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Baicalein Protects against 6-OHDA-Induced Neurotoxicity through Activation of Keap1/Nrf2/HO-1 and Involving PKCα and PI3K/AKT Signaling Pathways

Zaijun Zhang,[†,‡,§,+] Wei Cui,[∥,+] Guohui Li,[⊥] Shuai Yuan,[†,‡] Daping Xu,[†,‡] Maggie P.M. Hoi,[†,‡] Zhixiu Lin,[#] Juan Dou,[▽,○] Yifan Han,*[,∥] and Simon M.Y. Lee*[,†,‡]

[†]State Key Laboratory of Quality Research in Chinese Medicine (University of Macau), Macao SAR, China

[‡]Institute of Chinese Medical Sciences, University of Macau, Macao SAR, China

[§]Institute of New Drug Research, Guangdong Province Key Laboratory of Pharmacodynamic, Constituents of Traditional Chinese Medicine & New Drug Research, College of Pharmacy, Jinan University, Guangdong, China

[∥]Department of Applied Biology and Chemical Technology, Institute of Modern Medicine, The Hong Kong Polytechnic University, Hong Kong SAR, China

[⊥]Department of Chemistry, Hong Kong University, Hong Kong SAR, China

[#]School of Chinese Medicine, The Chinese University of Hong Kong, Hong Kong SAR, China

[▽]Department of Pediatrics, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

[○]Departments of Pharmacology and Neurology, Emory University School of Medicine, Atlanta, Georgia 30322, United States

ABSTRACT: Baicalein, one of the major flavonoids found in *Scutellaria baicalensis* Georgi, displays neuroprotective effects on experimental models of Parkinson's disease (PD) in vitro and in vivo. Although the antioxidative and/or anti-inflammatory activity of baicalein likely contributes to these neuroprotective effects, other modes of action remain largely uncharacterized. In the present study, baicalein pretreatment significantly prevented cells from 6-hydroxydopamine (6-OHDA)-induced damage by attenuating cellular apoptosis. However, post-treatment with baicalein did not show any restorative effect against 6-OHDA-induced cellular damage. We found that baicalein increased transcriptional factor NF-E2-related factor 2 (Nrf2)/hemo oxygenase 1(HO-1) protein expression and decreased Kelch-like ECH-associated protein 1 (Keap1) in a time- and concentration-dependent manner in PC12 cells. In addition, baicalein induced Nrf2 nuclear translocation and enhanced antioxidant response element (ARE) transcriptional activity, which conferred cytoprotection against 6-OHDA-induced oxidative injury. Moreover, we demonstrated that cytoprotective effects of baicalein could be attenuated by Nrf2 siRNA transfection and the HO-1 inhibitor zinc protoporphyrin (Znpp) as well as the proteasome inhibitor MG132. Furthermore, PKCα and AKT protein phosphorylation were up-regulated by baicalein pretreatment, and selective inhibitors targeted to PKC, PI3K, and AKT could block the cytoprotective effects of baicalein. Taken together, our results indicate that baicalein prevented PC12 cells from 6-OHDA-induced oxidative damage via the activation of Keap1/Nrf2/HO-1, and it also involves the PKCα and PI3K/AKT signaling pathway. Ultimately, the neuroprotective effects of baicalein may endue baicalein as a promising candidate for the prevention of PD.

KEYWORDS: *Parkinson's disease, oxidative stress, 6-OHDA, baicalein, neuroprotection, Nrf2*

INTRODUCTION

Parkinson's disease (PD) is characterized by a selective and progressive loss of dopaminergic neurons in the central nervous system (CNS), resulting in motor deficits. Although the molecular mechanisms responsible for this neuronal death remain unclear, accumulating evidence has demonstrated that oxidative stress plays a pivotal role in dopaminergic cell death in PD.[1] 6-OHDA, a hydroxylated analogue of the natural neurotransmitter dopamine, induces cytotoxicity selective for catecholaminergic neurons due to its uptake into the neuron via dopamine and noradrenergic transporter.[2] Previous studies have demonstrated that 6-OHDA induces neurodegeneration through the processing of hydrogen peroxide and hydroxyl radicals in the presence of iron and seems to be toxic to the mitochondrial complex I, leading to the formation of superoxide free radicals.[3] Furthermore, studies have suggested that 6-OHDA treatment reduces the content of striatal glutathione (GSH) and enzyme activity of superoxide dismutase (SOD) and increases lipid peroxidation and malondialdehyde (MDA) levels.[4]

Previous studies have revealed the protective effects of Nrf2 activation in the reduction of oxidative stress in various models of neurodegenerative disorders.[5] In addition, the loss of Nrf2 is associated with increased vulnerability to 6-OHDA both in vitro and in vivo.[6] The transplantation of overexpressing Nrf2 in astroglial cells resulted in a significant reduction in the susceptibility to 6-OHDA neurotoxicity.[7] Therefore, these findings suggest that Nrf2 represents a promising target for PD treatment.

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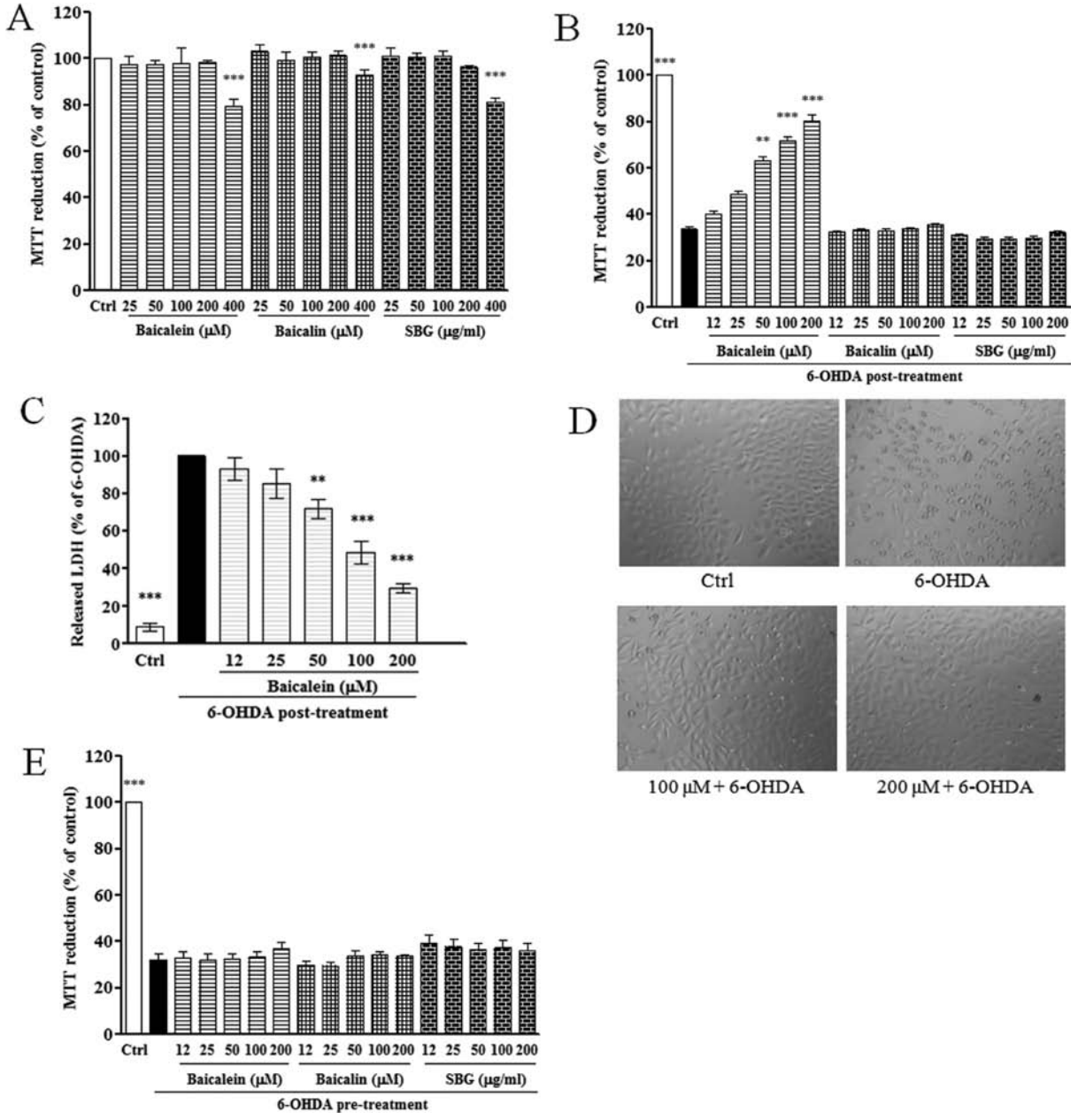

Figure 1. Effect of baicalein on 6-OHDA-induced PC12 cells damage. PC12 cells were plated in 96-well plate, treated with baicalein or vehicle for 12 h, prior or after exposure to 1 mM 6-OHDA. (A) Cytotoxicity evaluation of baicalein, baicalin, and SBG for 12 h of treatment on PC12 cells. (B) PC12 cells were pretreated with baicalein, baicalin, and SBG for 12 h prior to 6-OHDA exposure for another 8 h. The cell viability was measured by MTT assay. (C) Effect of baicalein pretreatment on 6-OHDA-induced LDH release. (D) Morphological changes in PC12 cells were observed by phase contrast microscopy. (E) PC12 cells were pretreated with 1 mM 6-OHDA for 2 h followed by 12 h of treatment with baicalein. The cell viability was measured by MTT assay. Data from three times independent experiments were expressed as means ± SEMs; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs 6-OHDA treatment alone, respectively.

For instance, a previous study reported that L-deprenyl (Selegiline), a pharmacological agent applied in the clinical treatment of PD, may exert neuroprotective effects, in part, through the stimulation of Nrf2 activity.[8]

Flavonoids are a family of polyphenolic compounds found ubiquitously in fruits and vegetables as well as in food products and beverages derived from plants. Recently, several natural flavonoids, such as curcumin,[9] luteolin,[10] and (−)-epigallocatechin-3-gallate (EGCG),[11] were found to exert beneficial effects through activation of the Nrf2/HO-1 signaling pathway. Baicalein, one of the main flavonoids extracted from the root of *Scutellaria baicalensis* Georgi (SBG), exerts several pharmacological effects, including protective effects associated with the cardiovascular system,[12] antibacterial and antiviral activity,[13,14] and anticancer activity.[15] In the CNS, it showed that baicalein exerted protective effects against neuronal death induced by cerebral ischemia,[16] enhanced learning and memory in rats,[17] and prevented against neuronal damage induced by 6-OHDA or MPTP in both in vitro and in vivo models of PD.[18−22]

Previous studies suggested that the mechanism of action underlying the neuroprotective effects of baicalein were associated with antioxidative action,[18−20,22] the attenuation of mitochondrial dysfunction, antiapoptotic effects, inhibition of JNK activation,[18] anti-inflammatory action,[20,22] and inhibition of

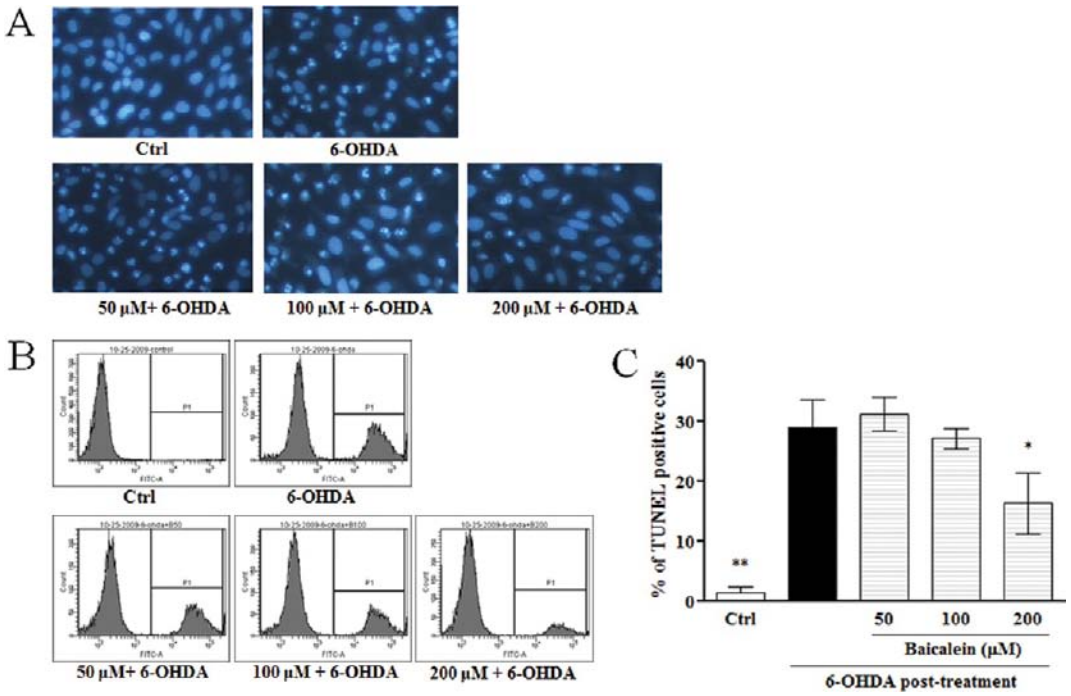

Figure 2. Baicalein inhibits 6-OHDA-induced apoptosis in PC12. PC12 cells were seeded in six-well plates and treated with baicalein (50−200 μM) or vehicle for 12 h, and then, the medium was replaced with 1 mM 6-OHDA or vehicle for 8 h. (A) Apoptotic cells were detected by Hoechst 33342 staining and were observed under fluorescent microscopy (20×). (B) TUNEL assays were performed according to the manufacturer's instructions and were analyzed by flow cytometry. (C) Quantificative analysis of TUNEL assay. *P < 0.05 vs 6-OHDA treatment alone.

α-synuclein oligomerization and fibrillation.[23] However, relatively little is known concerning the effect of baicalein on Nrf2-ARE and related upstream signaling pathways, such as protein kinase C (PKC)[24] and phosphatidylinositol 3-kinase (PI3K)/AKT.[25] In the present study, we investigated the cytoprotective mechanism of baicalein against 6-OHDA-induced oxidative damage in PC12 cells.

MATERIALS AND METHODS

Reagents. Baicalein, baicalin, and sulforaphane (SFN) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The ethanolic extract of SBG was purchased from Chengdu MUST Biotechnology. Co., Ltd. (Chengdu, China). 6-OHDA and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Opti-MEM I Reduced-Serum Medium was purchased from Molecular Probes/Invitrogen (Eugene, OR). F-12K, horse serum, fetal bovine serum, penicillin, and streptomycin were supplied by Gibco BRL (Grand Island, NY). Antibodies against phospho-PKCα, PKC, phospho-Akt, Akt, keap1, and β-actin were purchased from Cell Signaling Technology (Beverly, MA), and the monoclonal antibody against HO-1 was purchased from Abcam (Cambridge, MA). The monoclonal antibody against Nrf2 was purchased from Novus Biologicals (Littleton, CO). Phorbol 12-myristate 13-acetate (PMA), Wortmannin, AKT inhibitor IV, and HO-1 inhibitor (zinc protoporphyrin, ZnPP) were obtained from Calbiochem (San Diego, CA). All other chemicals and reagents used in this study were of analytical grade.

PC12 Cell Line Culture. Rat pheochromocytoma cells (PC12) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained at 37 °C in an incubator with 95% humidified atmosphere and 5% CO_2 and were cultured in F-12K medium containing 15% (v/v) heat-inactivated horse serum, 2.5% (v/v) FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cell culture media were replaced every other day. Baicalein was dissolved in dimethylsulfoxide (DMSO), and the stock solutions (100 mM) were added directly to the culture media. The control cells were treated with medium containing DMSO only. The final concentration of solvent was always less than 0.3%.

Primary Cerebellar Granule Neurons (CGNs) Culture. CGNs were prepared from 8 day old Sprague−Dawley rats (The Animal Care Facility, The Hong Kong Polytechnic University), as described in a previous publication.[26] Briefly, neurons were seeded at a density of 2.7×10^5 cells/mL in basal modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, and penicillin (100 units/mL) and streptomycin (100 μg/mL). Cytosine arabinoside (10 μM) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. With the use of this protocol, more than 95% of the cultured cells were granule neurons. After 7 days, CGNs cultured in vitro have acquired several features of mature neurons.[27] Therefore, experiments were performed at 8 days in vitro (DIV).

MTT Assay. PC12 cells were plated at a density of 1×10^4 cells/100 μL per well in 96-well plates. After the cells were starved, the serum-free medium was replaced with fresh low-serum medium (0.5% FBS) containing various concentrations of baicalein or vehicle, and then, the cells were maintained for 12 h. The cells were then exposed to 1 mM 6-OHDA or vehicle in fresh low-serum medium for 8 h. The cell viability was measured by the MTT assay. The absorbance was measured with a WallacVictor3 V microplate reader at 570 nm (Perkin-Elmer, The Netherlands).

Lactate Dehydrogenase (LDH) Assay. The cell viability was also determined by measuring the activity of LDH released into the incubation medium released following damage to the cellular membranes. Total and released LDH activities were measured according to the instructions provided by the Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany). Absorbance was detected using a microplate reader at 490 nm. LDH released was normalized to LDH release of 6-OHDA group, and the results were shown as a percentage of the 6-OHDA group.

Fluorescein Diacetate (FDA)/Propidium Iodide (PI) Double Staining Assay. Viable granule neurons were stained with fluorescein formed from FDA, which is de-esterified only by living cells. PI can penetrate the cell membranes of dead cells to intercalate into double-stranded nucleic acids. Briefly, after incubation with 10 μg/mL FDA and

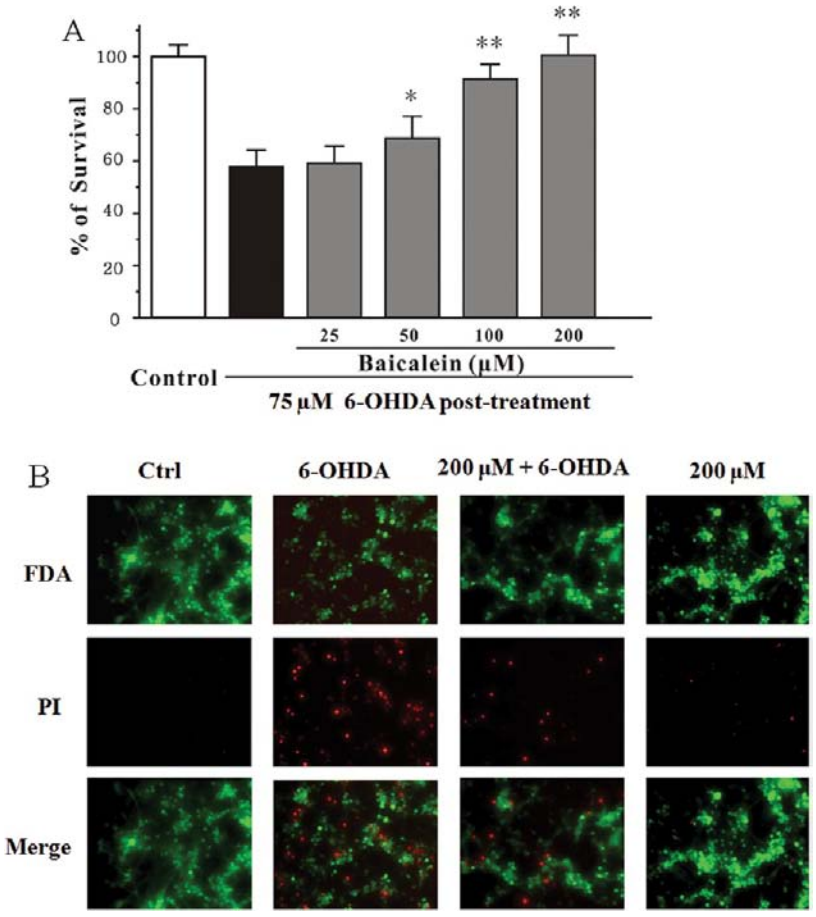

Figure 3. Baicalein prevents 6-OHDA-induced primary cultured CGNs death. CGNs were treated with baicalein at the indicated concentrations for 2 h and then exposed to 75 μM for another 24 h. (A) Cell viability was measured by MTT assay. Data from three times independent experiments were expressed as means ± SEMs; *P < 0.05 and **P < 0.01 vs 6-OHDA treatment alone, respectively. (B) Baicalein blocked neuronal loss and reversed the morphological alterations induced by 6-OHDA. CGNs were preincubated with or without 200 μM baicalein and then exposed to 75 μM 6-OHDA for 2 h. Two hours after the 6-OHDA challenge, CGNs were assayed with FDA/PI double staining. The neurons were also visualized by Hoechst 33342 staining. The cell morphological alterations were visualized using a fluorescence microscope (40× magnification).

5 μg/mL PI for 15 min, the neurons were examined and photographed using UV light microscopy. The photographs were then compared with neurons photographed under phase contrast microscopy.

Hoechst 33342 Staining. PC12 cells were seeded in a six-well plate at a density of 2×10^5 cells per well in 2 mL of medium. After treatment, the cells were washed with ice-cold PBS, stained with Hoechst 33342 (10 μg/mL) with RNase (5 μg/mL) in PBS for 15 min at room temperature, and were then fixed for 15 min in 1% (w/v) paraformaldehyde. Images were recorded with a fluorescence microscope (Carl Zeiss, Axiovert 200, Taunton, MA) equipped with a camera (Carl Zeiss, AxioCam HRc).

Flow Cytometric TUNEL Analysis. The TUNEL assay was performed using the APO-BrdU TUNEL Assay Kit (Molecular Probes) according to the manufacturer's instructions. After they were stained, cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). TUNEL-positive cells were considered apoptotic.

Immunofluoresence. The cells were washed three times with ice-cold PBS and were fixed by 4% paraformaldehyde for 15 min at room temperature. The cells were then washed three times with PBS (containing 1% BSA) and were blocked with blocking buffer (containing 0.3% Triton X-100 and 1% BSA in PBS) for 45 min at room temperature. The cells were incubated with anti-Nrf2 antibody (1:200 diluted in blocking buffer) overnight at 4 °C. The cells were then washed three times with PBS and were incubated with Alexa Fluor 488-conjugated antibody (1:200) (Invitrogen) for 45 min at 37 °C. To visualize the nuclei, the cells were counterstained 300 nM DAPI for 10 min. The fluorescence images were captured using a fluorescence microscope.

Nrf2 Transcriptional Activity. The Nrf2 transcriptional activity was monitored by Cignal Antioxidant Response Reporter (luc) Kit (SABiosciences, Frederick, MD). PC12 cells were seeded on a 96-well plate at a density of 8×10^3 cells per well. After 24 h, 150 ng of the ARE reporter construct was transfected using the SureFECT transfection reagent according to manufacturer's protocol (SABiosciences). Thirty hours after transfection, the cells were treated with vehicle (0.1% DMSO) or baicalein for 12 h in low-serum medium. Firefly and Renilla luciferase activity was determined using the Dual-Glo luciferase assay system from Promega (Madison, WI) according to the manufacturer's instructions.

Preparation of Whole Cell, Cytosolic, and Nuclear Extracts. For the whole cell protein extraction, PC12 cells were washed with ice-cold PBS for three times and then with RIPA lysis buffer containing 1% PMSF (Sigma) and 1% Protease Inhibitor Cocktail (Roche Applied Science) and were incubated for 30 min on ice. Cell lysates were centrifuged at 12500g for 20 min at 4 °C, and the supernatant was collected and stored at −80 °C. The subcellular fractionation was conducted according to manufacturer's protocol of nuclear extraction kit (Cayman Chemical, Ann Arbor, MI). The protein content was assayed using the BCA protein quantification kit.

Nrf2 siRNA Transfection. PC12 cells were seeded with 8×10^3 cells per well in 96-well plates or 2×10^5cells per well in six-well plates with growth medium. Until they achieved 40−60% confluence after incubation, cells were transfected with Nrf2 siRNA or scrambled RNA (Santa Cruz Biotechnology, Santa Cruz, CA) using SureFECT transfection reagent according to the manufacturer's instructions

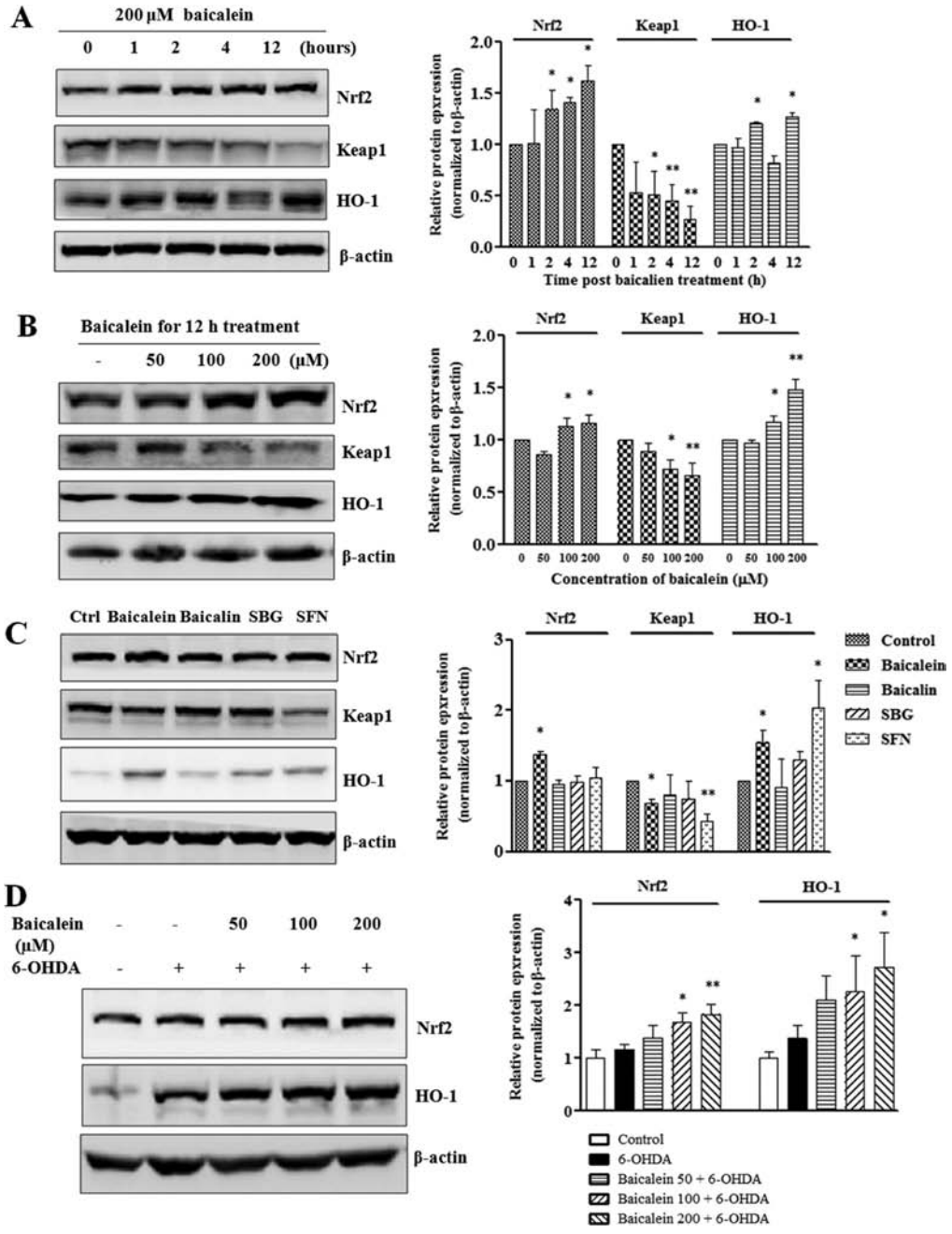

Figure 4. Baicalein activates Nrf2/HO-1 signaling pathway in the PC12 cells treated with or without 6-OHDA. (A and B) Baicalein increases Nrf2/HO-1 protein expression and decreases Keap1 protein expression in a time- and concentration-dependent manner. PC12 cells were treated alone with baicalein at the indicated times (A) or concentrations (B). The proteins were then prepared for Western blotting with antibodies that recognize Nrf2 and Keap1 and HO-1. (C) Comparison of 200 μM baicalein, 200 μM baicalin, 200 μg/mL SBG, and 20 μM SFN for 12 h of treatment on regulation of Nrf2, Keap1, and HO-1 proteins expression. Data from three times independent experiments were expressed as means ± SEMs; $^*P < 0.05$ and $^{**}P < 0.01$ vs untreated control (untreated group), respectively. (D) Baicalein pretreatment concentration dependently increases Nrf2 and HO-1 protein expression in 6-OHDA-treated PC12 cells. Data from three times independent experiments were expressed as means ± SEMs; $^*P < 0.05$ and $^{**}P < 0.01$ vs 6-OHDA treatment group, respectively.

(SABiosciences). After drug treatment, cell viability was determined by the MTT assay in a 96-well plate. The protein extraction was performed in six-well plate for further Western blotting analysis.

Western Blotting. Protein samples (30 μg) were resolved by SDS-PAGE and transferred to PVDF membranes. The blots were analyzed with the appropriate primary antibodies (1:1000), and peroxidase-conjugated secondary antibodies (1:2500) were used to detect the proteins of interest by enhanced chemiluminescence.

Determination of SOD, Catalase, and MDA Levels in PC12 Cells. The SOD, catalase, and MDA levels in PC12 cells were quantified by commercial kits (Beyotime Institute of Biotechnology, Beijing, China). After treatment, the cells were harvested using a cell scraper and were lysed by RIPA buffer on ice for 15 min. Lysed cells were centrifuged at 12000*g* for 20 min to remove debris. The supernatant was subjected to measurement of SOD, catalase, and MDA levels as well as protein content according to the manufacturer's protocol. Those levels were normalized to milligrams of protein in the samples. To avoid detergents in RIPA buffer affecting measurement of SOD and catalase activities, an equal volume of RIPA buffer to test sample was used as a blank control in the SOD and catalase test.

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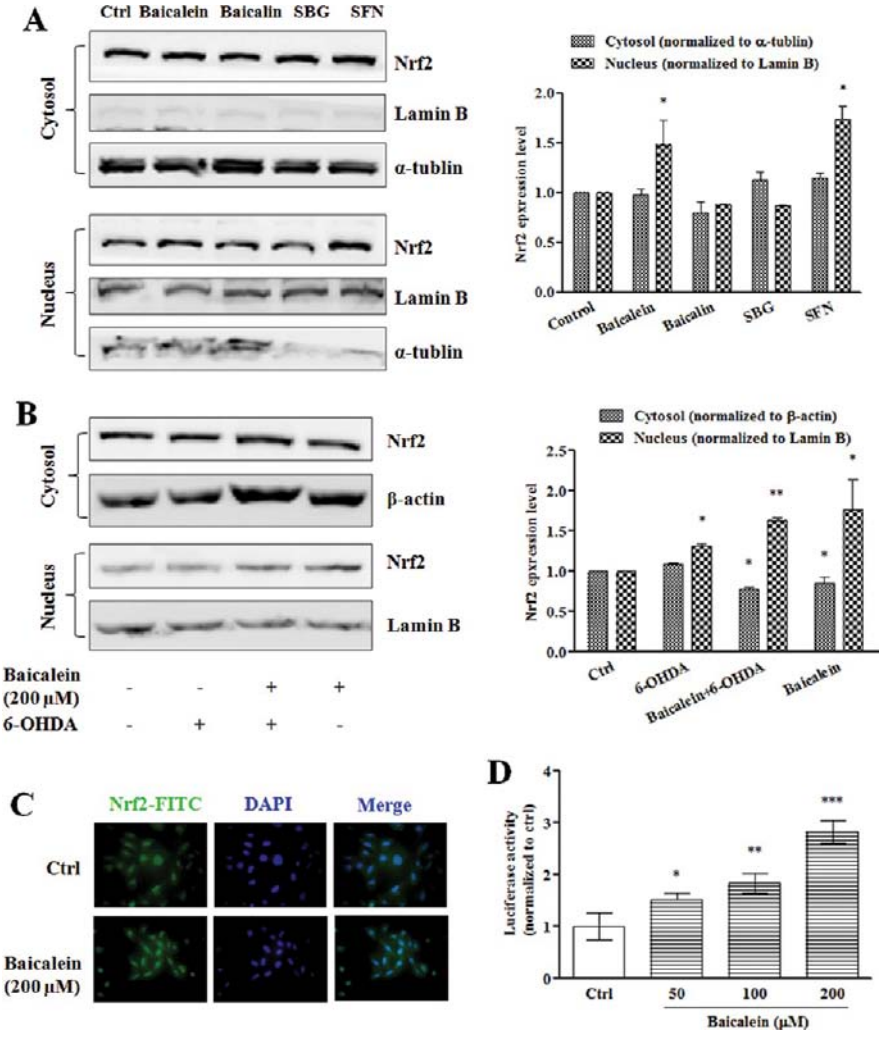

Figure 5. Baicalein induces Nrf2 nuclear translocation and increases its transcriptional activity. PC12 cells were pretreated with baicalein, baicalin, and SBG for 12 h, followed by incubation with 1 mM 6-OHDA for an additional 2 h. The cytosolic and nuclear extracts were prepared for Western blotting. (A) Comparison of 200 μM baicalein, 200 μM baicalin, 200 μg/mL SBG, and 20 μM SFN on nuclear Nrf2 protein expression. (B) Baicalein increased nuclear Nrf2 protein accumulation. (C) Fluorescent image (20×) of FITC-conjugated secondary antibody staining indicated the location of Nrf2 (green) by anti-Nrf2 antibody, DAPI staining indicated the location of the nucleus (blue), and the merged image of baicalein-treated cells shows the nuclear localization of Nrf2 protein. (D) PC12 cells were transfected with the ARE-reporter system plasmids, as described in the Materials and Methods, and then treated with baicalein (50−200 μM). After 12 h, the cells were harvested, and luciferase activities were measured. Data from three times independent experiments were expressed as means ± SEMs; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs control (untreated group), respectively.

SOD catalyzes the dismutation of superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2). The xanthine−xanthine oxidase system generates O_2^- that, in turn, reduced WST-1 to their corresponding formazan absorbing at 450 nm. After the addition of all solutions in each well as described in the protocol of manufacturer, the microplate was stirred thoroughly and then incubated at 37 °C for 20 min. The absorbance at 450 nm of the end point reaction (20 min) was measured by using a microplate reader. The percentage inhibition of each sample was calculated by using the following equation: $[(A1 - A3) - (AS - A2)]/(A1 - A3) \times 100$, where A1, A2, A3, and AS were the absorbance at 450 nm for uninhibited test, blank sample, blank reagent, and sample, respectively. The SOD activity was directly calculated using a plot of the percentage inhibition versus the amount of SOD in mg/mL. One unit of SOD activity was defined as the amount of the enzyme in a sample solution causing 50% inhibition (IC_{50}) of the rate of reduction of tetrazolium salt.

For measurement of catalase activity, briefly, 20 μL of cell lysates was added to a 96-well plate containing 180 μL of 50 mM phosphate buffer (pH 7.0). The reaction was started by the addition of 100 μL of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm.

The MDA level was assayed by spectrophotometrically measuring the thiobarbituric acid-reactive substances (TBARS) levels at 532 nm. Fifty microliters of cell lysates was mixed with 250 μL of tricholoroacetic acid (TBA) (10% w/v) solution followed by boiling in a water bath for 15 min. After they were cooled to room temperature, the samples were centrifuged at 3000 rpm for 10 min, and 200 μL of each sample supernatant was transferred to a test tube containing 100 μL of TBA solution (0.67% w/v). Each tube was then placed in boiling water for 15 min. After they were cooled to room temperature, samples were transferred to a 96-well plate, and the absorbance was measured at 532 nm with respect to the blank solution.

Statistical Analysis. Measurements were performed three times independently for multiple biological samples. The data were expressed as means ± standard errors of the mean (SEMs) and analyzed by GraphPad Prism 5.0 (GraphPad, San Diego, CA). One-way analysis of variance and Dunnett's test were used to evaluate the statistical differences. The value of statistical significance was set at $P < 0.05$.

RESULTS

Baicalein Prevents but Does Not Restore 6-OHDA-Induced Neurotoxicity in PC12 Cells. The evaluation of toxicity indicated that baicalein and baicalin up to 200 μM and SBG up to 200 μg/mL did not show toxic effects to PC12 cells (Figure 1A). Then, their cytoprotective and cytorestorative effects were assessed at nontoxic concentrations. At the initial stage of establishment of cytoprotective assay, the effects of serial concentrations (0.25−2 mM) and different treatment times of 6-OHDA on damage of PC12 cells were determined. Finally, the optimal concentration and treatment time of of 6-OHDA were found to be 1 mM and 8 h, respectively, which leads to damage of about 60% PC12 cells (data not shown). Therefore, this optimal 6-OHDA treatment condition on PC12 cells was used in subsequent experiments. The results from MTT assay demonstrated that pretreatment of PC12 cells with various concentrations of baicalein (12−200 μM), but not baicalin and SBG, could concentration dependently increase PC12 cells viability following 6-OHDA treatment (Figure 1B). Similarly, pretreatment with baicalein significantly reduced the LDH leakage caused by 6-OHDA (Figure 1C). Notably, cellular morphological observation also showed that baicalein prevented the loss of PC12 cells and reversed the morphological alterations, including cells detachment and the shrinkage of cell bodies induced by 6-OHDA (Figure 1D). However, in a post-treatment plan, neither of them showed a significantly restorative effect against 6-OHDA-induced PC12 cells damage (Figure 1E).

In addition, Hoechst 33342 staining and TUNEL assay was used to evaluate PC12 cells apoptosis. As shown in Figure 2A, normal cells were circular or elliptical with no nuclear condensation or fragmentation. In contrast, bright condensed dots indicative of apoptotic bodies were clearly identified in 6-OHDA-treated PC12 cells. Pretreatment with 200 μM baicalein significantly attenuated 6-OHDA-induced nuclear condensation. The result of TUNEL assay followed by flow cytometeric analysis was consistent with Hochest 33342 staining in that 200 μM baicalein significantly decreased TUNEL-positnive cells (Figure 2B,C).

Baicalein Prevents 6-OHDA-Induced Primary Cultured CGNs Death. The protective effect of baicalein was further assessed on primary cultured CGNs, and CGNs were pretreated with serial concentrations of baicalein (25−200 μM) for 12 h and then exposed to 6-OHDA for another 24 h. CGNs were found to be much more sensitive to 6-OHDA toxicity in that 75 μM 6-OHDA significantly decreased the cell survival rate. Baicalein prevented 6-OHDA-induced death of CGNs in a concentration-dependent manner (Figure 3A). FDA/PI double staining assay showed that baicalein significantly blocked 6-OHDA-induced loss of neurons and reversed 6-OHDA-induced morphological alterations, including unhealthy bodies and the compromised extensive neuritic network (Figure 3B).

Baicalein Activates Nrf2/HO-1 Signaling Pathway in the PC12 Cells Treated with or without 6-OHDA. Exposure of PC12 cells 200 μM baicalein resulted in a time-dependent increase in Nrf2 protein expression, and an increase in HO-1 expression was correlated with the changes seen in Nrf2 expression. However, expression of Keap1, the negative regulator of Nrf2, was decreased in a time-dependent manner (Figure 4A). PC12 cells were then treated with different concentrations of baicalein for 12 h, and baicalein up-regulated Nrf2/HO-1 protein expression and down-regulated Keap1 protein expression in a concentration-dependent manner, as shown in Figure 4B. We further compared the effect of baicalein (200 μM), baicalin (200 μM), and SBG (200 μg/mL) treatments on Nrf2/HO-1 induction; it was found that baicalein was more potent on altering these proteins than baicalin and SBG. Another well-studied Nrf2 activator SFN (20 μM), a phytochemical in plant foods, also significantly down-regulated Keap1 and up-regulated HO-1 expression (Figure 4C).

In addition, we investigated the effects of baicalein on Nrf2/HO-1 expressions in 6-OHDA-treated PC12 cells. The result showed that 6-OHDA slightly but not significantly changed the expression level of Nrf2 and HO-1, while baicalein concentration dependently increased the protein expression of Nrf2 and HO-1 as compared to 6-OHDA treatment alone (Figure 4D).

Baicalein Induces Nrf2 Nuclear Translocation and Transcriptional Activation. We further compared the effects of baicalein, baicalin, and SBG treatments on nuclear protein level of Nrf2 and examined whether baicalein induced Nrf2 nuclear translocation and increased its transcriptional activity. Subcellular fractions were prepared for Western blotting analysis, baicalein and SFN, but not baicalin or SBG could increase the nuclear Nrf2 protein level (Figure 5A). Moreover, no matter baicalein treatment with or without 6-OHDA could significantly decrease the cytosolic Nrf2 protein level and increase the nuclear level of Nrf2, while 6-OHDA treatment alone mildly increased the nuclear expression level of Nrf2 (Figure 5B). The cellular immunofluorescence with Nrf2-specific antibody also demonstrated that baicalein increased Nrf2 nuclear accumulation (Figure 5C). Moreover, we analyzed the induction of baicalein on a luciferase reporter containing three tandem sequences of the Nrf2-dependent antioxidant response element (ARE-LUC). Baicalein increased the transcriptional activity of Nrf2-ARE in a concentration-dependent manner (Figure 5D).

Baicalein Rescues the Loss of Antioxidant Enzyme Activities and Reduces Lipid Peroxidation in 6-OHDA-Treated PC12 Cells. We found that the treatment of PC12 cells with 6-OHDA caused a dramatic decrease in the activities of SOD and catalase but an increase in the intracellular MDA level. In this experimental condition, the pretreatment with baicalein significantly rescued the decrease of SOD and catalase activity and reduced intracellular MDA levels in a concentration-dependent manner (Table 1).

Table 1. Effect of Baicalein on Activities of Antioxidant Enzymes and Lipid Peroxidation in 6-OHDA-Treated PC12 Cells[a]

	U/mg protein		
groups	SOD	catalase	MDA (μmol/mg protein)
control	68.60 ± 2.23***	1.67 ± 0.60*	0.77 ± 0.19***
6-OHDA	8.62 ± 2.82	0.72 ± 0.05	3.44 ± 1.99
50 μM + 6-OHDA	24.62 ± 1.25**	1.17 ± 0.02*	2.66 ± 0.62
100 μM + 6-OHDA	37.48 ± 5.73**	1.27 ± 0.18*	2.34 ± 0.28
200 μM + 6-OHDA	67.26 ± 26.38***	1.76 ± 0.36**	1.13 ± 0.7*

[a]PC12 cells were pretreated with the indicated concentrations of baicalein for 12 h and then exposed to 1 mM 6-OHDA for 2 h. Cells were then harvested and lysed for the determination of SOD, catalase, and MDA level. Data from three times independent experiments were expressed as means ± SEMs. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs 6-OHDA treatment alone, respectively.

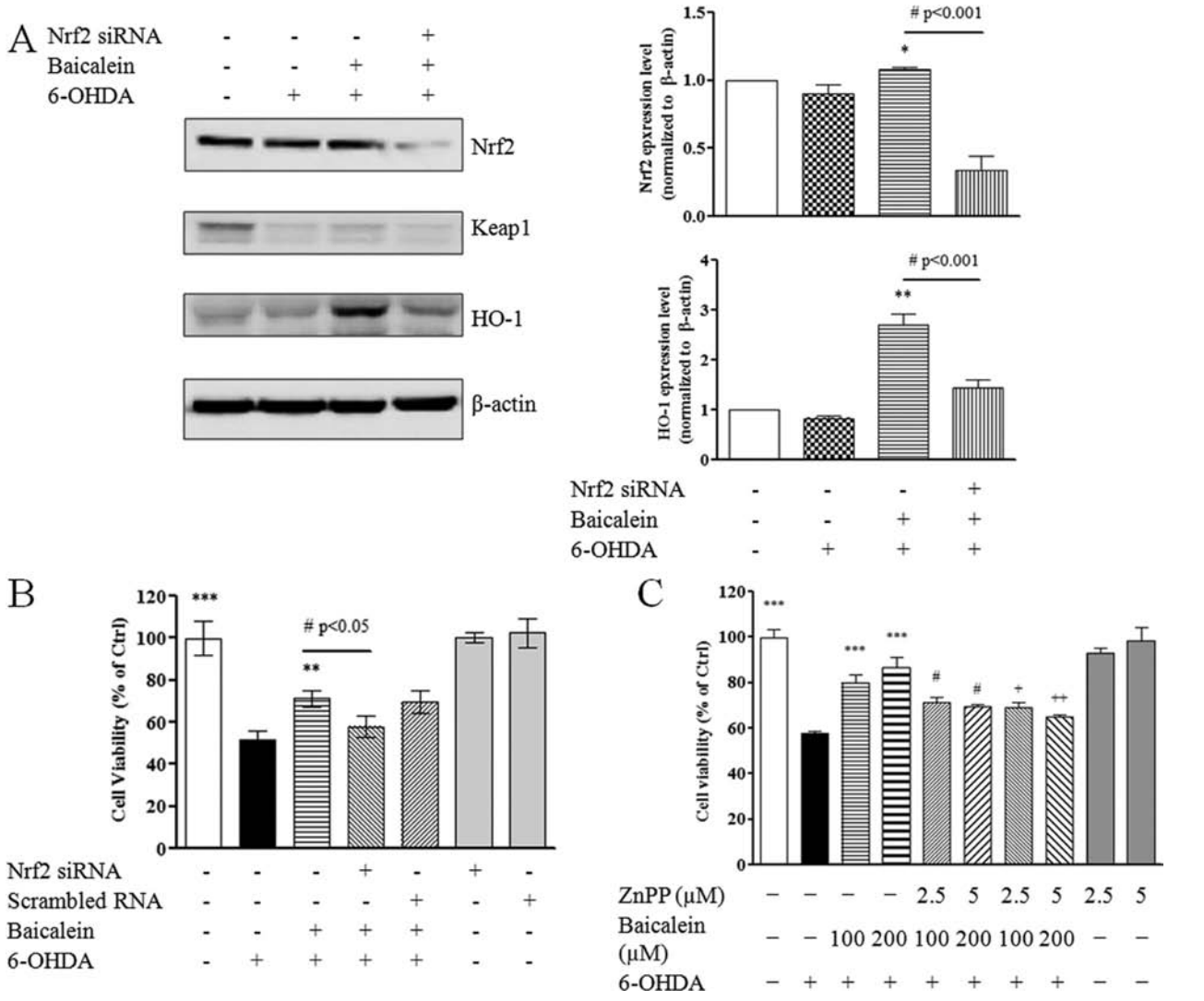

Figure 6. Nrf2 siRNA and HO-1 inhibitor (Znpp) attenuate the cytoprotective effect of baicalein. (A and B) Nrf2 siRNA transfection down-regulates Nrf2/HO-1 proteins expression and blocks cytoprotection of baicalein. Cells were transfected with Nrf2 siRNA, as described in the Materials and Methods, and exposed to baicalein (200 μM) for 12 h. The cells were then subjected to 6-OHDA challenge. (A) After 2 h of 6-OHDA treatment, proteins were prepared for Western blotting analysis. (B) After exposure to 6-OHDA for 8 h, the MTT assay was performed to measure cell viability. (C) HO-1 inhibitor (Znpp) attenuates the cytoprotective effect of baicalein. PC12 cells were pretreated with Znpp (2.5 or 5 μM) for 1 h followed by exposure to baicalein and 6-OHDA, and the MTT assay was performed to measure cell viability. Data from three times independent experiments were expressed as means ± SEMs. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs 6-OHDA treatment alone, respectively. $^{\#,+}P < 0.05$ and $^{+2}P < 0.01$ vs baicalein plus 6-OHDA treatment group, respectively.

Nrf2 siRNA and HO-1 Inhibitor Block the Cytoprotection of Baicalein in 6-OHDA-Treated PC12 Cells. To better define the cytoprotective role of Nrf2/HO-1 activation associated with baicalein, we tested the effect of Nrf2 siRNA transfection on baicalein-induced protection against 6-OHDA-damaged PC12 cells. We found that Nrf2 siRNA transfection dramatically decreased Nrf2 protein expression as well as the downstream signaling HO-1, a key protein transcriptional regulated by Nrf2 (Figure 6A). Notably, the silencing of Nrf2 protein did not significantly affect Keap1 protein expression. In a cell viability test, Nrf2 siRNA transfection significantly blocked the cytoprotection of baicalein; however, a scrambled RNA transfection that was used as a negative control did not yield any detectable effect. Both Nrf2 siRNA and scrambled control RNA transfection alone did not affect the PC12 cell viability (Figure 6B). To confirm whether the protective effect of baicalein is related to its induction of definitely involved HO-1 expression, we determined the blocking effect of a HO-1 inhibitor called ZnPP on in vitro neuroprotective effect of baicalein. The results showed that ZnPP could clearly abrogated the protective effect of baicalein on 6-OHDA-induced cytotoxicity (Figure 6C), suggesting that the cytoprotective effect of baicalein is mediated through HO-1 induction.

Proteasome Inhibitor MG132 Reverses the Down-regulation of Keap1 and Blocks the Cytoprotective Effect of Baicalein. MG132, a proteasome inhibitor,[28] pretreatment significantly reversed the decreases in Keap1 and increases of HO-1 expression that were induced by baicalein (Figure 7A). In addition, blocking the proteasome system by MG132 significantly attenuated the cytoprotection of baicalein against 6-OHDA-induced PC12 cells damage (Figure 7B).

Activation of PKCα and AKT Signaling Pathways Is Possibly Involved in Cytoprotection of Baicalein. Baicalein increased the protein phosphorelation of PKCα and AKT time

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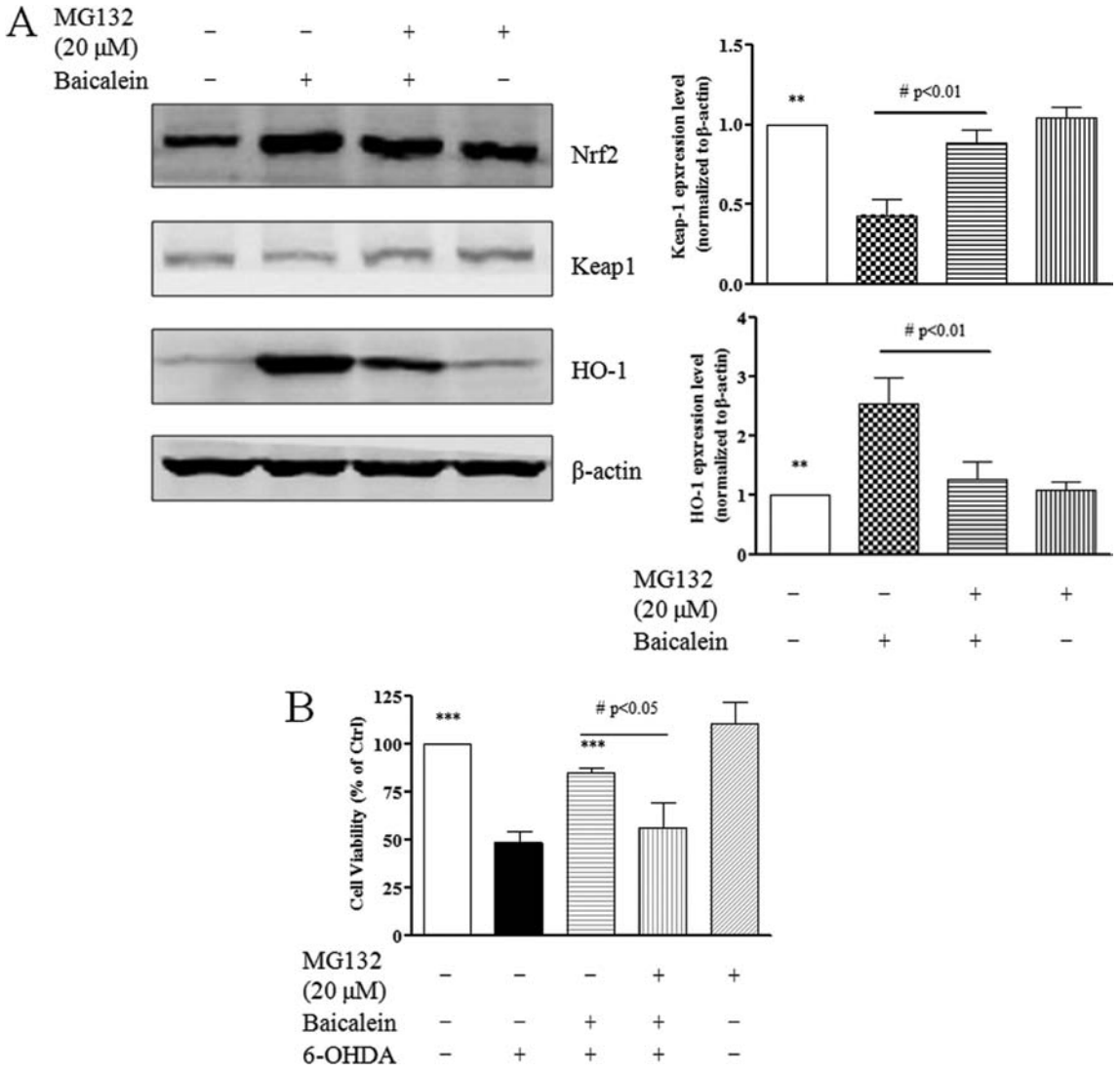

Figure 7. MG132 inhibits baicalein-induced Keap1 protein degradation and blocks its cytoprotective effect. (A) PC12 cells were pretreated with 20 μM MG132 for 1 h and then incubated with 200 μM baicalein for another 12 h, and proteins were prepared for Western blotting analysis. *P < 0.05 and **P < 0.01 vs baicalein treatment alone. (B) PC12 cells were pretreated with 20 μM MG132 for 1 h prior to incubation with 200 μM baicalein for another 12 h. The cells were then exposed to 6-OHDA to induce cell damage, and the MTT assay was performed to determine cell viability. Data from three times independent experiments were expressed as means ± SEMs; ***P < 0.001 vs 6-OHDA treatment alone.

dependently within 2 h of treatment, and baicalein treatment for 2 h up-regulated the protein phosphorelation of PKCα and AKT in a concentration-dependent manner (Figure 8A,B). To further confirm whether the activation of PKCα/AKT was involved in the cytoprotective effects related to baicalein, cells were pretreated with specific PKC inhibitor (PMA),[29] PI3K inhibitor (wortmannin),[30] and AKT inhibitor (AKTi IV, Calbiochem, Cat. No. 124011) for 1 h prior to addition of baicalein. We found that all of the inhibitors could significantly attenuate the protective effects of baicalein against 6-OHDA-induced PC12 cell damage (Figure 8C).

■ DISCUSSION

This study provides evidence that baicalein can exert neuroprotective effect through the activation of the Keap1/Nrf2 pathway and the subsequent up-regulating HO-1 and possibly other class II enzymes, such as SOD and catalase. To our knowledge, the present study is the first to reveal that baicalein activates Keap1/Nrf2/HO-1 signaling in PC12 cells and exerts an antioxidative defense mechanism against 6-OHDA-induced neurotoxicity. Therefore, this study implies that the Nrf2/HO-1 pathway may represent a pharmacological target and that baicalein might be a candidate for the prevention of neurodegeneration.

Baicalein significantly reduced cell injury at 50 μM, and the protective effect of baicalein was with a dose-dependent manner (Figure 1). However, 50 μM baicalein could not ameliorate DNA fragmentation, and the results showed that DNA fragmentation was inhibited by 200 μM baicalein (Figure 2). A previous study has been reported that exposure of PC12 cells to a low concentration of 6-OHDA results in apoptosis, whereas an increased concentration results in a mixture of apoptosis and necrosis. Higher concentrations of 6-OHDA result in a complete loss of cell viability and membrane integrity, which are characteristics of necrosis.[31] In present study, 1 mM 6-OHDA treatment resulted in more than 60% reduction of cell viability in MTT assay; however, only about 30% cellular apoptosis was observed. When the cells were prepared for apoptosis analysis,

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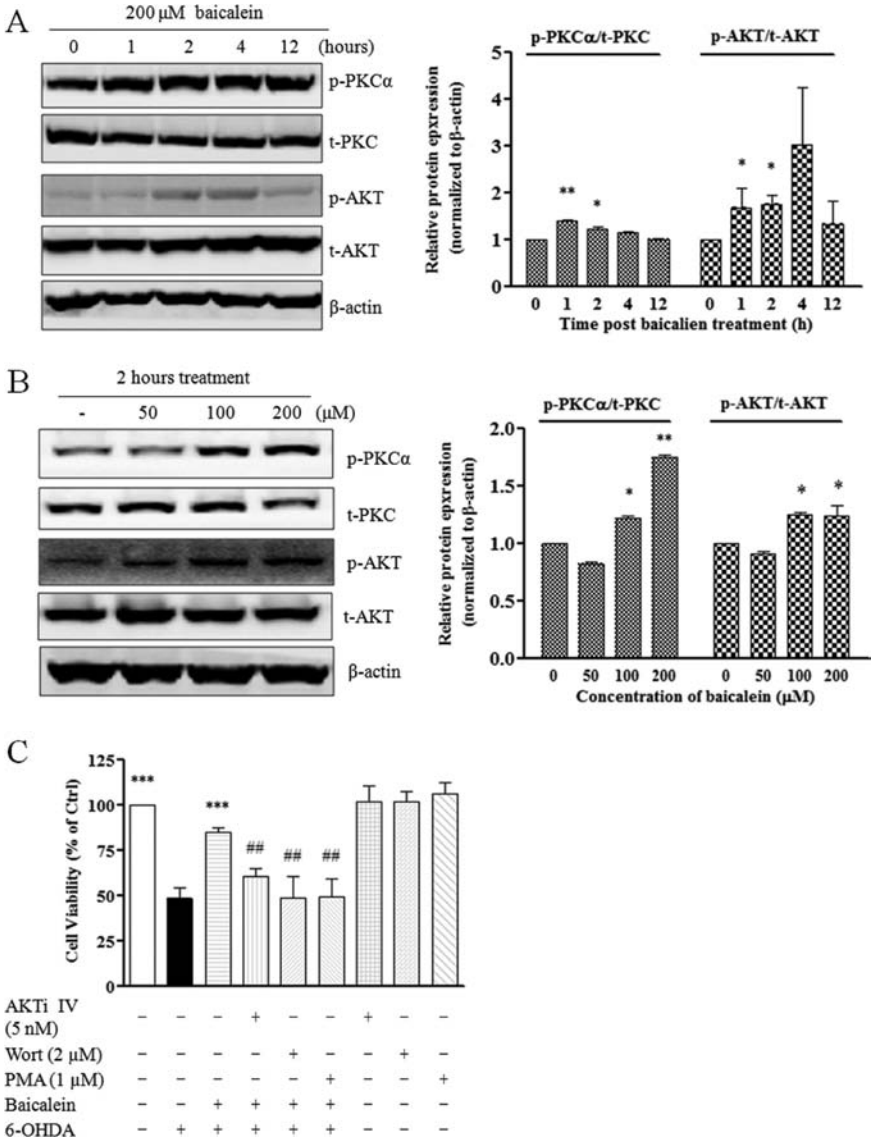

Figure 8. Involvement of PKCα/AKT pathways in the cytoprotection of baicalein. Time and concentration response of baicalein on activation of PKCα and AKT signaling pathway was determined. PC12 cells were treated with baicalein at the indicated times (A) or concentrations (B), and then, the proteins were prepared for Western blotting with activation-specific antibodies that recognize p-PKCα and p-Akt. (C) The pretreatment of PKCα, PI3K, and AKT inhibitors blocks the cytoprotective effect of baicalein. PC12 cells were pretreated with 5 nM AKT inhibitor IV (AKTi IV) or 2 μM wortmannin (Wort) or 1 μM PMA for 1 h prior to incubation with 200 μM baicalein for another 12 h and then were exposed to 6-OHDA to induce cell damage. The MTT assay was performed to determine cell viability. Data from three times independent experiments were expressed as means ± SEMs; $^{***}P < 0.001$ vs 6-OHDA treatment alone, $^{\#}P < 0.05$ vs baicalein + 6-OHDA treatment group.

the detached and dead cells were removed by washing with cold-PBS, and only the adherent cells were collected for apoptosis analysis. This might be the reason causing discrepancy between cytoprotection assessment by MTT assay and apoptosis analysis.

Increasing evidence supports a pivotal role for oxidative stress in neurodegenerative diseases such as PD.[1] The transcriptional up-regulation of cytoprotective genes represents one mechanism by which cells defend themselves against oxidative stress. Under oxidative stress conditions, the transcription factor Nrf2 binds to the ARE to induce antioxidant and phase II detoxification enzymes.[7] Previously, Jakel et al. demonstrated that 6-OHDA activates the Nrf2-ARE both in vivo and in vitro, which functions as part of a cellular defense mechanism to protect cells against ROS-mediated damage.[6] However, cell death still occurs in 6-OHDA-treated cells and other animal models. This effect may be due, in part, to the activation of the Nrf2-ARE system by 6-OHDA, which is not sufficient to prevent toxicity due to 6-OHDA. In the present study, we also found that 6-OHDA treatment slightly up-regulated the protein expression of Nrf2 and HO-1 (Figure 4D), increased the nuclear expression level of Nrf2 (Figure 5B), and decreased Keap1 expression (Figure 6A), which is a negative regulator of Nrf2. Such activation appears to be insufficient to defend cells against oxidative damage; therefore, more potent activators of Nrf2-ARE system are necessary to attribute cytoprotection.

Baicalein, baicalin, wogonin, and wogonoside, four major flavonoids with free radical scavenging and antioxidant activities, have been found in SBG. Baicalein and its glycoside baicalin are more effective antioxidants in this plant.[32] At the initial phase of present study, we compared the cytoprotective effect of SBG, baicalin, and baicalein on 6-OHDA-induced PC12 damage and Nrf2 induction activity, and it was found that baicalein exhibited

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the most potent cytoprotective and Nrf2 induction activity, revealing that the cytoprotective effect of baicalein might be associated with its Nrf2 induction activity.

Recently, a study has been reported that baicalein attenuates H_2O_2-induced mitochondrial oxidative stress by activating Nrf2-mediated MnSOD induction in Chinese hamster lung fibroblasts (V79-4) cells.[33] Here, we found that baicalein protected PC12 cells from 6-OHDA-induced oxidative insults. This protective effect, in part, is associated with an up-regulation of HO-1 expression. Along with other phase II enzymes, SOD and catalases serve as defense mechanism(s) against oxidative damage. Among the various cytoprotective enzymes, the protective functions of HO-1 have recently been emphasized,[34] and growing evidence suggests that the modulation of HO-1 expression represents a novel target for therapeutic intervention of neurodegeneration.[35] In the present study, baicalein treatment significantly increased HO-1 protein expression levels (Figure 4A,B) in a Nrf2- and Keap1-dependent manner, since the up-regulation of HO-1 can be reversed by the genetic silencing of Nrf2 (Figure 6A) and chemical blocking of Keap1 inactivation (Figure 7A).

Activation of Nrf2 by baicalein is possibly mediated by two different modes of action. First, baicalein could directly up-regulate the level of Nrf2 protein expression (Figure 4) and increase Nrf2 nuclear translocation and transcriptional activity (Figure 5). However, the mechanism(s) remain unknown regarding how baicalein acts on Keap1 to activate Nrf2/HO-1 pathway. Studies have established that Keap1 controls Nrf2 stability, which under nonstressing conditions constantly targets Nrf2 for degradation, and this interaction represents an attractive target for the regulation of Nrf2 signaling.[36] In this study, baicalein treatment dramatically decreased Keap1 protein expression in PC12 cells (Figure 4). Keap1 contains cysteine residues that are believed to regulate the association between Keap1 and Nrf2 stabilization.[36] Therefore, we hypothesize that baicalein could act on the cysteine thiol residue of Keap1. Previous studies suggest that curcumin, a flavonoid with a similar structure to that of baicalein, inactivates Keap1 through modifications of its cysteine thiols.[37] Therefore, a similar molecular mechanism may underlie Nrf2 stabilization exerted by baicalein. Through acting on Keap1, baicalein increased Keap1 degradation by the proteasome system and prevented Keap1-mediated inactivation of Nrf2. Thus, this interaction may release Nrf2 from the Nrf2−Kea1 complex and enhance its nuclear translocation in PC12 cells (Figure 5). In turn, Nrf2 binds to ARE of the HO-1 promoter to induce HO-1 expression. Consistent with this hypothesis, we demonstrated that baicalein-induced down-regulating Keap1 and increasing HO-1 expression was fully reversed by the proteasome inhibitor MG132 pretreatment (Figure 7A), which also blocked the protective effect of baicalein in 6-OHDA-induced PC12 cell damage (Figure 7B).

PKCα and PI3K/Akt activation are well-documented pathways involved in protection against oxidative stress. In addition, the PKCα and PI3K/Akt pathways are reported to be involved in the activation of Nrf2-dependent HO-1 expression.[24,25] The current experiments were designed to determine whether a possible role exists between the PKCα and PI3K/Akt pathways in the regulation of baicalein-induced Nrf2/HO-1 activation and subsequent protection against 6-OHDA-induced neurotoxicity. We found that baicalein significantly up-regulates protein phosphorylation of PKCα and Akt (Figure 8A,B). In addition, we applied specific inhibitors for PKCα, PI3K, and Akt and confirmed the involvement of PKCα and Akt in baicalein-induced protection against neurotoxicity of 6-OHDA (Figure 8C).

In conclusion, the present study demonstrated that baicalein effectively prevented against 6-OHDA-induced oxidative damage of PC12 cells via activation of Keap1/Nrf2/HO-1 and involvement of the PKCα and AKT cell survival signaling pathway. Although different neurodegenerative diseases, such as AD and PD, have different pathogenesis, they may share some similar pathological processes, for example, the generation of ROS, which further causes oxidative stress and subsequent damage to neuronal cells. Our findings suggest that baicalein might be a candidate for the prevention of neurodegenerative diseases.

AUTHOR INFORMATION

Corresponding Author

*Tel: (852)3400 8695. Fax: (852)2364 9932. E-mail: bcyfhan@polyu.edu.hk (Y.H.). Tel: (853)8397 4695. Fax: (853)2884 1358. E-mail: simonlee@umac.mo (S.M.-Y.L.).

Author Contributions

[+]Both authors contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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