instruments, Winooski, VT, USA). All the operations were carried out in low ambient light, and the materials used were protected with foil paper.

4.7. Glutathione Assay

As described by Hissin and Hilf [69], pellets were resuspended in phosphate-EDTA buffer (pH 8.0) and sonicated 3 times on ice for 10 s. The mixture was centrifuged at 4 °C at 2500 rpm for 10 min. After that, protein concentration was measured using the bicinchoninic method [68]. A solution of HClO₄ 1% *v/v* was added, and the samples were put in the vortex on ice for 5 min to allow precipitation. Samples were additionally centrifuged at 4 °C at 14,000 rpm for 10 min, and supernatants were used to measure GSH and GSSG. To measure GSH, 20 μ L of ortho-phthalaldehyde (OPT, 1 mg mL⁻¹ methanol) and 150 μ L of phosphate buffer (pH 8.0) were added to 50 μ L of each sample. GSSG concentration was determined by incubating supernatants with 3 μ L of N-ethylmaleimide (NEM) for 5 min and then adding 20 μ L of OPT and 150 μ L of NaOH buffer (pH 12.0). In both cases, fluorescence was measured after 15 min of incubation at an excitation wavelength of 350 nm and an emission wavelength of 420 nm using a microplate fluorescence reader (FLx800, Bio-Tek Instruments, Winooski, VT, USA). The data are expressed as the GSH/GSSG ratio.

4.8. Western Blot Assays

After treatments, SH-SY5Y cells were harvested and washed with PBS. Afterwards, cells were centrifuged at 2300 rpm for 5 min and whole-cell lysates were prepared with lysis buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 20 μ L/mL leupeptin, 10 μ L/mL pepstatin, 35 μ L/mL PMSF) on ice for 30 min. Following that, lysed cells were centrifuged at 1600 $\times$ g at 4 °C for 10 min.

To obtain nuclear extracts, cellular pellets were resuspended with the lysis buffer 10 mM HEPES (pH 7.9), 1 mM DTT, 10 mM KCl, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO₄, 10 mM Na₂MoO₄, 0.5 mM PMSF, 10 μ g/mL leupeptin and 1 μ g/mL pepstatin on ice for 15 min. After the incubation, 10% Nonidet P-40 was added and centrifuged at 13,000 rpm at 4 °C for 30 s to break cell membranes without damaging nuclear membranes. Then, the obtained pellet was resuspended in another lysis buffer (20 mM HEPES pH 7.9, 1 mM DTT, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.4 mM NaCl, 20% glicerol, 1 mM NaVO₄, 10 mM Na₂MoO₄, 0.5 mM PMSF, 10 μ g/mL leupeptin and 1 μ g/mL pepstatin) and then shook on vortex for 30 min at 4 °C. Eventually, samples were centrifuged at 4 °C for 5 min, and nuclear extracts were obtained (supernatants).

Protein extracts were separated using a 10% SDS-PAGE, transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA), and blocked for 60 min at room temperature with a blocking buffer containing 10% skimmed milk in PBS. Then, membranes were incubated for 1 h at room temperature with primary antibody against CAT (1:500), SOD1 (1:500), GR (1:500), GPx (1:500), β -actin (1:500), and Nrf2 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by 30 min washing with PBS-Tween solution (0.1% Tween). Eventually, membranes were incubated with anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. After being washed again for 30 min with PBS-Tween, membranes were incubated with ECL solution (Pierce™ ECL Western

Blotting Substrate) for 1 min. All Western blot experiments were performed in at least three independent biological replicates. β -actin was used as a Western blotting loading control. Densitometric analysis of the bands was carried out using Fiji ImageJ2 software (v. 1.54g) [70].

4.9. Statistical Analysis

Data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test with the software GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). A value of $p < 0.05$ was considered statistically significant.

5. Conclusions

Molecular docking is a valuable tool for predicting the binding affinity of compounds to a target, which can help prioritize scaffolds for further experimental evaluation. In this study, four compounds (1–4) were selected as potential inhibitors of the Keap1-Nrf2 protein–protein interaction and evaluated in SH-SY5Y cells under Fenton reagent-induced oxidative stress, a commonly used model for neurodegenerative disorders. Pre-treatment for 24 h produced the most pronounced protective effects, suggesting that the observed antioxidant response may rely on activation of endogenous cellular defense mechanisms rather than direct chemical scavenging.

The compounds appeared to exert antioxidant activity in part by promoting Nrf2 nuclear translocation, which was associated with upregulation of key antioxidant enzymes (CAT, SOD, GR, and GPx). This was accompanied by a partial restoration of glutathione homeostasis (GSH/GSSG ratio) and reductions in intracellular oxidative species and lipid peroxidation (TBARS) levels. Among the molecules tested, compound 3 showed the most consistent activity across several biochemical and functional endpoints, apparently primarily via Nrf2 activation, whereas other compounds displayed more selective effects. However, direct assays of protein–protein interactions were not performed, so additional mechanisms cannot be excluded.

These results support the hypothesis that pharmacological modulation of the Keap1-Nrf2 interaction may trigger a multi-component cellular antioxidant response. While further studies are required to elucidate the mechanisms and potential therapeutic relevance fully, these compounds represent promising starting points for the development of Nrf2-targeted strategies aimed at mitigating oxidative stress in neurodegenerative contexts. Future work will focus on scaffold optimization to improve efficacy, selectivity, and pharmacological properties, with in vivo validation needed to explore relevance in neurodegenerative contexts.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27041862/s1>.

Author Contributions: Conceptualization, S.D.V. and E.G.-B.; methodology, E.G.-B.; investigation, S.D.V., E.G.-B. and S.T.; resources, G.B. and M.P.G.-S.; writing—original draft preparation, S.D.V., E.G.-B. and M.G.C.; writing—review and editing, G.B. and M.P.G.-S.; supervision, G.B. and M.P.G.-S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by PRIN PNRR 2022 “P2022CKMPW—TACSI Driver: a multi-tasks platform to guide the Target identification, Assessment of the binding, Collection of natural products from waste, Synthesis of derivatives, and In vitro/In vivo polypharmacological profile evaluation of bioactive compounds.”, CUP D53D2301703001, missione 4 “Istruzione e ricerca” Componente 2 “Dalla ricerca all’impresa”—investimento 1.1 del Piano Nazionale di Ripresa e Resilienza (PNRR) finanziato dall’Unione Europea “Next Generation EU”.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

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

References

- Pieczyński, S.R.; Neustadt, J. Mitochondrial dysfunction and molecular pathways of disease. *Exp. Mol. Pathol.* **2007**, *83*, 84–92. [CrossRef] [PubMed]
- Lipinski, B. Hydroxyl radical and its scavengers in health and disease. *Oxid. Med. Cell. Longev.* **2011**, *2011*, 809696. [CrossRef] [PubMed]
- Valko, M.; Leibfriz, D.; Moncol, J.; Cronin, M.T.D.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44–84. [CrossRef] [PubMed]
- Ma, Q. Role of Nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* **2013**, *53*, 401–426. [CrossRef]
- Zhang, D.D. Mechanistic studies of the Nrf2-Keap1 signaling pathway. *Drug Metab. Rev.* **2006**, *38*, 769–789. [CrossRef]
- Huang, W.J.; Zhang, X.; Chen, W.W. Role of oxidative stress in Alzheimer's disease. *Biomed. Rep.* **2016**, *4*, 519–522. [CrossRef]
- Forman, H.J.; Zhang, H. Targeting oxidative stress in disease: Promise and limitations of antioxidant therapy. *Nat. Rev. Drug Discov.* **2021**, *20*, 689–709. Erratum in *Nat. Rev. Drug Discov.* **2021**, *20*, 652. <https://doi.org/10.1038/s41573-021-00267-5>. [CrossRef]
- Ray, P.D.; Huang, B.-W.; Tsuiji, Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signal.* **2012**, *24*, 981–990. [CrossRef]
- Dias, V.; Junn, E.; Mouradian, M.M. The role of oxidative stress in Parkinson's disease. *J. Park. Dis.* **2013**, *3*, 461–491. [CrossRef]
- Hassanzadeh, K.; Rahimmi, A. Oxidative stress and neuroinflammation in the story of Parkinson's disease: Could targeting these pathways write a good ending? *J. Cell. Physiol.* **2019**, *234*, 23–32. [CrossRef]
- Trist, B.G.; Hare, D.J.; Double, K.L. Oxidative stress in the aging substantia nigra and the etiology of Parkinson's disease. *Aging Cell* **2019**, *18*, e13031. [CrossRef]
- Rashmi, R.; Venugopal, R.B.; SubbaRao, M.V. Naturally occurring Nrf2 activators in the management of diabetes. *Nutr. Food Sci.* **2017**, *2*, 555–595. [CrossRef]
- Cassidy, L.; Fernandez, F.; Johnson, J.B.; Naiker, M.; Owoola, A.G.; Broszczak, D.A. Oxidative stress in Alzheimer's disease: A review on emergent natural polyphenolic therapeutics. *Complement. Ther. Med.* **2020**, *49*, 102294. [CrossRef] [PubMed]
- Cheignon, C.; Tomas, M.; Bonnefont-Rousselot, D.; Faller, P.; Hureau, C.; Collin, F. Oxidative stress and the amyloid beta peptide in Alzheimer's disease. *Redox Biol.* **2018**, *14*, 450–464. [CrossRef] [PubMed]
- Simunkova, M.; Alwasel, S.H.; Alhazza, I.M.; Jomova, K.; Kollar, V.; Rusko, M.; Valko, M. Management of oxidative stress and other pathologies in Alzheimer's disease. *Arch. Toxicol.* **2019**, *93*, 2491–2513. [CrossRef]
- Reuter, S.; Gupta, S.C.; Chaturvedi, M.M.; Aggarwal, B.B. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radic. Biol. Med.* **2010**, *49*, 1603–1616. [CrossRef]
- Wang, X.; Wang, W.; Li, L.; Perry, G.; Lee, H.-g.; Zhu, X. Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. *Biochim. Biophys. Acta* **2014**, *1842*, 1240–1247. [CrossRef]
- Cuadrado, A.; Manda, G.; Hassan, A.; Alcaraz, M.J.; Barbas, C.; Daiber, A.; Ghezzi, P.; León, R.; López, M.G.; Oliva, B.; et al. Transcription factor NRF2 as a therapeutic target for chronic diseases: A systems medicine approach. *Pharmacol. Rev.* **2018**, *70*, 348–383. [CrossRef]
- Dinkova-Kostova, A.T.; Abramov, A.Y. The emerging role of Nrf2 in mitochondrial function. *Free Radic. Biol. Med.* **2015**, *88*, 179–188. [CrossRef]
- Hayes, J.D.; Dinkova-Kostova, A.T. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem. Sci.* **2014**, *39*, 199–218. [CrossRef]
- Shih, A.Y.; Li, P.; Murphy, T.H. A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. *J. Neurosci.* **2005**, *25*, 10321–10335. [CrossRef] [PubMed]
- Jaramillo, M.C.; Zhang, D.D. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. *Genes Dev.* **2013**, *27*, 2179–2191. [CrossRef] [PubMed]
- Suzuki, T.; Motohashi, H.; Yamamoto, M. Toward clinical application of the Keap1–Nrf2 pathway. *Trends Pharmacol. Sci.* **2013**, *34*, 340–346. [CrossRef] [PubMed]
- Jain, A.D.; Potteti, H.; Richardson, B.G.; Kingsley, L.; Luciano, J.P.; Ryuzoji, A.F.; Lee, H.; Krunic, A.; Mesecar, A.D.; Reddy, S.P.; et al. Probing the structural requirements of non-electrophilic naphthalene-based Nrf2 activators. *Eur. J. Med. Chem.* **2015**, *103*, 252–268. [CrossRef]

25. Zhuang, C.; Narayanapillai, S.; Zhang, W.; Sham, Y.Y.; Xing, C. Rapid identification of Keap1–Nrf2 small-molecule inhibitors through structure-based virtual screening and hit-based substructure search. *J. Med. Chem.* **2014**, *57*, 1121–1126. [\[CrossRef\]](#)
26. Bello, M.; Morales-González, J.A. Molecular recognition between potential natural inhibitors of the Keap1–Nrf2 complex. *Int. J. Biol. Macromol.* **2017**, *105*, 981–992. [\[CrossRef\]](#)
27. Sun, H.; Zhu, J.; Lin, H.; Gu, K.; Feng, F. Recent progress in the development of small molecule Nrf2 modulators: A patent review (2012–2016). *Expert Opin. Ther. Pat.* **2017**, *27*, 763–785. [\[CrossRef\]](#)
28. Mou, Y.; Wen, S.; Li, Y.-X.; Gao, X.-X.; Zhang, X.; Jiang, Z.-Y. Recent progress in Keap1–Nrf2 protein–protein interaction inhibitors. *Eur. J. Med. Chem.* **2020**, *202*, 112532. [\[CrossRef\]](#)
29. Richardson, B.; Jain, A.; Speltz, T.; Moore, T. Non-electrophilic modulators of the canonical Keap1/Nrf2 pathway. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 2261–2268. [\[CrossRef\]](#)
30. Ma, B.; Lucas, B.; Capacci, A.; Lin, E.Y.-S.; Jones, J.H.; Dechantsreiter, M.; Enyedy, I.; Marcotte, D.; Xiao, G.; Li, B.; et al. Design, synthesis and identification of novel, orally bioavailable non-covalent Nrf2 activators. *Bioorg. Med. Chem. Lett.* **2020**, *30*, 126852. [\[CrossRef\]](#)
31. Qin, X.; Hou, R.; Qu, Z.; Yu, J.; Zhang, W.; Ma, H.; Zhuang, C. Structure-based molecular hybridization design and synthesis of Keap1–Nrf2 inhibitors for anti-inflammatory treatment. *Bioorg. Chem.* **2025**, *158*, 108350. [\[CrossRef\]](#)
32. Crisman, E.; Duarte, P.; Dauden, E.; Cuadrado, A.; Rodríguez-Franco, M.I.; López, M.G.; León, R. KEAP1–NRF2 protein–protein interaction inhibitors: Design, pharmacological properties and therapeutic potential. *Med. Res. Rev.* **2023**, *43*, 237–287. [\[CrossRef\]](#)
33. *Schrödinger Release 2021-1 Glide v. 7.4*; Schrödinger LLC.: New York, NY, USA, 2021.
34. Friesner, R.A.; Banks, J.L.; Murphy, R.B.; Halgren, T.A.; Klicic, J.J.; Mainz, D.T.; Repasky, M.P.; Knoll, E.H.; Shelley, M.; Perry, J.K.; et al. Glide: A new approach for rapid, accurate docking and scoring. 1. method and assessment of docking accuracy. *J. Med. Chem.* **2004**, *47*, 1739–1749. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Friesner, R.A.; Murphy, R.B.; Repasky, M.P.; Frye, L.L.; Greenwood, J.R.; Halgren, T.A.; Sanschagrin, P.C.; Mainz, D.T. Extra Precision Glide: docking and scoring incorporating a model of hydrophobic enclosure for protein–ligand complexes. *J. Med. Chem.* **2006**, *49*, 6177–6196. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Halgren, T.A.; Murphy, R.B.; Friesner, R.A.; Beard, H.S.; Frye, L.L.; Pollard, W.T.; Banks, J.L. Glide: A new approach for rapid, accurate docking and scoring. 2. enrichment factors in database screening. *J. Med. Chem.* **2004**, *47*, 1750–1759. [\[CrossRef\]](#) [\[PubMed\]](#)
37. *Schrödinger Release 2021-1: LigPrep*; Schrödinger LLC.: New York, NY, USA, 2021.
38. Xie, H.-R.; Hu, L.-S.; Li, G.-Y. SH-SY5Y human neuroblastoma cell line: In vitro cell model of dopaminergic neurons in Parkinson's disease. *Chin. Med. J.* **2010**, *123*, 1086–1092. [\[CrossRef\]](#)
39. Zhang, C.; Li, C.; Chen, S.; Li, Z.; Ma, L.; Jia, X.; Wang, K.; Bao, J.; Liang, Y.; Chen, M.; et al. Hormetic effect of panaxatriol saponins confers neuroprotection in PC12 cells and zebrafish through PI3K/AKT/mTOR and AMPK/SIRT1/FOXO3 pathways. *Sci. Rep.* **2017**, *7*, 41082. [\[CrossRef\]](#)
40. Halliwell, B.; Adhikary, A.; Dingfelder, M.; Dizdaroglu, M. Hydroxyl radical is a significant player in oxidative DNA damage in vivo. *Chem. Soc. Rev.* **2021**, *50*, 8355–8360. [\[CrossRef\]](#)
41. Liu, Q.; Hu, S.; He, Y.; Zhang, J.; Zeng, X.; Gong, F.; Liang, L. The protective effects of Zhen-Wu-Tang against cisplatin-induced acute kidney injury in rats. *PLoS ONE* **2017**, *12*, e0179137. [\[CrossRef\]](#)
42. Li, W.K.; Wang, G.F.; Wang, T.M.; Li, Y.Y.; Li, Y.F.; Lu, X.Y.; Wang, Y.H.; Zhang, H.; Liu, P.; Wu, J.S.; et al. Protective effect of herbal medicine Huangqi decoction against chronic cholestatic liver injury by inhibiting bile acid-stimulated inflammation in DDC-induced mice. *Phytomedicine* **2019**, *62*, 152948. [\[CrossRef\]](#)
43. Kim, H.J.; Park, C.; Kim, G.-Y.; Park, E.K.; Jeon, Y.-J.; Kim, S.; Hwang, H.J.; Choi, Y.H. *Sargassum serratifolium* attenuates RANKL-induced osteoclast differentiation and oxidative stress through inhibition of NF- κ B and activation of the Nrf2/HO-1 signaling pathway. *Biosci. Trends* **2018**, *12*, 257–265. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Zhao, Q.; Mao, A.; Yan, J.; Sun, C.; Di, C.; Zhou, X.; Li, H.; Guo, R.; Zhang, H. Downregulation of Nrf2 promotes radiation-induced apoptosis through Nrf2 mediated Notch signaling in non-small cell lung cancer cells. *Int. J. Oncol.* **2016**, *48*, 765–773. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Li, X.; Wang, H.; Gao, Y.; Li, L.; Tang, C.; Wen, G.; Zhou, Y.; Zhou, M.; Mao, L.; Fan, Y. Protective effects of quercetin on mitochondrial biogenesis in experimental traumatic brain injury via the Nrf2 signaling pathway. *PLoS ONE* **2016**, *11*, e0164237. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Simpson, D.S.A.; Oliver, P.L. ROS generation in microglia: Understanding oxidative stress and inflammation in neurodegenerative disease. *Antioxidants* **2020**, *9*, 743. [\[CrossRef\]](#)
47. Singh, A.; Kukreti, R.; Saso, L.; Kukreti, S. Oxidative stress: A key modulator in neurodegenerative diseases. *Molecules* **2019**, *24*, 1583. [\[CrossRef\]](#)
48. Pompella, A.; Visvikis, A.; Paolicchi, A.; Tata, V.D.; Casini, A.F. The changing faces of glutathione, a cellular protagonist. *Biochem. Pharmacol.* **2003**, *66*, 1499–1503. [\[CrossRef\]](#)

49. Guo, Y.; Zhang, X.; Wu, F.-G. A graphene oxide-based switch-on fluorescent probe for glutathione detection and cancer diagnosis. *J. Colloid Interface Sci.* **2018**, *530*, 511–520. [\[CrossRef\]](#)
50. Jeong, C.-H.; Joo, S.H. Downregulation of reactive oxygen species in apoptosis. *J. Cancer Prev.* **2016**, *21*, 13–20. [\[CrossRef\]](#)
51. Kayanoki, Y.; Fujii, J.; Islam, K.N.; Suzuki, K.; Kawata, S.; Matsuzawa, Y.; Taniguchi, N. The protective role of glutathione peroxidase in apoptosis induced by reactive oxygen species. *J. Biochem.* **1996**, *119*, 817–822. [\[CrossRef\]](#)
52. Zhang, L.; Li, J.; Zong, L.; Chen, X.; Chen, K.; Jiang, Z.; Nan, L.; Li, X.; Li, W.; Shan, T.; et al. Reactive oxygen species and targeted therapy for pancreatic cancer. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 1616781. [\[CrossRef\]](#)
53. Ribas, V.; García-Ruiz, C.; Fernández-Checa, J.C. Glutathione and mitochondria. *Front. Pharmacol.* **2014**, *5*, 151. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Tsikas, D. Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. *Anal. Biochem.* **2017**, *524*, 13–30. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Dasgupta, A.; Klein, K. Methods for measuring oxidative stress in the laboratory. In *Antioxidants in Food, Vitamins and Supplements*; Dasgupta, A., Klein, K., Eds.; Elsevier: San Diego, CA, USA, 2014; pp. 19–40.
56. Dawn-Linsley, M.; Ekinci, F.J.; Ortiz, D.; Rogers, E.; Shea, T.B. Monitoring thiobarbituric acid-reactive substances (TBARs) as an assay for oxidative damage in neuronal cultures and central nervous system. *J. Neurosci. Methods* **2005**, *141*, 219–222. [\[CrossRef\]](#)
57. Cunha, M.P.; Martín-de-Saavedra, M.D.; Romero, A.; Egea, J.; Ludka, F.K.; Tasca, C.I.; Farina, M.; Rodrigues, A.L.S.; López, M.G. Both Creatine and Its Product Phosphocreatine Reduce Oxidative Stress and Afford Neuroprotection in an In Vitro Parkinson's Model. *ASN Neuro* **2014**, *6*, 1759091414554945. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Correa, F.; Ljunggren, E.; Patil, J.; Wang, X.; Hagberg, H.; Mallard, C.; Sandberg, M. Time-dependent effects of systemic lipopolysaccharide injection on regulators of antioxidant defence Nrf2 and PGC-1 α in the neonatal rat brain. *Neuroimmunomodulation* **2013**, *20*, 185–193. [\[CrossRef\]](#)
59. Liang, L.-P.; Pearson-Smith, J.N.; Huang, J.; Day, B.J.; Patel, M. Neuroprotective effects of a catalytic antioxidant in a rat nerve agent model. *Redox Biol.* **2019**, *20*, 275–284. [\[CrossRef\]](#)
60. Lo, S.C.; Li, X.; Henzl, M.T.; Beamer, L.J.; Hannink, M. Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. *EMBO J.* **2006**, *25*, 3605–3617. [\[CrossRef\]](#)
61. *Schrödinger Release 2021-1: Protein Preparation Wizard*; Schrödinger LLC.: New York, NY, 2021.
62. Madhavi Sastry, G.; Adzhigirey, M.; Day, T.; Annabhimoju, R.; Sherman, W. Protein and ligand preparation: Parameters, protocols, and influence on virtual screening enrichments. *J. Comput.-Aided Mol. Des.* **2013**, *27*, 221–234. [\[CrossRef\]](#)
63. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63. [\[CrossRef\]](#)
64. Lee, H.-S.; Jeong, G.-S. Protective Effects of 6,7,4'-Trihydroxyflavanone on Hypoxia-Induced Neurotoxicity by Enhancement of HO-1 through Nrf2 Signaling Pathway. *Antioxidants* **2021**, *10*, 341. [\[CrossRef\]](#)
65. Garcimartín, A.; Merino, J.J.; González, M.P.; Sánchez-Reus, M.I.; Sánchez-Muniz, F.J.; Bastida, S.; Benedí, J. Organic silicon protects human neuroblastoma SH-SY5Y cells against hydrogen peroxide effects. *BMC Complement. Altern. Med.* **2014**, *14*, 384. [\[CrossRef\]](#)
66. LeBel, C.P.; Ischiropoulos, H.; Bondy, S.C. Evaluation of the probe 2',7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* **1992**, *5*, 227–231. [\[CrossRef\]](#)
67. Uchiyama, M.; Mihara, M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.* **1978**, *86*, 271–278. [\[CrossRef\]](#)
68. Smith, P.K.; Krohn, R.I.; Hermanson, G.T.; Mallia, A.K.; Gartner, F.H.; Provenzano, M.D.; Fujimoto, E.K.; Goeke, N.M.; Olson, B.J.; Klenk, D.C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76–85. [\[CrossRef\]](#)
69. Hissin, P.J.; Hilf, R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* **1976**, *74*, 214–226. [\[CrossRef\]](#)
70. Rueden, C.T.; Schindelin, J.; Hiner, M.C.; DeZonia, B.E.; Walter, A.E.; Arena, E.T.; Eliceiri, K.W. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinform.* **2017**, *18*, 529. [\[CrossRef\]](#)

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.