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J. Agric. Food Chem., Just Accepted Manuscript • DOI: 10.1021/acs.jafc.6b01551 • Publication Date (Web): 18 Jul 2016

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Dimerumic Acid and Deferricoprogen Activate the Akt/HO-1 Pathway and
Prevent Apoptotic Cell Death in 6-OHDA-induced SH-SY5Y Cells

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ABSTRACT: Parkinson’s disease (PD) is a neurodegenerative disorder, which can be modeled using the neurotoxin 6-hydroxydopamine (6-OHDA) to generate oxidative stress. Here, we studied the effects of the antioxidants deferricoprogen (DFC) and dimerumic acid (DMA), produced by rice fermented with Monascus purpureus NTU 568, on 6-OHDA-induced apoptosis in SH-SY5Y cells and their potential protective mechanisms. DMA and DFC inhibited 6-OHDA-induced apoptosis and cellular reactive oxygen species (ROS) in SH-SY5Y human neuroblastoma cells. Molecular analysis demonstrated associated upregulation of the Ak mouse strain thymoma (Akt), heme oxygenase-1 (HO-1), and signal-regulated kinase (ERK) pathways along with inhibited phosphorylation of c-Jun N-terminal kinase (JNK) and p38 pathways and altered homodimeric glycoprotein, N-methyl-D-aspartate (NMDA) receptor, and immunoglobulin Fc receptor gene expression. These results suggested that the neuroprotection elicited by DMA and DFC against 6-OHDA-induced neurotoxicity was associated with the Akt, MAPK, and HO-1 pathways via regulating the gene expression of NMDA receptor, homodimeric glycoprotein, and immunoglobulin Fc receptor.

KEYWORDS: Monascus purpureus-fermented rice, Parkinson’s disease, apoptosis, dimerumic acid, deferricoprogen
INTRODUCTION

Parkinson’s disease (PD) is a degenerative nerve disease associated with the progressive loss of dopaminergic producing nerve cells in the substantia nigra pars compacta (SNpc). Treatment of levodopa is the therapeutic criterion for PD but is only effective for symptomatic relief in the past stages of the disease. Accordingly, there is a specific medicinal need for new curative for PD. Though there are not completely understood about the mechanisms accountable for the death of dopaminergic cell, multiply evidence from human post-mortem investigations and animal studies indicated that oxidative stress was an important role in initiating this process.\(^1\)\(^-\)\(^3\) Therefore, inhibition of oxidative stress might be a feasible therapeutic approach for PD.

A large number of PD models have been developed to assistant in the detection of neuroprotective curatives for PD. Many of these models utilize experimental neurotoxins like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA). 6-OHDA is widely used to induced \textit{in vitro} and \textit{in vivo} PD models and it could lead up oxidative stress.\(^4\) Previous reports have indicated that auto-oxidation contributed to the formation of reactive oxygen species (ROS).\(^5\) Moreover, ROS were established to play a critical role in 6-OHDA-induced cell apoptosis.\(^6\) In addition, mitogen-activated protein kinases (MAPKs) are usually
related to nerve cell death and apoptosis,\(^7,^8\) whereas the Ak mouse strain thymoma (Akt) pathway is regarded to be essentiality for the protection of neurons from death and cell survival.\(^8,^9\) Accordingly, various studies have shown that MAPK pathway activation and Akt inhibition mediate 6-OHDA-induced neuronal damage.\(^8,^10\)

*Monascus purpureus*-fermented rice (red mold rice, RMR) has been indicated to be efficient for the administration of hypercholesterolemia, diabetes, hypertension, obesity, and Alzheimer’s disease as well as for the prevention of cancer.\(^11^-^17\) Our research group has been found that extracts of rice which was fermented with *M. purpureus* NTU 568 suppressed amyloid β (Aβ)-induced neuron cytotoxicity via combined anti-inflammatory and antioxidant mechanisms.\(^18\) RMR contains some bioactive compounds which were pigments, polyketide monacolins, deferricoprogen (DFC), and dimerumic acid (DMA). DMA and DFC potent radical scavenging activity and exhibit the structural features of iron chelating (siderophore).\(^19\) DMA in particular has been exhibited to be defendable versus cytotoxicity in rat hepatocytes which were induced by oxidative stress.\(^20\) Previously, we demonstrated that DMA and DFC may protect against 6-OHDA toxicity by inhibiting the formation of ROS and apoptosis in rodent cells of neural crest origin.\(^21\) Furthermore, we showed that DMA and DFC-containing extract of rice which was fermented with *M. purpureus* NTU 568 (extracted with 50% ethanol, called R50E) may prevent degenerative nerve via
anti-inflammatory and anti-oxidative mechanisms, indicating its potential curative
efficacy for PD therapeutics.\textsuperscript{22}

In the present study, SH-SY5Y human neuroblastoma cells were exposed to
6-OHDA was used to be a model to assess the mechanisms fundamental the
cytoprotection generated by the secondary metabolites DMA and DFC extracted from
rice which was fermented with \textit{M. purpureus} NTU 568. Cell viability, apoptosis, ROS
formation, proteins and their modification states, and gene expression were examined.
MATERIALS AND METHODS

Materials. Potato Dextrose Agar (PDA) was purchased from Becton, Dickinson and Company (Sparks, MD, USA). N-acetyl cysteine (NAC) and 6-OHDA were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA, USA). All other assay kits were purchased from Cayman Chemical Co., Ltd. (Ann Arbor, MI, USA). The antibodies were purchased from Abcam Inc. (Cambridge, UK).

Preparation of Red Mold Rice (RMR). Long-grain rice was fermented with M. purpureus NTU 568 as described previously to obtain RMR. The RMR was further dried by freeze dryer and extracted with 50% ethanol.

Identification of Deferricoprogen (DFC) and Dimerumic Acid (DMA). The 50% ethanol extract of RMR, R50E, was separated and purified by MCI gel (CHP 20P, 75–150 µm; Mitsubishi Chemical Industries, Tokyo, Japan) to produce five fractions tagged R50E-1 through R50E-5 after filtration and concentration. The anti-oxidative activities of the five fractions were determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. R50E-3 had the highest DPPH scavenging activity, further separated and purified by Sephadex LH-20 gel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) to produce six fractions. Ultimately, the highest DPPH scavenging activities fractions were accumulated and
purified by preparative high performance liquid chromatography to acquire purified DMA and DFC.\textsuperscript{30}

**SH-SY5Y Cells Culture and Drug Treatment.** Ham’s F12 and MEM medium (1:1), contained 10 mM sodium pyruvate, 2 mM glutamine, and 15% fetal calf serum was used to culture SH-SY5Y human neuroblastoma cells at 37 °C with 95% air and 5% CO\textsubscript{2}. SH-SY5Y cells were plated onto 96-well plates at a density of 2 × 10\textsuperscript{4} cells each well. After 24 h of incubation, the growth media were attended with various concentrations of 6-OHDA. 6-OHDA was dissolved in 0.02% ascorbic acid saline as a stock solution (the final concentration was 25 mM). Cells were exposed to R50E, DMA, or DFC at different concentrations in the absence or presence of 6-OHDA for 24 h.

**Cell Viability Assay.** SH-SY5Y cells viability was assessed by the MTT method. Solution of 0.5 mg/mL MTT (final concentration) was added to each well after treatment with extract samples or 6-OHDA. After 4 h incubation, the MTT solution was threw out and added 200 µL DMSO to solubilize the formazan in each well. The absorbance was estimated with ELISA universal microplate reader (EXL 800, BIO-TEK Instrument Inc., Winooski, VT, USA) at 570 nm.

**ROS Assessment.** The level of cellular oxidative stress was established by the estimation of ROS. After drugs treatment, the collected cells were washed and
suspended in 1 mL PBS which containing 15 µM dichlorodihydrofluorescein diacetate (DCFH-DA), and incubated at 37 °C for 30 min. The cells were removed the excess DCFH-DA with PBS. The cell pellets were suspended with 200 µL PBS and the level of ROS was analyzed by flow cytometry (BD FACSCanto™, BD Biosciences, San Jose, CA, USA).

**Apoptosis Analysis via Annexin V-FITC/Propidium Iodide (PI)**

**Double-Labeled by Flow Cytometry.** The level of cell apoptosis was analyzed by the Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson). SH-SY5Y cells were treated with 6-OHDA in the presence or absence of DMA and DFC for 12 h. After incubation, the cells were washed with PBS, and then suspended in 1X binding buffer. The density of cell was adapted to $1 \times 10^6$ cells/mL. The cell suspension were mixed with Annexin V-FITC in 1.5 mL centrifuge tube, incubated in the dark for 30 min at 37 °C, and then washed with 1× binding buffer. PI (5 µL) was added to the mixtures, which were then analyzed using a flow cytometry (BD FACSCanto™). Cells negative for PI and positive for Annexin V were established as early apoptotic cells; cells positive for PI and Annexin V were established as late apoptotic cells.

**Western Immunoblotting.** Cells were lysed and the cell lysates were centrifuged (10,000 × g for 10 min) and the supernatant were assembled as the cell extract. The concentrations of protein in the cell extracts were established by Bio-Rad protein
assay kit. The cell extracts were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane. The 50 g/L nonfat dry milk solution used to block the PVDF membranes for 1 h and then the PVDF membranes were incubated with primary antibodies for 2 h. The membranes were washed with PBST and shaken in a solution of horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary antibody. Protein expression levels were recognized by an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA).

**Microarray Analysis.** Total RNA was amplified using an Agilent Low Input Quick-Amp Labeling kit (Agilent Technologies, Santa Clara, CA, USA) and labeled with Cy3 (CyDye, Agilent) during the transcription process. Cy3-labeled cRNA was fragmented to a general size of approximately 50–100 nucleotides by incubation with fragmentation buffer for 30 min at 60 °C. The correspondingly fragmented labeled cRNA was then combined and hybridized to an Agilent SurePrint G3 Human Gene Exp V3 Array for 17 h at 65 °C. The microarrays were washed and blow-dried with a nitrogen gun, and then examined with an Agilent microarray scanner at 535 nm to detect Cy3. The images of scanner were assayed by Feature extraction10.5.1.1 software (Agilent), an image analysis and normalization software used to quantify signal and background intensities for each feature.
Statistical Analysis. For SPSS statistical analysis (Chicago, IL, USA), all data were analyzed using one-way analysis of variance followed by Dunnett’s test or the Mann-Whitney test. Significant differences were exhibited as $P < 0.05$. 
RESULTS

Identification of DMA and DFC from RMR. We purified DFC and DMA from R50E by preparative HPLC and characterized them using nuclear magnetic resonance (NMR) and mass spectrometry. The structures of DFC and DMA are presented in Figure 1.

DMA and DFC Protect SH-SY5Y Cells against 6-OHDA-Induced Cell Death. SH-SY5Y cells were treated with different concentrations of DMA or DFC (0, 5, and 10 µM) in the presence or absence of 6-OHDA (100 µM). These results indicated that DMA (5 and 10 µM) significantly increased cell survival rate to 61 and 91% (Figure 2) and that DFC (5 and 10 µM) significantly increased cell survival rate to 80 and 86% (Figure 2), suggesting that DMA and DFC decreased cytotoxicity in 6-OHDA-induced SH-SY5Y cells.

DMA and DFC Reduce the Formation of 6-OHDA-Induced ROS in SH-SY5Y Cells. Accumulation of intracellular and extracellular ROS is reflected a key mediator of cytotoxicity and a premier trigger of apoptotic signaling ensuing 6-OHDA treatment. To establish whether the protective effects of DMA and DFC were associated with inhibition of 6-OHDA-induced ROS formation in SH-SY5Y cells. DCFH-DA, an oxidation-sensitive fluorescent dye, was used to determine the intracellular ROS by flow cytometry. DFC or DMA (10 µM) significantly decreased
the mean fluorescence intensity in 6-OHDA-induced SH-SY5Y cells compared with
6-OHDA alone group (Figure 3).

**DMA and DFC Diminish 6-OHDA-Induced SH-SY5Y Cell Apoptosis.** To establish whether treatment with DMA and DFC inhibited 6-OHDA-induced apoptosis in neurons, the number of fluorescent apoptotic cells was quantified by annexin V-FITC/PI staining and assessed by flow cytometry. The percentage of apoptotic cells was significantly raised after a 12-h incubation with 6-OHDA at 100 µM (Figure 4). DMA and DFC treatment significantly reduced the percentage of apoptotic cells in 6-OHDA-induced SH-SY5Y cells compared with 6-OHDA alone group.

**Reversal of Akt Pathway- and Heme Oxygenase-1 (HO-1)-Mediated SH-SY5Y Cells Damage through the Protective Effects of DMA and DFC.** Neuronal damage from 6-OHDA primarily occurs through the Akt pathway and via a reduce in detoxifying enzymes such as HO-1,24,25 we assessed the protein levels of Akt and HO-1 in SH-SY5Y cells exposed to 6-OHDA and in the presence or absence of DMA or DFC. The results of protein immunoblot are shown in Figure 5. 6-OHDA reduced the ratio of phosphorylated Akt (p-Akt)/Akt and also reduced the levels of HO-1 in SH-SY5Y cells. Both DMA and DFC at 10 µM restored the levels of HO-1 and p-Akt/Akt ratio in 6-OHDA-treated SH-SY5Y cells. In addition, DMA and DFC
raised the \( p\)-Akt/Akt ratio compared with non-treated group in SH-SY5Y cells (Figure 5A). Data in Figure 5B showed that there were no significant differences in HO-1 levels between DMA alone group and non-treated group.

Reversal of Mitogen-Activated Protein Kinase (MAPK)-Mediated SH-SY5Y Cells Damage through the Protective Effects of DMA and DFC.

MAPKs were containing c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinases (ERK) have been studied to be associated with neuronal apoptosis and cell death.\(^\text{26,27}\) We therefore assessed the levels of these proteins in SH-SY5Y cells exposed to 6-OHDA and in the presence or absence of DMA or DFC.

The results of protein immunoblot are shown in Figure 6. 6-OHDA raised the ratios of \( p\)-JNK/JNK and \( p\)-p38/p38 in SH-SY5Y cells, whereas both DMA and DFC at 10 µM restored these ratios. In addition, both DMA and DFC at 10 µM raised the ratio of \( p\)-ERK/ERK in SH-SY5Y cells.

Microarray Analysis Identifies Distinct Changes in Gene Expression Following 6-OHDA and DMA or DFC Treatment. To investigate the molecular differences consequent to 6-OHDA, DMA, or DFC treatment, we performed microarray analysis to compare the global gene expression profiles of SH-SY5Y cells following treatment to 6-OHDA and DMA or DFC. Gene expression analysis was achieved using one-color hybridization to Agilent SurePrint G3 Human Gene Exp V3
microarrays. The difference between the normalized log ratio values for each gene on the array was estimated for the various treatment conditions in SH-SY5Y cells and is displayed as a comparison between untreated and 6-OHDA groups, 6-OHDA and 6-OHDA+DMA groups, and 6-OHDA and 6-OHDA+DFC groups.

In the 6-OHDA treatment group, 2.6-fold up-regulated glutamate receptor, ionotropic, N-methyl D-aspartate (NMDA) 2C (grin2c) gene expression (Figure 7A) and 2.2 fold down-regulated stanniocalcin 2 (stc2) gene expression (Figure 7B) were observed. In contrast, following co-treatment with DMA, grin2c was down-regulated 2.5 fold (Figure 7A) and stc2 was up-regulated 2.1 fold (Figure 7B) in 6-OHDA-induced SH-SY5Y cells. In turn, DFC might exert its protective function through Fc gamma R-mediated phagocytosis regulation, as the expression of the associated fcgr2a gene, which demonstrated 40.4 fold up-regulation in the 6-OHDA treatment group, was down-regulated 28.3 fold in 6-OHDA-treated SH-SY5Y cells co-treated with DFC (Figure 8).
DISCUSSION

In this study, we showed that DMA and DFC from *M. purpureus*-fermented rice reduced neurotoxicity in 6-OHDA-induced SH-SY5Y cells. These results indicate that the neuroprotective effect might be mediated by the reduction of intracellular ROS formation and the activation of HO-1, Akt, and ERK pathways. Furthermore, DMA and DFC inhibited the phosphorylation of the p38 and JNK pathway in SH-SY5Y cells. In addition, DMA and DFC might regulate the *grin2c, stc2*, and *fcgr2a* genes, respectively.

RMR has been reported that containing several bioactive components associating with chemoprevention. For example, extracts of rice which fermented by *M. purpureus* NTU 568 fermented repressed amyloid β (Aβ)-induced neurocytotoxicity via combined anti-inflammatory and antioxidant mechanisms. As R50E may prohibit neurodegeneration through anti-oxidative and anti-inflammatory mechanisms, it might thus have potential therapeutic value in PD.

In the present study, we investigated the neuroprotective effects of the R50E-extracted antioxidants DMA and DFC using human neuroblastoma-derived SH-SY5Y cells. DMA has been found anti-oxidant effects in primary hepatocytes, and ensured against hepatotoxicity via inhibiting oxidative stress. DMA also has been reported to be efficient in diminishing in a mouse model of carbon
tetrachloride-treated liver injury. Moreover, DMA inhibited the cell invasion and the
activation of H$_2$O$_2$-mediated MAPKs pathways in SW620 human colon cancer cells.

In comparison, DFC was found to directly remove DPPH free radicals at lesser doses
(IC$_{50} = 15.37$ µg/mL) and exhibited protective effects against the cell death and
cytotoxicity in citrinin-induced HEK-293 human embryonic kidney cells.

Furthermore, DMA and DFC may protect against 6-OHDA toxicity by inhibiting ROS
formation and apoptosis. Together, such evidence suggested that the secondary
metabolites of *M. purpureus*-fermented rice such as DFC and DMA exhibit
distinguished protective effects versus chemical and oxidative stresses.

The generation of ROS has been presented to being an important role in
neurodegenerative diseases such as PD. 6-OHDA, one of the most commonly
applied catecholaminergic neurotoxins, is known to generate ROS via intracellular
hydrogen peroxide production, leading to cell death. Moreover, the system of
nitrogen oxide (NOX) was established as the most significant intracellular source of
ROS other than mitochondria. Notably, our research group has previously
demonstrated that DMA and DFC treatment reduced the 6-OHDA-induced formation
of extracellular and intercellular ROS and decreased NADPH oxidase-2 expression in
differentiated PC-12 cells.

The SH-SY5Y human neuroblastoma cell line possesses many properties of
substantia nigra nerve cells and is widely applied to research the death of
dopamine-producing nerve cells.\textsuperscript{34} In the present study, we utilized SH-SY5Y cells
induced with 6-OHDA as a model system to examine the neuroprotective capacity of
DMA and DFC and investigate the role of inhibition of the ROS-generating system in
their neuroprotection. We established that exposure to 6-OHDA decreased SH-SY5Y
cell survival rate and increased the formation of intracellular ROS in SH-SY5Y cell
culture, inducing a combination of both necrotic and apoptotic cell death.\textsuperscript{4} Similarly,
this toxin was found to induce morphological changes in rat PC-12 cells that are
typical of apoptosis containing membrane blebbing, DNA fragmentation, and cell
shrinkage.\textsuperscript{35} In these cells, we demonstrated that DMA and DFC could reverse that
6-OHDA induced DNA fragmentation, activate caspase-3, down-regulate Bcl-2, and
up-regulate Bax.\textsuperscript{21} In the current study, we also found that DMA and DFC could
reduce and partially reverse the early apoptotic events of SH-SY5Y cells, suggesting
DMA and DFC could interrupt the 6-OHDA-induced apoptosis and oxidative stress in
these cells.

Akt is a critical role in essential cellular functions including cell survival and
proliferation via phosphorylating a type of enzymes such as detoxifying,
pro-apoptotic regulators, transcription factors, and antioxidant proteins.\textsuperscript{36} Certain
studies found that Akt plays an principal therapeutic role for curing stroke,
neurodegenerative diseases, and diabetes.\textsuperscript{36,37} In the present study, we established that SH-SY5Y cells exposed to 6-OHDA for 9 h reduced the phosphorylation of Akt, whereas DMA and DFC repaired Akt phosphorylation. Several cytoprotective enzymes regulated by the Akt cascade such as HO-1 has been shown to be a critical target in neuroprotective functions.\textsuperscript{38} Accelerating attestation established that a pharmacological inducer, HO-1, expressing may maximize the internal antioxidant potential of cells.\textsuperscript{39} Our data indicated that 6-OHDA reduced the levels of HO-1 in SH-SY5Y cells and that DMA and DFC were also able to reverse this decrease. Therefore, we propose that DMA and DFC induced Akt phosphorylation and further activated the expression of HO-1 to neuralize the neurotoxicity and reduced the neuronal injury in 6-OHDA-induced SH-SY5Y cells.

MAPK pathway plays critical roles in promoting neuronal cell survival and suppressing apoptosis via the inhibition and phosphorylation of several downstream substrates.\textsuperscript{40} A succession of studies have reported that MAPK signaling pathways represent anti-apoptotic and survival factors in multiple examples including the resistance against 6-OHDA-induced neurotoxicity.\textsuperscript{41} In the present study, we found increased phosphorylation of JNK and p-38 after exposed with 6-OHDA, whereas treated with DMA and DFC restored the phosphorylation of MAPK proteins in 6-OHDA-induced neurons. These results indicate that maintained MAPK activity
might be adverse for SH-SY5Y cells survival and that the MAPKs pathways play a important role in the neuroprotective effects of DMA and DFC in SH-SY5Y cells.

*grin2c* encodes a subunit of the NMDA, ion type glutamate (ionotropic glutamate) receptor, is found in the central nervous system (CNS) and which regulates the flow of primarily calcium, potassium, and sodium ions in and out of the cell. Accordingly, an abnormal increase of *grin2c* gene expression leads to the death of nerve cells. In this study, we found that DMA restored the elevated *grin2c* gene expression found in 6-OHDA-induced SH-SY5Y cells. *stc2* encodes a secreted homodimer glycoprotein and its expression has been associated with nerve cell growth and differentiation. Our data indicated that DMA also increased the reduced *stc2* gene expression observed in 6-OHDA-induced SH-SY5Y cells. These results indicate that DMA might function through the glutamate signaling pathway and cell surface receptor linked signal transduction regulation to protect against the 6-OHDA-induced apoptosis in SH-SY5Y cells, as the *grin2c* and *stc2* genes associated with these two pathways (Figure 7). In turn, *fcgr2a* encodes an immunoglobulin Fc receptor on the cell membrane, which has been shown to be associated with diseases of the central nervous system. In this study, we found that DFC restored the elevated *fcgr2a* gene expression seen in 6-OHDA-treated SH-SY5Y cells (Figure 8). These results indicate that DFC might exert its protective function through Fc gamma R-mediated
phagocytosis regulation to protect against the 6-OHDA-induced apoptosis in SH-SY5Y cells, as the **fcgr2a** gene associated pathway. Therefore, we suggest that DMA regulation of **grin2c** and **stc2** and DFC regulation of **fcgr2a** gene expression served to reduce the neuronal damage in 6-OHDA-induced SH-SY5Y cells.

In conclusion, our findings indicated that DMA and DFC were able to interrupt 6-OHDA-induced apoptosis and oxidative stress in SH-SY5Y cells. Moreover, DMA and DFC activated Akt phosphorylation, induced HO-1 expression, and reduced the phosphorylation of JNK and p-38 to counteract the neurotoxicity in 6-OHDA-induced SH-SY5Y cells. Furthermore, DMA and DFC regulated **grin2c**, **stc2**, and **fcgr2a** gene expression to ameliorate the neuronal damage (Figure 9). These results supplied critical implications for the development of mechanism-based therapies for PD. In addition, we anticipate that the DMA and DFC derived from *M. purpureus* fermentation might supply as a functional food for the prevention or treatment of PD.
This work was supported by the research grant (MOST 104-2320-B-002-056) from Ministry of Science and Technology, Taiwan.
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FIGURE CAPTIONS

**Figure 1.** Structures of dimerumeric acid (DMA) (A) and deferricoprogen (DFC) (B).

**Figure 2.** SH-SY5Y cell survival following DMA or DFC co-treatment with 6-OHDA. SH-SY5Y cells were exposed to 6-OHDA (100 µM) in the presence of DMA or DFC for 24 h, and cell viability was measured using the MTT assay. Data represent the means ± SD of survival as measured by cell viability of three independent experiments. *P < 0.05, compared with the control group, #P < 0.05, ##P < 0.01, compared with the 6-OHDA group only.

**Figure 3.** ROS level of SH-SY5Y cells by DMA or DFC co-treated with 6-OHDA for 4 h. SH-SY5Y cells were exposed to 6-OHDA (100 µM) in the absence or presence of 10 µM DMA or DFC for 4 h. Intracellular ROS were stained with dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min and analyzed by flow cytometry. Data represent the means ± SD of survival as measured by cell viability of three independent experiments. *P < 0.05, compared with the control group, #p < 0.05, compared with the 6-OHDA group.
Figure 4. DMA or DFC decreased apoptotic cells in 6-OHDA-treated differentiated SH-SY5Y cells. SH-SY5Y cells were exposed to 6-OHDA (100 µM) in the absence or presence of 10 µM DMA or DFC for 12 h. Apoptotic cells were stained with Annexin V-FITC/PI stain and analyzed by flow cytometry. Data represent the means ± SD of survival as measured by cell viability of three independent experiments. *P < 0.05, compared with the control group, #P < 0.05, compared with the 6-OHDA group only.

Figure 5. DFC or DMA increased 6-OHDA-mediated reduction of the p-Akt/Akt ratio (A) and HO-1 expression (B) in SH-SY5Y cells. SH-SY5Y cells were exposed to 6-OHDA (100 µM) in the absence or presence of 10 µM DMA or DFC for 9 and 15 h, respectively and the protein extracts were assayed by immunoblot to measure p-Akt, Akt, HO-1, and GAPDH expression. Data represent the means ± SD of survival as measured by cell viability of three independent experiments. **P < 0.01, compared with the control group, #P < 0.05, compared with the 6-OHDA group.

Figure 6. Both DFC and DMA decreased 6-OHDA-mediated enhancement of the MAPK ratio in SH-SY5Y cells. SH-SY5Y cells were exposed to 6-OHDA (100 µM) in the absence or presence of 10 µM DMA or DFC for 6 h. Protein extracts were assessed for p-p38, p38, p-JNK, JNK, p-ERK, and ERK expression by Western blot
analysis. Data represent the means ± SD of survival as measured by cell viability of three independent experiments. **P < 0.01, compared with the control group, #P < 0.05, ##P < 0.01, compared with the 6-OHDA group.

Figure 7. Changes in *grin2c* (A) and *Stc2* (B) gene expression in response to DMA regulation of 6-OHDA-induced neurotoxicity in SH-SY5Y cells. SH-SY5Y cells were exposed to 6-OHDA (100 µM) in the presence of DMA (10 µM) for 24 h. Coordinate changes in gene expression in SH-SY5Y cells were identified by expression array. O&C: 6-OHDA group compared with control group, DMA&O: DMA+6-OHDA group compared with 6-OHDA group.

Figure 8. Changes in *fcgr2a* gene expression in response to DFC mediation of 6-OHDA-induced neurotoxicity in SH-SY5Y cells. SH-SY5Y cells were exposed to 6-OHDA (100 µM) in the presence of DFC (10 µM) for 24 h. Coordinate changes in gene expression in SH-SY5Y cells were identified by expression array. O&C: 6-OHDA group compared with control group, DFC&O: DFC+6-OHDA group compared with 6-OHDA group.

Figure 9. Schematic drawing of DMA and DFC inhibition of 6-OHDA-induced
529 cytotoxicity in SH-SY5Y cells.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.

(A) Log2 Fold change

(B) Log2 Fold change

grin2c

stc2
Figure 8.
Figure 9.