Neuroprotective Role of Orientin on β-amyloid-induced Cell Death in SH-SY5Y Neuronal Cells via mTOR Signaling Pathway

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***Abstract*—** Alzheimer's disease (AD) is the world leading neurodegenerative disease (ND), characterized as progressively loss of neuronal cells in the brain, features as memory loss, cognitive impairment, and even death. Inhibiting beta amyloid (Aβ) production and clearance of Aβ could serve as therapeutic strategies for AD. There is substantial evidence that the mammalian target of rapamycin (mTOR) signaling, which is involved in the generation and clearance of Aβ, played a role in the etiology of AD. Dysregulation of mTOR signaling could decrease Aβ clearance in AD as mTORC1 is an autophagy inhibitor. Orientin has been shown to have a wide range of therapeutic benefits, including anti-inflammatory and neuroprotective effects. As a result, mTOR inhibition may promote lysosomal breakdown of Aβ through autophagy process. The main objective of the study was to determine the roles of orientin on β-amyloid-induced cell death in SHSY5Y neuronal cells via mTOR signaling pathway. The 70% confluent SH-SY5Y cells were pre-treated with 10 µM (½MNTD) and 20 µM (MNTD) of orientin for 24 hours, followed by exposure to the half maximal inhibitory concentration (IC50) of Aβ in the presence of orientin for another 24 hours. After which, the lipid peroxidation and mitochondrial membrane potential (Δψm) levels were measured using the Elabscience® Malondialdehyde (MDA) Colorimetric Assay Kit (Cell Samples) and Elabscience® Mitochondrial Membrane Potential Assay Kit (with JC-1), respectively. Additionally, the relative expressions of mTOR signaling proteins (p-mTOR 2481, p-Raptor, p-Rictor and GβL) was also determined using cell-based ELISA techniques. In the lipid peroxidation assay, cells pre-treated with orientin at both MNTD and ½MNTD with Aβ increased the relative MDA level as compared to the cells treated with Aβ alone and untreated cells. Contrarily, the treatment groups pre-treated with orientin at ½MNTD and MNTD with Aβ showed no significant increase in normalised JC-1 ratio (%) in the study on Δψm. As for determination of mTOR signaling proteins expression, a significant decrease in phosphorylation of p-mTOR 2481 in Aβ as compared to untreated cells while a significant increase was recorded in the phosphorylation of p-mTOR 2481 in treatment groups containing orientin MNTD and 25 µM indomethacin with Aβ as compared to cells treated with Aβ alone. Based on the findings of the present study, it can be concluded that orientin has a function in the mTOR signaling pathway, namely in downregulating the relative expression of p-mTOR signaling proteins. However, additional study on orientin's pharmacokinetics and bioavailability is needed before validating its usefulness as a treatment agent for AD.

***Keywords*—** Lipid peroxidation, Mitochondrial Membrane Potential, Neurodegenerative disease, Orientin.

I. Introduction

Alzheimer's disease (AD) is the most common kind of neurodegenerative disease (ND), which is characterized by the death of neuronal cells in the brain and a progressive decline in cognitive function. The most prominent neuropathological features of AD are thought to be amyloid beta (Aβ) plaque and neurofibrillary tangles (NFTs). It has also been shown that mitochondrial activity is disrupted in AD as a result of the lower mitochondrial membrane potential together with an increase in the formation of reactive oxygen species (ROS), subsequently, causes the damage of lipid membranes and leads to lipid peroxidation. In addition to this, the participation of the mammalian target of rapamycin, often known as mTOR, is thought to be impacted in Aβ. mTOR is a conserved protein kinase that regulates the balance between protein production and degradation [1]. It forms two different complexes: the mTOR complex 1 (mTORC1), which regulates protein homeostasis, inhibits autophagy, lipid metabolism and mitochondrial function and mTOR complex 2 (mTORC2), which regulates cellular shape, mitochondrial metabolism, and cellular proliferation [1]. mTOR then combines these signals to regulate ribosome synthesis, transcription, translation, and autophagy [1-2]. mTORC1 is an autophagy inhibitor, and dysregulation of mTOR signaling might reduce Aβ clearance in AD [3]. Several research have shown that the activation of mTOR results in the failure of Aβ removal from the brain as mTOR, an inhibitor of autophagy, will cause the disruption of Aβ clearance [3]. Furthermore, mTOR activation is also linked to aberrant Aβ production. Multiple research results support the hypothesis that chronic degeneration of the autophagy pathway is a significant contributing factor to the

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failure of Aβ clearance from the AD brain, and that the autophagy system is responsible for the fate of Aβ in the AD brain [1, 3]. As a result, inhibiting mTOR activity promotes autophagy, reduces the accumulation of Aβ aggregates, and facilitates the process of Aβ clearance. This study investigated the potential roles of orientin, a flavonoid, in regulating the mTOR signaling pathway.

II. MATERIALS AND METHODS

A. Cell Culture & Initiation of Treatments

SH-SY5Y cell line was obtained from the American Tissue Culture Collection (ATCC). The cells were grown in a Corning 25 cm2 tissue culture flask with media. These cells were incubated at 37°C with 5 % CO2 incubator. In this study, the treatments of cells were conducted as described by Law et al. [4]. After 24 hours of treatment, the lipid peroxidation assay, determination of mitochondrial membrane potential and cell-based ELISA for the determination of expression of mTOR signaling proteins were conducted.

B. Lipid Peroxidation Assay

To determine the level of lipid peroxidation in the treatment groups, the assay was conducted following the manufacturer's instruction from Malondialdehyde (MDA) Colorimetric Assay Kit (Cell Samples) (Elabscience® USA).

C. Determination of Mitochondrial Membrane Potential

Mitochondrial membrane potential was determined using Mitochondrial Membrane Potential Assay Kit (with JC-1) from Elabscience®(USA).

D. Determination of Expression of mTOR Signaling Proteins

In the determination of expression of mTOR signalling proteins, the cells were seeded into 96- well plate at the density of 1x 106 cells/mL. After the treatments, the medium was discarded, and wells were washed with 1x TBST for 5 minutes for 3 times on a shaker Ice-cold methanol was then added and incubated at 4°C for 20 minutes. The methanol was then discarded and washed with 1x TBST for 5 minutes thrice. Once done, 100 µL of 0.6 % H_2O_2 was added into the wells and incubated for 5 minutes on the shaker. After that, the H_2O_2 was discarded and washed with 1x TBST for 5 minutes thrice. BSA/TBS was then added into the wells and incubated for 1 hour at room temperature on a shaker. After 1 hour, the BSA/TBS was discarded and washed with 1x TBST for 5 minutes thrice. A total of 100 µL of primary antibody [Phospho-mTOR (Ser2481), mTOR (7C10), Raptor (24C12), Rictor (53A2), GβL (86B8), Phospho-Rictor (Thr1135) (D30A3) and Phospho-Raptor (Ser792)] from Cell Signaling Technology (USA) was then added into each of the wells and incubated overnight at 4°C. After overnight incubation, the primary antibody was discarded and 100 µL of secondary antibody Anti-rabbit IgG, HRP-linked Antibody from Cell Signaling Technology (USA) was added into each of the wells for 1 hour. After 1 hour, the secondary antibody was discarded and washed with 1x TBST for 5 minutes thrice. After that, 100 µL of TMB was added for 5 minutes followed by adding 100 µL of 500 mM sulphuric acid (H_2SO_4). Finally, the plates were read at 490 nm using Tecan Infinite 200 PRO (Switzerland) to determine the value of expression of mTOR signaling proteins. To determine the measurement of cell viability, the solution in 96-well plate was discarded and washed 3x with dH2O for 5 minutes each time. Plate was then dried using an air-dryer. Once dried, 100 µL of 0.04 % crystal violet freshly prepared in 4 % ethanol (EtOH) was added into the wells and incubated for 30 minutes. Then, 96-well plate was dried once again using an air-dryer. Once dried, 100 µL of 1 % SDS in dH2O was added into the wells and read at 595 nm using Tecan Infinite 200 PRO (Switzerland). The data from 490 and 595 nm was normalized to 595 nm. The data was then normalized to the untreated group, and then finally, to its total protein.

E. Statistical Analysis

All experiments within this study were completed in triplicate. The data were presented as means ± standard deviation. The data variance was calculated using SPSS version 16 and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test at $p<0.05$. A p-value < 0.05 was deemed statistically significant**.**

III. RESULTS AND DISCUSSION

A. Determination of Lipid Peroxidation

Deposition of Aβ may lead to lipid peroxidation, which can damage cells. When free radical species are present in the presence of free iron, lipid peroxidation occurs. As free radical species come into contact with free iron, a process known as lipid peroxidation occurs, which causes cell damage. Various previous studies have reported that flavonoid molecules are able to chelate iron, suggesting that they may be able to prevent lipid peroxidation [5]. This is due to the fact that flavonoids have a high reducibility and may be utilized as antioxidants, thus preventing oxidation and thereby minimizing iron overload-induced oxidative damage [5]. However, based on the results from the present studies, pre-treatment of cells with orientin at MNTD and ½MNTD in the presence of Aβ showed an elevated level of relative MDA when compared to untreated cells (Fig 1). These findings deduced that orientin at MNTD and ½MNTD have not shown any rescue effects to lower the relative MDA level of the cells when they were exposed to 53 uM of Aβ. These findings are contradicting with many studies, which reported that some flavonoids could behave as both antioxidants and prooxidants, depending on the concentration and free radical source. In a study by Durgo et al. who examined the influence of flavonoids on the formation of MDA after prolonged exposure of HEp2 and CK2 cells to nontoxic concentrations of flavonoids [6] revealed that luteolin and fisetin enhanced primary toxic effect of hydrogen peroxide. These two compounds behave as prooxidants showing synergistic effects with free radicals of different origin. This could possibly explain why there was a trend of increase in MDA levels when cells were pre-treated with orientin with Aβ even though there was statistically insignificant.

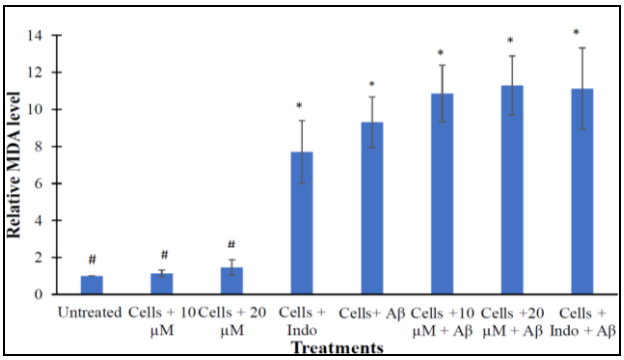

Fig 1 The effects of orientin at ½MNTD and MNTD on the relative MDA level in SH-SY5Y cells. The data shown means ± S.D. in triplicates. '*' denotes the treatment was significantly different from the untreated cells while '#' denotes the treatment was significantly different from the beta-amyloid analyzed using one-way analysis of variance followed by Tukey's multiple comparison test at $p<0.05$.

B. Determination of Mitochondrial Membrane Potential

Overproduction of Aβ in AD is associated with an increase in the number of damaged mitochondria, which induces oxidative stress, loss of ΔΨm, and ATP production [7]. Numerous AD patients display evidence of reduced ATP levels, increased oxidative stress, and decreased ΔΨm [98] in their brains. Neurological damage, inflammation, and ageing may all impair mitochondrial function by inducing fission, decreasing ΔΨm, and reducing ATP production [8].

In this study, flow cytometry was utilised to evaluate mitochondrial membrane potential by the double fluorescence staining of mitochondria by JC-1 as either green fluorescent J-monomers or red fluorescent aggregates. As shown in Fig 2 and Fig 3, the treatment cells receiving orientin at ½MNTD and MNTD exhibited a substantial reduction in normalized JC-1 ratio of 35.8 % and 22.1 %, respectively. This implies that orientin affects ΔΨm differently when Aβ is present in SH-SY5Y cells, since increasing orientin concentration leads to a reduction in ΔΨm. This contradicted with in a study by Lam et al., who demonstrated that ΔΨm levels were increased when orientin was introduced at ½MNTD and MNTD in H2O2 oxidative damage SH-SY5Y cells [9]. Pre-treatment of cells with orientin at MNTD and ½MNTD with Aβ increased the ΔΨm as compared to the Aβ group alone by 21.2 % and 7.2 % despite not being statistically significant, demonstrating a beneficial influence on cell survival. This finding is in accordance with the findings of Yu et al., whereby orientin significantly decreased the production of ROS in mice induced by Aβ [10]. Previous studies have also reported that orientin moderately improved the ΔΨm of neuronal cells, potentially via activating the Nrf2-ARE signaling pathways, and to help in preventing mitochondrial dysfunction [11]. Nrf2 is essential for the maintenance of cellular redox homeostasis. The ΔΨm and ATP production are affected by Nrf2 as it regulates the oxidation of fatty acids in mitochondria and helps to maintain the structural and functional integrity of mitochondria [12]. As the Nrf2/ARE signaling pathway is involved in the functioning of mTOR, there is reason to believe that Nrf2 activation may directly enhance cognitive abilities by reducing the amount of oxidative stress experienced [12-13].

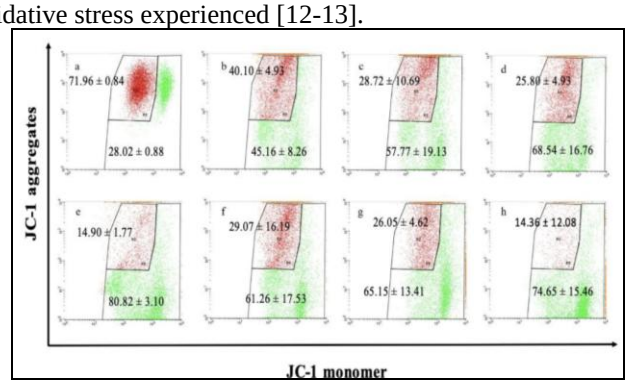

Fig 2 Mitochondria membrane potential analysis of orientin treatments on Aβ-stimulated SH-SY5Y ells using flow cytometry. (a) untreated cells; (b) cells + 10 µM orientin; (c) cells + 10 µM orientin (d) cells + indomethacin (e) Cells + Aβ; (f) Cells + 10 µM orientin + Aβ (g) Cells + 20 µM orientin + Aβ; (h) Cells + Indomethacin + Aβ

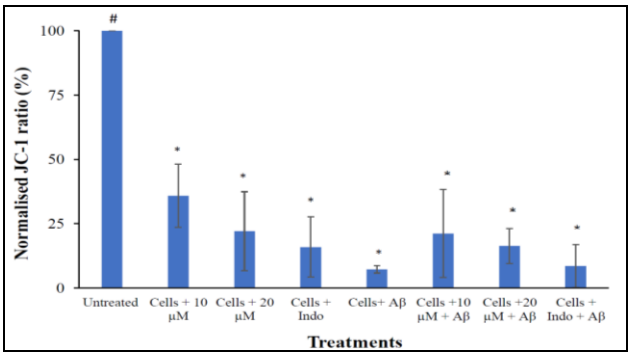

Fig 3 The effects on mitochondrial membrane potential upon Aβ-stimulation on SH-SH5Y cells. Bars indicate the means ± standard deviation. '*' denotes the treatment was significantly different from the untreated cells while '#' denotes the treatment was significantly different from the beta-amyloid analyzed using one-way analysis of variance followed by Tukey's multiple comparison test at $p<0.05$.

C. Expression of mTOR Signaling Proteins

In many different models of AD, the protein complex known as mammalian target of rapamycin 1 (mTORC1), which functions as a nutrition sensor and a fundamental controller of cell growth and proliferation, is also undergoing some changes (AD). A further essential component of the etiology of AD is an imbalance in the insulin/PI3K/Akt signaling pathway. A study observed that human AD brain samples had lower levels of overall mTORC1 and C2 protein [14].

In the treatment group of orientin at MNTD with 53 µM of Aβ, there was a significant increase in phosphorylation of mTOR (**p-mTOR**) as compared to 53 µM Aβ (Fig 4A). Cells pre-treated with orientin at MNTD with 53 µM Aβ showed almost similar relative expression level as the untreated cells, though the expression was not statistically significant. Similarly, orientin at ½MNTD with 53 µM Aβ did not show any significant change in phosphorylation as compared to 53 µM Aβ alone. It is clear that the combination of ½MNTD and 53 µM Aβ is not nearly as effective as the combination of MNTD and 53 µM Aβ in bringing about a shift in the phosphorylation. It is possible that this might be due to the

concentration of orientin is not sufficient to cause a change in the phosphorylation level.

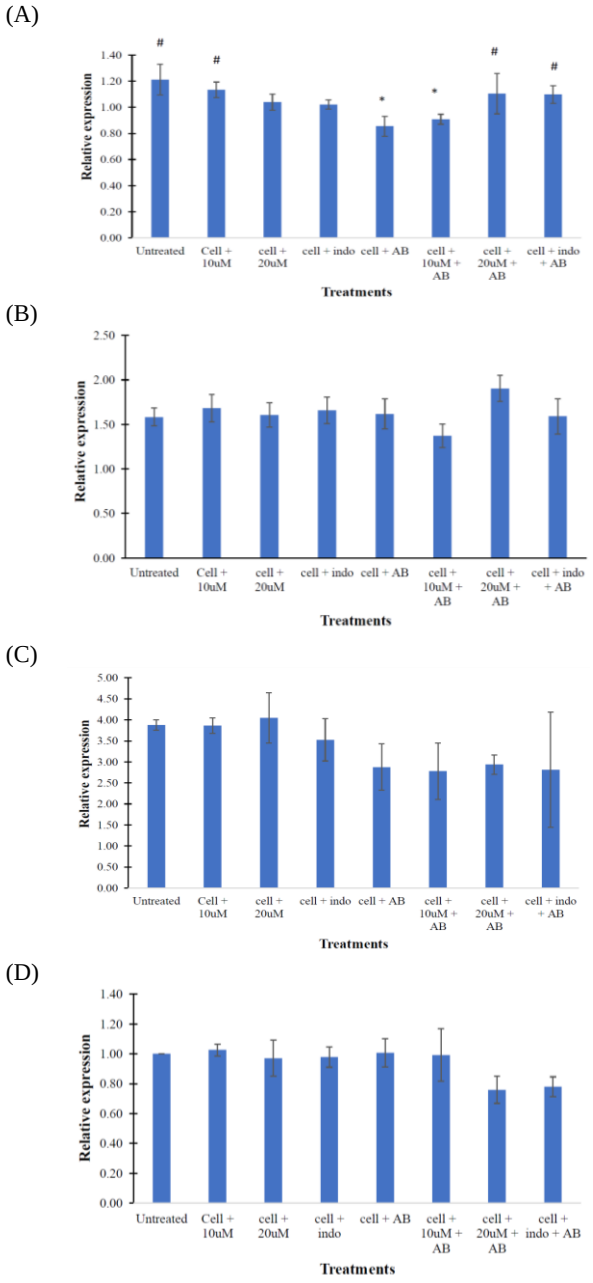

Fig 4 The effects of orientin at ½MNTD and MNTD on (A) p-mTOR 2481 relative to total mTOR (B) p-RAPTOR relative to total RAPTOR (C) p-RICTOR relative to total RICTOR (D) GBL protein expression in SH-SY5Y cells. The data shown are means ± S.D. in triplicates. '*' denotes the treatment was significantly different from the untreated cells while '#' denotes the treatment was significantly different from the beta-amyloid analysed using one-way analysis of variance followed by Tukey's multiple comparison test at $p<0.05$.

Eukaryotic initiation factor 4E-binding protein-1 and ribosomal protein S6 kinase are also shown to be associated with Regulatory-associated protein of mTOR or also known as raptor [15]. Raptor is involved in the maintenance of cell size and the expression of the mTOR protein [15]. The raptor-mTOR complex (TORC1) regulates growth via S6K1 and 4EBP1/PHAS [16]. In addition, AMPK suppresses mTORC1 by the phosphorylation of 59 raptor as raptor is able to disrupt the mTORC1 complex via the phosphorylation of TSC1/2 [15]. According to the findings of the present study, 53 µM Aβ and treatments did not produce any significant effects, and these effects are not reliant on **p-Raptor** (Fig 4B). In line with these findings, phosphoS792-Raptor and phospho-p70S6K levels were unaltered in their Aβ expression model, when study was conducted on AD brain samples from the prefrontal and temporal cortex [16].

mTORC2 is a complex that is resistant to rapamycin. It is made up of the proteins rictor, mTOR, GL/mLST8, PRR5/Protor, and Deptor [17]. It is not completely controlled by the nutrients in the body, but it is insulin sensitive and causes the activation of the Protein Kinase B through the insulin-like growth factor-1 (IGF-1) [17]. According to this study, 53 µM Aβ and orientin at ½MNTD and MNTD did not have any statistically significant impacts on the cells with 53 µM Aβ, and that the treatments are not dependent on **p-Rictor** (Fig 4C). The current findings are in accordance with the findings by Sun et al., in which the ratio of p-Rictor to total Rictor did not undergo any significant shifts throughout the course of AD [18]. This suggests that the effects of Aβ had no influence on the mTORC2 activity. It is possible that the lack of changes in p-rictor is related to the fact that the activation of the gene is contingent on the complexity of the cell lineage that was employed, the metabolic state, and the age of the cell. In their model of AD utilising SHSY cells, Lee et al. found that the expression levels of p-rictor were consistently inhibited [16].

In this study, there was no statistically significant link between GβL and Aβ, and the findings also showed that **GβL** is not dependent on orientin at ½MNTD and MNTD (Fig 4D). In addition to the findings obtained using p-rictor expression, it is possible to postulate that the involvement of mTORC2 in Aβ-induced SH-SY5Y cells is not significant and without effect [18-19]. The results of present study show that the activity of the mTORC1 gene has decreased p70 ribosomal S6 kinase (p70S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1), which are two essential signaling targets of mTORC1, and mLST8 is responsible for promoting the activity of mTOR kinase via their interactions. The fact that there was no substantial change in GβL as demonstrated in this study could be because the activity of mTORC1 was reduced. Consequently, the cells did not require GβL.

IV. CONCLUSIONS

The current study found that orientin at both MNTD and ½MNTD does not show any rescue attempts and statistical significance in decreasing the MDA levels when Aβ is present. Contrarily, the pre-treatment with orientin causes a significant increase in the MDA levels in SH-SY5Y cells. On the other hand, orientin shows promising efforts in increasing the mitochondrial membrane potential the cells pre-treated with orientin at MNTD and ½MNTD with Aβ increases the $\Delta\Psi m$ as compared to the Aβ treatment alone, despite not being statistically significant, demonstrating a beneficial

influence on cell survival. In terms of expression of mTOR signalling proteins, present studies showed that there is a statistically significant change in p-mTOR expression when orientin is administered in both concentrations while no significant relative expression was recorded for p-Raptor, p-Rictor and GBL proteins. Therefore, it is possible to make a conclusion that orientin does, in fact, play a role in the mTOR signalling pathway by controlling the expression of p-mTOR. Nevertheless, to further explore the therapeutic efficacy of orientin and expand its spectrum of applications, more research on its pharmacokinetics and bioavailability should be investigated in greater depths prior to confirming the roles of orientin as the therapeutic agent for AD.

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DATA AVAILABILITY STATEMENT

The data presented in this study are available upon request.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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