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ANTI-APOPTOTIC ACTIONS OF SAFINAMIDE IN 6-HYDROXYDOPAMINE-INDUCED CELL MODEL OF PARKINSON'S DISEASE

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder. Safinamide has been identified as a new therapeutic approach in the treatment of PD. In the present study, the effect of safinamide was studied on 6-hydroxydopamine (6-OHDA)-induced SHSY5Y cell model of PD. The 6-OHDA-challenged SH-SY5Y neuronal cells were treated with safinamide. Cell viability, reactive oxygen species (ROS) levels, and apoptosis were determined using dimethylthiazol-diphenyltetrazolium bromide (MTT) assay, dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay and cell cycle analysis, respectively. Treatment with safinamide slightly increased the cell viability, lowered the ROS levels and reduced apoptosis. However, all the results obtained were not statistically significant. In conclusion, safinamide possesses slight neuroprotective properties, achieved through anti-oxidative stress and anti-apoptotic properties.

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide, affecting more than 1% of individuals over 60 years old, leading to progressive disability that can be slowed down but never stopped by the currently available treatments. It is characterised by the selective loss of neuromelanin-containing dopaminergic neurons and the presence of Lewy bodies in the substantial nigra pars compacta of the patient. The aetiology of PD is still yet to be determined, but it is commonly accepted that the pathogenesis of PD involves an interplay of various detrimental cellular events such as oxidative stress, mitochondrial dysfunction, deficits in ubiquitin-proteasome system, lipid peroxidation, and apoptosis. PD is a chronic and progressive disease with four cardinal signs, namely resting tremor, rigidity, bradykinesia, and postural instability. Currently, there are still no specific laboratory tests or imaging techniques for diagnosing PD. There is no cure available for the disease, and symptomatic treatments rely on several classes of medications, with levodopa being the gold standard. Other classes of drugs include dopamine agonists, anticholinergic, catechol-O- methyltransferase inhibitors, and monoamine oxidase B (MAO-B) inhibitors. However, the current treatments available are designed to only restore dopaminergic activity in the dopamine-deplete striatum of PD patients, with certain extent of relieving motor symptoms. As of today, no established treatments are able to slow, stop, or modify the course of the disease [1].

Safinamide, namely (S)-(+)-2-[4-(3-fluorobenzyloxy-benzylamino) propionamide is one of the new drugs that was recently approved by U.S. Food and Drug Administration (FDA) in March 2017. Safinamide, a MAO-B inhibitor as well as a glutamate inhibitor, is currently indicated as an

adjunctive treatment to levodopa/carbidopa in patients with PD experiencing "off" episodes. The term "on" in levedopa treatment refers to the periods of improved mobility after treatment while periods of impaired motor function, in which patient responds poorly to levodopa are known as "off" time [2]. Several clinical trials had been conducted in the last decade, and studies on safinamide as an add-on therapy have suggested that it has significantly improved both motor and non-motor symptoms [3], lengthened "on" time with non-troublesome dyskinesia [4,5], reduced oxidative stress, and increased dopamine levels in the brain [6]. Interestingly, recent in vivo studies on PD have suggested that other than symptomatic relief of motor symptoms, safinamide confers significant neuroprotection against axonal degeneration and demyelination [7] and suppresses microglial activation, thus providing neuroprotection [8].

Despite having some evidence of safinamide being neuroprotective, no study had been conducted to explore the neuroprotective and anti-apoptotic actions of safinamide. Oxidative stress leading to apoptotic dopaminergic neuronal cell death has been known to play an essential role in the development of PD [9,10]. Therefore, this study was aimed to evaluate how safinamide protects the dopaminergic neurons from degeneration in 6-hydroxydopamine (6-OHDA)-induced cell model of PD. The 6-OHDA has been known to cause massive destruction of dopaminergic neurons [11]. It is widely used to induce models of PD because it reproduces the main pathological processes of PD in cells, such as oxidative stress, neurodegeneration, neuroinflammation, and apoptotic neuronal death [12]. The study focuses on evaluating the anti-apoptotic effects of safinamide in 6-OHDA-induced neuronal cells.

MATERIALS AND METHODS

Cell Culture and Treatment

SH-SY5Y cells (American Type Culture Collection, USA) were cultured in high glucose Dulbecco's Modified Eagle Medium (GibcoTM, USA), supplemented with 10% foetal bovine serum (Gibco, Brazil) and maintained at 37℃ with 5% carbon dioxide (CO2) in Galaxy S CO2 incubator (RS Biotech, UK).

A total of 4 treatment groups were prepared: control, 6-OHDA (25 µM; Sigma-Aldrich, USA) only, 6-OHDA with ½ maximum non-toxic dose (MNTD) of safinamide and 6-OHDA with MNTD of safinamide. These are the common treatment groups used to determine efficacy of drugs or compounds [13,14]. MNTD of safinamide was determined previously as 3 µM [15]. Safinamide was added simultaneously with 6-OHDA to the cells during treatment.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Assay

The SH-SY5Y cells with seeding density of 5.5×10^5 cells/mL were seeded into 96-well plates (TPP, Switzerland). The cells were treated as described previously and incubated at 37℃ with 5% CO_2 for 24 and 48 hours. Then, 20 µL of MTT (Calbiochem, USA) was added into each well and incubated at 37℃ with 5% CO_2 for 4 hours. The solution in well was removed and replaced with 100 µl of dimethyl sulfoxide, followed by reading absorbance at 570 nm in the dark.

Dichloro-dihydro-fluorescein Diacetate (DCFH-DA) Assay

The SH-SY5Y cells with seeding density of 4.5×10^5 cells/well were seeded into a 24-well plate (TPP, Switzerland) and treated for 24 hours. Then, the cells were collected and spun down at 1500 rpm for 5 minutes. A portion of the cells was transferred to a 96-well black plate (TPP, Switzerland) while the remaining cells were used for cell counting by Trypan blue staining method. Black plates were used in this assay because they absorb light and reduce background and crosstalk. DCFH-DA (40 µM) was added into each well and read using a SpectraMax microplate reader at the fluorescence excitation of 485 nm and emission of 525 nm. Fluorescence readings were taken at 0, 10, 20, and 30 minutes. The fluorescence readings were then normalised according to the respective cell concentration determined via the cell counting method, to calculate the relative fluorescence unit (RFU). This method is adapted from Yap *et al.* (2016) [16] and Yew *et al*. (2014) [17].

Cell Cycle Analysis

The SH-SY5Y cells (2.4×10^6) were seeded into 6×10 mm dishes (SPL lifescience, Korea) and exposed to the various treatment groups as described previously for 24 hours. The cells were then harvested, fixed with 70% of ethanol (Merck, Germany) and stored at 4℃ overnight. The cells were washed with phosphate-buffered saline (PBS; Biobasic, USA), spun down at 3000 rpm for 5 minutes at 4℃ and resuspended in 500 µL of PBS containing 15 µM of ribonuclease (Thermo Fisher Scientific, USA) and 20 µM of propidium iodide (Sigma Aldrich, USA). The cells were then transferred to Fluorescent Activated Cell Sorter (FACS) tubes (BD Bioscience, USA). Analysis of the percentage of cells at different phases of the cell cycle ($SubG_1$, G_0/G_1 and S phase) was performed using the FACSCalibur flow cytometer (BD Biosciences, USA) and the software Cell Quest Pro.

Statistical Analysis

Results were expressed as mean ± standard deviation and analysed by One-way ANOVA with Tukey's post-test using SPSS Statistics (IBM, USA). Data from MTT and DCFH-DA assays were obtained from at least 3 independent sets of study, while results of cell cycle analysis were analysed from a single experiment. Differences between groups were considered statistically significant at a value of $P \leq 0.05$.

RESULTS AND DISCUSSION

Determination of Neuroprotective Effect of Safinamide on 6-OHDA-Induced SH-SY5Y Cells

MTT assay was used to examine the cytotoxicity effects of 6-OHDA on SH-SY5Y cells for different durations (24 and 48 hours). As shown in Figure 1, 6-OHDA induced cell death in SH-SY5Y cells. It slightly decreased the cell viability,

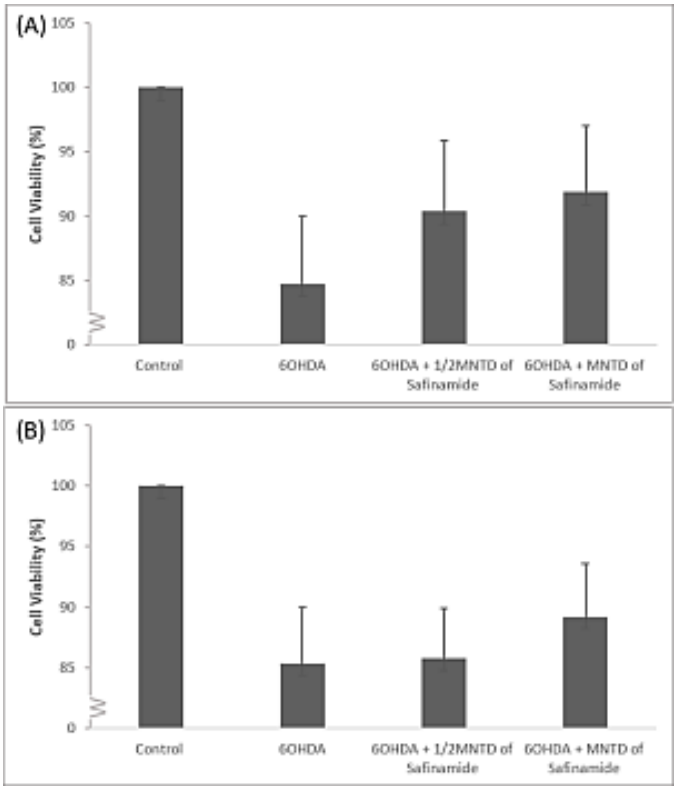

Figure 1. Effect of safinamide on cell viability in 6-OHDA-induced SH-SY5Y cells at (A) 24 hours and (B) 48 hours. Mean ± standard deviation of data from at least three independent experiments are shown. Differences between groups are not statistically significant

which mimicked the pathology present in PD patients in the early stage of the disease. To determine and compare the neuroprotective effects of safinamide at different concentrations, 6-OHDA-induced SH-SY5Y cells were treated with 1.5 μM (½ MNTD) and 3.0 μM (MNTD) of safinamide. MTT assay showed that safinamide slightly increased the SH-SY5Y cell viability as compared with the 6-OHDA-treated group in a dose-dependent manner, although not statistically significant ($P>0.05$). Cell viability was greater at 24 hours as compared to 48 hours after the treatment, indicating that safinamide might be more effective as a short-acting drug.

In PD, protection of dopaminergic neurons is needed in the development of effective therapies, as current therapies do not fully solve the degenerative progress of PD. Dopaminergic cells die progressively due to the presence of neurotoxins. Hence, drugs which are able to inhibit cell death are particularly important in PD. In the present in vitro study, safinamide had shown mild neuroprotective effects against the 6-OHDA-induced PD model. 6-OHDA, a dopamine neurotoxin, can induce oxidative stress that leads to apoptotic cell death [18]. Several studies have shown that safinamide presents with both neuroprotective and anti-inflammatory effects [7,8,19,20]. It is able to suppress microglial activation, hence protecting dopaminergic neurons from degeneration [8]. A review summarising the pharmacokinetics, and pharmacodynamics of safinamide has suggested that the drug prevented 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced toxicity [21]. Additionally, safinamide is known for its multi-modal actions, being both a MAO-B inhibitor and a glutamate inhibitor (through inducing the blockage of voltage-gated sodium channels). Taken together, safinamide could prevent

neuronal cell death, at the same time possesses other effects such as anti-inflammation and inhibits MAO-B or glutamate.

The mild neurorescue or neuroprotective effects of safinamide observed in the present study might be due to the fact that it is not a potent drug. According to FDA, safinamide is currently indicated as an adjuvant therapy to levodopa for PD patients that are experiencing "off" episodes [22], and rarely prescribed to patients as a standalone drug. Additionally, the present study tested safinamide at low concentrations, as toxicity study has shown that safinamide is a relative safe drug at low dose [23], hence causing the minimal effects of safinamide on the tested model. Future studies may continue to explore a higher concentration range of safinamide for its effect on cell viability.

Determination of Oxidative Stress in Safinamide Treated 6-OHDA-Induced SHSY5Y Cells

To determine the intracellular reactive oxygen species (ROS) scavenging activities of safinamide on 6-OHDA-induced SH-SY5Y cells, the DCFH-DA assay was carried out. The levels of ROS increased in cells upon treatment with 6-OHDA, as seen in Figure 2. Treatments with safinamide for 24 hours had moderately lowered the ROS levels within the cells, although the decrease is not statistically significant ($P>0.05$). Hence, the results suggest that safinamide might have mild antioxidant properties. The current study shows that safinamide reduced ROS levels in neuronal cells by about 1.5-fold at ½ MNTD, as compared to 6-OHDA-induced group. In another study, safinamide had shown to reduce ROS levels by about 1.2 – 2.7-fold in hydrogen peroxide-induced myoblasts [24].

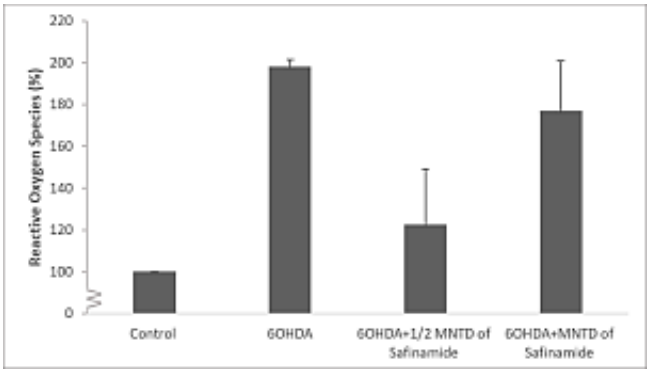

Figure 2. Effect of safinamide on oxidative stress in 6-OHDA-induced SH-SY5Y cells at 24 hours. Mean ± standard deviation of data from at least two independent experiments are shown. Differences between groups are not statistically significant

The loss of dopaminergic neurons depicted in the pathology of PD can be recapitulated in vitro through exposure to oxidative stress-inducing drug such as 6-OHDA. Increased oxidative stress within the cells will eventually lead to cell death, resulting in decreased cell viability. Safinamide had demonstrated antioxidant effects by decreasing the ROS levels in the SH-SY5Y cells as shown in the present study. Previous study also concluded that safinamide could reduce oxidative stress within cells, in which the drug decreased the ROS levels in the muscle fibres of mice [24].

Determination of Anti-Apoptotic Actions of Safinamide on 6-OHDA-Induced SH-SY5Y Cells

To determine the anti-apoptotic effect of safinamide, the treated SH-SY5Y cells were subjected to the analysis of cell cycle by flow cytometry. Cells present in the subG$_1$ phase of cell cycle are undergone apoptosis due to DNA fragmentation. The low percentage of apoptotic cells observed in the control as seen in Figure 3 was caused by spontaneous cell death. The SH-SY5Y cells treated with 6-OHDA served as a comparison against the safinamide treatment groups. From Figure 3, the apoptotic population of 6-OHDA group was 6.6-fold higher than the untreated control, suggesting that 6-OHDA induced apoptosis in SH-SY5Y cells. Treatment with safinamide at MNTD after 24 hours had resulted in a decrease of the percentage of apoptotic population. However, the cell population in subG1 phase was slightly increased when treated with ½ MNTD of safinamide. The comparisons were made against the 6-OHDA-induced SH-SY5Y cells. The results show that safinamide might prevent apoptosis at MNTD but not at ½ MNTD.

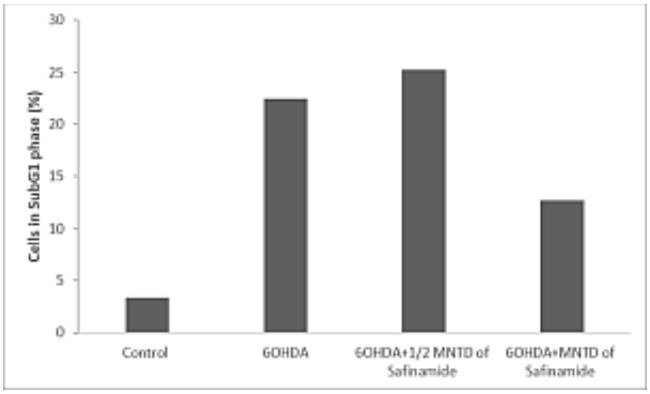

Figure 3. Effect of safinamide on apoptosis in 6-OHDA-induced SH-SY5Y cells at 24 hours

Apoptosis, a known process of programmed cell death, is initiated by various factors such as oxidative stress and mitochondrial dysfunction. Main pathway activated in PD is the intrinsic apoptotic pathway, while recent evidence has suggested that extrinsic pathway may be active as well, though its role remains unclear [25]. Safinamide that acts as a sodium channel inhibitor might have the ability to inhibit apoptosis as inhibition of sodium channels was found to inhibit apoptosis in motor neurons [26]. Safinamide was found to be a potent voltage and frequency dependent

blocker of skeletal muscle sodium channels, both *in vitro* and *in vivo* in another study [27].

CONCLUSION

Safinamide has shown to exhibit slight neuroprotective effects by reducing cell death, ameliorating oxidative stress and preventing apoptosis in the 6-OHDA-induced SH-SY5Y cells. However, the results were not statistically significant. Future studies are needed to explore the effects of safinamide at a wider range of concentrations. The current study tested the effects of safinamide at the low doses of ½ MNTD and MNTD, which might result in the low efficacy of the drug. Therefore, higher concentrations (with tolerable toxicity) that might show better drug effects should be tested. Since safinamide is currently indicated as an adjuvant therapy, effects of safinamide in combination with other drugs such as dopamine agonists, catechol O-methyltransferase inhibitors and anticholinergics should also be explored. In order to better elucidate the protective effects of safinamide, safinamide could be administered to the cells prior to addition of 6-OHDA. This could help to determine how well is safinamide in preventing the levels of damage caused by 6-OHDA. Lastly, *in vivo* studies and clinical trials are needed to be carried out to obtain more data on the use of the drug.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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