International Journal of Pharmacology

ISSN 1811-7775

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2019.933.943

Research Article
Anti-neuroinflammatory Effect of Newly Synthesized Iridium Metal Complexes in LPS-induced Microglia Cells

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Abstract

Background and Objective: Platinum compounds are largely replaced by iridium (Ir)-based metal complexes as these compounds display encouraging anticancer effects with less side effects. Iridium compounds exhibited notable properties against platelet activation, however, its effect anti-neuroinflammatory effect remain unknown. Therefore, a comparative anti-neuroinflammatory effect of novel iridium compounds (Ir-3, Ir-6 and Ir-11) were investigated in LPS-induced BV2 microglial cells. **Materials and Methods:** Quantitative real-time PCR was performed to detect the mRNA expression of COX-2, iNOS, TNF-α, IL-1β, IL-6 and anti-inflammatory cytokines Arg-1 and IL-10. Protein expression of iNOS and IL-1β was tested by Western blotting assay. The oxygen-glucose deprivation-reperfusion (OGD/R)-induced inflammation was also evaluated in BV2 cells. **Results:** The results showed that LPS-treated BV2 microglia had fewer branches that appeared to be resorbed into the cell body, whereas control microglia showed small soma with distal arborization. The LPS induced COX-2, iNOS, TNF-α, IL-1β and IL-6 and decreased Arg-1 and IL-10 mRNA expressions. Among the tested iridium compounds, Ir-6 attenuated the mRNA and protein expression of iNOS and T NF-α and protein expression of IL-1β. The OGD/R significantly increased COX-2, iNOS, TNF-α and IL-1β mRNA in BV2 microglia cells. **Conclusion:** These results suggest that Ir-6 can be consider as a novel anti-neuroinflammatory agent to treat neurodegenerative diseases via regulating cytokines and iNOS.

Key words: BV2 microglia cells, cytokines, iridium complex, oxygen glucose deprivation/reperfusion (OGD/R), SAR

Citation: Yen-Jen Chen, Kuo-Ching Yuan, Thanasekaran Jayakumar, Chih-Hsuan Hsia, Chih-Wei Hsia, Marappan Velusamy, Duen-Suey Chou and Shaw-Min Hou, 2019. Anti-neuroinflammatory effect of newly synthesized iridium metal complexes in LPS-induced microglia cells. Int. J. Pharmacol., 15: 933-943.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Inflammation triggers glial cells, generating proinflammatory cytokines and mediators, which harms neurons, induce the disease progression[1]. Induction of microglia activation alters cell morphology, proliferation, migration and produces inflammatory cytokines that additionally transmit inflammation in neurons[2]. Reactive oxygen species, nitric oxide (NO) and tumor necrosis factor-α (TNF-α) produced in the activated microglia could lead to toxic effects in neurons and intensified neuronal loss[3]. The BV2 microglial cells are the most commonly used cell lines for the neuroinflammation related drug research and development[4]. Bacterial lipopolysaccharide (LPS) induces microglia activation that produces pro inflammatory cytokines (IL-1β, IL-12 and TNF-α)[5]. Anti inflammatory mediators (e.g., IL-10 and TGF-β) and neurotrophic factors (IGF-1) produced by M2 like microglia induce tissue repair and growth stimulation. The LPS stimulated microglial activation triggers nuclear factor-κB (NF-κB), which induces various cytokines, such[6] as TNF-α, IL-1β and IL-6. Therefore, LPS-induced inflammatory events in microglia have been considered studying the characteristics of microglia-mediated inflammatory responses both in *in vitro* and *in vivo*.

Metal complexes are fascinating molecules in the field of inorganic and medicinal chemistry as they have concentrated much attention as an approach for the novel drug development[7]. Various anti-inflammatory drugs present in the market have enhanced their activities when bind with transition metal ions[8]. Copper-aspirin complex was found to be more effective anti-inflammatory effect than aspirin in rats[9]. Kale *et al.*[10] found zinc complex reduced carrageenan-induced inflammatory edema in rat paws. Studies also found different metal complexes of copper (II), cobalt (II) and manganese (II) exhibit strong anti-inflammatory activities[11].

Despite in recent decades, transition metal complexes such as ruthenium, iridium and rhodium have attracted much attention as anti-cancer agents, iridium complexes have been appealed as novel metal complex because of their distinctive properties in synthetic chemistry[12]. Recent studies have found some novel iridium-(III) complexes (Ir-3, Ir-6 and Ir-11) exhibit strong antiplatelet effects[13-15]. However, to our understanding, metal-based drug, especially iridium metals, has not been permitted for the treatment of inflammatory diseases. Therefore, anti-neuroinflammatory effect of Ir (III) complexes, such as [Ir(Cp*)(L3, 1-(2-pyridyl)-3-(3-methoxyphenyl)imidazo [1,5-a]pyridine) Cl]BF_4 (Ir-3), [Ir(Cp*)(L6, 1-(2-pyridyl)-3-(4-dimethylaminophenyl)imidazo[1,5-a]pyridine) Cl]BF_4 (Ir-6) and [Ir(Cp*)(L11, 1-(2-pyridyl)-3-(2-hydroxyphenyl)imidazo[1,5-a]pyridine) Cl]BF_4 (Ir-11) was evaluated in LPS-stimulated BV2 microglial cells. The structure-activity relationship (SAR) of iridium complex to their observed anti-neuroinflammatory effect was also described.

MATERIALS AND METHODS

This entire study was performed at the Department of Pharmacology, Taipei Medical University, Taipei, Taiwan from March, 2018-June, 2019.

Synthesis of metal complexes (Ir-3, Ir-6 and Ir-11): A series of 3 novel iridium complexes have been synthesized as shown in Fig. 1. Briefly, a mixture of metal precursor $[Ir(Cp^*)(Cl)_2]_2$ and the corresponding ligands (L) were taken in 1:2 ratio in dry methanol and stirred for 2 h at room temperature. Subsequently, 2.5 equivalents of NH_4BF_4 was added to the initial pale-yellow solution, it slowly changed the color to orange. After 12 h, the solution was evaporated and obtained residues were washed with diethyl ether (20 mL) and then dried under vacuum. The desired products were purified by crystallization from the dichloromethane/hexane mixture to obtain orange microcrystals.

Microglia BV2 cell culture: Professor WW-Lin (College of Medicine, National Taiwan University, Taiwan) gifted murine microglia cell line (BV2). The cells were maintained at 37°C and 5% CO_2/95% air in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 100 U mL^{-1} penicillin G and 100 mg mL^{-1} streptomycin.

Cell viability assay: Cell viability was measured by using MTT assay as described in previous study[16]. The BV2 cells were seeded into 24-well culture plates at 5×10^5 cells/well in DMEM containing 10% FBS at 37°C for 24 h. When cells attained 85% confluence, they were treated with various concentrations of Ir-3, Ir-6 and Ir-11 (5, 10 and 20 μmol L^{-1}) or solvent control (0.1% DMSO) for 30 min and then stimulated with LPS (1 μg mL^{-1}) for 24 h. The percentage of cell viability was measured by calculating the absorbance of treated-cells/absorbance of control cells×100%. The optical density was measured at 570 nm by a MRX absorbance reader (Dynex Technologies, Chantilly, VA, USA). In addition, the morphology of LPS treated cells was observed under a microscope.

Quantitative real-time PCR: The BV2 microglia cells (5×10^5 cells/well) were plated in 12-well culture plates, after they attained 85-90% confluence, cells were treated with

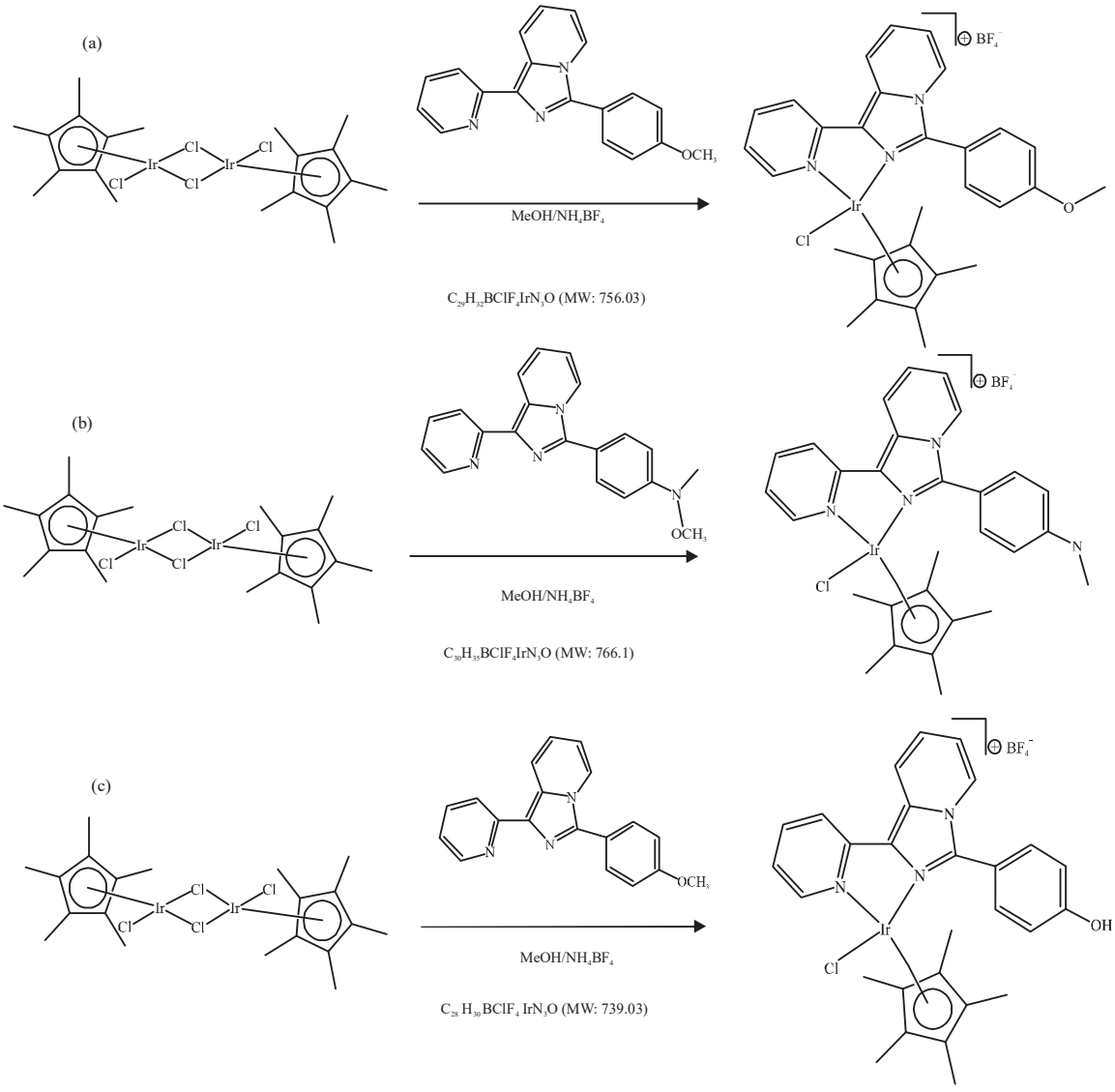

Fig. 1(a-c): Chemical structure and synthesis of Iridium compounds, Ir-3, Ir-6 and Ir-11. Scheme of synthesis of complex (a) [Ir (Cp*) L3 Cl]BF_4 (Ir-3), (b) [Ir(Cp*) L6 Cl]BF_4 (Ir-6) and (c) [Ir(Cp*) L11 Cl]BF_4 (Ir-11)

Table 1: Primers used for quantitative PCR

Factors name	Sequence (5'→3') forward	Sequence(5'→3') reverse
ARG1	GTGAAGAACCCACGGTCTGT	GCCAGAGATGCTTCCAACTG
iNOS	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
IL-1β	AACCTGCTGGTGTGTGACGTTC	CAGCACGAGGCTTTTTTGTTGT
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
COX-2	CCACTTCAAGGGAGTCTGGA	AGTCATCTGCTACGGGAGGA
TNF-α	TCTTCTGTCTACTGAACTTCGG	AAGATGATCTGAGTGTGAGGG
IL-6	CCTCTCTGCAAGAGACTTCCATCCA	GGCCGTGGTTGTCACCAGCA
GAPDH	GAACATCATCCCTGCATCCA	GCCAGTGAGCTTCCCGTTCA

LPS (1 μg mL^{-1}) for 24 h. Total RNA was extracted using RNA isolation kit (MACHEREY-NAGEL) according to the manufacturer's instruction. The RNA was transcribed into cDNA under Oligo (dT), Primers and SuperScrip First-Strand Synthesis System (Invitrogen). The RT-PCR was done by QuantiTect SYBR Green PCR Kit (QIAGEN) using forward and reverse primers sequences of the target proteins as shown in Table 1.

Western blotting analysis: The cells were pretreated with Ir-3, Ir-6 and Ir-11 (5 and 10 μmol L^{-1}) or 0.1% DMSO for 20 min and then stimulated with LPS (1 μg mL^{-1}). Subsequently, cell lysates were prepared in lysis buffer and proteins were extracted using this buffer. Equal amount of proteins (30 μg) were applied to 12% SDS-PAGE and the separated proteins were then electrophoretically transferred on to PVDF membranes (0.45 μm). Blots were incubated with the targeting primary antibodies against iNOS and IL-1β for 2 h and then incubated with HRP-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG for 1 h at room temperature. The blots were developed with ECL system to detect the immune-reactive bands.

Oxygen glucose deprivation/reperfusion (OGD/R): The OGD/R model was achieved according to the method described by Zhou *et al.*[17] Briefly, BV2 cells were washed three times with deoxygenated PBS and cultured in deoxygenated and glucose free OGD buffer (116 nmol L^{-1} NaCl, 1.8 nmol L^{-1} $CaCl_2$, 0.8 nmol L^{-1} $MgSO_4$, 5.4 nmol L^{-1} KCl, 1 nmol L^{-1} NaH_2PO_4, 14.7 nmol L^{-1} $NaHCO_3$, 10 nmol L^{-1} HEPES, pH 7.4) for 6 h in an anaerobic chamber (Forma, Thermo Scientific, USA) with 5% CO_2 and 95% N_2. At the end of the OGD period, all the cultures were placed under normoxic conditions (21% O_2 and 5% CO_2) and the media was quickly replaced with glucose-containing normal media and incubated at different time points (1, 4, 7 and 24 h). All the cultures were maintained in a humidified 37°C incubator.

Statistical analysis: All data are expressed as the means±standard error (SEM). Data were calculated using one-way analysis of variance (one-way ANOVA), if one-way ANOVA discovered significant differences among the group means, a subsequent comparison of Newman-Keuls method was performed. A $p<0.05$ was designated a statistically significant difference.

RESULTS

LPS alters the morphology of BV2 microglia: One microgram per milliliters LPS was added to BV2 microglia containing culture medium for different time points (1, 2 and 3 h). As shown in Fig. 2a, the morphology of control BV2 microglia showed small soma with distal arborization, characteristic of "ramified" microglia. The cells treated with LPS presented fewer branches that were shorter and or appeared to be resorbed into the cell body (Fig. 2b-d).

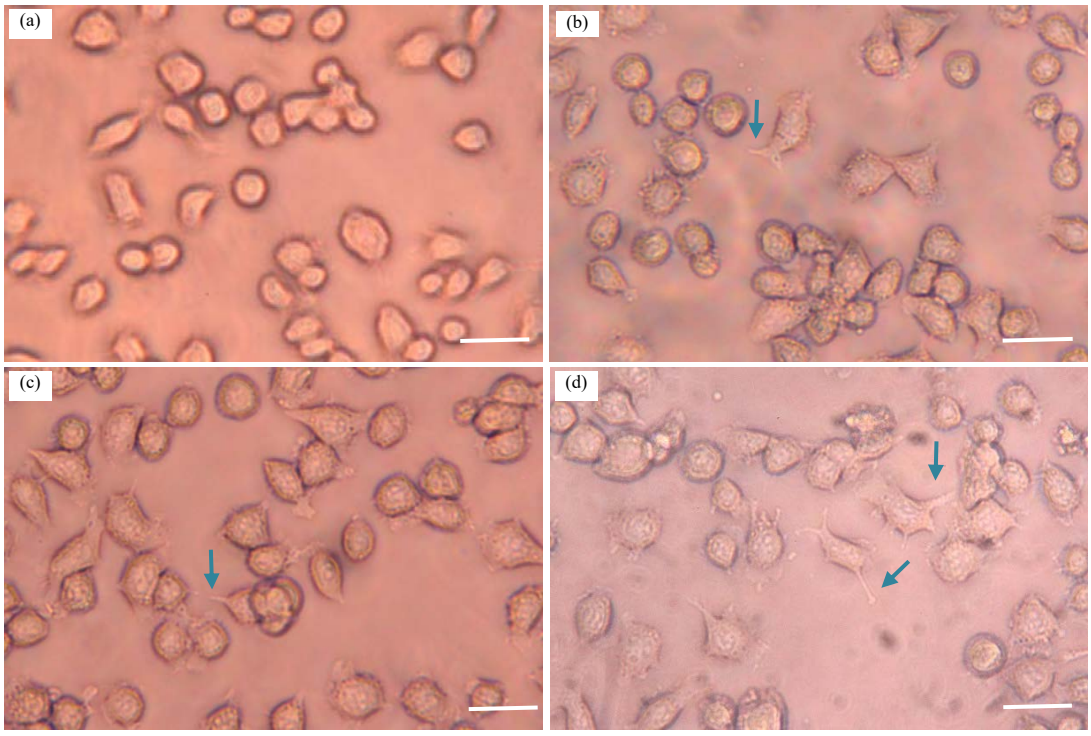

Fig. 2(a-d): Morphology of untreated normal and LPS treated BV2 cells (a) normal, LPS-induced (b) 1 h, (c) 2h and (d) 3h BV2 cells
All images were taken with low magnification (10x), scale bar = 100 μm, arrows indicate the changes in LPS-activated BV2 cells

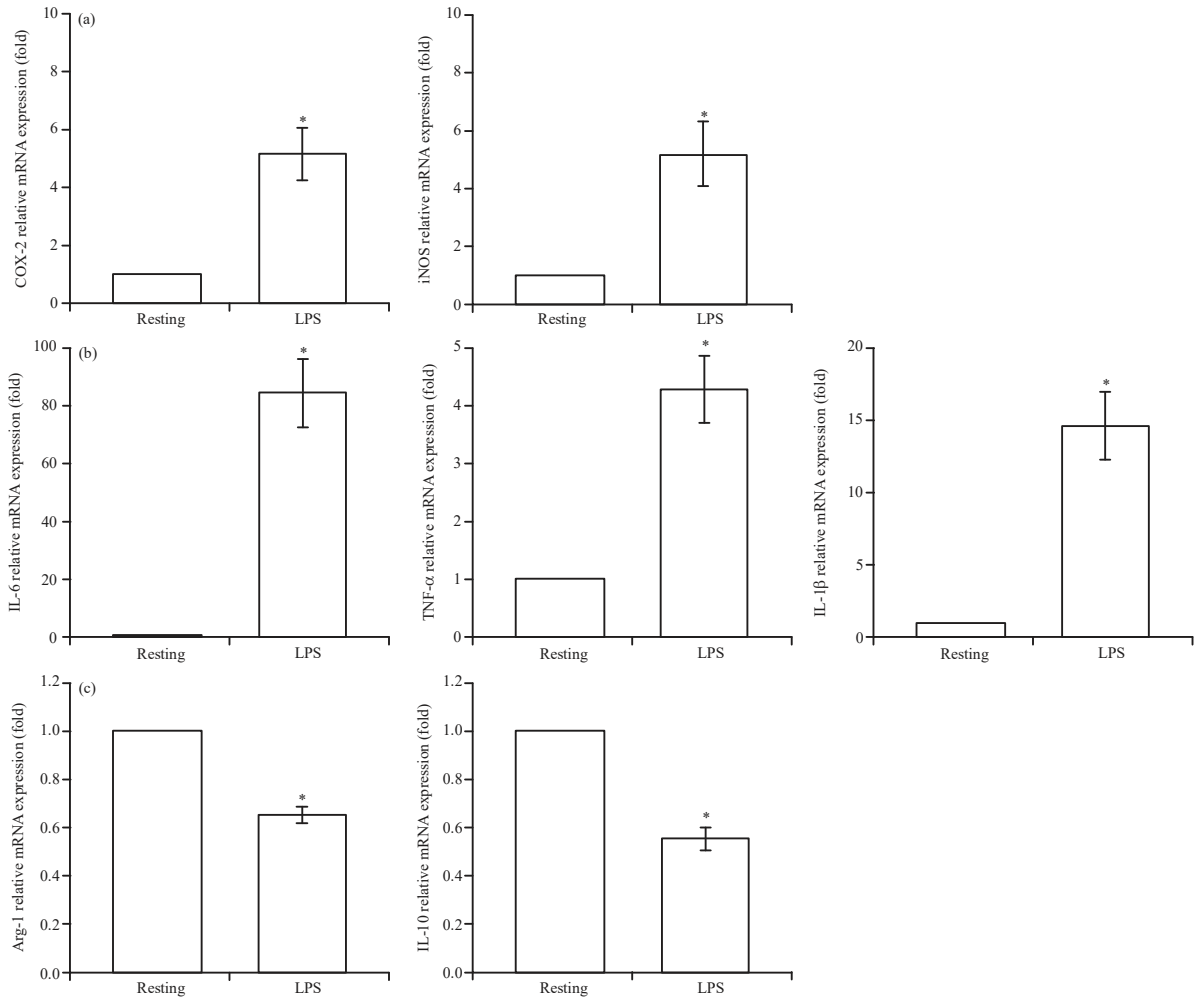

Fig. 3(a-c): Impact of LPS on mRNA expression of inflammatory enzymes, pro-inflammatory and anti-inflammatory cytokine in BV2 cells. mRNA expression of (a) inflammatory enzyme COX-2 and iNOS, (b) pro-inflammatory cytokines IL-6, TNF-α and IL-1β and (c) anti-inflammatory cytokines Arg-1 and IL-10 in normal and LPS-induced BV2 cells

*p<0.05 compared with the resting groups

LPS stimulated iNOS and COX-2 mRNA in BV2 cells: As COX-2 is an important mediator of inflammatory reactions, it inhibition has been shown to exert anti-inflammatory actions. The molecular cross-talk between iNOS and COX may regulate tissue homeostasis and hence contribute to pathophysiological processes. It has been suggested that COX-2 up-regulation was strictly dependent on iNOS expression. In this study, LPS induced iNOS mRNA expression more significantly ($p<0.01$) than that of the COX-2 ($p<0.05$) in BV2 cells (Fig. 3a).

LPS on pro-inflammatory cytokines in BV2 cells: IL-6, TNF-α and IL-1β are representative pro-inflammatory cytokines produced by microglia cells. To test the LPS-mediated pro-inflammatory process, the mRNA expression of IL-6, TNF-α and IL-1β were detected in BV2 cells. As shown in Fig. 3b, LPS significantly stimulated the mRNA expression of IL-6, TNF-α and IL-1β ($p<0.05$) when compared to LPS untreated control cells.

LPS reduced anti-inflammatory cytokine expression in BV2 cells: M1 microglia release proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β to induce inflammatory response. In contrast, M2 microglia produce anti-inflammatory Arg1, IL-10 and neurotrophic factors, such as nerve growth factor, which attenuate the inflammatory response In this study, LPS reduced Arg1 and IL-10 mRNA expression significantly ($p<0.05$) when compared to control BV2 cells (Fig. 3c).

Ir-6 complex attenuated LPS- induced iNOS and TNF-α mRNA expression: As shown in Fig. 4, Ir-6 significantly inhibited LPS-induced iNOS and TNF-α mRNA expression in BV2 cells in a concentration-dependent manner. More precisely, compared with control cells, LPS markedly increased iNOS and TNF-α mRNA expression, however, co-treatment with Ir-6 at 5 and 10 μmol L^{-1} significantly decreased their expression (Fig. 4b). Conversely, Ir-3 and Ir-11 did not effective on these mRNAs (Fig. 4a, c).

Ir-6 inhibited LPS-induced protein expression of iNOS and IL-1β in BV2 cells: Figure 5b indicated that Ir-6 concentration-dependently inhibited the LPS-induced increase in iNOS and IL-1β expression in BV2 cells. The decrease of these expressions in LPS-induced microglia was more apparent at 10 μmol L^{-1} pretreated cells. Moreover, Ir-3 or Ir-11 did not alter these proteins in LPS stimulated cells (Fig. 5a, c).

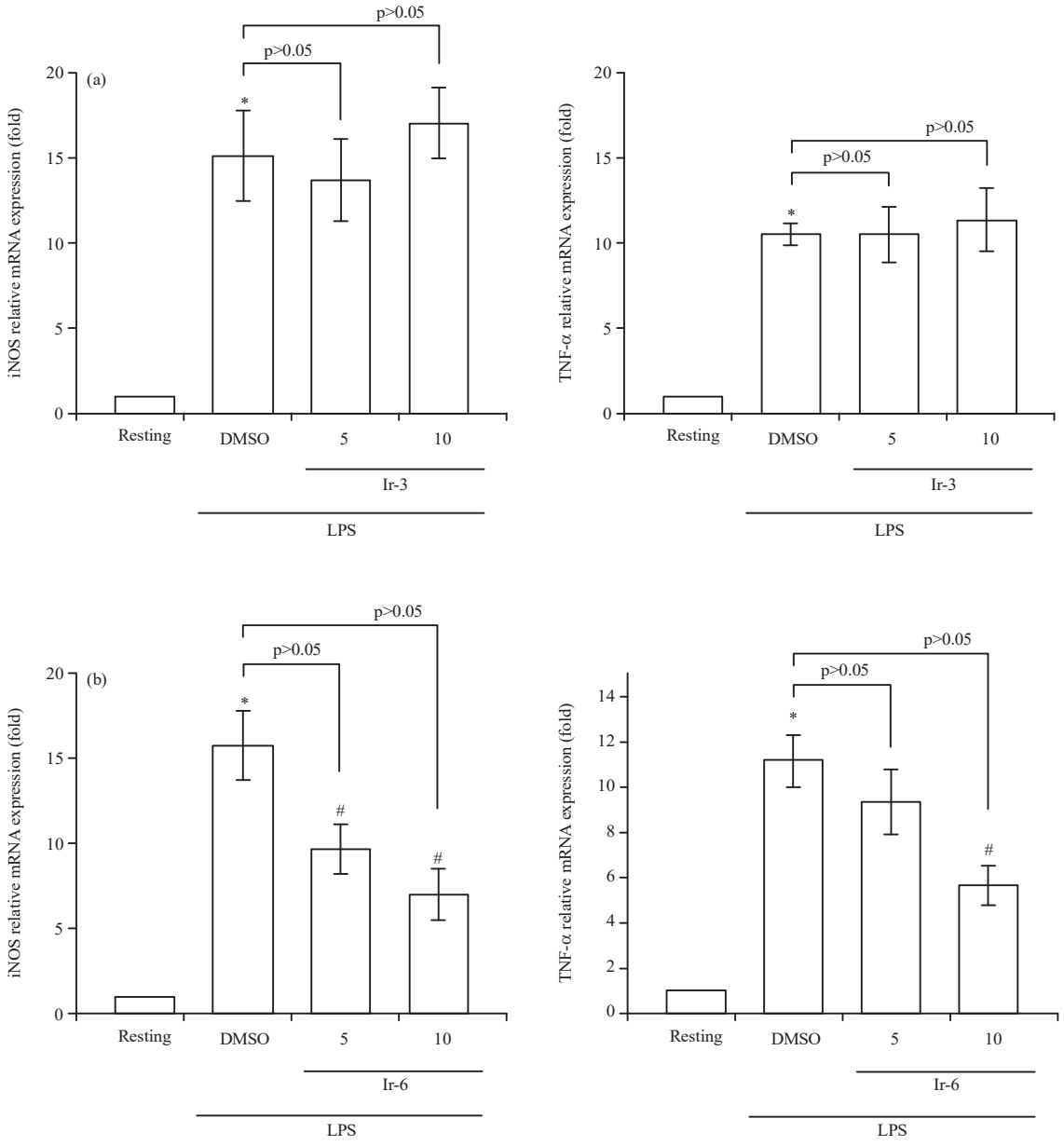

Fig. 4: Continue

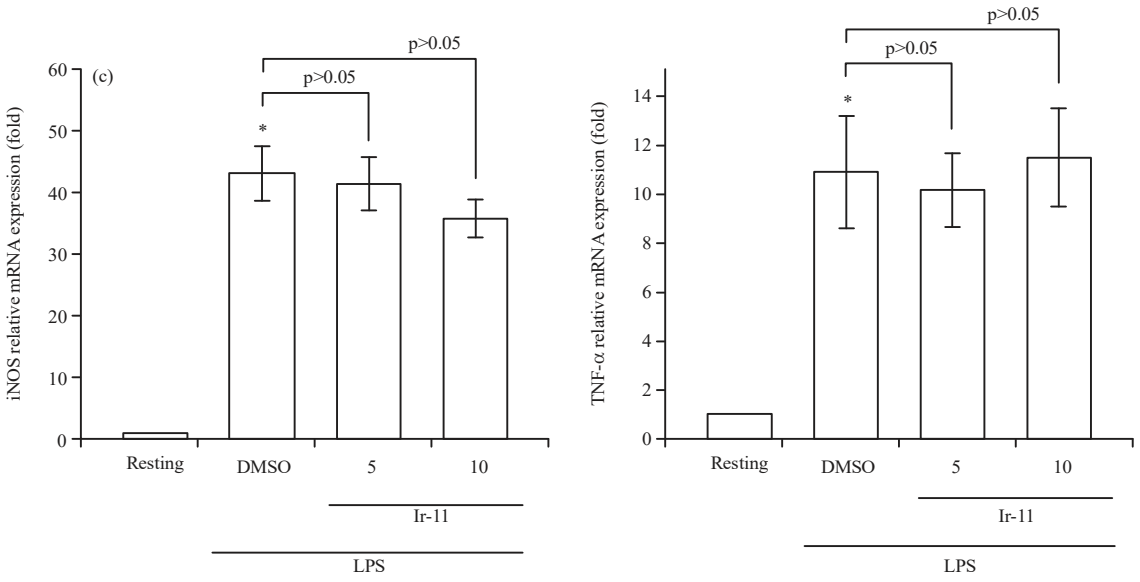

Fig. 4(a-c): Effect of iridium compounds on LPS-induced mRNA expression of iNOS and TNF-α in BV2 cells. Cells were pretreated with 5 and 10 μmol L^{-1} of (a) Ir-3, (b) Ir-6 and (c) Ir-11 for 30 min and then stimulated by LPS (1 μmol L^{-1}) for 24 h

mRNA expression of iNOS and TNF-α were evaluated as described in the methods, data are presented as the means±SEM (n = 4), *p<0.05 compared with the resting groups, #p<0.05 compared with the LPS-treated cells

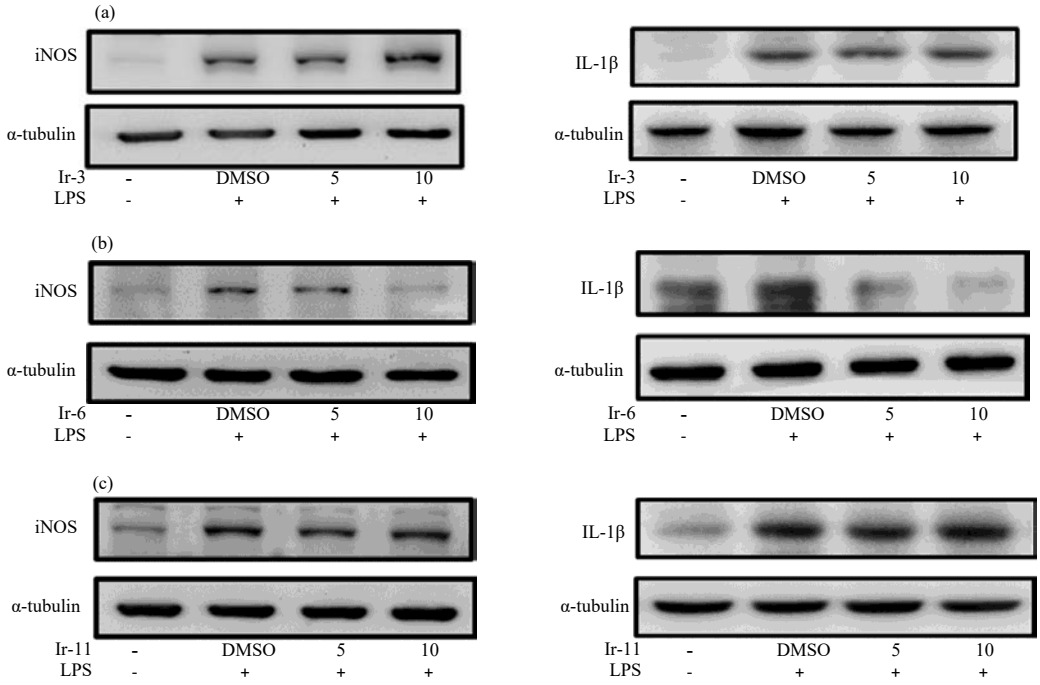

Fig. 5(a-c): Effect of iridium (III) compounds on LPS-induced protein expression of iNOS and IL-1β in BV2 cells. Cells were pretreated with 5 and 10 μmol L^{-1} of (a) Ir-3, (b) Ir-6 and (c) Ir-11 for 30 min and then stimulated by LPS (1 μg mL^{-1}) for 24 h

Protein expression of iNOS and IL-1β were evaluated as described in the methods, data are presented as the means±SEM (n = 4)

Ir-6 inhibited LPS-induced protein expression of iNOS and IL-1β in BV2 cells: Figure 5b indicated that Ir-6 concentration-dependently inhibited the LPS-induced increase in iNOS and IL-1β expression in BV2 cells. The

decrease of these expressions in LPS-induced microglia was more apparent at 10 μmol L^{-1} pretreated cells. Moreover, Ir-3 or Ir-11 did not alter these proteins in LPS stimulated cells (Fig. 5a, c).

Ir-6 recovered OGD/R-induced inflammation in microglia via inhibiting iNOS and COX-2 expression: As oxygen-glucose deprivation/reperfusion (OGD/R) model mimics stroke, here we examined the anti-inflammatory effect of iridium compounds on microglial cells. As shown in Fig. 6, OGD/R was implemented in BV2 cells. The mRNA expression of COX-2, iNOS, TNF-α and IL-1β significantly ($p<0.05$) upregulated in BV2 cells induced by OGD/R at 23 h reperfusion (Fig. 7a, b). As the results shown in Fig. 8a-c, OGD 23 h/R significantly increased iNOS and COX-2 expression compared to those in the control group. Among the tested iridium compounds, Ir-6 was significantly reduced OGD/R-induced iNOS and COX-2 in BV-2 cells (Fig. 8b).

DISCUSSION

Inflammatory reaction-mediated neuroinflammation plays a critical role in the development of neurodegenerative pathologies[18]. Many studies have shown that down regulation of microglia activation might protect against neurodegenerative diseases[19]. Organometallic iridium complexes have fascinated ample attention due to their exceptional properties of rich synthetic chemistry, variable oxidation states that are dominant under physiological conditions[12]. In the present study, the anti-neuroinflammatory effect of newly synthesized iridium complexes, Ir-3, Ir-6 and Ir-11 was investigated in LPS stimulated BV2 cells. We found that among the three tested Ir (III) complexes, Ir-6 has potent effects against LPS-induced BV2 cells via inhibiting LPS and OGD/R-induced iNOS and COX-2 and LPS-induced iNOS and TNF-α expression. These results propose that Ir-6 may act as a novel anti-inflammatory and microglia-modulating drug for neurodegenerative diseases.

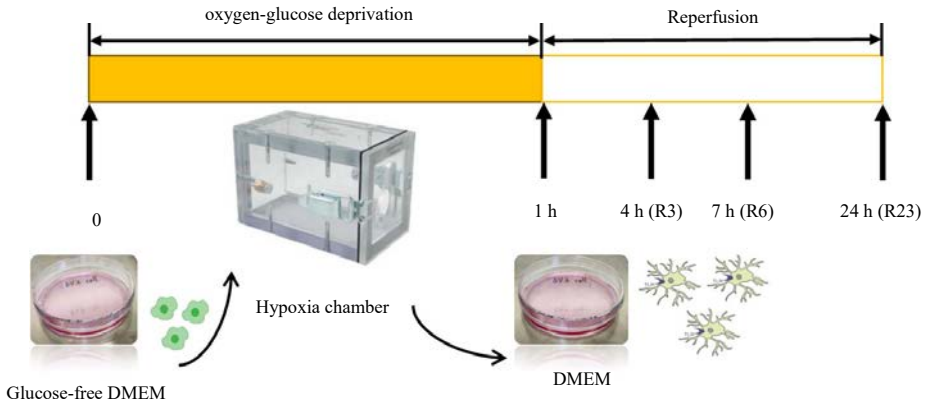

Fig. 6: Experimental design of oxygen and glucose deprivation/reperfusion (OGD/R)-induced inflammation in rat model
OGD/R was done by changing the medium with high-glucose DMEM containing 10% FBS and cells were shifted to a normoxic incubator with 95% air and 5% CO_2, R3, R6 and R23 indicated the time, which was required to induce reperfusion (3, 6 and 23 h)

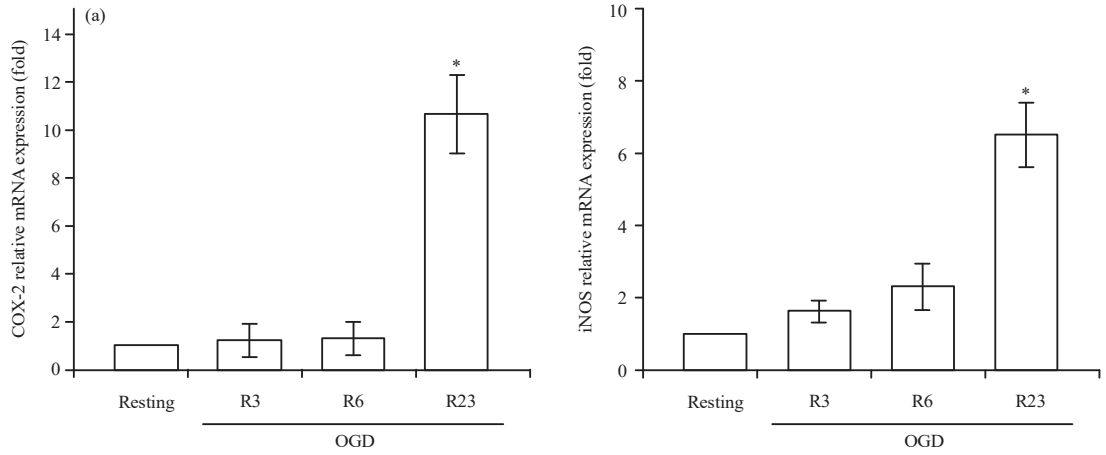

Fig. 7(a-b): Continue

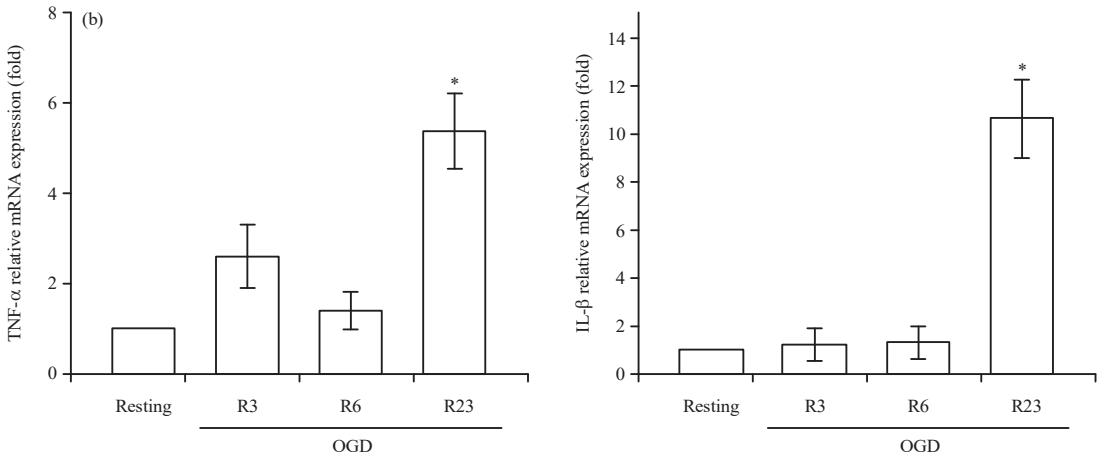

Fig. 7(a-b): mRNA expression of inflammatory enzymes and pro-inflammatory cytokines in OGD/R-activated microglia (a) mRNA expression of COX-2 and iNOS and (b) TNF-α and IL-1β in normal and OGD/R-induced BV2 cells

R3, R6 and R23 indicated the time, which was required to induce reperfusion (3, 6 and 23 h), *p<0.05 compared with the resting groups

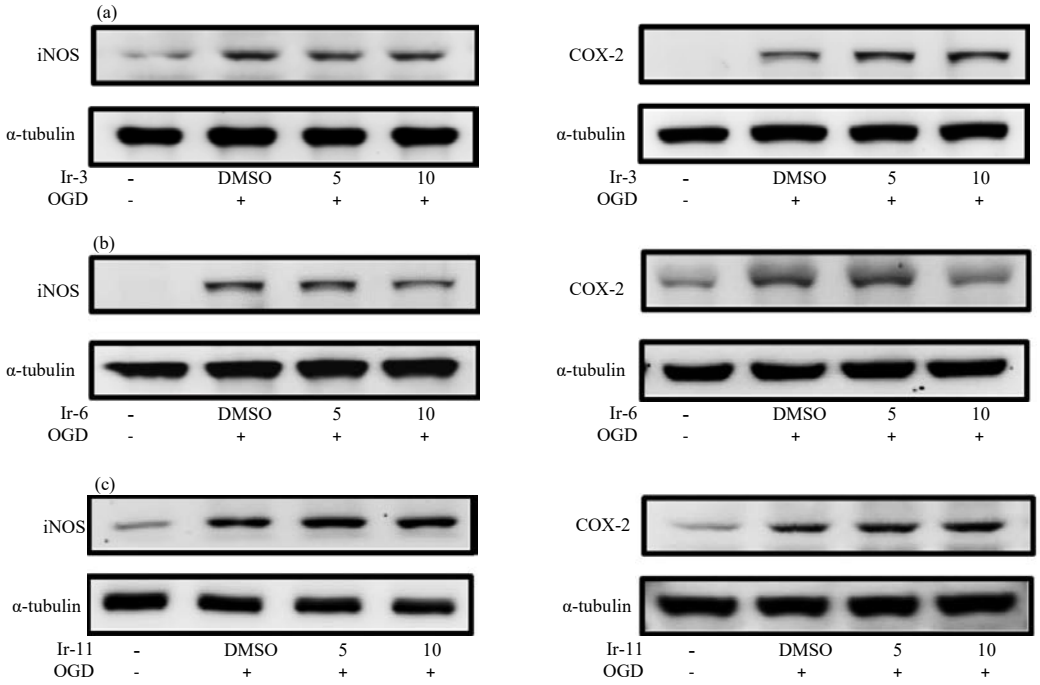

Fig. 8(a-c): Ir-6 condensed OGD/R-induced protein expression of iNOS and COX-2 in BV2 cells (a) Ir-3, (b) Ir-6 and (c) Ir-11 were added with OGD into the medium for 1 h and waited until the end of reperfusion time (23 h)

Protein expression of iNOS and COX-2 were evaluated as described in the methods, data are presented as the means±SEM (n = 4)

Enhanced expression of iNOS during the inflammatory response can increase the local production of NO, which has been suggested to be involved in neurodegenerative processes[20]. Pharmacological inhibition of iNOS has been shown to protect mice from spatial memory dysfunction and depressive-like behavior[21]. An increased mRNA and protein expression of iNOS and COX-2 have reported in the brains of LPS treated mice[22]. Similarly, in this study, LPS exposure in BV2 cells significantly elevated COX-2 and iNOS mRNA and protein expression. However, Ir-6 significantly inhibited the LPS-induced protein and mRNA expression of iNOS but Ir-3 and Ir-11 did not response on this inflammatory enzyme.

LPS-activated microglia secrete pro-inflammatory cytokines TNF-α, IL-1β and IL-6, all of which play major roles in

the inflammation-associated diseases[23]. TNF-α, a crucial mediator of the inflammatory response, activates the expression of chemokines, thereby affecting biological functions. The IL-6 is also an important pro-inflammatory cytokine in the immune response. The IL-1β is reported to regulate the progression of several neurodegenerative diseases. Therefore, inhibiting of these inflammatory cytokines might be an important strategy for the treatment of this disease. Our results showed that among the tested Ir (III) compounds, Ir-6 significantly inhibited the LPS-induced TNF-α and IL-1β protein expression and inhibited TNF-α mRNA expression. These results suggested that Ir-6 may inhibit neuroinflammation via suppressing the inflammatory cytokines.

This study also observed a significant decrease in the mRNA expression of Arg-1 and IL-10 in LPS-exposed BV2 cells. A previous study also found a decrease of Arg-1 and IL-10 in 1, 2 and 4 h LPS-induced cells compared to untreated control cells[24]. It has been documented that over activation of microglia during stroke augments brain damage. Using OGD/R to mimic stroke, this study explored the anti-inflammatory effect of iridium compounds on microglial cells and discovered the underlying mechanisms. The OGD for 1 h followed by reperfusion for 23 h significantly induced the expression of COX-2 and iNOS in BV-2 cells, which were inhibited by pretreatment with Ir-6.

Generally, it is stated that complexes that have high rate of hydrolysis exhibit better cytotoxicity than the complex with less hydrolyzing properties. For instance, a potent antiplatelet activity has been noted in bovine, ovine, caprine kappa-casein and their hydrolysates due to their strong hydrolytic actions[25]. Consequently, this study hypothesized that the observed anti-inflammatory activities of Ir-6 may essentially depend on the dimethyl (electron donating substitution) groups on the iridium ligands. Likewise, the lack of substitution on phenyl group in Ir-6 might be augmented the amount of hydrolytic property on its ligand system, this could be contributed major role to its detected potent anti-inflammatory effects.

CONCLUSION

This study demonstrated that BV2 microglia activated by LPS produced mRNA expressions of iNOS, COX-2, IL-6, TNF-α and IL-1β or reduced anti-inflammatory markers Arg-1 and IL-10. Moreover, among the three iridium compounds tested, only Ir-6 inhibits iNOS, TNF-α and IL-1β to suppress LPS-induced neuroinflammation. Moreover, OGD/R-induced expression of COX-2 and iNOS in BV-2 cells was inhibited by pretreatment with Ir-6. Therefore, iNOS, TNF-α and IL-1β may be the effective therapeutic for the anti-inflammatory potential for Ir-6. Altogether, these results direct that Ir-6 may be a chief novel iridium compound to project new anti-inflammatory drugs for the treatment of neurodegenerative diseases.

SIGNIFICANCE STATEMENT

Over activated microglia is involved in various kinds of neurodegenerative diseases. Destruction of microglial activation has emerged as a novel strategy for treatment of neuroinflammatory diseases. With this hypothesis, we investigate the protective effects of novel iridium compounds against LPS-induced inflammatory responses in BV2 microglia cells and demonstrate that iridium compounds significantly attenuate microglia activation. The significance of this study is to suggest that iridium compound could be a potential therapeutic agent for alleviating neuro-inflammatory diseases accompanied by activated microglia.

ACKNOWLEDGMENT

This work was supported by grants from the Ministry of Science and Technology of Taiwan (MOST-105-2314-B-038-068, MOST-106-2314-B-038-004 and MOST-106-2320-B-038-049-MY3), Taipei Medical University-Taipei Medical University Hospital (106TMU-TMUH-24) and the University Grants Commission, India (MRP-MAJOR-CHEM-2013-5144, 69/2014 F. No. 10-11/12UGC).

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