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The Synergistic Effects of LRRK1 Inhibition and TLR4 Activation on Apoptotic, Inflammatory Parameters and α-Synuclein Expression in HMC-3 and SH-SY5Y Cell Lines

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Abstract

LRRK1 protein is sole paralog of LRRK2 protein, which plays a role in autosomal dominant Parkinson's disease inheritance in humans. Both proteins share similar domain organization characteristics. Like LRRK2, LRRK1 also possesses kinase and GTPase activities. However, role of LRRK1 in pathogenesis of Parkinson's disease remains poorly understood. In this study, we aimed to evaluate cytokine expression levels and expression of apoptotic and inflammatory proteins in commonly used HMC-3 microglial and SH-SY5Y neuroblastoma cell lines following inhibition of LRRK1 by IN04 inhibitor. Cytokine expression was assessed through IL-6, apoptosis via caspase-3 activation, and proliferation and inflammation through p38 phosphorylation. Additionally, effect of IN04 on α-synuclein expression was evaluated. Furthermore, the synergistic effect of IN04 with LPS was investigated, as LPS induces inflammation through TLR4 activation. To this end, four experimental groups were established: control, IN04, LPS, and IN04+LPS. According to our results, IN04 treatment increased α-synuclein expression in both HMC-3 and SH-SY5Y cells. IN04 treatment elevated caspase-3 activation and p38 phosphorylation in HMC-3 cells, whereas interestingly, it decreased both parameters in SH-SY5Y cells. Moreover, IN04 treatment increased IL-6 expression in HMC-3 cells, while in SH-SY5Y cells, combination of IN04 with LPS led to an increase in IL-6 expression.

Keywords

Parkinson, Inflammation, LRRK1, Apoptosis

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HMC-3 ve SH-SY5Y Hücre Hatlarında LRRK1 İnhibisyonunun ve TLR4 Aktivasyonunun, Apoptotik, İnflamatuar Parametreler ve α-Sinüklein Ekspresyonu Üzerindeki Sinerjistik Etkileri

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Özet

LRRK1 proteini insanda otozomal dominant Parkinson kalıtımında rol oynayan LRRK2 proteinin tek paralogudur. Her iki protein domain organizasyonu açısından benzer özelliklere sahiptir. LRRK1 proteini de LRRK2 proteini gibi kinaz ve GTPaz aktivitesine sahiptir. LRRK1 proteinin Parkinson patogenezindeki rolü tam anlaşılamamıştır. Bu sebeple yapmış olduğumuz çalışmada LRRK1 proteinin IN04 inhibitörü ile inhibe edilmesi sonucu Parkinson araştırmalarında yaygın olarak kullanılan HMC-3 mikroglia hattı ve SH-SY5Y nöroblastoma hücre hatları üzerindeki sitokin ekspresyon düzeylerinin değerlendirilmesi ve apoptotik, inflamatuvar proteinlerin ekspresyon düzeylerinin belirlenmesi hedeflenmiştir. Sitokin ekspreyonu IL-6 üzerinden, apoptoz kaspaz-3 aktivasyonu üzerinden, proliferasyon ve inflamasyon p38 fosforilasyonu üzerinden değerlendirilmiştir. Ayrıca IN04'un α-sinüklein ekspresyonu üzerinden etkisi değerlendirilmiştir. Bunun yanında LPS uygulanması sonucu IN04' ün LPS ile sinerjistik etkisi tespit edilmiştir. LPS, TLR4 aktivasyonuna yol açarak inflamasyon sürecini başlatmaktadır. Bu amaçla yapmış olduğumuz çalışmada kontrol, IN04, LPS, IN04+LPS olmak üzere 4 adet deney grubu oluşturulmuştur. Elde ettiğimiz verilere göre IN04 uygulaması sonucu HMC-3 ve SH-SY5Y hücrelerinde α-sinüklein ekspresyonu artmaktadır. IN04 uygulaması HMC-3 hücrelerinde kaspaz-3 aktivasyonunu ve p38 fosforilasyonunu artırırken, ilginç bir şekilde SH-SY5Y hücrelerinde kaspaz-3 aktivasyonu ve p38 fosforilasyonunu azaltmaktadır. IN04 uygulamasına bağlı olarak HMC-3 hücrelerinde IL-6 ekspreyonu artarken, SH-SY5Y hücrelerinde IN04'un LPS ile kombinasyonu sonucu IL-6 ekspresyonu artmaktadır.

Anahtar kelimeler

Parkinson,
İnflamasyon,
LRRK1,
Apoptoz

1. INTRODUCTION

Parkinson's disease is one of the most common neurodegenerative disorders worldwide. Common symptoms observed in Parkinson's pathogenesis include tremor, postural instability, and sleep disturbances [1]. At the molecular level, the loss of function of dopaminergic neurons in the substantia nigra pars compacta leads to Parkinson's disease. The degeneration of dopaminergic neurons in this region can involve disruptions in mitochondrial signaling pathways, the formation of α-synuclein aggregates, and impairments in lysosomal pathways [2]. From a genetic perspective, the main genes implicated in autosomal Parkinson's include LRRK2, α-synuclein, VPS35, PINK1, and Parkin [3]. Clinical genetic studies by Zimprich and colleagues identified that mutations in the LRRK2 gene cause autosomal Parkinson's disease [4]. The LRRK2 protein possesses dual enzymatic activity, comprising both kinase and GTPase functions. Mutations in the kinase domain of LRRK2 lead to hyperactive kinase activity, contributing to Parkinson's pathogenesis. Additionally, mutations in the GTPase domain affect protein dimerization and play a crucial role in regulating kinase activity [5].

The functions of LRRK1, the sole human paralog of LRRK2, in the brain and its role in Parkinson's pathogenesis are not fully understood. Similar to LRRK2, LRRK1 exhibits kinase and GTPase activity. LRRK1 was first identified in the U2OS cell line as a proliferation factor [6]. Primarily, LRRK1 has been associated with osteosclerotic diseases and osteopetrosis [7]. Studies in LRRK1 knockout (KO) mouse models revealed altered bone structure, indicating that LRRK1 is essential for normal bone development [8]. Daniel Korr and colleagues demonstrated that LRRK1 is an active kinase capable of GTP hydrolysis [9]. Rab7, a known substrate of LRRK1 [10], is essential for intracellular transport, autophagy, and endocytosis/exocytosis. However, the specific stage of transport at which LRRK1-mediated phosphorylation of Rab7 is activated remains unknown. Furthermore, treatment with CCCP in COS7 cells was shown to promote the interaction of LRRK1 with Atg13 [11]. In LRRK1 KO cells, autophagosomes accumulate, indicating a blockage in autophagy progression.

The IN04 inhibitor, developed for LRRK1, was identified through molecular docking studies and experimentally shown to prevent ATP binding to LRRK1 [12]. Unlike LRRK2, LRRK1 exhibits lower affinity for intracellular microtubules. Moreover, while PKC kinases activate LRRK1 by phosphorylating its COR domain [13], LRRK2 activity is regulated by PKA kinases [14]. One of the key shared features of LRRK1 and LRRK2 is the phosphorylation of Rab proteins at the switch II position. Specifically, LRRK1 phosphorylates Rab7 at serine 72, whereas LRRK2 phosphorylates Rab8a and Rab10 at serines 72 and 73 [15], respectively. Unlike LRRK2, LRRK1 lacks an ARM domain. LRRK2 activity is regulated by 14-3-3 chaperone proteins via phosphorylation sites within its ARM domain [16], whereas 14-3-3 proteins do not influence LRRK1 function. The most notable functions of LRRK1 include phosphorylation of Rab7 and regulation of the EGFR pathway [17]. LRRK1 kinase activity has been shown to play a critical role in the incorporation of EGF into intraluminal vesicles [17]. EGF was initially identified as a neuronal differentiation factor [18], suggesting that LRRK1 may have a potential role in brain development and neuronal differentiation.

The aim of this study was to evaluate the inflammatory and apoptotic parameters in SH-SY5Y and HMC-3 cells, which are commonly used in Parkinson's research, following LRRK1 kinase inhibition by IN04. Additionally, we investigated to assess α-synuclein expression in the same cell lines. To further elucidate the role of LRRK1 in α-synuclein expression, synergistic effects in combination with LPS treatment were also evaluated.

2. MATERIAL AND METHOD

2.1 Chemicals, Reagents and Preparation and Application of IN04 and LPS

Dulbecco's Modified Eagle Medium (DMEM), Penicillin/Streptomycin, and Fetal Bovine Serum used for cell culture were obtained from Gibco. Caspase-3 (BIO-TECHNE, NB100-56708, 1:1000), phospho-p38 (p-p38; Santa Cruz, sc-9211, 1:1000), p38 (Santa Cruz, sc-8690, 1:1000), and GAPDH (Cell Signaling Technology, #2118) antibodies were purchased from the respective suppliers. All primer antibodies were used at a 1:1000 dilution according to the manufacturer's instructions. Seconder antibodies obtained from Cell Signaling Technology (Anti-rabbit IgG, HRP-linked Antibody #7074, Anti-mouse IgG, AP-linked Antibody #7056).

TLR4 antagonist lipopolysaccharide (LPS) was obtained from Invivogen (LPS from E. Coli O111:B4, tlrl-3pelps) and LRRK1 inhibitor IN04 was purchased from InvivoChem (#V22210). IN04 was dissolved in DMSO to prepare a stock solution with a final concentration of 10 mM during IN04 treatment, care was taken to ensure that the final concentration of DMSO in the cell culture medium did not exceed 0.1%. LPS was diluted with sterile distilled water from a 1 mg/mL stock solution before use. Both IN04 and LPS stock solutions were stored at −20 °C after dilution.

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2.2 Experimental Groups

In this study, all experiments were performed in 6 groups of HMC-3 and SH-SY5Y cell lines as (i) control group treated only with 0.1% DMSO, (ii) IN04 group treated with 10 μM IN04 for 24 hour , (iii) IN04 + LPS group first treated with 10 μM IN04 for 24 hours and then with 250 ng/mL LPS for 6 hours, and (iv) LPS group treated with 250 ng/mL LPS for 6 hours.

2.3 Cell Culture

The SH-SY5Y and HMC-3 cells were preserved in freezing medium containing 8% DMSO in a liquid nitrogen tank. After being retrieved from the nitrogen tank, the cells were cultured in 25 cm² and 75 cm² culture flasks in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin under regular cell culture condition at 37 °C in a humidified incubator with 5% CO_2. Upon reaching approximately 80% confluence, the cells were subcultured using Trypsin-EDTA into new sterile cell culture flasks. Cell viability was periodically assessed using trypan blue staining and light microscopy.

2.4 Cell Proliferation Assay

SH-SY5Y and HMC-3 cells were seeded into 96-well culture plates at a density of 1×10^4 cells per well. After allowing the cells to proliferate for 24 hours, the respective treatment agents were applied for an additional 24 hours and 6 hours. At the end of the drug and stimulant treatment periods, MTT solution (0.5 mg/ml) was added to each well, and the plates were incubated for 4 hours under conventional cell culture conditions. After incubation, the culture medium was carefully removed from each well. The insoluble formazan crystals were then dissolved by adding 100 µL of DMSO to each well. The resulting formazan crystals were solubilized by pipetting to ensure homogenization. Absorbance was measured at 570 nm using a microplate spectrophotometer (Agilent BioTek Synergy HTX Multimode Reader, Santa Clara, California).

2.5 RT PCR Analysis

The cell groups treated with the active compound in 6-well plates were collected using Trypsin-EDTA, with 1×10^6 cells per group, and washed twice with PBS. Total RNA was isolated from the cell groups using the OneStep RNA Reagent Kit (BioBasic, Toronto, Canada) according to the manufacturer's protocol. The purity of the isolated RNA with respect to DNA and protein contamination was assessed using a NanoDrop spectrophotometer. RNA samples obtained from the cell groups were reverse transcribed into complementary DNA (cDNA) following the manufacturer's instructions for the cDNA Reverse Transcription Kit (Applied Biosystems, California, USA). For cDNA synthesis, 1 µg of total RNA was used as the initial input. The obtained cDNAs were subjected to PCR amplification using the specific primers for IL-6 gene forward 5' CCACCGGGAACGAAAGAGAA 3' and reverse 5' TCTTCTCCTGGGGGTACTGG 3', for α-synuclein gene forward 5' TATCTGTACCTGCCCCCACT 3' and reverse 5' GCCACAAAATCCACAGCACA 3' and for GAPDH gene as a loading control forward 5' GTCTCCTCTGACTTCAACAGCG 3' and reverse 5' ACCACCCTGTTGCTGTAGCCAA 3'. The PCR program was carried out following Phusion™ High-Fidelity PCR Kit protocol (New England Biolabs, USA) as initial denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 20 s, annealing at 65 °C for 20 s, and extension at 72 °C for 30 s. A final extension step was performed at 72 °C for 10 min. Upon completion of the program, samples were held at a constant temperature of +4 °C. The PCR products were loaded on 1.2% agarose gels containing EtBr in presence of 6× DNA loading dye for electrophoresis. The gene expression bands captured using a Bio-Rad ChemiDoc imaging system (USA) were quantified according to the respective GAPDH gene expression for normalization.

2.6 Protein Sample Preparation and Immunoblotting

To extract proteins, a total of 1×10^6 cells were collected from each cell group were first washed with PBS and then homogenized in a lysis buffer containing 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 50 mM HEPES, sodium orthovanadate, sodium pyrophosphate, sodium fluoride, and β-glycerophosphate on ice for 30 minutes by vortexing every 10 minutes. Following the centrifugation at 12,000 rpm for 10 minutes at +4 °C, protein concentrations were determined using a BCA assay kit (Thermofisher, USA) in the supernatants. For each group, 40 µg of protein was separated on 4–10% SDS-PAGE gels at 100 V. The proteins were then transferred onto PVDF membranes at 90 V for 90 minutes. The membranes were blocked with 5% BSA in TBS-T solution for 30 minutes and subsequently incubated first with the p-38 (Santa Cruz, Heidelberg, Germany), p-p38 (Santa Cruz, Heidelberg, Germany), caspase-3 (Bio-Techne, Minnesota, USA), GAPDH (Cell Signaling Technology, Massachusetts, USA) primary antibodies, overnight and then with the secondary antibody(Cell Signaling Technology, Massachusetts, USA) at room temperature for 2 hours. Protein bands were visualized using an ECL substrate kit (BioRAD, Vienna, Austria), and images were captured using a ChemiDoc MP imaging system (BioRAD, Vienna, Austria). Band intensities were quantified using ImageJ software (version 1.53) processing software, (National Institutes of Health, Bethesda, MD, USA).

2.7 Statistic Analysis

Data were statistically analyzed to compare differences among multiple groups using the Kruskal–Wallis and one-way ANOVA tests. For pairwise comparisons, the Mann–Whitney U test was applied. The homogeneity of data distribution was assessed through post hoc analysis, for which Tukey's test was used. Statistical analyses were performed using GraphPad Prism version 8.0.

3.RESULTS

3.1 Evaluation of Cell Cytotoxicity of IN04 on HMC-3 and SH-SY5Y Cells

Based on the data obtained following 24-hour treatments with 2.5, 5, 10, 20, 40, and 60 µM concentrations of IN04, the 10 µM dose was determined to be the optimal concentration for subsequent experiments. IN04 exhibited similar effects on cell viability in both SH-SY5Y and HMC-3 cell lines. No significant difference was observed between the untreated control group and the control group treated with 0.1% DMSO. Cell viability of untreated control groups was considered as 100%. Treatment with 0.1% DMSO resulted in 96% cell viability in HMC-3 cells. Similarly, the viability of HMC-3 cells treated with 0.1% DMSO was determined to be 97%. In SH-SY5Y cells, treatment with 2.5 µM, 5 µM, 10 µM, 20 µM, 40 µM, and 60 µM IN04 resulted in cell viabilities of 84%, 70.6%, 56.3%, 50.4%, 31.3%, and 21.2%, respectively. Likewise, treatment of SH-SY5Y cells with 2.5 µM, 5 µM, 10 µM, 20 µM, 40 µM, and 60 µM IN04 yielded viabilities of 85.2%, 65.1%, 49.2%, 41.3%, 33.4%, and 15.6%, respectively. In both cell lines, treatment with 10 µM IN04 reduced cell viability by approximately half, indicating an IC_{50} value around this concentration. According to the results of the MTT assay, 10 µM IN04 was selected for use in the following experiments.

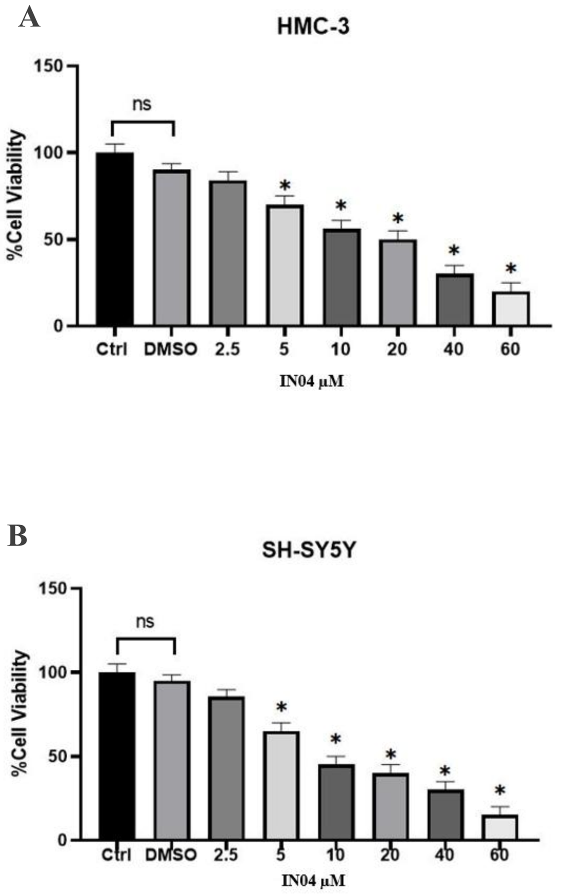


Figure 1. IN04 suppresses HMC-3 and SH-SY5Y cell proliferation. Cell viability graphs showing changes in cell proliferation of (A) HMC-3 and (B) SH-SY5Y cell lines following treatment with the indicated doses of IN04. Data are expressed as mean ± SD of three independent experiments. Statistically significant differences were defined as $p < 0.05$.

3.2 IN04 increased α-synuclein mRNA levels SH-SY5Y and HMC-3 Cells

Evaluation of α-synuclein expression in the neuronal cell lines HMC-3 and SH-SY5Y revealed that treatment with IN04 and LPS significantly upregulated α-synuclein levels in both cell types. In HMC-3 cells, treatment with IN04 alone resulted in a 3.3-fold increase in α-synuclein mRNA expression compared to the control group. Combined treatment with IN04 and LPS further elevated expression to 4.1-fold, while LPS alone induced a 4.4-fold increase (Figure 2A). Similarly, in SH-SY5Y cells, IN04 treatment alone led to a 2.1-fold increase, and the combination of IN04 and LPS produced a 2.4-fold increase in a-synuclein levels. LPS treatment alone resulted in a 2.36-fold increase (Figure 2B). Overall, these findings suggest that IN04 induces an increase in a-synuclein expression that is comparable to, and in some cases approaches, the effect of LPS treatment.

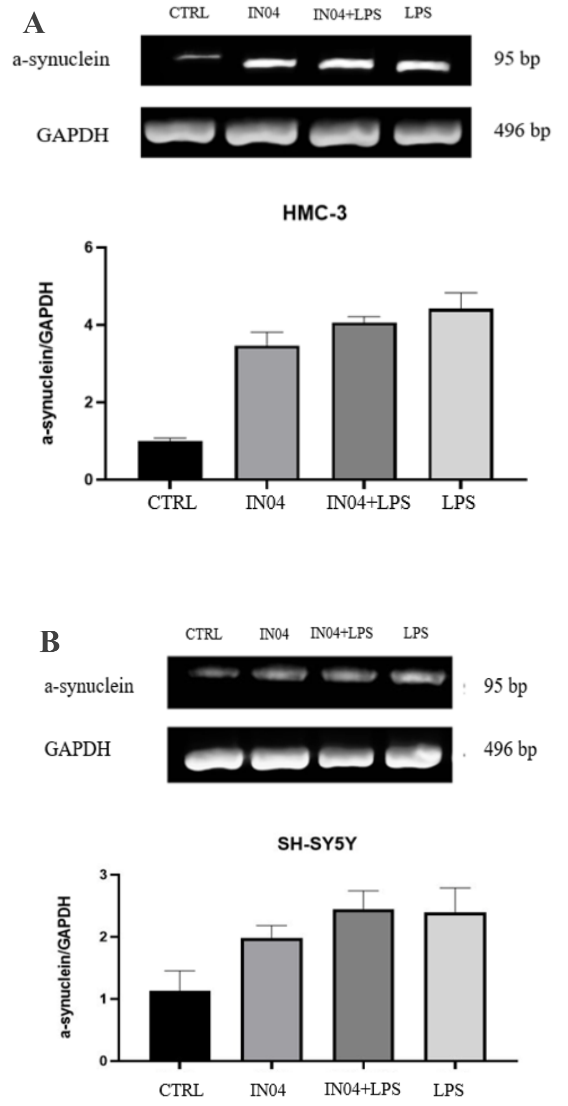


Figure 2. Effects of IN04 on α-synuclein mRNA expression in neuronal cell lines, (A) HMC-3 and (B) SH-SY5Y. mRNA levels were normalized to GAPDH and expressed as fold change relative to the control group. Data are given as mean ± SD of three independent experiments. Statistical significance was defined as $p < 0.05$.

3.3 Effects of INO4 p38 Phosphorylation on HMC-3 and SH-SY5Y Cells

Analysis of p38 phosphorylation revealed that IN04 treatment inhibited p38 phosphorylation in SH-SY5Y cells, whereas in HMC-3 cells, p38 phosphorylation was significantly elevated only in the IN04-treated group. In HMC-3 cells, p38 phosphorylation increased 1.77-fold in the IN04-treated group, while the combined IN04+LPS treatment resulted in a 0.95-fold increase, and LPS treatment alone led to a 1.1-fold increase (Figure 3A). This reduction in the combined treatment group may be due to the reversible effect of IN04 when LPS was applied in fresh medium following IN04 treatment. Additionally, low-dose and short-duration LPS treatment did not significantly affect p38 phosphorylation in HMC-3 cells. In SH-SY5Y neuroblastoma cells, p38 phosphorylation was measured as 0.75-fold in the IN04-treated group, 1.55-fold in the IN04+LPS group, and 1.8-fold in the LPS-only group (Figure 3B). Since SH-SY5Y cells are neuroblastoma cells, inhibition of LRRK1 by IN04 may reduce p38 phosphorylation, thereby suppressing proliferation. In contrast, LPS treatment enhanced p38 phosphorylation in these cells.

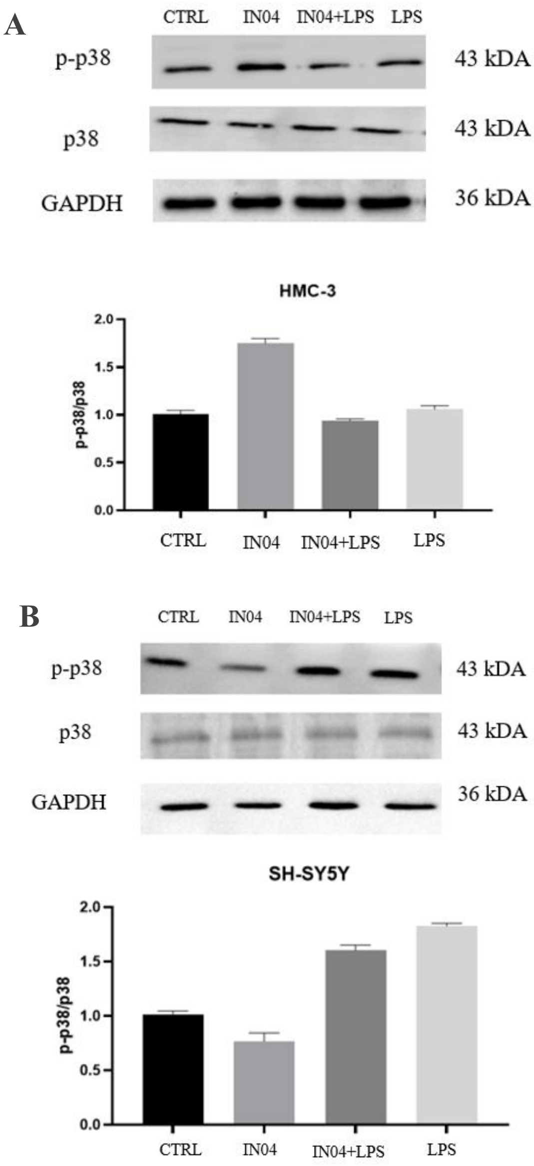


Figure 3. IN04 modulates p38 phosphorylation in neuronal cell lines. (A) HMC-3 and (B) SH-SY5Y. Phosphorylated p38 (p-p38) levels were normalized to total p38 and GAPDH according to the control group. Data represent the mean ± SD of three independent experiments. Statistically significant differences are indicated as $p < 0.05$.

3.4 Effects of IN04 Caspase-3 Activation on HMC-3 and SH-SY5Y cells

Our findings indicate that IN04 treatment induces significant caspase-3 activation in microglial cells. In the IN04 + LPS and LPS groups, caspase-3 activation was lower compared to the group treated with IN04 alone. In HMC-3 cells, caspase-3 activation increased 3.1-fold following IN04 treatment, whereas combined IN04+LPS treatment resulted in a 2-fold increase, and LPS treatment alone led to a 1.7-fold increase (Figure 4A). The pronounced increase in caspase-3 activation by IN04 suggests that LRRK1 may play a role in microglial apoptosis and during Parkinson's disease progression.

In SH-SY5Y cells, IN04 treatment alone caused a 0.8-fold increase in caspase-3 activation, the combined IN04+LPS treatment led to a 1.7-fold increase, and LPS treatment alone resulted in a 2.1-fold increase (Figure 4B). Interestingly, LPS treatment appeared to elevate total caspase-3 expression in SH-SY5Y cells. It is suggested that increasing the dose and duration of LPS treatment may further enhance caspase-3 activation.

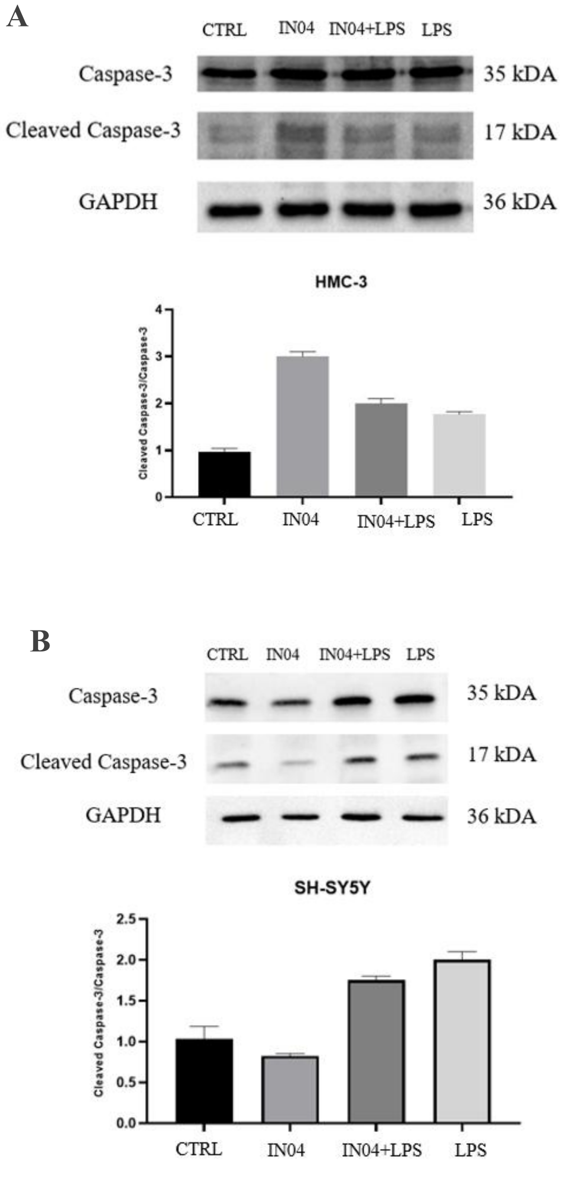
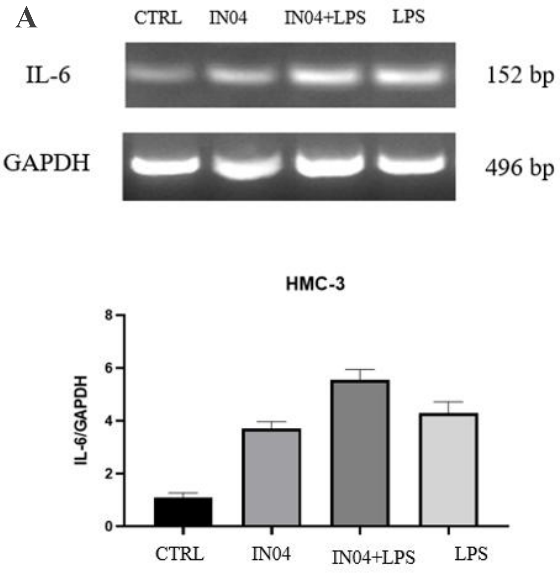


Figure 4. The effect of IN04 on caspase-3 activation was assessed by Western blot analysis in (A) HMC-3 and (B) SH-SY5Y cell lines. The ratio of cleaved caspase-3 to total caspase-3 was normalized to GAPDH and presented as fold changes relative to the control. Statistically significant differences are indicated as $p < 0.05$.

3.5 Effects of IN04 IL-6 mRNA Levels on HMC-3 and SH-SY5Y Cells

IN04 treatment significantly upregulated IL-6 expression in HMC-3 cells. In SH-SY5Y cells, IN04 and LPS acted synergistically to enhance IL-6 levels, while HMC-3 cells consistently showed elevated IL-6 in response to IN04. In HMC-3 cells, IL-6 expression increased 3.7-fold following IN04 treatment, 5.5-fold with combined IN04+LPS treatment, and 4.6-fold with LPS treatment alone (Figure 5A). These results indicate a synergistic effect of IN04 and LPS on IL-6 expression in HMC-3 cells.

In SH-SY5Y cells, IL-6 expression increased 1.1-fold with IN04 treatment, 1.5-fold with IN04+LPS treatment, and 1.2-fold with LPS treatment alone (Figure 5B). Considering the generally low IL-6 production in SH-SY5Y cells, the combined IN04+LPS treatment induced a modest increase in IL-6 expression.



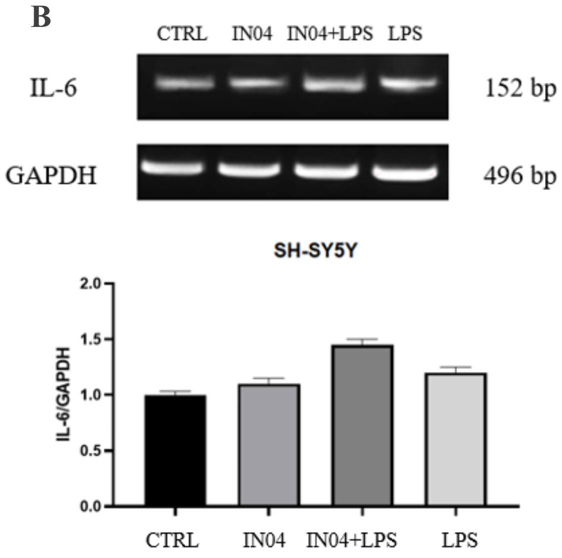


Figure 5. IN04 modulates IL-6 mRNA expression in neuronal cell lines, (A) HMC-3 and (B) SH-SY5Y. IL-6 mRNA levels were normalized to GAPDH and expressed as fold change relative to the control group. Data represent the mean ± SD of three independent experiments. Statistically significant differences are indicated as $p <$ 0.05.

4.DISCUSSION

In addition to mitochondrial proteins and proteins involved in the mitophagy pathway, it is known that neuronal apoptosis and inflammation play a critical role in the development process of Parkinson's disease. Following the discovery that mutations in the *LRRK2* protein lead to autosomal dominant Parkinson's disease, studies have focused on the mechanistic regulations of *LRRK2*. The *LRRK1* protein possesses a domain organization similar to *LRRK2*, except that *LRRK1* lacks the ARM domain found in *LRRK2*. The role of *LRRK1* in Parkinson's disease progression has not been fully elucidated. However, recent studies have demonstrated that *LRRK1* tightly regulates the EGFR pathway, suggesting a potential influence of *LRRK1* on microglial regulation. Furthermore, independent studies have shown that *LRRK1* phosphorylates the *Rab7* protein at the S72 position. *Rab7* activation is known to affect lysosomal activity and endolysosomal trafficking [19]. Phosphorylated and thereby activated *Rab7* enhances α-synuclein clearance. However, how an increase in *LRRK1* expression or kinase activity affects α-synuclein degradation remains unclear [20].

In our study, it was observed that treatment with IN04, an *LRRK1* inhibitor, increased α-synuclein levels at the mRNA level. This increase was detected in both SH-SY5Y and HMC-3 cells, suggesting that *LRRK1* may be required for α-synuclein turnover. In RNA-based multi-omic datasets derived from Alzheimer's disease patients, *LRRK1* has been found to localize to mitochondria. The same study also reported that *LRRK1* is among the proteins involved in immune activation in Alzheimer's disease samples [21]. Considering the strong link between Parkinson's disease and mitochondrial regulation, the precise role of *LRRK1* in mitochondrial dysfunction in Parkinson's remains to be determined. Interestingly, large cohort screenings of Parkinson's patients for *LRRK1* mutations have not yielded significant findings, suggesting that *LRRK1* may not have a direct genetic effect on Parkinson's disease [22].

The concentration of 16 µM has been determined as the dose at which *LRRK1* kinase activity is completely inhibited [23]. In the microglial cell line HMC-3, IN04 treatment increased caspase-3 activation. Interestingly, combined treatment with IN04 and LPS produce a synergistic effect on caspase-3 activation. The Parkinson's disease–associated *LRRK2* mutation Y1699C in the GTPase domain is known to activate caspase-3 [24]. These findings suggest that enzymatic regulations of *LRRK* proteins may be linked to programmed cell death, i.e., apoptosis. Like *LRRK2*, *LRRK1* exhibits GTPase activity by hydrolyzing GTP to GDP; however, the effect of *LRRK1*'s GTPase activity on its kinase function remains less understood compared to *LRRK2*.

Treatment of both HMC-3 and SH-SY5Y cells with IN04 resulted in increased α-synuclein expression at the mRNA level. In HMC-3 cells, IN04-induced α-synuclein upregulation was not further enhanced by co-treatment with LPS, and a similar observation was made in SH-SY5Y cells. The IN04 compound was developed based on homology modeling and virtual screening studies against *LRRK1*. Some *LRRK2* inhibitors are also known to inhibit *LRRK1* activity. However, to elucidate *LRRK1*'s cellular functions more clearly, it is essential to develop *LRRK1*-specific inhibitors.

LRRK1 is a dimeric protein with GTPase activity that occurs within the Roc domain. Developing inhibitors specifically targeting the *LRRK1*-specific Roc domain would allow for a better understanding of *LRRK1*-mediated regulation of cellular processes through its GTPase activity.

Mainly, p38 phosphorylation affects cell proliferation, differentiation, growth, and inflammatory processes [25]. Our study indicates that microglial cells are sensitive to IN04 treatment regarding p38 phosphorylation, whereas in SH-SY5Y neuroblastoma cells, IN04 inhibits p38 phosphorylation. *LRRK1* kinase activity may thus be necessary for SH-SY5Y proliferation. Although SH-SY5Y cells are commonly used in Parkinson's research, they are a neuroblastoma-derived cancer cell line and therefore differ from normal neuronal cells in certain characteristics.

IL-6 is an important cytokine involved in secondary immune responses, secreted by T cells to regulate B-cell stimulation [26] . Immune response and inflammation play a major role in Parkinson's disease. Individuals with autoimmune diseases such as systemic lupus or NMDAR encephalitis have a higher risk of developing Parkinson's. Genome-wide studies have identified *LRRK1* among the genetic loci associated with systemic lupus and NMDAR encephalitis [27], [28], suggesting that *LRRK1* might also influence Parkinson's disease development via autoimmunity. In our study, IN04 treatment significantly increased IL-6 expression in microglial cells. However, in neuroblastoma cells, IN04 alone did not increase IL-6 expression but markedly enhanced it when combined with LPS. Investigating cytokine responses in *LRRK1* knockout models or under *LRRK1* inhibition in response to stimulants such as LPS will contribute to understanding the role of *LRRK1* in the immune system.

5. CONCLUSION

In conclusion, our study demonstrates that inhibition of *LRRK1* promotes inflammation and induces apoptosis. Furthermore, inhibition of *LRRK1* with IN04 led to increased α-synuclein expression in microglial and neuroblastoma cell line models. These findings suggest a potential link between *LRRK1* kinase activity and Parkinson's disease. Additionally, *LRRK1* inhibition was found to enhance microglial inflammation and significantly induce microglial apoptosis. Further advanced studies are required to better understand the effects of *LRRK1* on the immune system, autophagy, apoptosis, and inflammation.

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