Full Paper

DJ-1–Mediated Protective Effect of Protocatechuic Aldehyde Against Oxidative Stress in SH-SY5Y Cells

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Abstract. *DJ-1* was identified as a causal gene for a familial form of early onset Parkinson's disease (PD), *park 7*. DJ-1 plays roles in transcriptional regulation and the anti-oxidative stress reaction. In this study, we found that protocatechuic aldehyde (PAL), a traditional Chinese medicine compound, bound to DJ-1 in vitro and that PAL protected SH-SY5Y cells but not DJ-1–knockdown SH-SY5Y cells from oxidative stress–induced cell death, indicating that the protective effect of PAL is mediated by DJ-1. Furthermore, PAL inhibited production of reactive oxygen species and the inhibition was abated in DJ-1–knockdown cells. PAL increased and decreased phosphorylation of AKT and PTEN, respectively, in SH-SY5Y cells, suggesting that the AKT pathway is one of the specific signaling pathways in PAL-induced neuroprotection. Moreover, PAL prevented superfluous oxidation of cysteine 106 of DJ-1, an essential amino acid for DJ-1's function. The present study demonstrates that PAL has potential neuroprotective effects through DJ-1.

Keywords: DJ-1, AKT, cell death, oxidative stress, Parkinson's disease

Introduction

Parkinson's disease (PD) is characterized by progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and affects 1% – 2% of people over the age of 60 years (1). Although the mechanisms underlying neurodegeneration in PD are unknown, the identification of genes linked to rare forms of familial PD have provided crucial insights into mechanisms of the pathogenesis of PD and new strategies for treatment of PD.

DJ-1 was first identified by our group as a novel oncogene that transformed mouse NIH3T3 cells in cooperation with activated *ras* (2) and was later found to be a causative gene for a familial form of Parkinson's disease (3). DJ-1 has multiple functions, including transcriptional

regulation (4 – 13), anti-oxidative stress function (14 – 16), mitochondrial regulation (17 – 19), and functions as a chaperone (20, 21) and as protease (22 – 24).

While neuroprotective strategies for PD therapy remain uncertain, current treatment for PD consists of symptomatic therapies to ameliorate clinical manifestations. L-Dopa remains the most effective drug for treating symptoms of PD. Motor and mental complications associated with chronic L-dopa usage, however, appear in most patients (25). Moreover, there is controversy about the neurotoxicity of L-dopa (26). It is therefore necessary to develop new drugs, especially drugs with neuroprotective effects, for treatment of PD.

Based on the discovery of a mutated *DJ-1* gene in familial parkinsonism and based on the function of DJ-1, we aim to find new-generation antiparkinson drugs that target DJ-1 protein and offer hope for cure of PD. In this study, we identified a DJ-1–binding compound, protocatechuic aldehyde (PAL), which is a traditional Chinese medicine compound. PAL protected SH-SY5Y cells against oxidative stress–induced death through activation

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of the Akt pathway concomitant with attenuation of excessive oxidation of DJ-1.

Materials and Methods

PAL

PAL was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and HPLC analysis showed that its purity was more than 98%.

Cell culture

Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum in a 5% CO₂ humidified atmosphere at 37°C.

Binding of PAL to DJ-1

Fixation of PAL on a sensor chip of a quartz crystal microbalance (QCM) (Affinix Q; Initium, Tokyo) was carried out as follows: The sensor chip was washed with a solution (volumes of H₂O₂ and sulfonic acid being 1:3) and incubated with 4 μ l of 1 μ M PAL dissolved in chloroform until the solution evaporated. The sensor chip fixed with PAL was set on the QCM and immersed in a PBS solution. Wild-type DJ-1 that had been expressed in and prepared from *E. coli* or bovine serum albumin (BSA) was injected into the PBS solution to a final concentration of 1 mg/l, and the frequency of the sensor chip was measured according to the manufacturer's protocol.

Cell viability assay

MTT assays using a Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto) were carried out to evaluate cell viability. SH-SY5Y cells (1×10^4 cells/100 μ l/well) in 96-well plates were pretreated with 0, 5, 10, or 20 μ M of PAL for 24 h, and 10- μ l volumes of various concentrations of H₂O₂ were added to the culture media in 96-well plates. After the plates had been incubated for 3 h, 10 μ l of a CCK-8 solution was added to each well and plates were incubated for another 4 h. Cell viability was then measured at an absorbance of 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). All of the results were normalized to values measured without cell culture.

Knockdown of DJ-1 in SH-SY5Y cells by siRNA

To knock down DJ-1 expression, siRNA targeting the DJ-1 gene (DJ-1 siRNA; Hokkaido System Science, Sapporo) was transfected into SH-SY5Y cells in 96-well plates (2×10^4 /well) using a microporator (MP-100; Digital Bio Technology, Seoul, Korea). The nucleotide sequences for siRNA targeting DJ-1 were as follows: 5'-

UGGAGACGGUCAUCCCUGUdTdT-3' (upper strand) and 3'-dTdTACCUCUGCCAGUAGGGACA-5' (lower strand). A nonspecific siRNA (control siRNA, Qiagen) was used as a negative control. At 48 h after transfection, the cells were incubated with various concentrations of PAL for 24 h, and the DJ-1 siRNA-transfected and control siRNA-transfected cells were treated with H₂O₂ for 2 and 1 h, respectively, and subjected to MTT assays.

Measurement of reactive oxygen species (ROS)

SH-SY5Y cells on cover glasses in 6-well plates were treated with PAL for 24 h and then treated with H₂O₂ for 1 h to induce oxidative stress. 2',7'-Dichlorodihydrofluorescein diacetate (H₂DCFDA) (Molecular Probes, Eugene, OR, USA) was added to the medium to a final concentration of 10 μ M. After incubation of the cells for 20 min, ROS images were observed under a scanning microscope (Biozero BZ-8000; Keyence, Osaka).

Western blot analysis

To examine the expression levels of proteins in SH-SY5Y cells, proteins were extracted from cells with a buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.5), and 0.5% NP-40. Proteins were then separated on a 12% polyacrylamide gel containing SDS and subjected to Western blot analysis with the respective antibodies. The following antibodies were used: anti-phospho-AKT (Ser473) (1:1000; Cell Signaling, Beverly, MA, USA), anti-AKT (1:1000, Cell Signaling), anti-phospho-PTEN (Ser380/Thr382/Thr383/Ser385) (1:1000; Novus Biologicals, Littleton, CO, USA), anti-PTEN (1:1000, Cell Signaling), anti-DJ-1 (1:1000; MBL, Nagoya), and anti-actin (1:10000; Chemicon, Temecula, CA, USA). Proteins on the membrane were reacted with an IRDye800 (Rockland, Philadelphia, PA, USA) or Alexafluor 680-conjugated secondary antibody (Molecular Probes) and visualized by using an infrared imaging system (Odyssey; LI-COR Biosciences, Lincoln, NE, USA).

Identification of the oxidative state of cysteine 106 (C106) of DJ-1 by MALDI-TOF/TOF-MS analysis

SH-SY5Y cells were treated with 20 μ M PAL for 24 h and then with 350 μ M H₂O₂ for 7 h. Proteins in cell extracts were immunoprecipitated with a rabbit anti-DJ-1 polyclonal antibody (final concentration of 2 μ g/ml) and separated on a 12.5% polyacrylamide gel. After the gel had been stained with Coomassie brilliant blue, a band corresponding to DJ-1 was cut out, reduced, alkylated with a buffer containing iodoacetamide, and digested with trypsin. Peptide solutions were mixed with α -cyano-4-hydroxycinnamic acid and applied onto an AnchorChip

target plate (AnchorChip™, Bruker Daltonik GmbH, Bremen, Germany). Mass measurements were carried out on an Ultraflex II-18 TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Mass spectra of the C106-containing peptide spanning 100 – 122 amino acids were obtained and analyzed with flex analysis software (Bruker Daltonics, version 2.4). A list of peptide mass fingerprinting was obtained for each sample and searched against the NCBI database by using a Mascot algorithm (Matrix Science, Boston, MA, USA). The rabbit anti-DJ-1 polyclonal antibody was prepared by us as described previously (2).

Statistical analyses

All of the experiments were repeated more than three times. Statistical significance was determined by using analysis of variance (one-way ANOVA) followed by Tukey's method, and data are presented as means $\pm$ S.D.

Results

Binding of PAL to DJ-1

The chemical structure of PAL is shown in Fig. 1. Binding activity of PAL to recombinant DJ-1 was examined using 27-MHz QCM (Affinix Q, Initium), which is a highly sensitive mass-measuring apparatus. A QCM Au electrode/sensor chip was coated with PAL and immersed in a PBS solution. The amount of protein binding to PAL was determined from frequency changes due to changes in mass on the electrode (with sensitivity in the order of subnanograms). As can be seen in Fig. 2, QCM showed decreasing frequency, indicating binding of PAL to recombinant DJ-1. The binding constant (K_d) of PAL to DJ-1 was calculated to be 9.167×10^{-8} M. No binding activity of PAL to BSA was found (Fig. 2B).

Effects of PAL on oxidative stress-induced cell death

To examine protective effects of PAL on H_2O_2 -induced cell death, SH-SY5Y cells were pretreated with various concentrations of PAL for 24 h; treated with 0, 325, 350, and 375 μ M H_2O_2 for 3 h; and then subjected to MTT assays. While H_2O_2 induced dose-dependent cytotoxicity in SH-SY5Y cells without pretreatment of PAL, pretreatment of cells with PAL was found to attenuate cell death caused by H_2O_2 in a dose-dependent manner (Fig. 3A).

To determine the specificity of PAL toward DJ-1, the effects of PAL on oxidative stress-induced cell death were examined using DJ-1-knockdown SH-SY5Y cells. To do that, SH-SY5Y cells were transfected with siRNA targeting human DJ-1 (DJ-1 siRNA) or with nonspecific siRNA (control siRNA). At 48 h after transfection, SH-SY5Y cells transfected with DJ-1 siRNA or control siRNA were pretreated with PAL for 24 h and then

Fig. 1. Chemical structure of protocatechuic aldehyde (PAL).

A. WT-DJ-1

B. BSA

Fig. 2. Binding activity of PAL to DJ-1. Binding of PAL to wild-type DJ-1(A) and BSA (B) was examined by using a quartz crystal microbalance as described in Materials and Methods. Δ EF indicates decreased frequency of a sensor chip. Experiments were carried out four times.

treated with various concentrations of H_2O_2 for 1 or 2 h, respectively. Since DJ-1-knockdown cells were more susceptible to H_2O_2 treatment than were control siRNA-transfected cells as described previously (15, 27) and since almost all of the DJ-1-knockdown cells died after cells were treated with H_2O_2 for 2 h, DJ-1-knockdown cells were treated with H_2O_2 for a shorter time than were control siRNA-transfected cells. Western blot analysis showed about 80% reduction of DJ-1 expression in DJ-1-knockdown SH-SY5Y cells compared with that in control siRNA-transfected SH-SY5Y cells (Fig. 3B). The results showed that while there was little protective activity of PAL in DJ-1-knockdown SH-SY5Y cells (Fig. 3C), PAL significantly protected control siRNA-

Fig. 3. DJ-1-specific protective activity of PAL against oxidative stress. **A:** SH-SY5Y cells were pretreated with 0, 5, 10, and 20 μM PAL for 24 h and then treated with various concentrations of H₂O₂ for 3 h and subjected to MTT assays as described in Materials and Methods. Significance: **P* < 0.05 and ***P* < 0.01, compared to control cells without PAL and H₂O₂. **B:** SH-SY5Y cells were transfected with siRNA targeting the *DJ-1* gene (DJ-1 siRNA) and non-specific siRNA (control siRNA) using a microporator (MP-100). At 49 h after transfection, the medium was changed and cells were incubated with various concentrations of PAL for 24 h. Protein extracts were prepared and the expression levels of DJ-1 and GAPDH were analyzed by Western blotting with anti-DJ-1 and anti-GAPDH antibodies (left panel). Quantification of intensity of bands is shown in the right panel. Cont and KD indicate control siRNA- and DJ-1 siRNA-transfected cells, respectively. **C, D:** SH-SY5Y cells were transfected with DJ-1 siRNA (**C**) or with control siRNA (**D**) as described in the legend of panel B; pretreated with 0, 5, 10, and 20 μM PAL; and then treated with 0, 300, 325, and 350 μM H₂O₂ for 1 or 2 h, respectively. Cells were then subjected to MTT assays as described in Materials and Methods. Significance: **P* < 0.05 and ***P* < 0.01, compared to control cells without PAL and H₂O₂. All of the experiments were carried out 4–5 times.

transfected SH-SY5Y cells from H₂O₂-induced cell death in a dose-dependent manner (Fig. 3D), indicating DJ-1-dependent activity of PAL.

Inhibition of production of intracellular ROS by PAL

When the level of ROS in cells exceeds the antioxidant capacity of the cells, oxidative stress will lead to cell damage. In order to examine the ability of PAL to inhibit production of intracellular ROS, SH-SY5Y cells that had been transfected with DJ-1 siRNA or with control siRNA were treated with H₂O₂ in the presence or absence of PAL and were stained with H₂DCFDA, which is a fluorescent probe to monitor ROS production. Incubation with H₂O₂ alone induced marked intracellular ROS production and resulted in strong fluorescence in both control SH-SY5Y cells and DJ-1-knockdown SH-SY5Y cells, and the level of fluorescence in DJ-1-knockdown cells

was stronger than that in control cells (Fig. 4: c and d). Pretreatment of cells with 20 μM PAL significantly inhibited ROS production in control siRNA-transfected SH-SY5Y cells but not in DJ-1 siRNA-transfected cells (Fig. 4: e and f), again suggesting a DJ-1-dependent effect of PAL on ROS production.

Activation of the AKT pathway by PAL

Since the AKT signaling pathway plays a central role in neuronal survival and since DJ-1 activates the AKT pathway by inhibiting PTEN, a negative regulator of the AKT pathway, the expression levels of phosphorylated proteins related to the AKT pathway in PAL-treated cells were examined by Western blot analyses. As shown in Fig. 5A, the level of phosphorylated AKT in PAL (+) / H₂O₂ (–)-treated SH-SY5Y cells was significantly increased to 183% compared to that in PAL (–) / H₂O₂

Fig. 4. Effects of PAL on production of ROS. SH-SY5Y cells were transfected with DJ-1 siRNA or with control siRNA as described in the legend of Fig. 3B. At 48 h after transfection, SH-SY5Y cells were treated with 20 μ M PAL for 24 h and then with 300 μ M H₂O₂ for 1 h. Cells were incubated with 10 μ M DCFH-DA for 20 min at 37°C and visualized under a scanning microscope. Scale bars represent 50 μ m of size images. Cont and KD indicate control siRNA- and DJ-1 siRNA-transfected cells, respectively. Experiments were carried out three times.

Fig. 5. Effects of PAL on the AKT signaling pathway. A: SH-SY5Y cells were treated with 20 μ M PAL for 24 h and then treated with 300 μ M H₂O₂ for 7 h. Protein extracts were prepared from cells and expression levels of total AKT, phosphorylated AKT, total PTEN, phosphorylated PTEN, DJ-1, and actin were examined by Western blotting with respective antibodies as described in Materials and Methods. B – D: Intensities of protein bands on membranes were quantified from three independent experiments and are shown as ratios of pAKT/AKT/actin, pPTEN/PTEN/actin, and DJ-1/actin (B, C, and D, respectively). Actin was used as a loading control. Significance: * P < 0.05, ** P < 0.01. Experiments were carried out three times.

(–)-treated cells (Fig. 5B). After exposure of cells to H_2O_2 , on the other hand, the level of phosphorylated AKT in the PAL (+) / H_2O_2 (+)-treated cells was increased to 295% compared to that in PAL (–) / H_2O_2 (+)-treated cells. The expression level of PTEN was then examined. As shown in Fig. 5C, the expression level of phosphorylated PTEN was reduced in PAL (+) / H_2O_2 (–)-treated SH-SY5Y cells, but there was no difference in cells that had been treated with PAL regardless of exposure of cells to H_2O_2 . The expression level of DJ-1 was increased in PAL-treated cells (Fig. 5D). These results suggest that PAL increased DJ-1 expression, leading to activation of the AKT pathway.

Effect of PAL on the oxidative state of C106 of DJ-1

Studies conducted by several reports, including us, have indicated that DJ-1 activity is regulated by the oxidative state of C106 of DJ-1. To examine the oxidative state of C106, SH-SY5Y cells pretreated with PAL were exposed to H_2O_2 . Proteins in cell extracts were then immunoprecipitated with an anti-DJ-1 antibody and precipitates were separated on a polyacrylamide gel. A protein band corresponding to DJ-1 was cut out, digested with trypsin, and subjected to MALDI-TOF/TOF-MS analysis. Peptides containing C106 had four peaks that correspond to reduced and oxidized C106 as SOH, SO_2H , and SO_3H forms (Fig. 6A). In the PAL (–) / H_2O_2 (–) and PAL (+) / H_2O_2 (–) groups, the sum of reduced and SOH forms of C106 was 71.7% of the total forms and the SO_3H form was 9.0% of the total forms (Fig. 6B). While treatment of cells with H_2O_2 alone increased the ratio of SO_3H form to 31.2%, it was decreased to about 21.1% after pretreatment with PAL (Fig. 6B). These results suggest that PAL inhibited formation of the SO_3H form of C106, an inactive form of DJ-1, resulting in accumulation of active DJ-1, thereby preventing H_2O_2 -induced cell death.

Discussion

In the present study, we found that PAL has protective effects against H_2O_2 -induced cell death in SH-SY5Y cells. PAL, a traditional Chinese medicine compound, exists in many traditional Chinese medicinal herbs, such as the leaves of *Stenoloma Chusanum* (L.) Ching and *Ilex chinensis* Sims and the roots of *Salvia miltiorrhiza*. These herbs have been widely used in Chinese medicine for treatment of cardiovascular diseases and burns and as antidotes. The pharmacology of PAL is, however, still unclear. Some pharmacological studies have shown that PAL possesses a number of biological activities, including inhibition of HBV DNA replication and HBV antigen expression (28) and inhibition of cytokine-induced

VCAM-1 and ICAM-1 expression (29). In this study, we found that PAL has DJ-1-dependent anti-oxidative activity.

Our group has identified DJ-1-binding compounds and assessed their binding activity by using QCM (27, 30). We used the same method to determine the DJ-1-binding character of PAL. An in vivo study demonstrated that pretreatment of SH-SY5Y cells with PAL inhibited cell death induced by H_2O_2 in a concentration-dependent manner. In DJ-1-knockdown cells, however, PAL lost the protective activity regardless of the concentration of PAL, suggesting that PAL prevents oxidative stress-induced cell death in a DJ-1-dependent manner. Redox imbalance caused by accumulation of excessive ROS results in mitochondrial dysfunction, protein misfolding, genetic mutation, and finally cell death (31). These are thought to be important factors that trigger the onset of Parkinson's disease. Suppression of the production of ROS by antioxidants might be an effective strategy for inhibiting oxidative stress-induced cell death. PAL decreased the level of ROS in SH-SY5Y cells, and DJ-1 dependency of PAL in reduction of the ROS level was further verified by using DJ-1-knockdown SH-SY5Y cells.

The AKT signaling pathway is activated by phosphatidylinositol 3-kinase (PI3K) in response to insulin, growth factors, cytokines, and cell stress (32 – 35). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3), and this is reversed by PIP3 phosphatase PTEN. Substrates phosphorylated by AKT contribute to activation of various cellular processes, such as cell survival, growth, proliferation, glucose uptake, metabolism, and angiogenesis. DJ-1 modulates activation of the AKT pathway in response to oxidative stress (36) and decreases PTEN activity by binding to PTEN, resulting in stimulation of phosphorylation of AKT, especially under oxidative stress conditions (37, 38). Both the levels of total and active/phosphorylated AKT were decreased in dopaminergic neurons of the midbrain in PD patients, suggesting a defective PI3K–AKT signaling pathway (39). In this study, we found that PAL activated the AKT pathway in SH-SY5Y cells. Western blot analyses showed that PAL up-regulated pAKT (Ser473) level in SH-SY5Y cells (Fig. 5B). Moreover, pretreatment of cells with PAL significantly increased AKT phosphorylation after cells were exposed to H_2O_2 (Fig. 5B). Concomitant with activation of AKT, PAL decreased the level of PTEN phosphorylation and increased the level of DJ-1 in SH-SY5Y cells (Fig. 5: C and D). Although the mechanism underlying up-regulation of DJ-1 expression by PAL is not clear at present, we have found that the expression of DJ-1 is up-regulated at the transcription level by wild-

Fig. 6. Effect of PAL on the oxidative status of DJ-1 in SH-SY5Y cells. A: SH-SY5Y cells were treated with 20 μ M PAL for 24 h and then with 350 μ M H₂O₂ for 7 h. Cell extracts prepared from cells were immunoprecipitated with an anti-DJ-1 antibody and the precipitates were separated on a 12.5% polyacrylamide gel. A stained band corresponding to DJ-1 was analyzed by MALDI-TOF/TOF-MS as described in Materials and Methods. Cysteine residues in the reduced state and oxidized to sulfenic and sulfinic acids but not sulfonic acid were covalently modified with iodoacetamide, which gives a shift of 56 daltons of C106-containing peptides spanning 100 – 122 amino acids. Signals at m/z 2267, 2283, 2299, and 2258 correspond to peptides with reduced (-SH), sulfenic acid (-SOH), sulfinic acid (-SO₂H), and sulfonic acid (-SO₃H) forms of C106, respectively. Experiments were carried out three times. B: Ratios of reduced and oxidized forms of C106 are shown.

type DJ-1 itself but not by C106-mutant DJ-1 (data not shown), indicating auto-regulation of DJ-1 expression. Since DJ-1-binding compounds, including PAL, rendered the oxidative status of C106 of DJ-1 to a reduced form, an active form of DJ-1 (ref. 27 and Fig. 6), it is therefore thought that DJ-1 activated by PAL induces the expression of the *DJ-1* gene and that the resultant DJ-1 protein then increases the level of phosphorylated AKT, resulting in inactivation of PTEN in cells that had been treated with PAL alone. In H₂O₂-treated cells, on the other hand, the effect of PAL on levels of phosphorylated PTEN and DJ-1 was not significantly changed. The expression of DJ-1 is induced by oxidative stress (14–16, 40). If H₂O₂ treatment of cells under the condition used in Fig. 5 almost saturates the induced expression level of DJ-1, the effect of PAL on levels of phosphorylated PTEN and DJ-1 might not be observed. The results suggest that PAL contributes to cell survival via activation of the AKT pathway in a DJ-1–dependent manner. Since detailed mechanisms underlying regulation of DJ-1 expression by PAL are not clear, further studies will be needed.

Cysteine is one of the most sensitive amino acids responding to the redox state due to the sulfhydryl group on the cysteine side chain. DJ-1 has three cysteine residues at amino acid numbers 46, 53, and 106 (C46, C53, and C106, respectively). C106 is the most sensitive cysteine residue among the three cysteines of DJ-1 against oxidative stress and the oxidized modification renders isoelectric points of DJ-1 into acidic points (40). Oxidation of C106 of DJ-1 regulates chaperone activity of DJ-1 and DJ-1–dependent dopamine synthesis (12, 21). In this study, we found that the level of the oxidized form of C106 as SO₃H that had been increased by treatment of cells with H₂O₂ was decreased in cells pretreated with PAL concomitant with an increase in the level of the reduced form of C106, suggesting that DJ-1 at least with the reduced form of C106 is an active form and that DJ-1 with the SO₃H form of C106 is an inactive form. It is therefore thought that PAL prevents superfluous oxidation of DJ-1.

Taken together, our results indicate that PAL has an anti-oxidative stress function by activating the AKT pathway in a DJ-1–dependent manner. We will further investigate the protective effects of PAL against oxidative stress-induced cell death using other types of oxidative stress agents such as 6-OHDA or MPP⁺ in cell and animal models of Parkinson's disease in order to find a potential pharmaceutical reagent for Parkinson's disease.

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