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Research Article

Anti-neuroinflammatory mechanism of safinamide in inhibiting lipopolysaccharide-induced microglial activation

Jashann Ashwyn[1], Yong Qi Leong[2], Brianna[1], Khuen Yen Ng[2], Soi Moi Chye[1], Anna Pick Kiong Ling[1], Kenny Gah Leong Voon[3], Yin Yin Ooi[4], Yee Lian Tiong[5], Rhun Yian Koh[1*]

[1] International Medical University, No. 126, Jalan Jalil Perkasa 19, Bukit Jalil, Kuala Lumpur, Malaysia
[2] Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, Subang Jaya, Selangor, Malaysia
[3] University of Nottingham Malaysia, Jalan Broga, Semenyih, Selangor, Malaysia
[4] Taylor's University, 1, Jalan Taylors, Subang Jaya, Selangor, Malaysia
[5] UCSI University, No. 1, UCSI Heights, Jalan Puncak Menara Gading, Taman Connaught, Cheras, Wilayah Persekutuan Kuala Lumpur, Malaysia

ABSTRACT

Neuroinflammation is an inflammatory response in the central nervous system that may lead to neurodegenerative diseases, such as Parkinson's disease (PD). PD is the second most common neurodegenerative disorder with a high prevalence among elderly individuals. Microglia, which are associated with neuroprotection, are activated during inflammation, resulting in damage to dopaminergic neurons in the substantia nigra. Based on previous studies, safinamide can provide neuroprotection to dopaminergic neurons by inhibiting microglial activation. Hence, this study aims to investigate the anti-neuroinflammatory mechanism of safinamide in inhibiting lipopolysaccharide (LPS)-induced microglial activation. 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of safinamide on BV-2 (microglial) cells. Maximum non-toxic dose (MNTD) and half MNTD of safinamide were then calculated. To determine whether safinamide could rescue lipopolysaccharide-treated BV-2 cells from cell death and oxidative stress, MTT assay and dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay were performed, respectively. Enzyme-linked immunosorbent assay (ELISA) was performed to investigate the involvement of STAT1/NF-kappa B pathway proteins in the activation of microglia. The MNTD of safinamide was determined to be 29.5±10.66 µM. Safinamide was not able to rescue BV-2 cells from LPS-induced cell death. Nevertheless, a slight reduction of reactive oxygen species levels was noted when LPS-induced BV-2 cells were treated with safinamide. There was a slight decrease in protein expression of STAT1, NF-kappa B, iNOS and COX-2 in the LPS-induced BV-2 cells after treatment with safinamide. While safinamide did not rescue BV-2 cells from cell death, safinamide has been shown to slightly reduce oxidative stress in BV-2 cells.

Keywords:
Safinamide, Anti-inflammation, Microglial cells, STAT1/NF-kappa B pathway, Oxidative stress

1. INTRODUCTION

Neuroinflammation is an inflammatory response in the central nervous system (CNS) mediated by cytokines, secondary messenger molecules, chemokines, and reactive oxygen species (ROS). These inflammatory mediators are released due to microglial activation. Neuroinflammation is initiated by microglia. Under steady-state conditions, microglia remain inactive through interactions with neighbouring neuronal cells. Inciting factors such as invading pathogens, neuronal injuries and toxic compounds contribute to the activation of microglia. The activation of microglia is usually caused by pathogen-associated molecular patterns and/or endogenous damage-associated molecular patterns. The activated microglia undergo phenotypical and morphological changes and through the

***Corresponding author:**
*Rhun Yian Koh Email: rhunyian_koh@imu.edu.my

release of various pro-inflammatory mediators, cause neurotoxicity and potentially lead to neurodegenerative diseases such as Parkinson's disease (PD)[1]. A clear understanding between dopaminergic neuron cell death and microglial activation has yet to be established. Microglial activation is thought to either be a cause or a consequence in the disease process of PD. The presence of reactive microglia has been noted in toxic-induced and transgenic mouse models of PD. In the last few years, many studies have suggested that the chronic inflammatory disease process of PD resulted in neuronal cell death. This is because the microglia synthesising excessive amounts of pro-inflammatory cytokines and reactive oxygen species. In summary, a plethora of studies have suggested that microglial activation correlates with PD progression and induces dopaminergic neuronal cell toxicity[2-3].

PD is the second most common progressive neurodegenerative disease after Alzheimer's disease[4]. Mean age of onset is approximately 60 years in most cases and the incidence of PD increases rapidly with age and peaks at 80[5]. Thirty-five percent of PD patients lived more than six years[6]. It is a complex multifactorial disease with the pathognomonic sign of Lewy bodies in the substantia nigra and a selective loss of dopaminergic neurons[7]. These neurons secrete dopamine, which interacts with structures within the basal ganglia and facilitate movement. With the loss of dopamine, PD is marked by cogwheel rigidity, resting tremors, bradykinesia, postural instability and gait impairment[8].

PD remains as an idiopathic disease and its pathogenesis still remains unclear[9]. However, evidence suggests that microglial activation or otherwise known as microgliosis plays a major role in the pathogenesis of PD[10]. Microglia usually exhibit a neuroprotective role within the CNS. However, in many neurodegenerative diseases, including PD, microglia exhibit a neurotoxic effect that serves as the cause of neuropathology[11]. Proteins synthesised (such as alpha-synuclein) and deposit themselves within the dopaminergic neurons through an intracellular cascade (e.g. nuclear factor, NF-kappa B pathway), interact with the microglia and get activated[12].

Most therapies are only able to address the dopamine deficiency and temporarily alleviate the motor symptoms of PD. Dopaminergic replacement therapy with levodopa is the gold standard treatment for PD, especially during early stages. Alternatives to levodopa, such as dopamine agonists, monoamine oxidase B inhibitors (rasagiline and selegiline) and amantadine are given, and in many cases, given adjunct to levodopa. However, these alternatives are only able to postpone the development of an adverse effect known as levodopa-induced dyskinesia (involuntary movements). Currently, only symptomatic therapies are available to treat PD[12-14].

In the recent decade, a newly approved drug named safinamide is currently in development as an add-on therapy to both dopamine agonists and levodopa[15-16]. In a recent double-blind placebo-controlled randomised trial, safinamide, as an add-on therapy to levodopa, significantly reduced troublesome dyskinesia in PD patients[17]. Safinamide is categorised as a selective and reversible inhibitor of monoamine oxidase (MAO) type B[18]. It has multiple modes of action, including dopamine modulation, calcium channel modulation, sodium channel inhibition and glutamate release inhibition. As dopamine is metabolised by MAO-B enzyme in the synaptic space, inhibiting MAO-B enzyme is considered a well-established approach. Hence, safinamide is used to reversibly inhibit MAO-B enzyme leading to increased synaptic dopamine levels[19].

Microglial activation is associated with a multitude of signalling pathways, including signal transducer and activator of transcription 1 (STAT1) and NF-kappa B, which play a role in regulating inflammatory responses and cell death. The activation of these signalling cascades resulted in the production of pro-inflammatory and inflammatory mediators[20-21]. STAT1 is usually triggered by hypoxia or oxidative stress in microglia[21-22]. Microglia responded to ROS and undergone phenotypical changes from ramified (steady-state microglia) to amoeboid form (activated microglia, M1 phenotype). STAT1 is a redox-sensitive protein and is sensitive to oxidative stress. The ROS activated STAT1 through S-gluthathionylation and this resulted in the transcription and production of pro-inflammatory cytokines (inducible nitric oxide synthase, iNOS and cyclooxygenase-2, COX-2), which resulted in neuroinflammation[21]. The NF-kappa B pathway is an additional signalling pathway that contributed to the overproduction of various pro-inflammatory mediators. This pathway is associated with inflammation, immune responses, cell cycle and survival. NF-kappa B played the role as the central regulator of neuroinflammation-associated disease pathogenesis. Pathogens, endotoxins, ischaemia, and oxidative stress functioned as triggers for the NF-kappa B signalling pathway[23-25].

In a recent *in vivo* PD study, other than symptomatic relief, safinamide has been shown to display neuroprotection effect through the reduction of microglial activation[26]. However, the neuroprotective properties of safinamide and its mechanism are not completely understood[27]. Therefore, this study evaluated the anti-neuroinflammatory mechanism of safinamide in inhibiting lipopolysaccharide (LPS)-induced microglial activation.

2. MATERIALS AND METHODS

2.1. Cell culture

BV-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), with a supplement of 10% foetal bovine serum (FBS; Gibco, South America) and maintained at 37°C with 5% carbon

dioxide (CO_2) in a humidified atmosphere.

2.2. Determination of maximum non-toxic dose (MNTD) and half MNTD of safinamide on BV-2 cells

Cytotoxicity of safinamide on BV-2 cells was determined using MTT assay. Safinamide mesylate salt powder (Sigma-Aldrich, USA) was reconstituted into a solution using phosphate buffered saline (PBS; Biotech, Canada). Cells were treated with six different concentrations of safinamide mesylate salt (Sigma-Aldrich, USA), which was obtained from a two-fold serial dilution from 50 µM safinamide to a concentration of 1.563 µM. Untreated cells were served as the control group. BV-2 cells at the seeding density of $5x10^4$ cells/mL were seeded into a 96-well plate. The plate was then incubated at 37°C with 5% CO_2 supply to reach a confluency of at least 80% before treating the cells with various concentrations of safinamide. After treatment, the plate was incubated at 37°C with 5% CO_2 supply for 48 hours. Subsequently, 20 µL of MTT (Calbiochem, USA) was added into each well and the plate was incubated for 4 hours at 37°C with 5% CO_2 supply. All the solutions were removed before adding 100 µL of dimethyl sulfate (DMSO) (Sigma-Aldrich, USA) to dissolve the precipitates, and the plate was read at 570 nm and 630 nm using a microplate reader. A graph on percentage of cell viability was plotted with the values obtained along with a graph of percentage of cytotoxicity. From this graph, the maximum non-toxic dose (MNTD) and ½ MNTD suitable to be used for BV-2 cells was determined.

2.3. Determination of the effects of safinamide on cell viability in LPS-induced BV-2 cells

LPS was used to induce oxidative stress buildup and cell death in the present study. To assess the effects of safinamide on cell viability in LPS-induced BV-2 cells, cells were treated with LPS concurrently with safinamide at MNTD and ½ MNTD for 48 hours. The four different treatment groups are as follows: control, 1 µg/mL LPS, 1 µg/mL LPS+½ MNTD of safinamide, and 1 µg/mL LPS+MNTD of safinamide. Percentage of cell viability in these four different groups were determined with MTT assay as described before.

2.4. Evaluation of reactive oxygen species (ROS) levels in LPS-induced BV-2 cells treated with safinamide

The intracellular ROS levels were identified using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence dye. BV-2 cells with a density of $5x10^4$ cells/mL were seeded into a 96-well black plate (Sigma-Aldrich, USA). After 24 hours, the cells were treated with 100 µL of MNTD or ½ MNTD safinamide with 1 µg/mL LPS. After the treatment, the plate was incubated at 37°C with 5% CO2 supply for 48 hours. After that, all the solutions were removed. One hundred µL of DCFH-DA fluorescence dye, which was prepared at a concentration of 10 µM, was added into each well. Fluorescence readings were taken immediately at 10-minutes interval using a microplate reader at the excitation wavelength of 485 nm and emission wavelength of 535 nm. Fluorescence readings of each treatment group were normalised to the corresponding readings obtained from MTT assay. The treated group was compared to the untreated control for the identification of fold change in ROS production.

2.5. Determination of the effects of safinamide on STAT1/NF-kappa B pathway in LPS-induced BV-2 cells

Proteins involved in the STAT1/NF-kappa B pathway were analysed with enzyme-linked immunosorbent assay (ELISA). ELISA is a method to analyse proteins through specific antigen-antibody binding. Proteins analysed were STAT1, NF-kappa B, iNOS and COX-2. BV-2 cells were seeded on 96-well plate with a seeding density of $5x10^4$ cells/mL and incubated at 37°C with 5% CO_2 supplement to reach a confluency of at least 70% before treating the cells with safinamide and LPS for 48 hours. Wells for secondary antibodies (staining control) were treated as in the control group. All solutions in each well were then removed, and 100 µL of -20°C methanol was added into each well to fix the cells for 20 minutes, before washing with Tris buffer saline (Bio Rad, USA) with Tween 20 detergent (TBST) (Calbiochem, USA) for 3 times at 5 minutes each. Then, each well was incubated with 100 µL 0.6% hydrogen peroxide (H_2O_2; Calbiochem, USA) for 30 minutes in dark. One hundred µL of 3% bovine serum albumin (BSA; Cell Signalling, USA) was added into each well and the plate was incubated for 1 hour at room temperature after washing with TBST as mentioned previously. The BSA was then removed from all wells except those for secondary antibodies.

One hundred µL of primary antibodies were then added into each well and the plate was incubated overnight at 4°C. Following this, cells were washed with TBST as mentioned previously before the incubation with secondary antibodies (anti-rabbit IgG, HRP-linked antibody 7074, Cell Signalling, USA) for 1 hour at room temperature. All primary antibodies were purchased from Cell Signalling, USA: STAT1, rabbit mAb #14994; NF-kappa B, rabbit mAb #8242; COX-2, rabbit mAb #12282; and iNOS, rabbit mAb #13120. One hundred µL of 3,3',5,5'-tetramethylbenzidine (TMB; Nacalai Tesque, Japan) was added into each well. As solutions turned blue, 100 µL of 500 mM sulfuric acid (H_2SO_4; Fisher Scientific, USA) was added into each well to stop the reaction. The plate was read at 450 nm with a microplate reader. Subsequently, the solution in each well was discarded before washing with distilled water 3 times at 5 minutes each. The plate was dried with an air-dryer. One

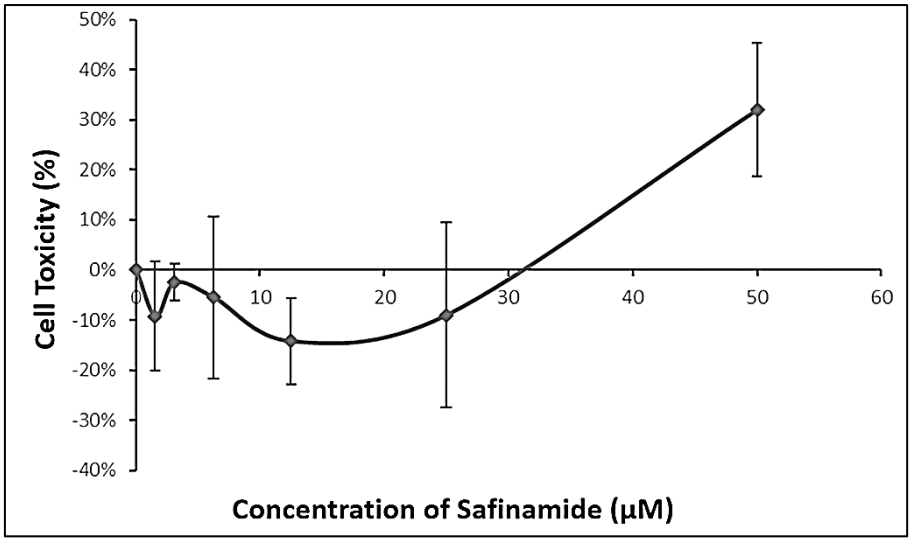

Figure 1. Cytotoxic effect of safinamide on BV-2 cells. Mean±standard deviation of data from three independent experiments are shown.

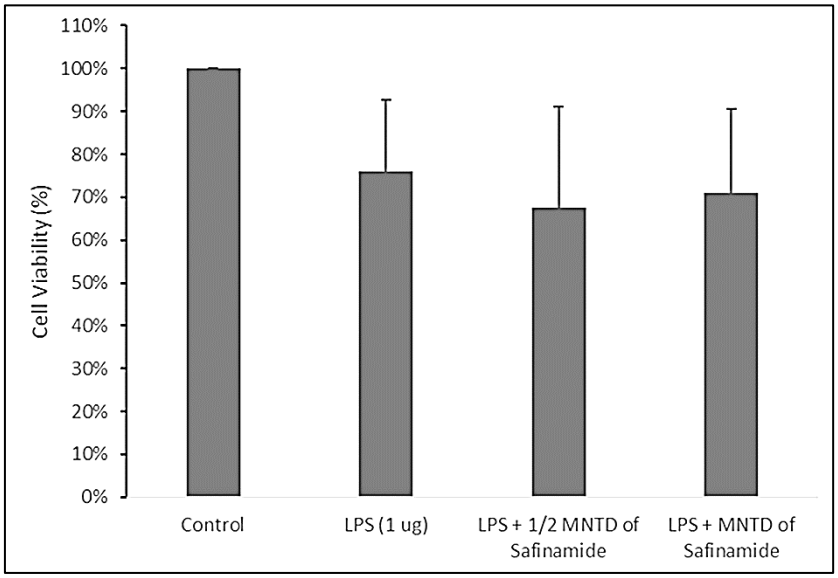

Figure 2. Effect of safinamide on the viability of LPS-induced BV-2 cells at 48 hours. Mean±standard deviation of data from three independent experiments are shown. Differences between groups are not statistically significant (p>0.05).

hundred µL of 0.04% crystal violet (Merck, USA) in 4% ethanol (Bumi-Pharma Sdn. Bhd, Malaysia) was added into each well and incubated for 30 minutes at room temperature. The plate was washed under running tap water and dried with an air dryer. One hundred µL of 1% sodium dodecyl sulfate (SDS; OmniPur, USA) was added into each well, and the plate was tapped gently to fully dissolve the precipitate before reading the plate at 595 nm with a microplate reader. The readings obtained from ELISA were normalised to the readings obtained from the crystal violet staining.

2.6. Statistical analysis

All values were expressed as mean±standard deviation from three independent experiments. ANOVA was used for data analysis and the differences were considered statistically significant at a value of p<0.05.

3. RESULTS

3.1. Evaluation of cytotoxicity of safinamide on BV-2 cells and determination of MNTD and half MNTD

MTT assay was performed to determine the cytotoxicity of safinamide on BV-2 cells. The MNTD was determined from Figure 1. The MNTD of safinamide was calculated to be 29.5±10.66 µM.

3.2. Effects of safinamide on cell viability in LPS-induced BV-2 cells

This experiment was done to ascertain whether safinamide is able to rescue the BV-2 cells from cell death. Based on Figure 2, BV-2 cells treated with LPS only experienced a slight reduction (p>0.05) in the cell viability by approximately 25% compared to the control.

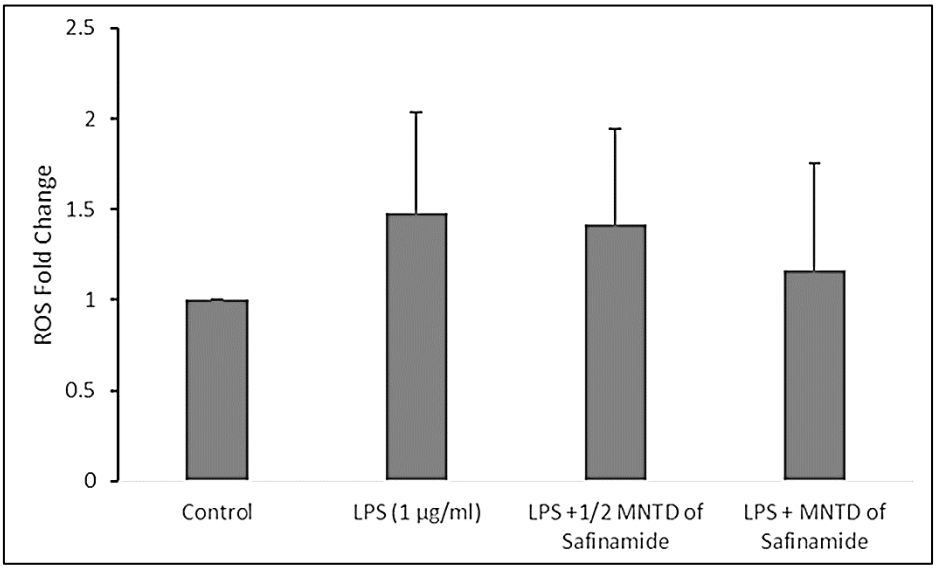

Figure 3. Effect of safinamide on level of oxidative stress in LPS induced-BV-2 cells at 48 hours. Mean±standard deviation of data from three independent experiments are shown. Differences between groups are not statistically significant (p>0.05).

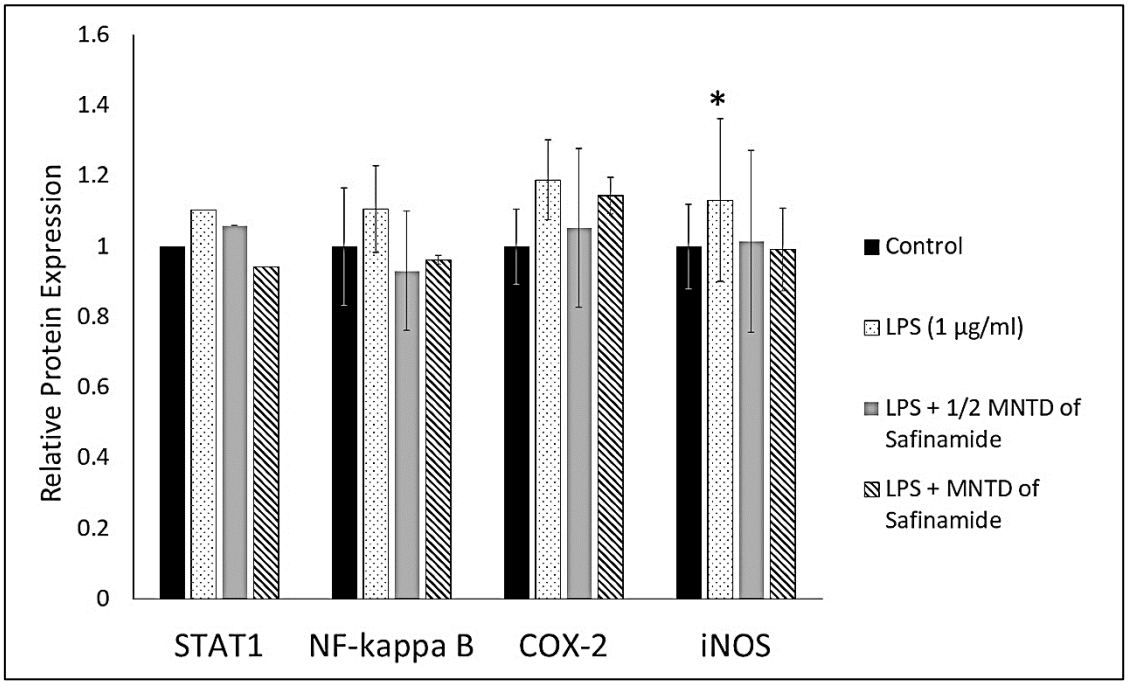

Figure 4. Effect of safinamide on downregulating Stat1, NF-kappa B, COX-2 and iNOS in LPS-induced BV-2 cells at 48 hours. Mean±standard deviation of data from three independent experiments for each protein is shown. *=p<0.05, compared to its control.

The cell viability of both the LPS+safinamide at MNTD and LPS+safinamide at ½ MNTD groups were slightly reduced (p>0.05) compared to the group treated with LPS only.

3.3. Effects of safinamide on ROS levels in LPS-induced BV-2 cells

The group induced with LPS only displayed a slight increase (p>0.05) of ROS of about 1.5 fold change compared to the control group. Both LPS+½ MNTD, and LPS+MNTD of safinamide groups have a slight decrease (p>0.05) in their fold change compared to the group with LPS only. The fold change of LPS+MNTD of safinamide was slightly lower (p>0.05) compared to LPS+½ MNTD of safinamide (Figure 3).

3.4. Effects of safinamide on STAT1/NF-kappa B pathway in LPS-induced BV-2 cells

ELISA was used to determine the proteins involved in the anti-oxidative effect of safinamide on microglial activation. From Figure 4, LPS slightly increased the relative protein expression of STAT1, NF-kappa B, and COX-2 and significantly increased the relative protein expression for iNOS compared to their control groups.

All the four proteins showed a slight reduction in their relative protein expression in the LPS+½ MNTD and LPS+MNTD of safinamide groups, when compared to the LPS only group. The LPS+MNTD of safinamide group appeared to have a greater reduction in its relative protein expression (STAT1) compared to the LPS+½ MNTD of safinamide group. On the other hand, the LPS+½ MNTD of safinamide group displayed a greater reduction in its relative protein expression (NF-kappa B) compared to the LPS+MNTD group. Similarly, the LPS+½ MNTD of safinamide group displayed a greater reduction in its relative protein expression (COX-2) compared to the LPS+MNTD of safinamide group. Nevertheless, the LPS+MNTD of safinamide group appeared to have a greater reduction in its relative protein expression (iNOS) compared to the LPS+½ MNTD of safinamide group. All four results did not have statistical significance ($p>0.05$) between their groups with the exception of iNOS, where statistical significance ($p<0.05$) was noted between the group treated with LPS only and the control group.

4. DISCUSSION

Previous studies have noted the use of 1 µg/ml LPS to activate microglia[28-30]. LPS is an endotoxin[31]. The LPS model is a commonly used model to activate microglia. It is able to replicate similar characteristics of PD including microglial activation and dopaminergic neuronal cell death in the substantia nigra[32-36]. A recent *in vivo* study indicated that the use of 1 µg/mL LPS was sufficient to activate BV-2 cells and promote disease progression in models of neurodegeneration[29]. Furthermore, LPS was shown to interact with the toll-like receptors (TLRs) present on the surface of microglia and mimic the activation of microglia seen in PD[37]. Pathways associated with microglial activation were upregulated by LPS. For instance, LPS is known to cause oxidative stress (ROS) in microglia, which in turn results in the expression of pro-inflammatory genes through the NF-kappa B pathway[28].

In this study, the cytotoxicity of safinamide mesylate salt was evaluate and MNTD of 29.5±10.66 µM was acquired. Based on Figure 1, negative values are seen at the lower concentrations of safinamide. Hence, the results suggested that safinamide was not toxic to the BV-2 cells at the concentrations tested, and that the MNTD used in this present study is comparable or lower than the doses used in other studies. It is worth noting that safinamide, a newly approved drug, have been tested on animal models in the past studies[38].

Based on Figure 2, LPS reduces cell viability compared to the control. Results showed that safinamide failed to rescue the BV-2 cells from cell death. Cell death would allow the release of more pro-inflammatory mediators which may perpetuate more cell death. The failure of safinamide in rescuing BV-2 cells indicates that this process of cell destruction cannot be halted. The differences between the LPS+safinamide groups and LPS only group were not statistically significant, and difference was negligible. This might be due to the multiple modes of action of safinamide, such as MAO-B inhibition, inhibition of glutamate release and calcium modulation that are not related to the regulation of cell death. Safinamide might not be able to rescue BV-2 cells from cell death through these modes of action. As safinamide is classified as an MAO-B inhibitor, cell rescue effects may be independent of MAO-B inhibition (based on previous studies)[16].

Safinamide as an MAO-B inhibitor is able to reduce oxidative stress by preventing the formation of toxic free radicals[16]. Safinamide displayed its ability in reducing the basal levels of oxidative stress in untreated BV-2 cells. Hence, LPS was added to the BV-2 cells to assess whether safinamide was capable of reducing the ROS fold change. Based on Figure 3, LPS+½ MNTD and LPS+MNTD groups displayed a slight reduction of the ROS fold change compared to the LPS only group. Safinamide has anti-oxidative properties similar to other MAO-B inhibitors[39-40]. Based on a previous *in vitro* study, safinamide is able to diminish the formation of ROS and increase the levels of glutathione in LPS-induced microglia[41]. The increase in glutathione levels is able to promote the expression of antioxidative molecules. Furthermore, safinamide induces the blockade of sodium channels that may be associated with the anti-oxidative properties of safinamide[41].

This study investigated the association of oxidative stress and microglial activation in safinamide-treated cells through the four proteins, STAT1, NF-kappa B, iNOS and COX-2. Based on Figure 4, the addition of safinamide to the LPS-induced BV-2 cells was able to reduce the relative protein expressions in all the four proteins. The specific functions of these four proteins are summarised in Table 1[21,25,41-43].

The interplay between these four proteins was essential in allowing microglial activation resulting in dopaminergic neurotoxicity. STAT1 protein is phosphorylated by Janus kinases (JAKs), receptor-associated tyrosine kinase proteins. The phosphorylated form of STAT1 enters the nucleus of the microglia and binds to specific promotor regions in DNA associated with iNOS and COX-2 transcription. Oxidative stress due to LPS, induces the aberrant activation of STAT1, resulting in an overproduction of iNOS and COX-2[44-45]. This is further supported by previous studies that explored the JAK/STAT pathway. A study reported that ROS directly activates STAT1 in microglia. STAT1 is considered a redox-sensitive protein and is therefore sensitive to oxidative stress[46]. Based on Figure 3, a decrease in oxidative stress was noted when ½ MNTD and MNTD of safinamide was added to the LPS-induced BV2 cells. Hence, safinamide may possess anti-oxidative properties,

Table 1. Specific functions of STAT1, NF-kappa B, iNOS and COX-2 in microglia activation.

Proteins	Physiological function	Microglial activation
STAT1	Cell apoptosis, cell survival, immune response, and proliferation	Binds to specific promoter regions in DNA activating transcription of target genes involved with coding iNOS and COX-2.
NF-kappa B	Immune response, injury response, glutamate clearance, and cellular metabolism	Chronic NF-kappa B activation elevates neuroinflammation and neuronal cell death.
iNOS	Neurotransmission, vascular function, host defense, and immune regulation	Overproduction of both inflammatory proteins
COX-2	Synthesis of the prostaglandins associated with acute and chronic inflammatory states	

which allowed it to reduce oxidative stress and thus reducing the hyperstimulation of STAT1.

Secondly, NF-kappa B is initiated by the degradation of IκB proteins. This occured due to the activation of IκB kinases. Signal molecules such as ROS, cytokines, growth factors and stress agents are able to trigger IκB kinases. Through the degradation of IκB proteins, NF-kappa B complex was released and translocated into the nucleus where it bound to specific promoter regions of the DNA and allowed the transcription of various pro-inflammatory cytokines, such as COX-2, TNF-α and IL-1β[24-25]. Based on Figure 3, safinamide was able to reduce the oxidative stress induced by LPS and therefore reduced the production of these inflammatory mediators.

Based on Figure 4, the relative protein expression of iNOS and COX-2 is reduced in safinamide-treated groups in comparison to the LPS only group. The hyperstimulation of both STAT1 and NF-kappa B contributes to the overproduction of iNOS and COX-2. Therefore, a decrease in oxidative stress due to the addition of safinamide had resulted in a decrease in the amount of iNOS and COX-2.

Safinamide is as an approved drugs that has multiple modes of action. It is a selective third-generation reversible MAO-B inhibitor[47]. MAO-B is an enzyme that degrades dopamine via deamination to convert it into 3,4-dihydroxyphenylacetic acid (DOPAC). The inhibition of this enzyme reduces the degradation of dopamine therefore prolonging the presence of dopamine within the brain[48]. The reversible action is important as this enables the MAO-B enzyme activity to recover by stopping safinamide administration, unlike selegiline and rasagiline, where they irreversibly inhibit MAO-B. Furthermore, safinamide also inhibits the release of glutamate by blocking sodium and calcium channel modulation, thus reducing glutaminergic transmission. The present findings suggest that safinamide not only acted as a MAO-B inhibitor, it also exhibited modest anti-oxidative effects. The results suggested that safinamide could be neuroprotective, in accordance to the findings from a previous study[20]. This effect might add benefits to patients who have been taking this medication.

5. CONCLUSIONS

To conclude, no disease-modifying treatments are able to slow down or cease the disease progression of PD. Past *in vivo* and *in vitro* studies indicated the potential of safinamide in possessing neuroprotective properties. In this study, the neuroprotective effects of safinamide against LPS-induced microglial activation was investigated and showed that safinamide has not been able to rescue BV-2 cells from cell death. Nevertheless, safinamide has been shown to slightly reduce oxidative stress in BV-2 cells possibly due to the regulation of STAT1, NF-kappa B, iNOS and COX-2.

For future studies, a broader concentration range of safinamide may be explored. Furthermore, other neuroinflammatory markers associated with anti-oxidative properties of safinamide may be investigated. As safinamide possesses more than one mechanism of action other than MAO-B inhibition, other mechanisms should be studied too. Lastly, the effects of safinamide adjunct with other medications should be investigated in future studies.

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Conflict of interest
None to declare.

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None to declare.

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B: drafting the article, final approval of the version to be published.
KYN: Conception or design of the work, final approval of the version to be published.
SMC: Conception or design of the work, final approval of the version to be published.
APKL: Conception or design of the work, final approval of the version to be published.
KGLV: Conception or design of the work, final approval of the version to be published.
YYO: Critical revision of the article, final approval of the version to be published.
YLT: Critical revision of the article, final approval of the version to be published.
RYK: Conception or design of the work, drafting the article, critical revision of the article, final approval of the version to be published.

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