MODULATION OF MULTIPLE NEUROINFLAMMATORY SIGNALLING PATHWAYS BY THE DIETARY GLYCOSIDIC FLAVONOID TILIROSIDE

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Copyright Statement

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Publications

This work based publications:

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Uchechukwu P Okorji, **Ravikanth Velagapudi**, Abdelmeneim El-Bakoush, Bernd L Fiebich, Olumayokun A Olajide: Antimalarial Drug Artemether Inhibits Neuroinflammation in BV2 Microglia Through Nrf2-Dependent Mechanisms. Molecular Neurobiology 11/2015; DOI:10.1007/s12035-015-9543-1.

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Ravikanth Velagapudi, Gina Baco, Sunjeet Khela, Uchechukwu Okorji, Olumayokun Olajide: Pomegranate inhibits neuroinflammation and amyloidogenesis

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Abstract

Hyperactivated microglia plays a key role in regulating neuroinflammatory responses which propagate damage to neurons. In recent years, substantial attention has been paid in identifying new strategies to abrogate neuroinflammation. Tiliroside, a dietary glycosidic flavonoid found in several medicinal and dietary plants is known to possess anti-inflammatory and antioxidant activities. This study is aimed at investigating the molecular mechanisms involved in the inhibition of neuroinflammation by the tiliroside.

Neuroinflammation inhibitory effects of tiliroside (2-6 μM) were investigated in BV2 microglia stimulated with a combination of LPS (100 ng/ml) and IFNγ (5 ng/ml). Results show that tiliroside significantly reduced the production of pro-inflammatory cytokines IL-6, TNFα, IL-1β while increasing the production of anti-inflammatory cytokine IL-10 in LPS/IFNγ-stimulated BV2 microglia. The compound reduced NO production in LPS/IFNγ-stimulated BV2 cells through inhibition of iNOS protein expression. Tiliroside also suppressed COX-2 protein expression and inhibited PGE_2 production in activated microglia. Western blotting and functional experiments revealed that inhibition of neuroinflammation by tiliroside was shown to be mediated through inhibition of NF-κB and p38 MAPK signalling pathways. Also, the compound activated SIRT1 and inhibited the expression of acetylated-NF-κB-p65 in LPS/IFNγ-activated BV2 microglia. Further experiments revealed that inhibition of neuroinflammation by tiliroside is not dependent on SIRT1. Tiliroside increased the levels of Nrf2, HO-1 and NQO1 antioxidant proteins, indicating an activation of the Nrf2 protective mechanisms in the microglia. Furthermore, transfection of BV2 cells with Nrf2 siRNA resulted in the loss of anti-inflammatory activities of tiliroside. Results of neurotoxicity experiments showed that neuroinflammation-induced neurodegeneration, DNA fragmentation, ROS generation and calcium accumulation were significantly reduced in HT22 neurons when exposed to conditioned medium from BV2 microglia that were pre-treated with tiliroside prior to stimulation with LPS/IFNγ.

Results from this study suggest that tiliroside inhibits neuroinflammation in LPS/IFNγ-activated BV2 microglia by targeting NF-κB and p38 MAPK signalling pathways. Furthermore, the compound activated Nrf2 antioxidant mechanisms in the

microglia, which appears to contribute to its anti-inflammatory activity. The study also established that tiliroside protects HT22 neurons from neuroinflammation-induced toxicity.

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Abbreviations

AP-1: Activator protein-1

AKT: Protein Kinase B (PKB)

AA: Arachidonic acid

AD: Alzheimer's disease

ANOVA: Analysis of variance

ALS: Amyotrophic lateral sclerosis

ARE: Antioxidant response elements

ATP: Adenosine triphosphate

BACE1: Beta-secretase-1

BSA: Bovine serum albumin

BAD: Bcl-2 associated death promoter

BDNF: Brain-derived neurotrophic factor

BBB: Blood-brain barrier

CNS: Central nervous system

COX: Cyclooxygenase

COX-1: Cyclooxygenase 1

COX-2: Cyclooxygenase 2

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

EMSA: Electrophilic mobility shift assay

ERK: Extracellular signal-regulated kinase

eNOS: Endothelial nitric oxide synthase

EGF: endothelial growth factor

GSK-3β: Glycogen synthase kinase 3 beta

GST: Glutathione S-transferase

HO-1: Heme oxygenase-1

IFNγ: Interferon gamma

ICAM-1: intracellular adhesion molecule-1

IκB: inhibitors of Kappa B

IKK: inhibitor of NF-kappa B kinase

IL-1β: Interlukin-1 beta

IL-10: Interlukin-10

iNOS: inducible nitric oxide synthase

IRAK1: interleukin-1 receptor-associated kinase-1

IRAK4: interleukin-1 receptor-associated kinase-4

(IRF)3: interferon regulatory factor 3

JNK: c-jun N-terminal kinase

LBP: LPS binding protein

LDS: Lithium Dodecyl Sulphate

LPS: Lipopolysaccharide

MAPK: mitogen activated protein kinase

MKP-1: MAPK phosphatase -1

MMP9: Matrix metalloproteinase-9

mRNA: messenger RNA

MS: Multiple Sclerosis

MyD88: myeloid differentiation primary response gene 88

NADPH-oxidase: nicotinamide adenine dinucleotide phosphate-oxidase

NEMO: NF-κB essential modifier

nNOS: neuronal nitric oxide synthase

NED: N-1-napthylethylenediamine dinucleotide phosphate

NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells

NEMO: NF-κB essential modulator

Nrf2: Nuclear factor erythroid 2-related factor 2

NFT: Neurofibrillary tangles

NQO1: NAD(P)H: quinone oxide synthase

PARP: poly ADP-ribose polymerase

p38 MAPK: p38 mitogen activated protein kinase

p65: Transcription factor p65 (RelA)

p50: NF-KappaB1

PAMPs: pathogen-associated molecular patterns

PDK-1: phosphatidylinositol-dependent kinase 1

PDGF platelet-derived growth factor

PGE_2 : Prostaglandin E2

PIP3: phosphatidylinositol 3,4,5-tiphosphate

PGES: Prostaglandin E synthase

PI3K: phosphoinositide 3-kinase

PMSF: Phenyl methyl sulphonyl fluoride

PKC: protein kinase C

PVDF: Polyvinylidene fluoride

PD: Parkinson's disease

ROS: reactive oxygen species

RNA: Ribonucleic acid

rpm: Rotation per minute

STAT-1: signal transducer and activator of transcription-1

SDS: Sodium dodecyl sulphate

TAB: TAK1-binding protein

TAK1: transforming growth factor- β-activated kinase 1

TBK1: TANK binding kinase 1

TIR: Toll-interleukin-1 receptor

TIRAP: TIR domain-containing adaptor protein

TLRs: Toll-like receptors

TLR4: Toll-like receptor 4

TNFα: tumour necrosis factor alpha

TNFR: tumour necrosis factor receptor

TRAF6: TNF receptor-associated factor 6

TRAM: TRIF-related adaptor molecule

TRIF: TIR domain-containing adaptor inducing IFN-β

TBST: Tris buffered saline with Tween 20

TGF-β: Transforming growth factor-β

-/-: Gene deletion

ºC: Celsius degree

µg: Microgram

µl: Microliter

µM: Micro molar

ng/ml: nanogram/millilitre

Chapter 1. General introduction

Inflammation is a physiological response that is triggered by the immune system, especially macrophages in the body to remove any harmful stimuli such as pathogens, damaged cells, local injury and irritants by releasing inflammatory mediators such as cytokines, platelets and chemokines. The word inflammation was derived from Latin "inflammo", which means "I set a light or I ignite" (Didonato et al. 2012). Such immediate or acute immune response by the body is beneficial; however, sustained or chronic production of inflammatory mediators like cytokines is thought to be the starting point for several fatal diseases like obesity, cancer, coronary artery diseases and neurodegenerative diseases (Zhang & An 2007). Tumour necrosis factor (TNF), interleukins (IL), interferon's (IFN), haemopoietic growth factor (HGF) and growth factor (GF) are historically termed as cytokines. Moreover, these pro-inflammatory cytokines are critical in the initiation of various inflammatory responses in the body.

1.1 Immunology of the central nervous system

The mammalian central nervous system (CNS) is a vital organ that has been considered as 'an immune privileged site', protected from the influx of peripheral immune cells and inflammatory mediators by an intact blood-brain barrier (BBB) (Fonseca et al. 2014). However, evidence from recent studies suggests that during a variety of CNS pathologies the brain has shown to be interactive with the peripheral immune system (Wohleb & Godbout 2013). It has been well demonstrated and accepted that disruption of BBB leads to this effective interaction (Konczol et al. 2013). Also, the presence of peripheral macrophages and immune cells in the CNS activates brain parenchymal cells such as microglia and astrocytes, which further initiate the production of inflammatory mediators like cytokines and chemokines. This acute inflammatory response by microglia seems to protect neurons in the CNS. However, uncontrolled release of cytokines could damage surveillance capacity of the microglia that eventually induces neuronal death (Abbott et al. 2010) and (Carvey et al. 2009).

1.1.1 Neuroinflammation: a pathological perspective

Inflammation is an acute or chronic reaction of tissues in the brain to the local injury (Streit et al. 2004). Acute inflammation is a defensive response specifically referred

to the accumulation of activated glial cells that comprises the immediate response to an injurious agent that resolves and repairs the damaged site. Chronic inflammation results from a broad spectrum of stimuli that are persistent or sustained cycles of injury and response. The deleterious effects of immunological microglia and astrocyte activation contribute to neurodestructive effects thus worsening the disease process through their actions (Streit et al. 2004) and (Peterson & Toborek 2014).

Microglia are the resident immune macrophages of the CNS that play a complex role in various immunological functions and neural activities. Under physiological conditions, ramified microglia are shown to regulate homoeostasis by controlling the production of cytokines, brain-derived neurotrophic factor (BDNF) and transforming growth factor (TGF) β in the brain (Miranda et al. 2015). In contrast, under pathological events, such as infection, local injury, ischemia, altered neuronal function, toxic insults and inflammation, ramified microglia becomes activated and changes to an amoeboid morphology and produce excess inflammatory mediators (Knott et al. 2000) and (Montgomery & Bowers 2012). In addition, microglia are known to recognise a variety of molecular targets, such as nucleotides, lipoproteins, abnormally processed, modified or aggregated proteins (e.g., Aβ) and various inflammatory cytokines which are considered as the strongest inducers (Michelle L. Block et al. 2007). Depending on the phenotype of the microglia, they can exert a variety of functional properties, which may be either neurotoxic or neuroprotective.

However, under severe pathological circumstances, microglia become hyper-activated and can induce uncontrolled detrimental neurotoxic effects by the excess production of a vast array of cytotoxic factors. Such as pro-inflammatory cytokines, chemokines like MCP-1α, MIP-α and CXCL-8, nitric oxide (NO), prostaglandin E2 (PGE_2) and neurotoxic reactive oxygen species (ROS) (Hamadi et al. 2016), (Kim & Cho 2008) and (Zhang & An 2007). Furthermore, pro-inflammatory cytokines such as interleukin-1beta (IL-1β), interleukin 6 (IL-6), and tumour necrosis factor α (TNFα), primarily mediate and facilitate a broad spectrum of neural activities and inflammatory processes in the brain (Sahu et al. 2013), (Kaushik et al. 2013) and (Lynch et al. 2004). Particularly, during the early period of neurodegenerative disease development, the role of activated pro-inflammatory cytokines could be diverse, ranging from adjacent microglial activation to neuronal death or damage in

the CNS (Gibbons & Dragunow 2006). Due to these neurotoxic mediators, understanding the causes and defining the characteristics of deleterious microglial activation in neurodegenerative disease has become a recent focus of research.

1.2 Role of neuroinflammation in neurodegenerative diseases

Neuroinflammation has been identified as a primary suspect in the pathologies of several neurodegenerative CNS disorders. Such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and tauopathies (Zecca et al. 2004), (Bal-Price & Brown 2001) and (Hsieh & Yang 2013). Due to the complexity of the events in neuroinflammation, it is unclear whether these responses from microglial activation and astrogliosis are beneficial, detrimental or neutral against neighbouring neurons. Studies in various animal models of neurodegenerative diseases revealed that chronic microglial activation and associated neuroinflammation is primarily involved in the progression of neuronal damage and death (Jurgens & Johnson 2012) and (Geller et al. 2001). In murine models of PD and ALS as well as in human AD brain tissue samples, upregulation of various microglial pro-inflammatory cytokines and other neurotoxic factors was observed. These studies further suggest that active microglia are the principle mediators of neurodegenerative conditions (Hamadi et al. 2016), (Mitra et al. 2015) and (Trepanier & Milgram 2010).

In adverse neurological diseases, neurons experience a primary insult that predisposes them to cell death, probably through elevated levels of pro-inflammatory cytokines and ROS-mediated oxidative stress (Zhang et al. 2016). Also, excess nitric oxide production during chronic inflammatory conditions by activated glia can induce neuronal death by causing selective inhibition of mitochondrial cytochrome C oxidase in neurons releasing high levels of ROS into the cytoplasm (Watanabe et al. 2000) and (Uttara et al. 2009). Some epidemiological studies have also confirmed the presence of toxic inflammatory mediators like TNFα, IL-1β, IL-6, ROS and RNS in the cerebrospinal fluid (CSF) of patients with PD (Poloni et al. 2000).

In addition, elevated levels of TNFα mRNA and its associated dopaminergic neuronal death were observed in the substantia nigra of rodent midbrain within the hours of administration of two neurotoxins, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This study further highlights the

deleterious effects of inflammatory mediators on neuronal viability (Barcia et al. 2005) and (Zhang et al. 2016). These studies also revealed the extent of TNFR1 and TNFR2 receptor expression on the neurons and it is assumed that excess TNFα produced during inflammation will bind to these receptors and induce neuronal death in the brain.

It has been hypothesised that pro-inflammatory cytokines such as TNFα and IL 1β from hyperactive microglia are capable of causing redox imbalance and DNA strand breakage in neurons (Vauzour et al. 2015) and (Michelle L. Block et al. 2007). Also, elevated microglial activation in various regions of the brain such as basal ganglia, striatum, frontal and temporal cortical regions was observed from patients with idiopathic PD when compared to healthy age-matched controls (Gerhard et al. 2006). These observations help to understand that persistent activation of microglia in the midbrain are due to the elevated levels of inflammatory cytokines and oxidative stress acting in an autocrine or paracrine manner to potentiate neurodegenerative responses.

Additional evidence that neuroinflammation is intimately involved in neuronal degeneration is the blood-brain barrier studies. It is evident from several cell culture and animal studies that the permeability of the BBB increases with age and excess cytokine production (e.g. TNFα) during inflammation and the likelihood of peripheral immune cell infiltration into the CNS (Abbott et al. 2010) and (Tajes et al. 2014). This means that cytokines produced by brain-resident microglia may not be acting alone in mediating neuron cell death, but peripheral circulating neurotoxic factors may also involve in inducing neuronal dysfunction and in facilitating the development of various neurodegenerative diseases.

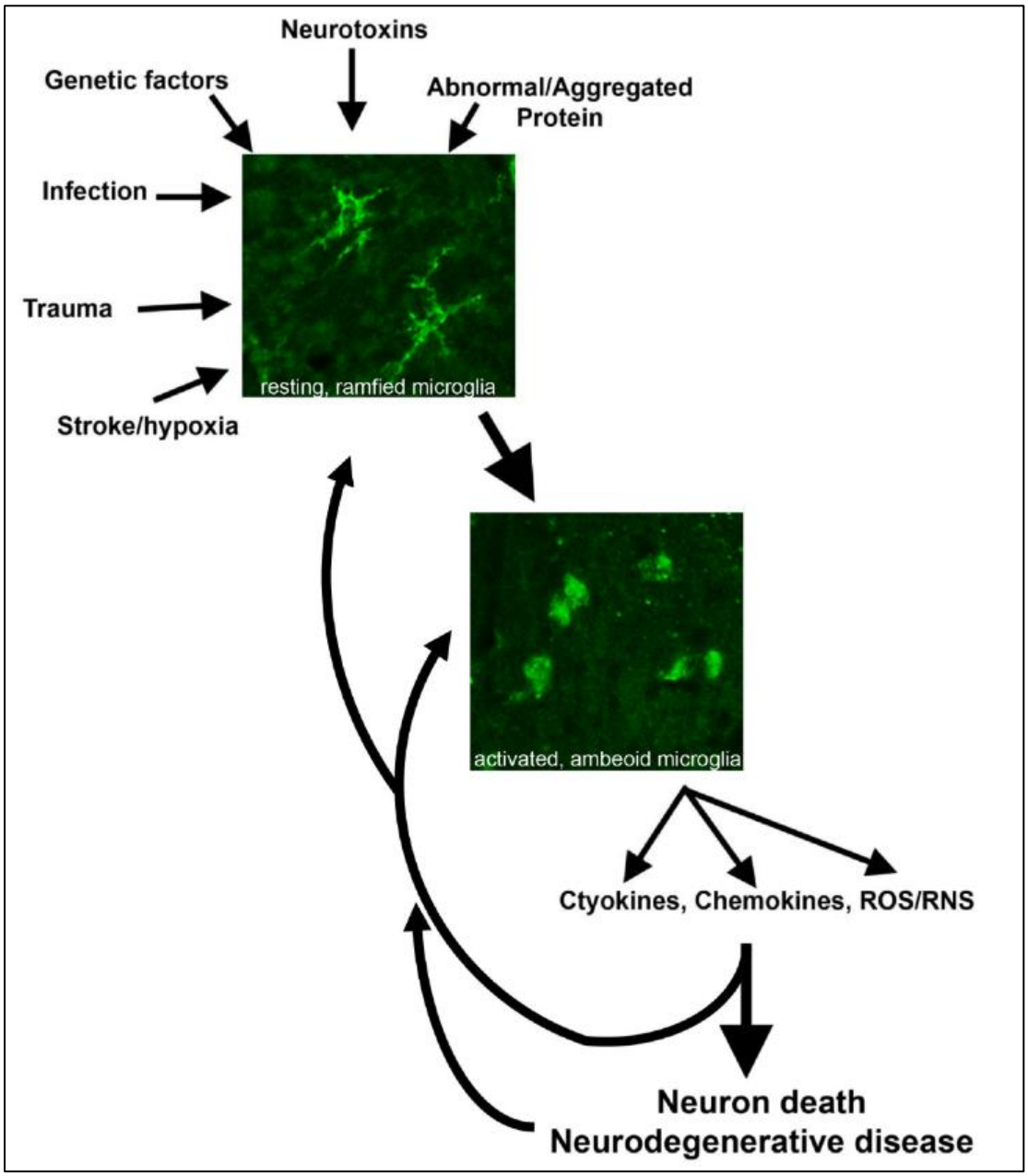

Figure 1.1 Self-propelling cycle of neuroinflammation and its associated neuronal death. Source:- (Frank-Cannon et al. 2009). RNS: Reactive nitrogen species, ROS: Reactive oxygen species.

1.4 Role of pro-inflammatory cytokines and other inflammatory mediators in neuroinflammation

Cytokines released at low levels during normal physiological situations are shown to mediate survival signals between microglia and adjacent neurons/astrocytes in the CNS. These are divided into pro-inflammatory and anti-inflammatory cytokines, which facilitate and inhibit inflammatory responses, respectively. Among all the pro-inflammatory cytokines TNFα, IL-1β and IL-6 are most widely investigated in neuroinflammation processes (Ramanan et al. 2008) and (McGeer & McGeer 2015). These have been shown to play a central role in initiating and regulating the cytokine cascade during an inflammatory response and have been shown to release consistently by activated microglia, along with other neurotoxic substances. Specifically, TNFα activates various pro-apoptotic signalling mediators such as caspases, MAPK (i.e. p38 MAPK, JNk1/2), NF-κB and several other inflammatory genes, while IL-6 and IL-1β induce morphological alterations in the microglia (Hehlgans & Pfeffer 2005) and (Park & Bowers 2010).

In addition, recent studies showed that when microglia stimulated with fibrillar Aβ, excess production of pro-inflammatory cytokines was observed and further studies show activated phagocytic oxidase (PHOX), that increased the levels of superoxide, peroxynitrite and damaged adjacent neurons (Chan et al. 2009) and (Brown & Neher 2010). Also, these pro-inflammatory cytokines have shown to activate adjacent microglia and astrocytes in the CNS (Park & Bowers 2010). It appears from the studies conducted by various investigators that specific inhibition of cytokine production in the neuroinflammation by active microglia may serve as a better strategy to ablate neurodegenerative conditions. Pro-inflammatory cytokines that have attracted a lot of attention in recent years were discussed below.

1.4.1 Tumour necrosis factor alpha (TNFα)

Tumour necrosis factor alpha (TNFα) is one of the important pro-inflammatory cytokine released by microglia that has shown to employ both homeostatic and pathophysiological roles in the CNS (Olmos & Llad 2014). In the healthy brain, the broad spectrum of regulatory functions by TNFα involves food and water intake, memory and learning, synaptic plasticity, sleep and astrocyte-induced synaptic strengthening (Kim et al. 2016). However, hyperactivated microglia release

significant amounts of TNFα which is shown to be involved in several neurological disorders such as AD and PD (Zhang & An 2007).

Initially, TNFα cytokine is synthesised as transmembrane protein (tmTNFα) which is later cleaved by matrix metalloprotease TNFα-converting enzyme (TACE) to release soluble TNFα (sTNFα) homotrimer (Idriss & Naismith 2000). The typical signal transduction of TNFα involves binding of sTNFα to two distinct surface receptors. Those are TNFα receptor 1 (TNFR1) and TNFα receptor 2 (TNFR2). These receptors are entirely different in their expression pattern and binding affinity for TNFα, however, the significant difference is the presence of cytoplasmic death domain tail on TNFR1, as this motif is entirely missing in TNFR2 (MacEwan 2002) and (Montgomery & Bowers 2012).

Studies have shown that activation of TNFR1 signalling results in the induction of various intrinsic signal transduction pathways. Such as nuclear factor-kappa B (NF-κB), c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein kinase (p38 MAPK) (Adli et al. 2010) while TNFR2 activation has shown to maintain homoeostasis functions of the CNS microenvironment (MacEwan 2002). Also, TNFR signalling pathways control the expressions of several inflammatory genes; particularly those regulated by the NF-κB signalling have shown to possess anti-apoptotic effects at low levels. Studies have demonstrated that cytokine interferon gamma (IFNγ) is a potent inducer of TNFα signalling along with other external stimuli such as LPS in the microglia (Ye et al. 2001) and (Merrill & Benveniste 1996).

Although neurons and astrocytes can produce low levels of TNFα, it is assumed that microglia is the primary source of this cytokine during neuroinflammation (Figure 1.2). The endogenous IFNγ induces microglial TNFα production and releases via activating IFNγ-surface receptors. TNFα that is released by microglia promotes the activation of TNFR1 signalling to induce excess pro-inflammatory cytokines and also shown to activate release of glutamate from hemi channels of microglial gap junctions (Olmos & Llad 2014). Furthermore, microglial TNFα activates TNFR signalling on astrocytes to induce glutamate exocytosis by blocking the glutamate uptake, thus increasing extracellular glutamate levels (McCoy & Tansey 2008). TNFα released from active microglia has both neuroprotective (at low concentrations) and

neurotoxic effects (high levels) related to the various signalling pathways activated by their receptors.

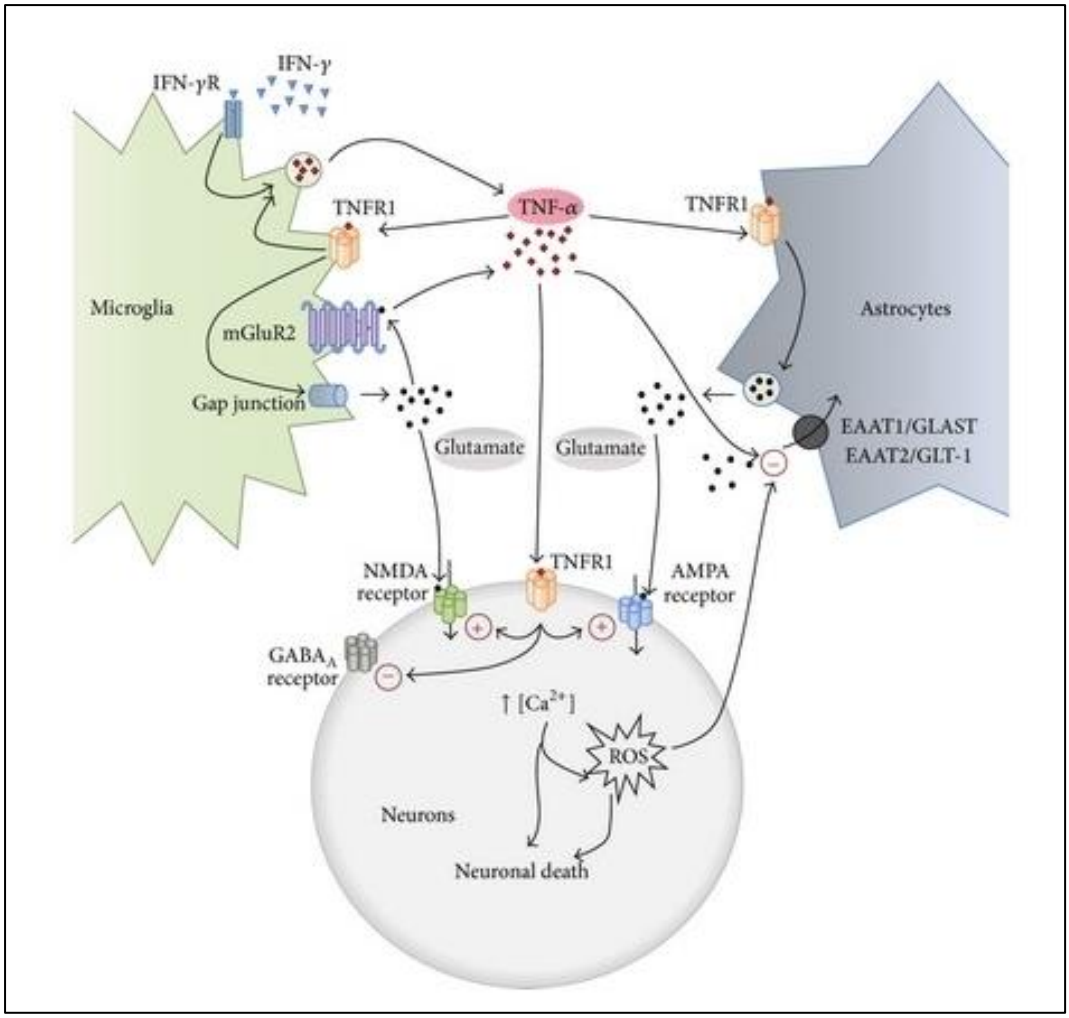

Figure 1.2 Tumour necrosis factor alpha (TNFα) signalling in the microglia. Source:- (Olmos & Llad 2014).

IFN: Interforns, TNFR1: Tumor necrosis factor receptor 1, mGLUR2: Metabotropic glutamate receptor 2, EAAT1: Excitatory amino acid transporter 1, GLAST: GLutamate ASpartate Transporter, GLT-1: Glutamate transporter 1, AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, NMDA: N-methyl-D-aspartate receptor, GABA: gamma-aminobutyric acid.

1.4.2 Interleukins

Interleukins are pro-inflammatory cytokines, which are expressed at low levels in healthy CNS, but increases very rapidly in response to the pathogenic invasion in the CNS or when treated with exogenous LPS, IFNγ or TNFα (Rothwell & Luheshi 2000). These are particularly synthesised by glial cells, and astrocytes, as well as by neurons and has been shown to regulate various cellular functions in the brain. Out of several interleukins produced by active microglia during neuroinflammatory conditions, IL-6 and IL-1β are widely studied (Akdis et al. 2011).

Accumulation of IL-6 and IL-1β are implicated in the progression of chronic neurological diseases including AD and PD, as well as acute neuroinflammatory conditions such as traumatic brain injury and stroke (Halle et al. 2008) and (Lynch et al. 2004). Also, these circulating interleukins upregulate the production of prostaglandins and other neurotoxic mediators in the CNS and are therefore considered as master regulators of neuroinflammation (Liu et al. 2012). Interleukin's, once secreted from microglia and astrocytes, it can further stimulate its production in an autocrine or paracrine fashion via binding to IL-1 surface receptor superfamily (IL-1Rs) (Araujo et al. 2009).

Pro-inflammatory cytokine	CNS origin	Effects on neurons	Synaptic effects	Effects on Aβ	Effects on tau	References
TNF-α	Microglia, astrocytes neurons	Pro-apoptotic; prevent apoptosis	Synaptic excitotoxicity; LTP ↓	↑ Aβ synthesis; ↓ Aβ clearance	↑ tau hyperphosphorylation	(74,75)
IL-1β	Microglial, astrocytes	Neuronal death and damage↑	LTP ↓ synaptic plasticity↓	↑ Aβ synthesis ↓ Aβ-related pathology	↑ tau phosphorylation ↓ tau pathology	(76-79)
IL-6	Microglial astrocytes endothelial cells	Rescue neurons	LTP ↓ prevents synaptic loss	↓ Aβ deposition	↑ tau phosphorylation	(80-82)
IL-18	Activated microglia, astrocytes and ependymal cells	Pro-apoptotic	↓ the induction of LTP	↑ production of APP ↑Aβ	↑ hyperphosphorylation of tau	(83-85)

CNS, central nervous system; TNF-α, tumour necrosis factor alpha; IL, interleukin; LTP, long term potentiation; Aβ, amyloid beta; ↑↓, increase or decrease.

Figure 1.3 Association of various pro-inflammatory cytokines with neurodegenerative diseases (Yuste et al. 2015) .

1.4.3 Nitric oxide

Nitric oxide (NO) is a potent signalling molecule, discovered nearly two decades ago and since then its vasodilator, neuromodulator and inflammatory mediator roles were continuously reported by various groups (Knowles & Moncada 1994) and (Calabrese et al. 2007). It has been suggested that upregulation in the NO signalling is involved in the pathogenesis of various neurodegenerative diseases such as AD, PD and MS, as well as ALS (Zecca et al. 2004), (Doherty 2011) and (Peterson & Toborek 2014).

The actual problem starts with the imbalance in NO production during inflammatory insults, where microglia becomes hyperactivated. In mammals, NO is mainly synthesised by nitric oxide synthases (NOS) through the conversion of L-arginine to NO and L-citrulline. Traditionally, NO is synthesised by three forms of NOS in the CNS: neuronal NOS (nNOS) or NOS1, inducible NOS (iNOS) or NOS2, endothelial NOS (eNOS) or NOS3 (Figure 1.4). The activity of nNOS and eNOS typically depend on intracellular calcium (Ca^{2+}-dependant) and are widely expressed in synaptic spines, astrocytes and the loose connective tissue surrounding blood vessels in the brain.

Although, the expression of iNOS (Ca^{2+}-independent) is not constitutive in the CNS, glial cells are shown to express this isoform in excess during pathological conditions such as in response to inflammatory stimuli (Alderton et al. 2001) and (Yuste et al. 2015). Figure 1.4 shows various steps involved in the production of NO from different isoforms. High levels of nitric oxide were synthesised following the transcriptional expression of inducible NOS (iNOS) mediated by intrinsic signalling pathways in microglial cells and astrocytes after cytokine exposure (Yuste et al. 2015).The role of NO and its associated reactive nitrogen species (RNS) in neuroinflammation was well studied using various animal models (Uttara et al. 2009). It appears that excess NO released by active microglia during neuroinflammatory conditions blocks the reuptake of glutamate, and facilitate neuronal death (Rao et al. 2012) and (Kim et al. 2009). The upregulation of iNOS isoform at the transcriptional level and its mediated NO production was observed during the neuroinflammatory process. Also it appears that several transcription factors are implicated in transactivation of iNOS gene during neuroinflammatory pathologies, among them NF-κB and AP-1 are the most important (Hoesel & Schmid 2013) and (Kim et al. 2015).

In microglia, this NF-κB-mediated iNOS expression triggers several intrinsic pathways related to RNS formation, caspase and nNOS signalling activities (Kim et al. 2009) and (Uttara et al. 2009). Several studies have demonstrated that inhibition of LPS-induced NF-κB signalling and its mediated iNOS and NO production will ablate microglial activation in neuroinflammatory conditions (Fiebich et al. 2002), (Xu et al. 2014), (Onasanwo et al. 2016) and (Kao et al. 2010).

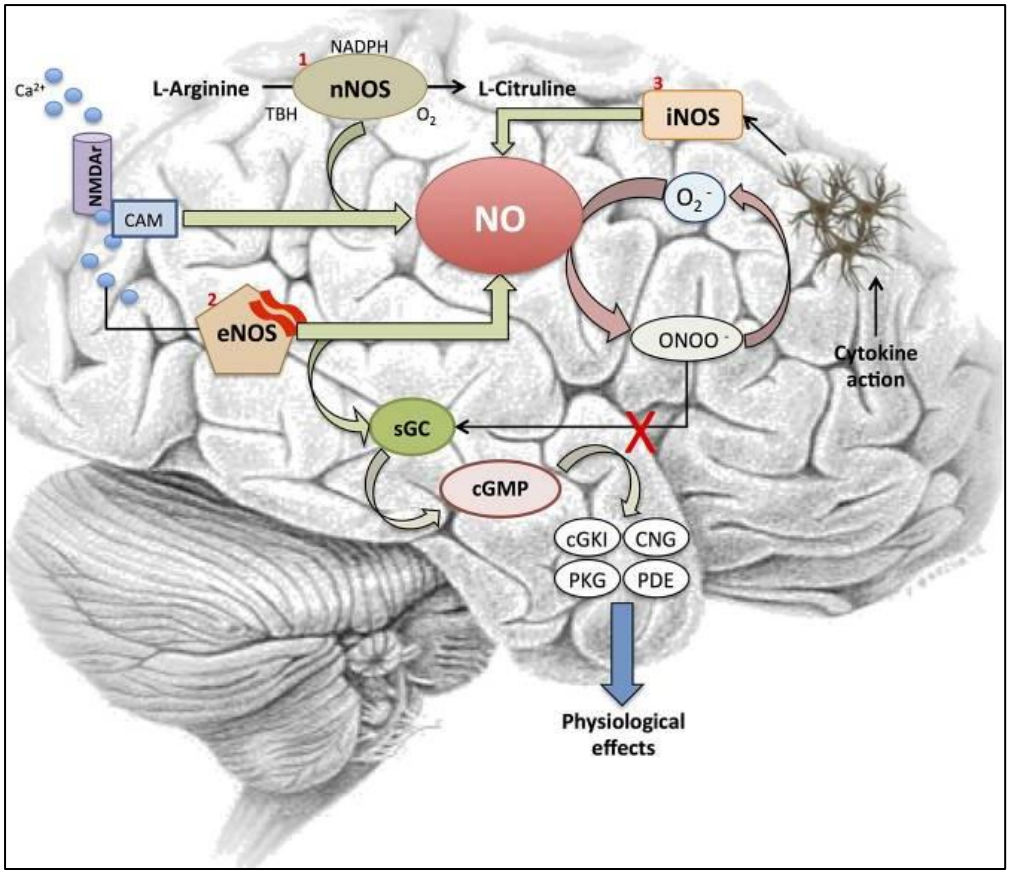

Figure 1.4 Nitric oxide signalling cascade. Source:- (Yuste et al. 2015). TBH: Tyramine beta-hydroxylase, NADPH: Nicotinamide adenine dinucleotide phosphate, nNOS: Neuronal nitric oxide synthase, iNOS: Inducible nitric oxide synthase , eNOS: Endothelial nitric oxide synthase, CAM: Calmodulin, sGC: Soluble Guanylate Cyclase, cGMP: Cyclic guanosine monophosphate, cGKI: cGMP-dependent protein kinase 1, PKG: cGMP-dependent protein kinase or Protein Kinase G, CNG: Cyclic nucleotide-gated, PDE: phosphodiesterase.

1.4.4 Prostaglandins

Two types of cyclooxygenases (COX) have been identified, COX-1 and COX-2. In which, COX-1 is a membrane-associated enzyme and constitutively expressed in most tissues of the brain and its mediated end products contribute to normal physiological functions. Interestingly, COX-2 is an inducible enzyme that is expressed in microglia, neurons, astrocytes and endothelia, which is responsible for the pathological production of prostaglandins (PGs) in response to pro-inflammatory stimuli such as endotoxins, cytokines, brain injury and other growth factors (Yang & Chen 2008) and (Maes 2012).

It has been well documented that COX enzymes regulate the first step of the conversion of arachidonic acid (AA) into unstable intermediate PGG_2/PGH_2 (Chattopadhyay et al. 2010), (de Oliveira et al. 2012) and (Calvello et al. 2012). Finally, in the presence of various cell-specific synthases, several biologically active prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2), as well as thromboxane A_2 (TXA_2) are formed from PGH_2 (Figure 1.5). It has been reported by Chen et al. that COX-2 plays a vital role in regulating the production of PGE_2 and PGI_2 in the brain hippocampus, while COX-1 mediates the conversion of PGD_2, TXA_2 and $PGF_{2\alpha}$ from PGH_2 (Chen et al. 2002). This study also demonstrated for the first time that PGE_2 is heavily involved in maintaining the synaptic transmission, membrane excitability, integration, and plasticity in the hippocampus.

Three types of prostaglandin E synthases (PGESs) are involved in the conversion of PGE_2 from PGH_2, microsomal PGES-1 (mPGES-1), PGES-2 (mPGES-2) and cytosolic PGES (cPGES) (Breyer et al. 2001) and (Sugimoto & Narumiya 2007). While mPGES-1 is primarily coupled with COX-2 signalling, cytosolic PGES has been shown to be associated with the COX-1 pathway, and lastly, mPGES-2 is a constitutive enzyme released into the cytoplasm (Narumiya et al. 1999) and (Sugimoto & Narumiya 2007). When hippocampal slices are challenged with LPS and IL-1β, elevated COX-2 expression followed by mPGES-1 and PGE_2 production was observed suggesting that mPGES-1 mediates neuroinflammatory process in the CNS (Yang et al. 2008) and (Yang & Chen 2008). Therefore, novel compounds targeting the mPGES may hold a great potential as alternatives to traditional non-steroidal anti-inflammatory drugs (NSAIDs) and COX inhibitors for alleviating or treating neuroinflammation-associated neurodegenerative diseases.

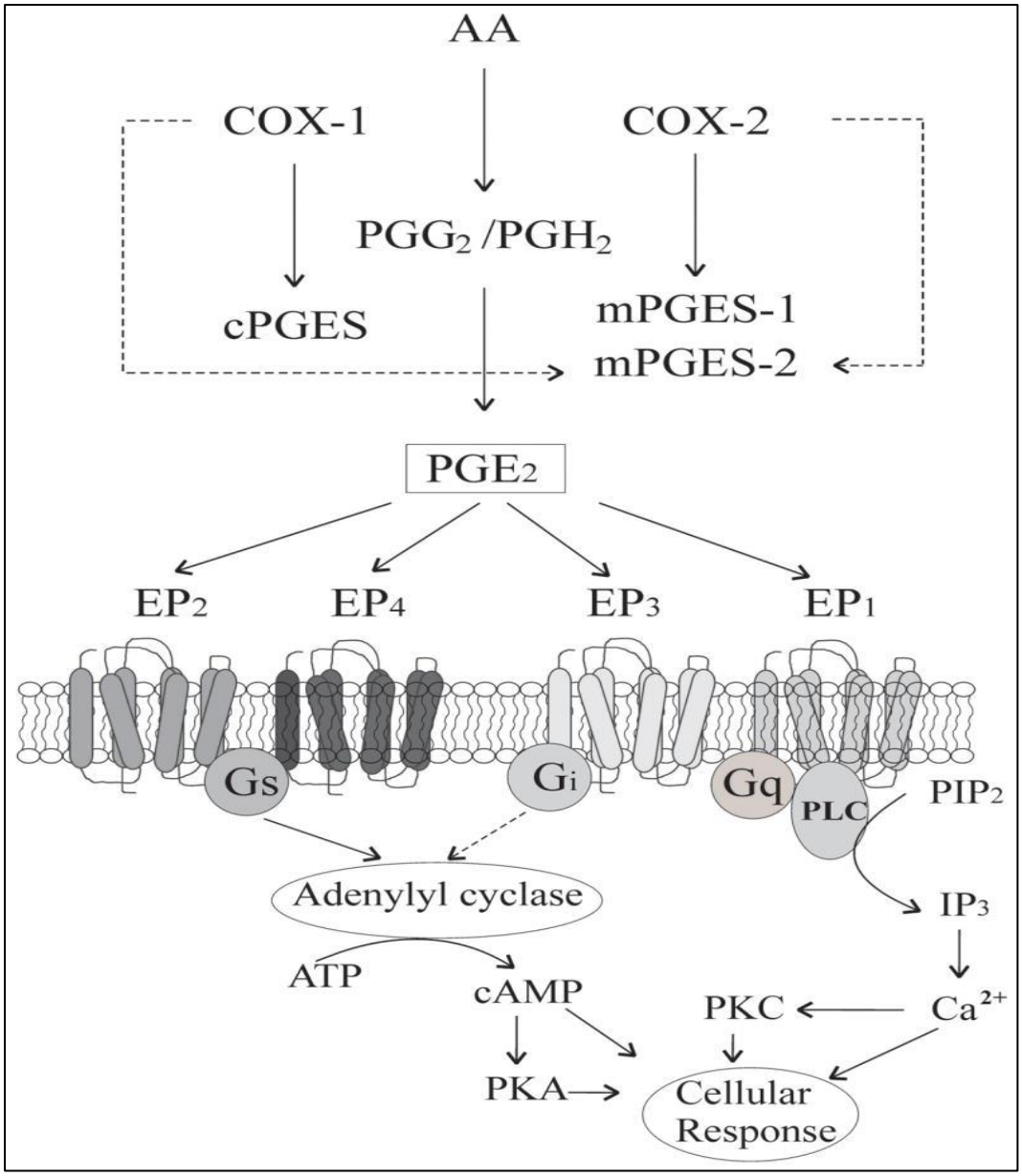

Figure 1.5 Prostaglandins signalling pathway and its specific membrane receptors. Source:- (Yang & Chen 2008). AA: Arachidonic acid, COX: Cyclooxygenase, cPGES: Cytosolic prostaglandin E2 synthase, mPGES: Microsomal prostaglandin E synthase, EP: Prostaglandin E2 receptor, PIP_2 : Phosphatidylinositol 4,5-bisphosphate, IP_3 : Inositol trisphosphate, PKC: Protein kinase C, ATP: Adenosine triphosphate, PLC: Phospholipase C, PKA: Protein kinase A.

It has been well documented that four subtypes of PGE2 receptors (EPs) that belong to the family of seven-transmembrane-domain G-protein-coupled receptors (GPCRs) are identified. All receptors EP_1, EP_2, EP_3 and EP_4 with unique cellular actions and signal transduction profiles (Shibuya et al. 1999) and (Sugimoto & Narumiya 2007) (Figure 1.5). For example, activation of EP_1 receptors increases intracellular calcium levels that are coupled with activation of inositol triphosphate (IP_3) and protein kinase C (PKC) signalling cascades (Zonta et al. 2003). Studies have shown that in neuroinflammation these signalling pathways play a vital role in controlling the transcription of various inflammatory cytokines (Kumar et al. 2003) and (Wang et al. 2014). On the other hand, activation of EP_2 and EP_4 receptors are shown to couple with cAMP and PKA pathways leading to an increase in the accumulation of cAMP (Streit et al. 2004) and (Shibuya et al. 1999). Animal studies conducted by Zhu et al. revealed that all four subtypes of PGE_2 receptors are heterogeneously expressed in microglia, neurons and astroglial cells in the regions of hippocampus and cortex (Zhu et al. 2005). These studies explains the elevated microglial pro-inflammatory cytokines and it's mediated neuronal toxicity when stimulated with exogenous inflammatory stimuli. Further evidence from other studies shows that known PKC inhibitor completely blocked the expression of COX-2 and the production of PGE_2 in LPS-treated rat hippocampal slices, strengthens the link between PKC signalling and ERK, p38MAPK and NF-κB signal transduction pathways (Sang et al. 2005) and (Jazwa et al. 2011).

Several epidemiological studies indicate that conventional anti-inflammatory therapies using NSAIDs can reduce the risk of developing neuroinflammation and its mediated neurotoxicity (Zhang & An 2007), (Trepanier & Milgram 2010), (Chattopadhyay et al. 2010) and (Vernieri et al. 2013). However, NSAIDs particularly COX-2 inhibitors (e.g. Celecoxib and Valdecoxib) have shown little or no therapeutic effect in the AD patients (Sano et al. 1997) and (Aisen et al. 2003). Also, long-term treatment with NSAIDs has been limited by gastrointestinal, cardiovascular or renal complications, as well as increased ability of the patient to tolerate the drugs (Minghetti et al. 2004) and (Chen et al. 2003). As an alternative, investigators are using different strategies to design new drugs that have been primarily focused on downstream targets of the COX-2 pathway (e.g., mPGES-1, EPs) respectively.

EP receptor	Signalling pathways	CRC cells	Endothelial cells	Smooth Muscle cells
EP1	Gαq→PLCβ→PKC →IP_3→Ca^{2+} release	Proliferation[50], Immunosuppression[51], EMT[52]	HIF1 activation[45]	contraction[53]
EP2	Gαs→AC→cAMP→PKA	Initiation, Proliferation, EMT[54], genomic instability[55]	Migration, survival[56], tubologenesis[57]	Proliferation[58], hyaluronan synthesis[46] Inhibition of calcium release[59]
EP3	Gαi AC→cAMP→PKA G12/13→Rho	Inhibition of proliferation[60], Metastasis[44]	HIF1 activation[45], PGE2 synthesis, tubologenesis[61]	Contraction[53], migration[62]
EP4	Gαs→AC→cAMP→PKA G^{α}i AC→cAMP→PK	EGFR-transactivation, cell growth[63], proliferation[64], survival[65], EMT[66], metastasis[67], chemokine expression[68]	Migration, tubulogenesis[69], cell-cell adhesion[70], CXCL8 induction[71], ICAM1 expression, immunosuppression[70], differentiation of EC precursors[72]	Inhibition of calcium release[59]

Figure 1.6 Table highlighting various prostaglandin receptors and their signalling pathways in the brain environment (Zecca et al. 2004). cPGES: Cytosolic prostaglandin E2 synthase, mPGES: Microsomal prostaglandin E synthase, EP: Prostaglandin E2 receptor, PIP_2 : Phosphatidylinositol 4,5-bisphosphate, IP_3 : Inositol trisphosphate, PKC: Protein kinase C, ATP: Adenosine triphosphate, PLC: Phospholipase C, PKA: Protein kinase A.

1.4.5 Reactive oxygen species (ROS)

Microglial induction of reactive oxygen species (ROS) during neuropathological conditions was shown to be more predominant compared to other neurotoxic mediators (Lee & Yang 2012) and (Zhang et al. 2016). In general, mitochondrial ROS at low concentrations are widely recognised to perform physiological functions such as cell proliferation, apoptosis, cell survival and differentiation (Dröge 2002) and (Bernhardi & Eugenín 2012). Excessive production of ROS is termed as oxidative stress. The primary source of ROS production is mitochondria and other specialised enzymes such as Xanthine oxidase (Xox), NADPH oxidase (NOX), P450 enzyme and inducible COX and NOS. ROS generated through these pathways were thought to be responsible for its deleterious effects on the CNS (Melo et al. 2011) and (Yuste et al. 2015). Also it is well established that generation of ROS is more predominant when inflammatory target proteins such as cytosolic phospholipase A2 (cPLA2), matrix metalloproteinase-9 (MMP-9), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are activated by various inflammatory stimulants (Wang et al. 2009), (Dröge 2002) and (Zhang & An 2007).

Studies indicate that in response to oxidative stress, microglia and astrocytes further release various inflammatory mediators that in turn trigger severe pathological events in the brain (Uttara et al. 2009) and (Zecca et al. 2004). For example, accumulation of ROS has shown to induce redox-sensitive transcription factors such as NF-κB and activator protein-1 (AP-1) in microglia, which further promotes more neuroinflammation and could be translated to functional deficits, such as cognitive impairment (Wang et al. 2014) and (Didonato et al. 2012).

Reactive oxygen species that are mainly responsible for inducing oxidative stress are hydroxyl radical ($\cdot OH^-$), singlet oxygen, superoxide anion (O_2^-), hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2). Furthermore, reactive nitrogen species (RNS) involve peroxynitrite ($ONOO^-$) and nitric oxide (NO) (Bernhardi et al. 2015) and (Hsieh & Yang 2013). As shown in Figure 1.7, several pro-inflammatory factors released by activated microglia during inflammation reacts with molecular oxygen (O_2) in the presence of various oxidase enzymes to produce highly reactive ROS and RNS. At low concentrations, these ROS and RNS are essential to perform physiological functions (e.g. killing invading microorganisms), however, they become detrimental at high levels (Chrissobolis & Faraci 2008) and (Melo et al. 2011). Apart

from neurodegenerative diseases, oxidative stress has also shown to mediate the pathogenesis of cardiovascular disorders such as stroke (Sandberg et al. 2014).

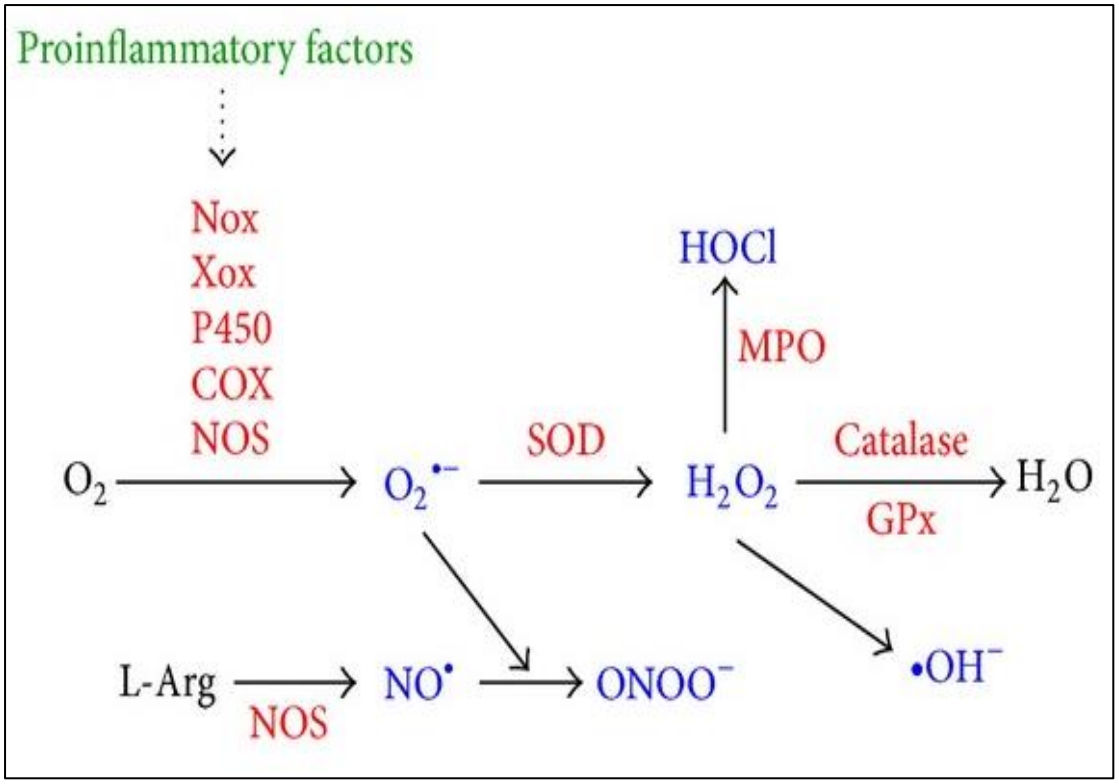

Figure 1.7 Generation and metabolism of reactive oxygen and nitrogen species. Source:- (Hsieh & Yang 2013). Nox: NADPH oxides, Xox: Xanthine oxidase, COX: Cyclooxygenase, NOS: Nitric oxide synthase, SOD: Superoxide dismutase, MPO: Myeloperoxidase, GPx: Glutathione peroxidase, L-Arg: L-arginine.

Also, it is widely accepted that the term oxidative stress defines an imbalance between the generation of ROS and the antioxidant systems that protect deleterious effects of ROS (Anand & Babu 2013) and (Halliwell 2006). Since mitochondria are the site of electron transport chain, it appears that defects in these physiological mechanisms induce excess ROS generation.

In contrast, induction of several antioxidant systems and proteins such as superoxide dismutase (SOD), catalases or Nrf2-induced heme oxygenase-1 (HO-1) may reduce ROS generation and attenuate the inflammatory response, as well as DNA damage induced by oxidative stress (Figure 1.8) (W. Li et al. 2008), (X. Li et al. 2008) and (Martin et al. 2004). However, studies have shown that during neuroinflammation the balance appears to be tipped in favour of oxidative stress and therefore a reduction in the expression of antioxidant proteins was observed (Peterson & Toborek 2014). Glutathione (GSH) has been recognised as an important cellular antioxidant that is mainly produced by microglia during oxidative stress. Modulations in the levels of GSH have been implicated in various stress mediated neurodegenerative diseases (Roychowdhury et al. 2003) and (Kim et al. 2004). GSH is abundant in glial cells and is involved in scavenging ROS and peroxynitrite produced during neuroinflammation. Curcumin, a natural dietary compound has been shown to inhibit H_2O_2 induced oxidative stress via increasing the levels of GSH in mice (Kim et al. 2004). Also, a study on post-mortem brain of AD patients has revealed decreased levels of GSH in some regions of the brain when compared with control, which emphasises the pivotal role of GSH in neurodegenerative conditions (Gu et al. 1998). Thus strategies that target to activate antioxidant mechanisms could bring hyperactive microglia to resting state.

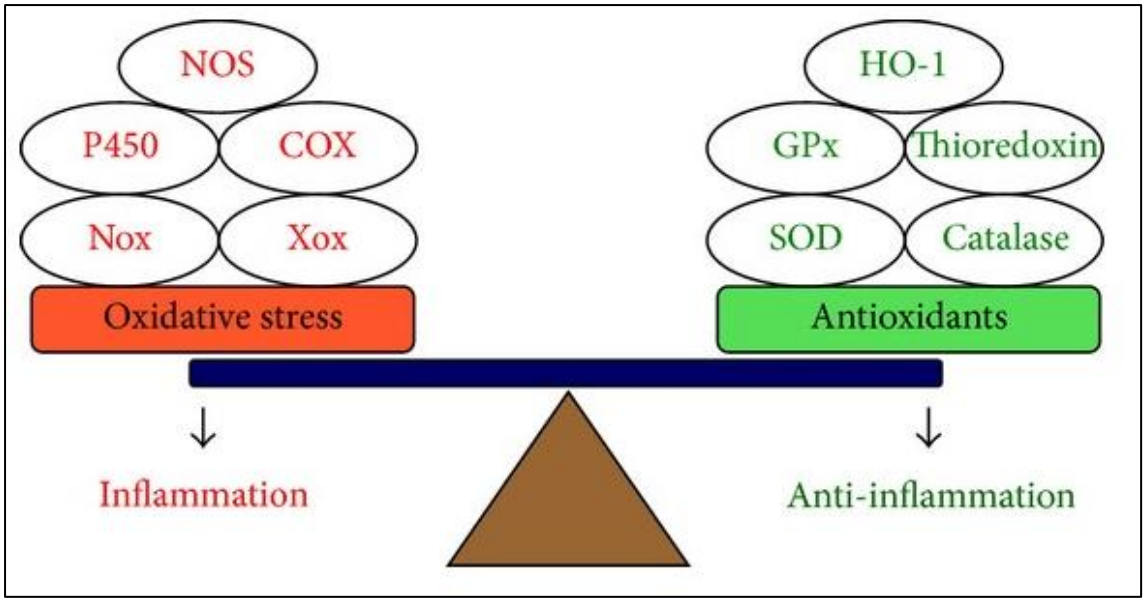

Figure 1.8 Balance between ROS/RNS mediated oxidative stress and antioxidant proteins. Source:- (Hsieh & Yang 2013). **Nox: NADPH oxides, Xox: Xanthine oxidase, COX: Cyclooxygenase, NOS: Nitric oxide synthase, SOD: Superoxide dismutase, HO-1: Heme oxygenase 1, GPx: Glutathione peroxidase.**

1.5 Anti-inflammatory cytokines

Accumulating evidence has demonstrated that pro-inflammatory cytokines produced by hyperactive microglia are the key components involved in the pathogenesis of various neurodegenerative diseases (Kettenmann et al. 2011) and (Jurgens & Johnson 2012). On the other hand, microglia tends to release anti-inflammatory cytokines that have shown to control the deleterious effects of pro-inflammatory cytokines in the CNS (Kim et al. 2016) and (Guillot-Sestier et al. 2015). In general, the acute release of pro-inflammatory cytokines benefits CNS by attacking invading pathogens. However, a sustained increase of these cytokines damages surrounding tissues causing dysfunction and ultimately deterioration of healthy microglia and neurons. The primary functional role of the anti-inflammatory cytokines that includes interleukin (IL)-1 receptor antagonists such as IL-4, IL-10, IL-11, and IL-13, as well as transforming growth factor-β1 (TGF-β1) is to down-regulate the sustained inflammatory toxicity-induced by cytokines and initiate tissues reconstruction. It can be hypothesised that microglial anti-inflammatory cytokines possess protective effects against neuroinflammation by blocking the harmful effects of pro-inflammatory cytokines. It also appears that deficiency of these anti-inflammatory cytokines in the brain may promote the risk of developing progressive neurodegenerative diseases like AD and PD (Richwine et al. 2009) and (Opal & DePalo 2000).

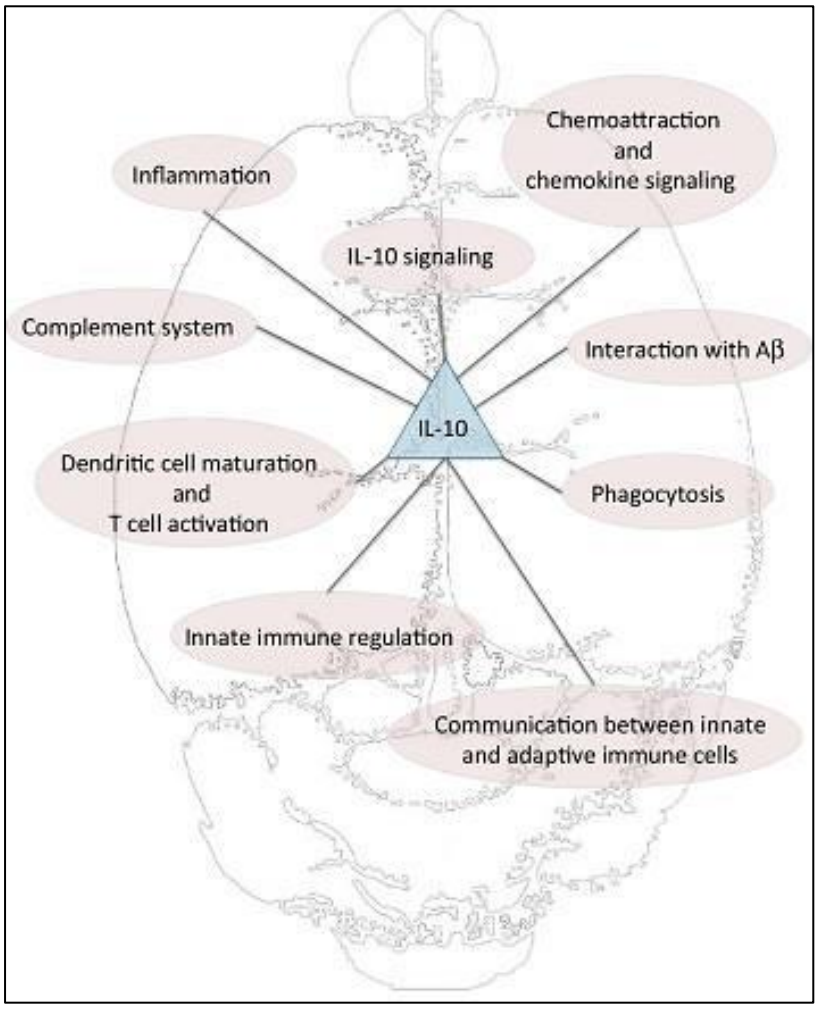

Figure 1.9 Various roles of anti-inflammatory cytokine IL-10

Source:- (Guillot-Sestier et al. 2015)

Among all the anti-inflammatory cytokines, IL-10 is widely studied due to its potent anti-inflammatory, cellular survival and anti-apoptotic properties (Chakrabarty et al. 2015) and (Richwine et al. 2009) (Figure 1.9). Studies have shown that IL-10 is capable of repressing various inflammatory cytokines such as TNF-α, IL-6 and IL-1β in LPS-activated microglia (Lynch et al. 2004). Also, IL-10 can upregulate endogenous anti-cytokines and down-regulate the expression of pro-inflammatory cytokine receptors in the brain (Park et al. 2007) and (McGeer & McGeer 2015). Moreover, acute administration of IL-10 protein in diverse animal models has been shown to suppress the development of spinally-mediated pain facilitation and also counter-regulated the function and production of various inflammatory mediators (Richwine et al. 2009) and (de Miranda et al. 2015). On the other hand blocking the production of IL-10 has shown to reverse the neuropathological behaviours in the murine models (Park et al. 2007). On top of that, recent clinical studies also indicate that low concentrations of IL-10 and IL-4 in the blood of patients with neuropathic pain highlights the pivotal roles of the anti-inflammatory cytokines (Zhang & An 2007) and (Chatterjee et al. 2014). From all these observations, it is understood that no direct evidence has been obtained about the other anti-inflammatory cytokines in the brain during neuroinflammatory conditions, where further investigation is required.

1.6 Microglial nuclear factor kappa B (NF-κB) signalling in neuroinflammation

The nuclear transcription factor NF-κB was first discovered by David Baltimore (Sen & Baltimore 1986). It was initially identified as an inducible transcription factor in peripheral lymphocytes. However, further research on NF-κB signalling has revealed its essential role in the pathological process associated with chronic neuroinflammation and neurodegeneration (Didonato et al. 2012). In mammals, NF-κB family typically comprises of several transcription factors that are equipped with Rel-homology domains (RHDs). These domain's bind to specific DNA sequences in the promoter regions of inflammatory genes known as kappa B (κB) sites (Adli et al. 2010) and (Hayden & Ghosh 2012). So far, five different NF-κB transcription factors were discovered in mammalian cells: p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) (Chen & Greene 2004) and (Didonato et al. 2012) (Figure 1.10). Out of these, RelB, c-Rel, and p65 are known to contain C-terminal transcription activation domains that facilitate the recruitment of target

inflammatory gene expression, while p52 and p50 form heterodimers with p65, c-Rel, or RelB to activate the transcription of other target genes (Hayden & Ghosh 2011).

In normal/resting cells, these NF-κB complexes will be located mainly in the cytoplasm inhibited by inhibitory kinase protein complex (IκB). So far, seven different types of IκB inhibitors equipped with ankyrin repeats were identified: IκBα, IκBβ, IκBε, IκBζ, p100, p105 and IκBns (Karin 2009) (Figure 1.10). With the help of these ankyrin repeats IκB inhibitors can hold the NF-κB dimers in the cytoplasm, which prevents the nuclear localisation of p65 subunit (Chen & Greene 2004). Upon activation of membrane TLR4 and innate receptors by inflammatory stimuli such as LPS, with the association of myeloid differentiation primary response 88 (MYD88), a signal transducing adaptor protein and TNF-receptor-associated factor 6 (TRAF6) becomes activated. This complex further triggers the induction/nuclear translocation of NF-κB via rapid phosphorylation of specific serine residues of IκB by a multi inhibitory kinase kinase protein complex (IKK) along with MAPK signalling (Kawai & Akira 2007) and (Takeda & Akira 2004). Ideally, these IKK complexes contain two subtypes of active kinases: IKKα and IKKβ, and a regulatory protein NEMO (NF-κB essential modifier, also termed as IKKγ) (Lawrence et al. 2005) and (Kopitar-Jerala 2015). Once IκB becomes phosphorylated by the IKK complex, the remaining kinase protein gets degraded by cytoplasmic proteasomes followed by translocation of NF-κB dimers (p50/p65) into the nucleus (Karin 1999). Which means for NF-κB to become fully active requires the phosphorylation and subsequent degradation of IκB, hence, inhibiting the phosphorylation of this kinase ultimately inhibits NF-κB's transcriptional activity.

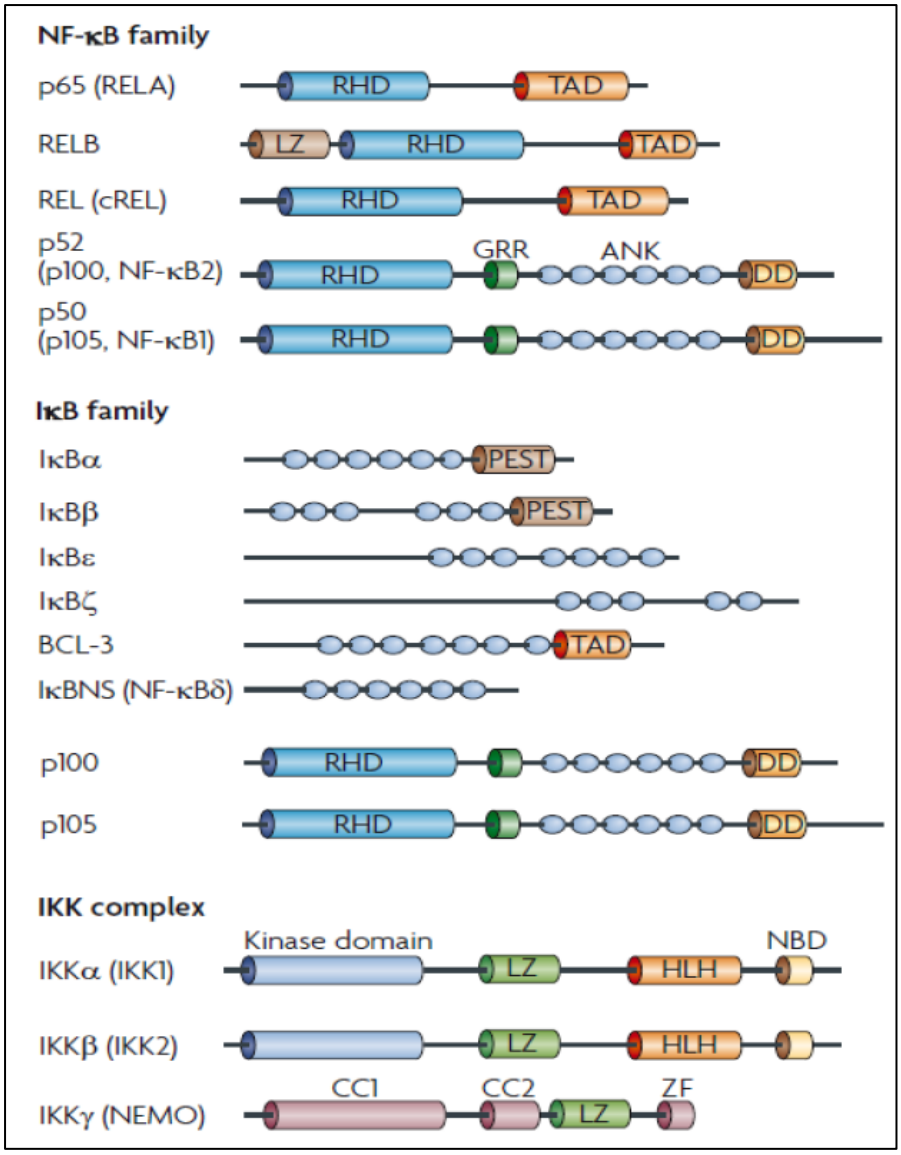

Figure 1.10 Members of the NF-κB, IκB and IKK protein families. Source:- (Hayden & Ghosh 2011). **RHD: Rel homology domain, TAD: Topologically associating domain, ANK: Ankyrin repeat, DD: Death domain, PEST: proline glutamic acid, serine and threonine sequence, NBD: Cyclic nucleotide-binding domain, LZ: leucine zipper, HLH: helix-loop-helix domain, IκB: inhibitor of kappa B , IKK; IκB kinase, ZF: Zinc finger domain, CC1/2: coiled-coil domain 1 and 2.**

Once in the nucleus, NF-κB enables the transcription of several genes that encode chemokines, pro-inflammatory cytokines, COX-2 and iNOS by binding to specific κB sites on DNA (Wang et al. 2014). Another interesting aspect in this classical NF-κB pathway is the transcription of IκBα protein in the nucleus, as this newly synthesised kinase help to detach NF-κB-p65 from the DNA and export the complex back to the cytoplasm (Didonato et al. 2012) and (Sun 2011). Studies conducted by Gupta et al. showed that NF-κB is capable of activating around 500 genes that are implicated in the inflammatory responses (Gupta et al. 2010). This ability of NF-κB signalling to control the transcription of multiple genes involved in various brain-related diseases makes it a novel target to block neuroinflammation and its associated neurodegenerative conditions. Due to the multiple steps involved in the regulation, NF-κB signalling pathway can be potentially aimed at various levels such as toll-like receptors, inhibitory kinases, phosphatases, nuclear translocation and DNA binding, as well as post-translational modifications in the nucleus (Figure 1.11).

Furthermore, these NF-κB-dependent pro-inflammatory cytokines, such as TNFα and IL-1β tend to activate surface receptors on microglia and adjacent neurons. Also, studies have shown that cytokines activate astrocytes that in turn induce intracellular NF-κB signalling; hence it turns into a vicious cycle that constantly produces neurotoxic mediators that induce neuronal death (Lawrence et al. 2005). In contrast, several cytokines like IL-10, TGFβ, glycogen synthase kinase-3 (GSK-3β) and IL-4 are shown to regulate NF-κB signalling negatively via blocking peroxisome proliferator-activated receptor (PPAR)-γ-mediated mechanisms (Paintlia et al. 2006) and (Kaltschmidt et al. 2005). In the nucleus, p50/ p65 subunits of NF-κB has to undergo several post-translational modifications like acetylation and methylation to regulate the transcription of the pro-inflammatory genes (Chen & Greene 2005) and (Zhu et al. 2011) which were discussed in detail in section 1.8.

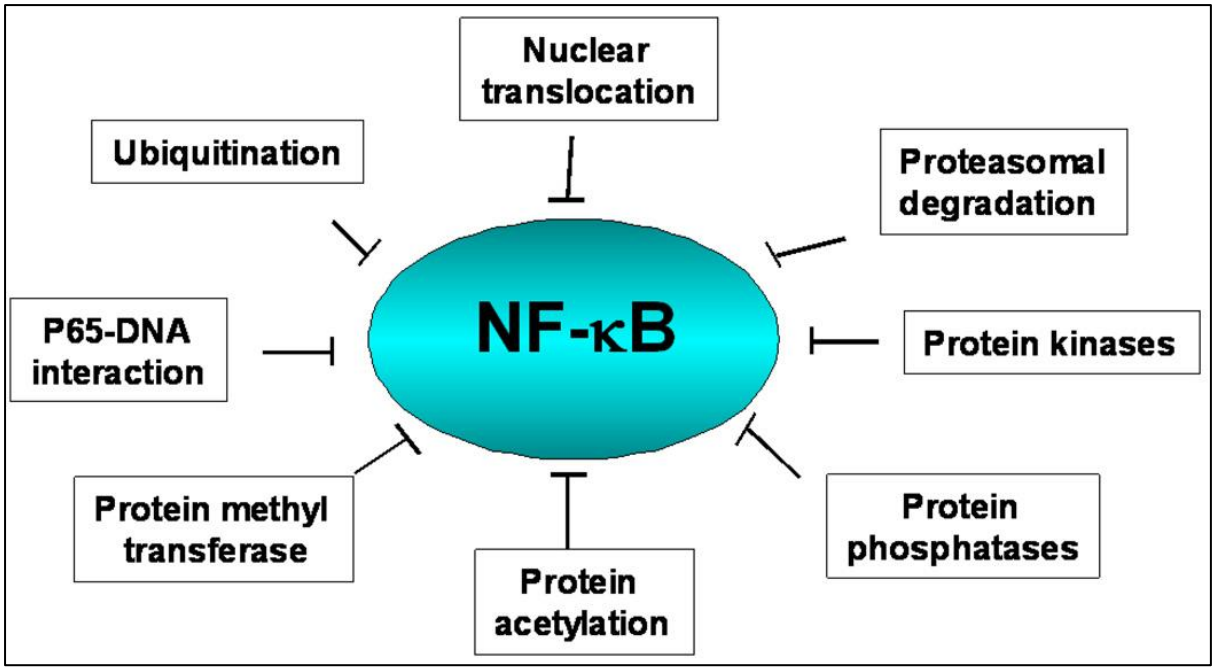

Figure 1.11 Potential targets for inhibiting NF-κB activation

Source:-(Gupta et al. 2010)

1.7 Microglial p38 mitogen-activated protein kinase (MAPK) signalling in neuroinflammation

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases which are critical for regulation of the production of inflammatory cytokines. Some pathogenic, extracellular stimuli and bacterial products such as LPS are shown to activate MAPKs cascades via toll-like receptors or TNF receptor family and ultimately leading to the transcription of various inflammatory genes such as iNOS, TNFα and IL-1β (Cuadrado et al. 2010) and (Onyango et al. 2005). The use of MAPK inhibitors emerges as an attractive strategy in neuroinflammatory conditions because they are capable of reducing the transcription and synthesis of pro-inflammatory cytokines in microglia (Zeng et al. 2010), (Xu et al. 2014) and (Zhu et al. 2014).

In mammalian cells three major groups of MAP kinases were identified: ERK1/2, JNK, and p38 MAP kinase, where ERK1 and 2 are typically activated by MAP kinase kinase 1 (MKK1) and MKK2, JNK is activated by MKK4 and MKK7 and finally p38 MAPK by MKK3 and MKK6. These kinases are critical regulators of various pro-inflammatory cytokines in the pathologies of neuroinflammation related neurodegenerative diseases (Kaminska 2005) and (Munoz & Ammit 2010). Among these, p38 MAPK signalling has been shown to play a central role due to its importance in the activation and production of neurotoxic mediators that are involved in the progression of neuroinflammation (Kim et al. 2004).

Activation of microglial membrane toll-like receptor 4 (TLR4) (e.g. by LPS) triggers p38 MAPK signalling (Didonato et al. 2012) and (Lawrence et al. 2005). This activation and expression of TLR4 further induce the phosphorylation of myeloid differentiation primary response gene 88 (MyD88) followed by TRAF6 activation. Studies have shown that in activated microglia, MyD88-dependent pathway is used by most TLRs to recruit IL-1R-associated kinase (IRAK)-1 and IRAK-4 and TRAF6 (Yamamoto et al. 2003) and (Coban et al. 2007). Afterwards, the dissociation of this complex takes place leading TRAF6 to associate and activates transforming growth factor β-activated kinase (TAK1) via TAK1-binding protein 1. Further activates the transcription factors NF-κB and activator protein 1 (AP-1) through the IKK complex and the MAPK pathway, respectively (Karin & Gallagher 2009). This activated TAK1 complex further triggers phosphorylation of mitogen-activated protein kinase kinase

kinases (MAPKKKs). These MAPKKK serine/threonine kinases get phosphorylated and further activates MAPK kinases (MKKs) such as MKK3, MKK4 and MKK6 which in turn phosphorylate p38 MAPK at Thr180 and Tyr182 residues.

Activated p38 further phosphorylates and activates serine and threonine residues of its kinase substrates, mitogen-activated protein kinase (MAPK)-activated protein kinases (MKs) such as MK2, MK3 and MK4 (Coban et al. 2007). Studies have shown that LPS-induced MK2 is involved in regulating the production of TNFα, IL-6 and IL-8 and other cytokines in microglia (Duraisamy et al. 2008), (Fyhrquist et al. 2010) and (Fyhrquist et al. 2010). When mouse models challenged with bacterial lipopolysaccharide showed excessive levels of endogenous MK2 in frontal cortex and hippocampus regions suggest the critical role of MK2 in the neuroinflammation (Corrêa et al. 2012). Moreover, targeted deletion of MK2 gene in mouse models resulted in a massive decrease in the production of TNFα when treated with LPS and D-galactosamine-induced shock (Kotlyarov et al. 1999). The intervention of these essential pathways may serve as a therapeutic approach for treating inflammatory neurological diseases. For example, the specific p38 MAPK inhibitor, SB203580 markedly reduced NO, PGE_2 production and iNOS protein expression in LPS-induced BV2 microglia. Furthermore, orally administered p38 inhibitor abolished cerebral IL-1β release in LPS-challenged mice models (Xing et al. 2011b). Also, several other studies have found that inhibition of p38 MAPK and its associated kinases such as MKK3/6 and MK2 would resolve neuroinflammation.

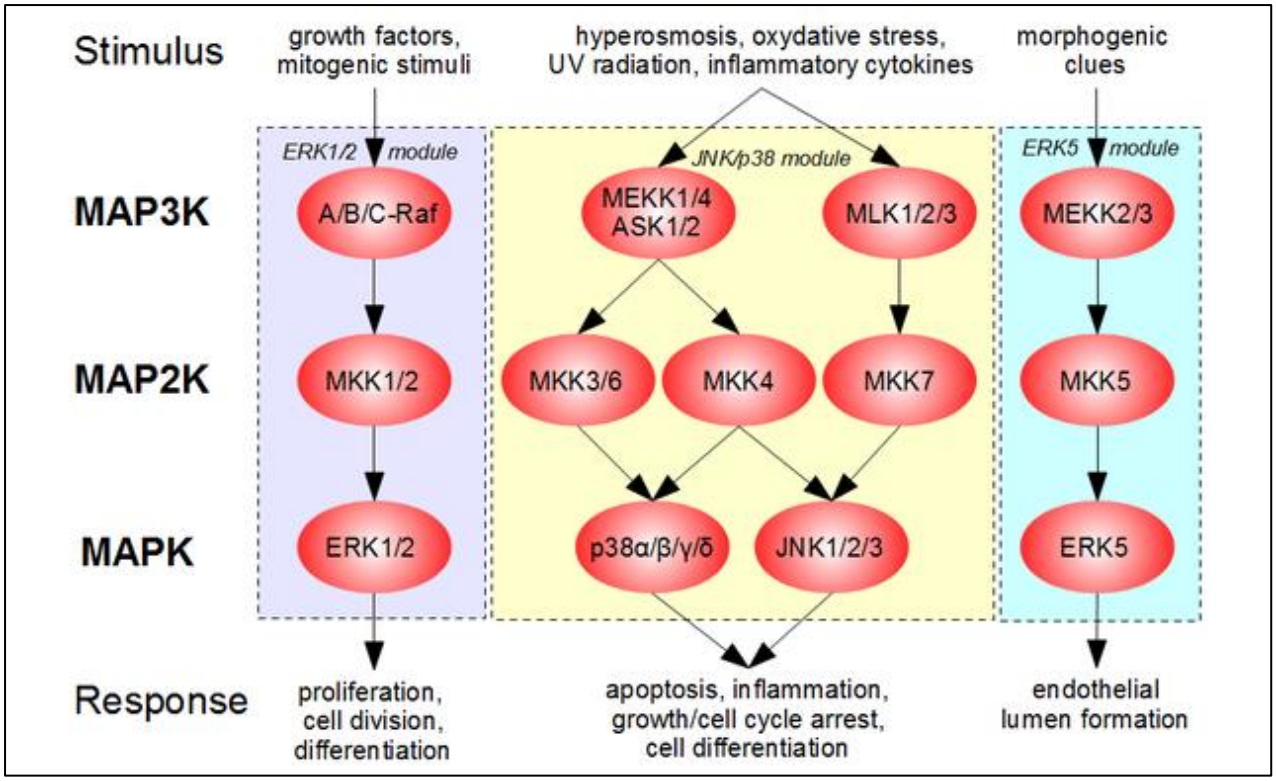

Figure 1.12 An overview of brain MAPK signalling cascades (Coban et al. 2007). **MAPK: mitogen-activated protein kinase, Raf: Rapidly Accelerated Fibrosarcoma, MKK: Mitogen-activated protein kinase kinase, MLK: Mixed Lineage Kinase, JNK: c-Jun N-terminal kinases, ERK: extracellular signal–regulated kinases.**

1.8 Role of Sirtuin 1 (SIRT1) in the microglia

The mammalian sirtuins are nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases that have traditionally been linked with neuroinflammation and its mediated neurodegenerative conditions. In general, sirtuins are referred as class III histone deacetylases (HDACs) that play a significant role in mediating critical cellular and physiological processes such as ageing, senescence and low-grade inflammation.

Among seven sirtuins, SIRT1 has been reported to be involved in promoting longevity in various species. Thus, pharmacological activation of sirtuins, particularly SIRT1 has shown to modulate neuroinflammatory conditions (Nimmagadda et al. 2013). In neuroinflammation, NF-κB complex has to undergo post-translational modifications to become fully active, which includes reversible acetylation of NF-κBp65 subunit by CREB-binding acetyltransferases, p300 and phosphorylation by protein kinase A (Chen Lf et al. 2001) and (Chen et al. 2005). However, studies have shown that SIRT1 deacetylase negatively regulates NF-κB signalling via deacetylation of Lys310 residue of RelA/p65 subunit (Chen et al. 2005) and (Jieming Ye et al. 2013), which means activation of SIRT1protein inhibits NF-κB signalling by promoting deacetylation of the p65 subunit. In addition, SIRT1 has been shown to deacetylate histones (H3 and H4) as well as other transcription factors such as p53, FOXO and Ku70 in glial cells (Outeiro et al. 2008). Moreover, overexpression of SIRT1 has shown to prevent neuronal death in various cell culture models and reduces hippocampal degeneration in animal models of AD, PD and ALS (Cho et al. 2015) and (Kim et al. 2007). Thus, compounds that pharmacologically promote the expression of SIRT1 could culminate deleterious effects of active microglia and ultimately protect neurons. For instance, resveratrol, a potent SIRT1 activator, has been shown to regulate immune responses in the LPS-stimulated microglia via NF-κB signalling by inhibiting the transactivation of NF-κB complex. Further studies revealed resveratrol promotes deacetylation of Lysine 310 residue of NF-κB -p65 subunit (Cho et al. 2015), (Haigis & Sinclair 2011)and (Ye et al. 2013).

Interestingly, chronic oxidative stress encountered during neuroinflammatory conditions apparently down-regulates the activity and expression of SIRT1 (Salminen et al. 2013). While its activation has shown to inhibit pro-inflammatory

cytokines in LPS-activated microglia (Cho et al. 2015), (Li et al. 2015) and (Nimmagadda et al. 2013). In contrast, reduction of SIRT1 protein expression was observed when endothelial cells were incubated with LPS. This condition was accompanied by significant increase in the acetylation of NF-κB-p65 and enhanced NF-κB transcriptional activity (Yang et al. 2012) and (J Ye et al. 2013), this further highlights the role of SIRT1 during neuroinflammation.

SIRT1 has been shown to be involved in a variety of pathophysiological processes like cell metabolism, inflammation and DNA repair, as well as cell growth. In macrophages, knockdown of SIRT1 gene lead to an increase of pro-inflammatory cytokines, whereas its activation has reversed the productions of TNFα, IL-1β and monocyte chemoattractant protein (MCP-1) (Rajendrasozhan et al. 2012), (Zhu et al. 2011) and (Yang et al. 2007). Also, there is abundant literature indicating that the functional dysregulation of SIRT1 is associated with many age-related neurodegenerative diseases like AD and PD (Haigis & Sinclair 2011) and (Guarente 2011), which means upregulating the activity of sirtuins could protect against damage from oxidative stress induced by inflammatory stimuli.

1.9 Role of nuclear factor erythroid 2-related factor 2 (Nrf2) in the microglia

Reduction of brain oxidative stress induced by neuroinflammatory conditions could be achieved by activation of various antioxidant mechanisms. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and its associated antioxidant response element (ARE) pathway is a primary sensor and is known as a master regulator of oxidative stress via its ability to modulate the expression of several redox-regulated antioxidant and detoxifying genes such as HO-1 and NQO1.

Activation of this pathway has been shown to be beneficial in several animal models of neuroinflammation and neurodegenerative disorders. In contrast, loss of these antioxidant defence systems has been observed in postmortem brains of patients with neurodegenerative conditions. For example, in regions of substantial nigra and dopaminergic neurons of AD and PD patients, lower levels of Nrf2 and its associated proteins were noticed, while increased load of free radicals was observed compared to age-matched controls (Bogdanov et al. 2008) and (Gan & Johnson 2014). Therefore, activating Nrf2 signalling provides a valuable target for the treatment of neuroinflammation associated neurological diseases.

Among the Nrf2 mediated stress proteins, heme-oxygenase 1 (HO-1) gained decent attention due to its pivotal role in modulating the phenotype of microglia during neuroinflammation. Genetically altered and dysregulated HO-1 protein have been associated with the susceptibility and pathogenesis of neurodegenerative diseases like AD, as well in brain ageing (Markesbery 1997) and (Pappolla et al. 1998). Interestingly, during tau abnormalities, clusters of microglia were observed around the neurons along with increased levels of Nrf2 and its mediated HO-1, suggesting that patient's brain is attempting to limit microgliosis by activating antioxidant mechanisms (Wakabayashi et al. 2010). In line with these findings, anti-inflammatory compounds like schizandrin C and several other small molecules have been shown to activate HO-1 protein in the microglia (Zeng et al. 2012), (Foresti et al. 2013), (Jazwa et al. 2011) and (Motohashi & Yamamoto 2004).

It has been well documented that Nrf2 activity is mainly regulated by cellular inhibitor Keap1. Further research on this antioxidant protein showed that disruption of Nrf2/Keap1 interaction is an essential process to push Nrf2 into nucleus (Hancock et al. 2012) and (Turpaev 2013). This crucial step is primarily targeted by medical interventions to produce specific Nrf2 inducers. Surprisingly, cancer is an exception to the benefits that are associated by Nrf2. In cancer cells, cytoprotective mechanism induced by Nrf2 has shown to be hijacked and used against gaining resistance to radiotherapy and chemotherapy. Levels of Nrf2 have proved to be elevated in several types of cancers such as endometrial, lung, breast and pancreatic cancer (Soini et al. 2014), (Hartikainen et al. 2012) and (Liu et al. 2013).

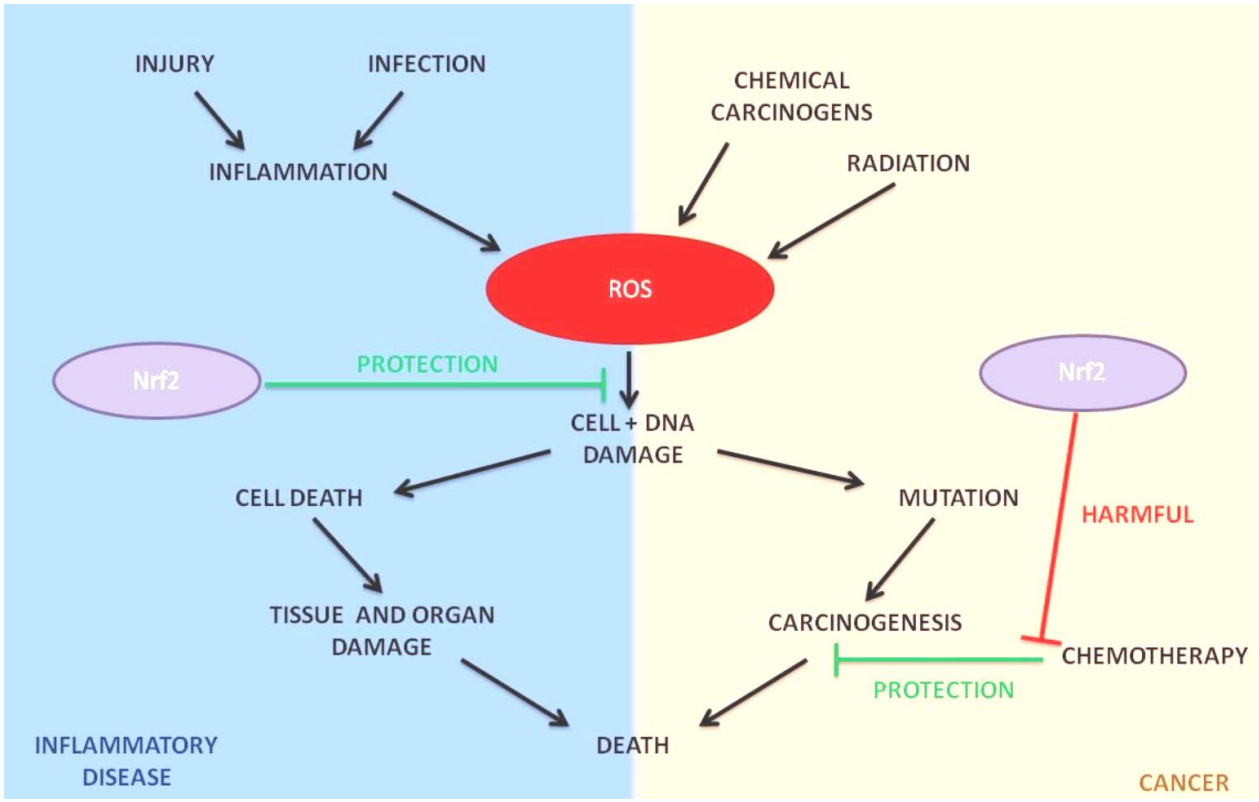

Figure 1.13 The dual role of Nrf2 and its mediated antioxidant proteins (Zeng et al. 2012). Nrf2: nuclear factor erythroid 2 related factor 2, HO-1: Heme oxygenase 1, NQO1: NAD(P)H dehydrogenase [quinone] 1, ARE: antioxidant response element, ROS: Reactive oxygen species.

1.10 BV2 microglia as an *invitro* experimental model for neuroinflammation

Mammalian microglia are the brain resident phagocyte-like cells with an important role in immunity, toxicology and inflammatory neuropathologies. As neuroinflammation is an expanding area of biomedical research, the role of microglia in the inflammatory pathologies has been extensively studied in various animal models and primary cell cultures. Due to the limited proliferation capacity of rodent primary microglia, they have to be freshly prepared for each experiment. For example, to assess various signalling or disease mechanisms in neuroinflammation, at least 15-30 new rodent's brains are required to yield enough cells to perform the desired experiments, which have a large impact on animal consumption. To overcome this challenge, a cell line that can be subcultured routinely would be highly desirable to save animals, expensive consumables and preferably time (Chan et al. 2007) and (Krause & Muller 2010).

Several studies have shown that immortalised murine microglial cell line BV2 could accelerate research on neuroinflammation and its mediated neurological diseases, provided that this particular cell line reduces the necessity of animal experiments and continuous fresh cell preparations (Lund et al. 2005) and (Henn et al. 2009). In an extension of these studies, it has been demonstrated that BV2 cell line is capable of reproducing the *in vivo* situation with high fidelity (Vehmas et al. 2003) and (Giulian & Baker 1986). Immortalised BV2 cells are generated by infecting mouse microglial cells with *v-raf/v-myc* (J2 virus) carrying retrovirus (Blasi et al. 1985). BV2 microglia cells when stimulated with LPS and other inflammatory stimuli, shown to induce the release of nitric oxide via upregulation of iNOS and superoxide anions via activation of NADPH oxidase (NOX) systems (Boje & Arora 1992) and (Ramanan et al. 2008). Interestingly, when rat primary microglia are treated with LPS, secreted lesser but substantial production of NO and iNOS protein expression, as well as reduced NADPH oxidase activation was observed (Colton & Gilbert 1987) and (Chan et al. 2007). This data introduced bias against usage of rat primary microglia in neuroinflammation research as iNOS induced NO production is considered as an important mediator in the pathologies of inflammation.

Further experiments to test the expression levels of Iba-1, a glial activation marker revealed that BV2 microglia are capable of producing high levels of Iba-1(Horvath et al. 2008). Since then, BV2 microglia have been used for several pharmacological

and toxicological studies, in phagocytosis, as well as for many immunological related discoveries (Lund et al. 2005) and (Hirt & Leist 2003). In addition, Henn et al. and his group examined the phenotypes of BV2 cells and confirmed that these cells are the perfect alternative for primary cultures (Henn et al. 2009). They also found that in response to LPS, 90% of inflammatory genes induced by BV2 cells were also produced by primary microglia; however, the upregulation of genes in the primary microglia were far less pronounced than in BV2.

N9 microglia cells are another popular retroviral-immortalised cell line that shares similar phenotypical characteristics with primary mouse microglia (Hickman et al. 2008). Also, when challenged with LPS, these cells have shown to upregulate the pro-inflammatory genes similar to BV2, including iNOS, IL-6, COX-2, TNFα and IL-1β (Stansley et al. 2012). In line with these invitro models, human immortalised microglia (HMO6) are rarely used in the neuroscience research; as it is harder to obtain these cells. They have to be derived from human embryos and can be difficult to get access as these will develop ethical and legal issues (Nagai et al. 2001). HMO6 cells were shown to express IL-1β, IL-6, IL-8, IL-10, IL-12, IL-15 and TNFα when challenged with various inflammatory or bacterial stimuli similar to BV2 microglial phenotype (Stansley et al. 2012). Out of all the *invitro* models, immortalised BV2 microglia appear to be the perfect model to study the neuroinflammation inhibitory effects of the novel compounds using various inflammatory stimuli.

Drugs or effects	Immune effects	Model
Lipopolysaccharide induced	Mediate generation of NO and TNF-α through p38	In vitro (primary rat and human microglia)
Aβ-induced	Mediate neuroinflammation through microglial p38	In vivo (rat brain)
Aβ-induced	Mediate glutamate excitotoxicity through p38	In vitro (primary rat astrocytes)
TNF-α/IL-1β-induced	Modulate iNOS induction through JNK1 Modulate iNOS induction through ERK	In vitro (astrocytes)
JNK inhibitory peptide (JIP)	Reduce NADPH oxidase-mediated H_2O_2 productivity through JNK	In vitro (BV-2 microglia)
JNK inhibitor, D-JNKI-1	The involvement of the JNK pathway on Tau pathology and cognitive deficits	In vivo (6-month-old SAMP8 mice)
Inhibition of phosphorylation of JNK	Suppresses Aβ-induced ER stress and upregulates prosurvival of mitochondrial proteins	In vitro (rat hippocampus)
HO-1 activator, CoppIX	Reverse iNOS/NO upregulation and HO-1 downregulation through JAK1/JNK/STAT1 signaling pathway	In vitro (BV-2 microglia)
p38β knockout	Mediate proinflammatory cytokines and neuron death through p38α but p38β MAPK is dispensable	In vitro (WT microglia co-culture with WT neurons)
Overexpression of JNK	Induce neurite extension	In vitro (primary neurons or PC12 cells)
Hyperphosphorylated Tau	Mediate amounts of aggregated Tau through p38	In vivo (transgenic mice)

Figure 1.14 Table summarizing various *in vivo* and *in vitro* models to study neuroinfoammation inhibitory effects of the novel small molecules. Aβ: amyloid β, ER: endoplasmic reticulum, ERK: extracellular signal-regulated kinase, HO-1: heme oxygenase-1, IL-1β: interleukin 1β, iNOS: inducible nitric oxide synthase, JAK: Janus Kinase, JNK: c-Jun N-terminal kinase, MAPK: mitogen-activated protein kinase, NO: nitric oxide, SAMP8: senescence-accelerated prone mouse 8, TNF-α: tumor necrosis factor-α, WT: wild type.

1.11 Pharmacology of flavonoids

Flavonoids belong to a broad class of secondary polyphenolic natural compounds that are widely distributed in the seeds, fruits, leaves, and flowers, as well as stem barks of plants. Till date, over 4000 flavonoids were identified with significant chelating and antioxidant properties (Heim et al. 2002) and (Christensen et al. 2008). In plants, these hydroxylated phenolic substances are likely to be synthesised by phenylpropanoid pathway in response to herbivores, ultraviolet radiation and microbial infection (Middleton 1998). The health benefits of these flavonoids were reported by various epidemiological studies (Calderon-Montano et al. 2011) and (Hartman et al. 2006). Flavonoids that predominantly exist in different fruits, wines, cocoa, tea and vegetables are known to exhibit free radical scavenging activity. Along with these effects, flavonoids were also shown to possess antioxidant activities, anti-inflammatory activities, coronary heart disease prevention, hepatoprotective, anticancer and antiviral activities (Xi et al. 2012), (Ishige et al. 2001) and (Zafrilla et al. 2001). Since the uptake of these dietary ingredients that contains flavonoids is a part of human diet, there is a need to evaluate the biological activity, bioavailability and metabolism of these flavonoids. So far several studies have highlighted that the pharmacological actions exert by the flavonoids are structure dependent (Hisashi Matsuda et al. 2002) and (Kumar et al. 2013).

Chemically, flavonoids are a large group of compounds having a benzo-γ-pyrone structure in common. However they are differentiated based on the arrangements of hydroxyl, methoxy, and glycosidic side groups on a fifteen carbon skeleton that contains two benzene rings (A and B) linked to heterocyclic pyrone ring (C) (Figure 1.15) (Rice-Evans et al. 1996). They can be further divided into various classes such as flavones (e.g. fruit skins, red wine, red pepper and tomato skin), flavonols (e.g. black tea, berry fruits, linden, rose hip, olive oil and broccoli), flavanones or dihydroflavons (e.g. grape fruits, citrus fruits, lemons and oranges), flavanols (e.g. tea) and isoflavones (e.g. soya bean) (Figure 1.16) (Ishige et al. 2001), (Xi et al. 2012) and (Kumar et al. 2013). All these subtypes of flavonoids occur in food as O-glycosides, aglycones and methylated derivatives that primarily differ in the levels of oxidation and substitution of A, B and C rings. Being phytochemicals, flavonoids are impossible to be synthesised my humans and animals (Sun et al. 2010). However,

once consumed, human intestinal microbiota hydrolysis these glycosidic units of dietary flavonoids.

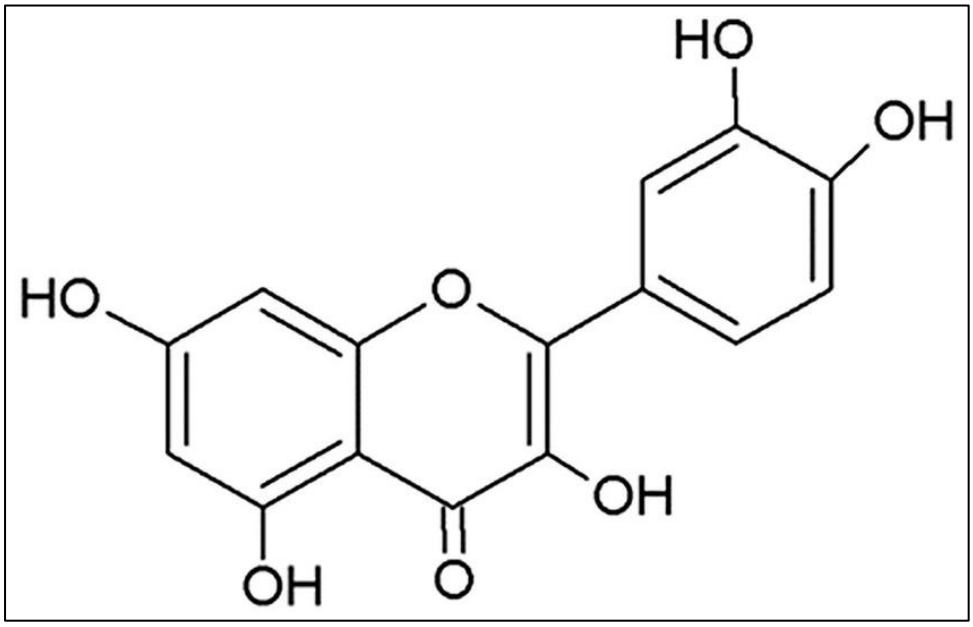

Figure 1.15 Basic nuclear structure of flavonoid

Source:- (Heim et al. 2002)

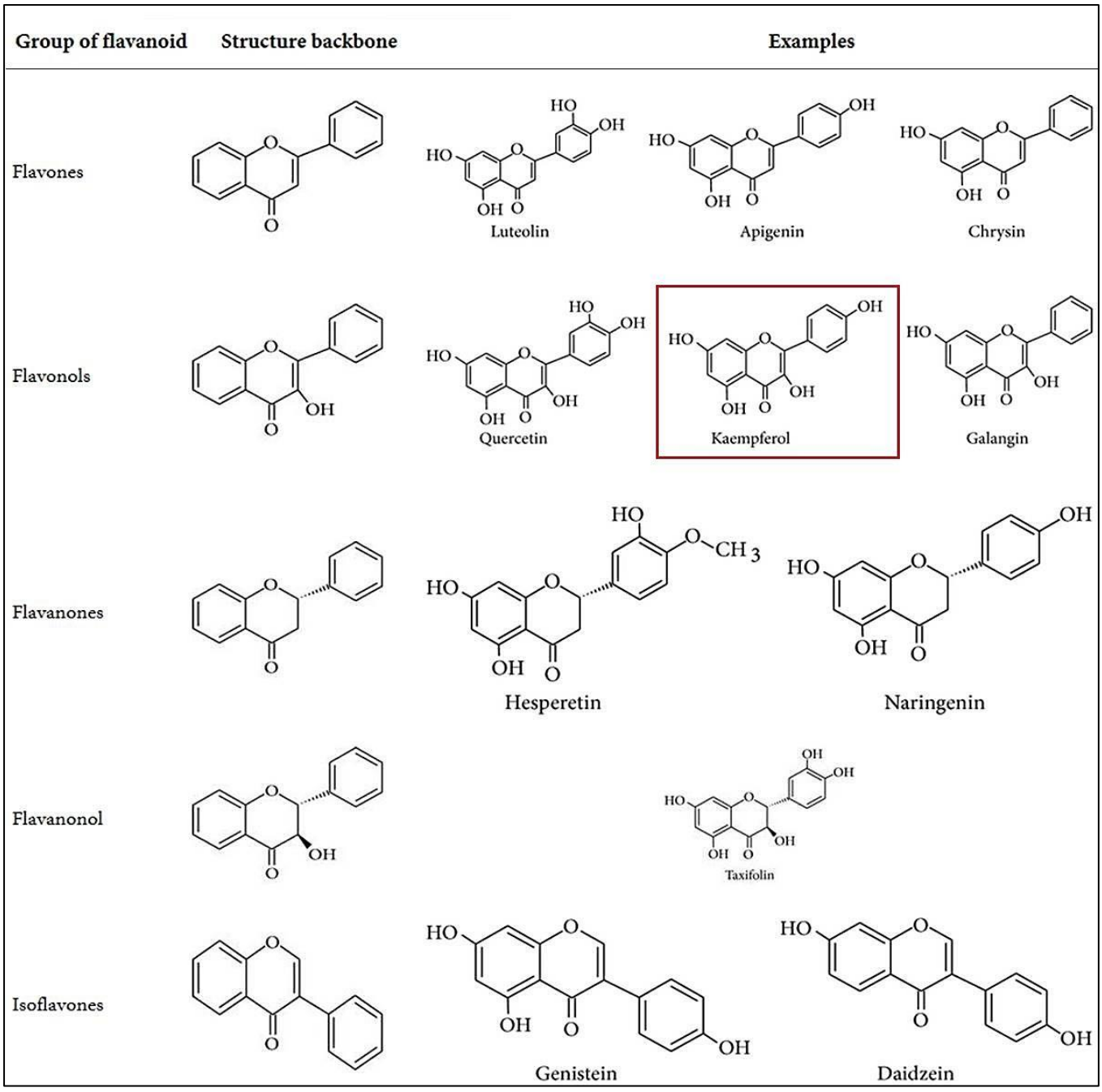

Figure 1.16 Classification and basic structure of flavonoids with examples

Source:- (Kumar et al. 2013)

Several *in vitro* and *in vivo* investigations have revealed the plausible biological properties exhibited by dietary flavonoids to confer neuroinflammation and other brain-related diseases such as dementia, AD, ALS and PD (Heim et al. 2002). Among these, the flavonoid kaempferol (highlighted in the Figure 1.16) gained attention in recent years due to its health benefits and existence in a wide variety of dietary supplements such as strawberries, rose hip, leek, onion, sweet potato and so forth (Kim & Choi 2013). Numerous evidence prove that kaempferol and its glycosides like tiliroside possess anti-inflammatory activities both invitro and in animals (Kim et al. 2010), (Medeiros et al. 2009), (Roth et al. 1999) and (S. Park et al. 2011).

1.11.1 Tiliroside

Tiliroside (kaempferol 3-O-β-D-(6″-O-coumaroyl) glucopyranoside) (Figure 1.17) is a dietary glycosidic flavonoid derived from kaempferol and is found in several medicinal and nutritional plants such as linden, olive oil, broccoli, rose hip and strawberry. Several studies have shown that tiliroside could be extracted from various plants, flowers and seeds (Table 1). For example, ethanolic extract of *Agrimonia pilosa Ledeb* (Rosaceae; AP) has shown to contain tiliroside as a major component (Jin et al. 2016).

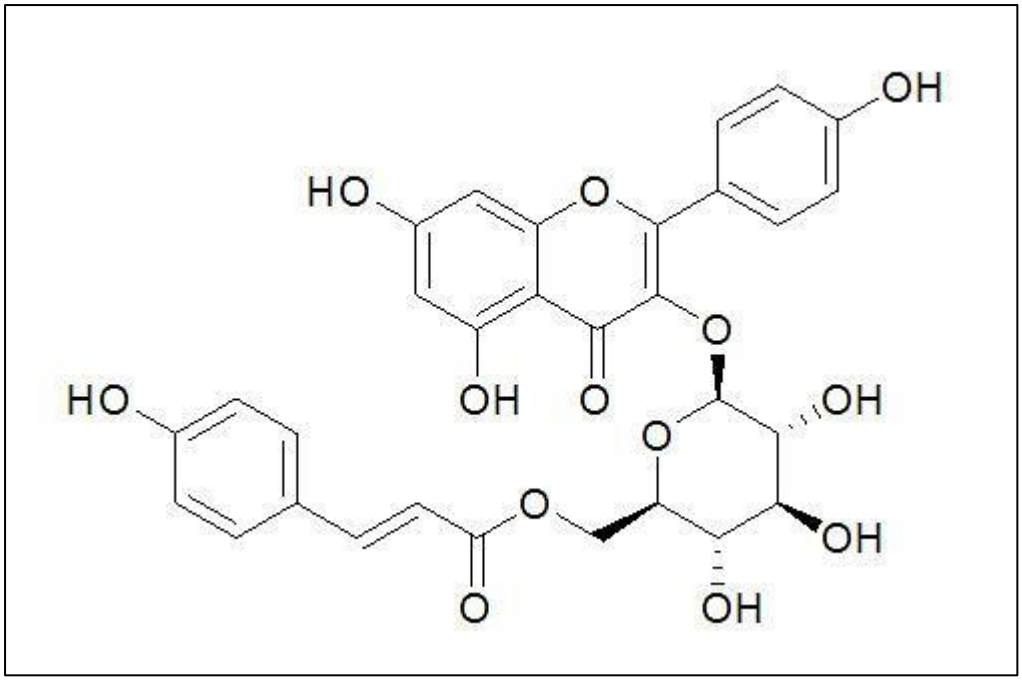

Figure 1.17 Chemical structure of tiliroside

Source :- (Jin et al. 2016)

Also, pharmacological research on this compound revealed that it exhibits excellent anti-diabetic properties via enhancing the consumption of glucose by insulin-resistant HepG2 cells (Qiao et al. 2011) and (Zhu et al. 2010). In addition to these effects, tiliroside also possesses vasorelaxant and anti-hypertensive effects. However, the exact mechanism of action is not known. Tiliroside has been shown to inhibit mouse ear inflammation induced by 12-O-tetradecano and mouse paw oedema when challenged with phospholipase A. These observations seem to suggest that tiliroside possess anti-inflammatory effects that warrant further research. Most recent studies on LPS-stimulated macrophages revealed the anti-inflammatory activities of tiliroside (Jin et al. 2016), although its precise mechanism of action in LPS/IFNγ-induced neuroinflammation is not studied yet. From all the previous studies on tiliroside it appears that the inhibitory effects are investigated when it is in the form of extract; however, this is the first study to examine its inhibitory activity of neuroinflammation directly.

Several reports have suggested the possible mechanisms of action of dietary flavonoids in various cellular models, however to what extent these beneficial effects can be extrapolated to humans is yet to understood (Holiman et al. 2001.). To fully comprehend the biological fate of the flavonoids, it is vital to have a thorough understanding of its bioavailability, absorption, metabolism and pharmacokinetics. Studies on humans conducted by Hollman et al. revealed that quercetin glucosides in the onions were readily absorbed in the small intestine via sodium-dependent glucose transporters (SGLT) (Hollman et al. 1995). Also, the same group has reported high levels of oxidatively metabolised quercetin in the blood plasma levels. Another study conducted by Oliveira et al. on quercetin and kaempferol revealed that both flavonoids were extensively metabolised into glucuronides and absorbed in the small intestine via UDP-glucuronosyltransferase isoform UGT1A9 (Oliveira et al. 2002). However, the extent of absorption and metabolism of tiliroside, a glycosidic derivative of kaempferol was not clear. Therefore, further studies were required to assess the bioavailability, metabolism and absorption of tiliroside in humans.

Species	Family	Tiliroside	Reference
Agrimonia pilosa Ledeb	Rosaceae	Kaempferol 3-O-β-D-glucopyranoside-6-p-coumaril ester	(Jin et al. 2016)
Helichrysum italicum	Asteraceae	Kaempferol 3-O-β-D -(6''-E-p-coumaroyl)-glucopyranoside	(Sala et al. 2003)
Magnolia fargesii	Magnoliaceae	Kaempferol 3-O-β-D-(6''-O-coumaroyl)glucopyranoside	(Jung et al. 1998)
Rosa canina	Rosaceae	Kaempferol 3-O-β-D-(6''-E-p-coumaroyl)-glucopyranoside	(Ninomiya et al. 2007)
Rubus idaeus	Rosaceae	Kaempferol, kaempferol 3-O-β-D-galactosides, kaempferol 3-O-β-L-arabinopyranoside and kaempferol 3-O-β-D-(6''-E-p-coumaroyl)- glucoside	(Zafrilla et al. 2001)
Tilia argentea	Tiliaceae	Kaempferol 3,7-O-β-di-rhamnoside and kaempferol 3-O-β-D-(6''-E-p-coumaroyl)-glucopyranoside	(H Matsuda et al. 2002)
Waltheria indica	Sterculiaceae	Kaempferol 3-O-β-D-(6''-O-coumaroyl)glucopyranoside	(Rao et al. 2005)

Table 1 Plant species that contain tiliroside (glycoside of kaempferol)

Source:- (Calderon-Montano et al. 2011)

1.12 Gap in knowledge

So far, very few studies have shown the anti-inflammatory effects of the dietary flavonoid tiliroside, although those investigations were very preliminary. Till now precise mechanism of action of tiliroside against neuroinflammation is not known.

1.13 Objectives of this study

This study is aimed at investigating neuroinflammation inhibitory activities of tiliroside in LPS/IFNγ-stimulated BV2 microglia

1.13.1 Specific Aims

1. To examine the neuroinflammation inhibitory activity of tiliroside in BV2 microglia that are stimulated with the combination of LPS and IFNγ.

2. To evaluate whether inhibitory actions of the compound on LPS/IFNγ-induced neuroinflammation are due to interference with NF-κB and p38 MAPK signalling in microglia.

3. Investigate the effects of tiliroside on Nrf2/HO-1/NQO1 activation and examine whether this antioxidant mechanism is involved in the anti-neuroinflammatory activity of the compound.

4. To examine whether tiliroside blocks the HT22 neuronal toxicity induced by neuroinflammation.

Chapter 2. Materials and methods

2.1 BV2 mouse microglia

Mouse microglia has shown high functional plasticity when activated with external inflammatory stimuli such as lipopolysaccharide (LPS) and gamma interferon. BV2 cells have been widely used to study molecular mechanisms in immunity, neuroinflammation, neurodegeneration and toxicology studies. The most important feature is, BV2 cells have an excellent inflammatory related gene expression pattern, which makes it appropriate for the current research. BV2 microglia cell line ICLC ATL03001 obtained from Interlab Cell Line Collection, Banca Biologicae Cell Factory, Italy. BV2 microglia are immortalised murine cell lines cultured in Roswell Park Memorial Institute medium 1640 (RPMI) supplemented with 10% foetal bovine serum (FBS) (Sigma), 2 mM L-glutamine (Sigma), 100 mM sodium pyruvate (Sigma), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma) in a 5% CO_2 incubator at 37ºC. Cells were passaged twice a week in 75 cm^2 filter-capped vented flasks. Once confluent, flasks were washed with phosphate-buffered saline (PBS) and trypsinised with 2 ml of 0.25% trypsin-EDTA solution. Trypsinisation was terminated by adding 8 ml of complete RPMI medium, followed by centrifugation at 1200 rpm for 5 minutes. Once the BV2 cell pellet was formed, the medium was aspirated and resuspended in fresh complete medium. Cells were then seeded out at a concentration of 2×10^5 cells /ml in various cell culture plates respectively.

2.1.1 Drugs and treatment

Tiliroside was purchased from Sigma and prepared in DMSO (Sigma). Primary stock of 100 mM of the compound was made and stored in small aliquots at -80°C. A working stock of 10 mM was prepared from aliquots of the original stock. The combination of (lipopolysaccharide) LPS (100 ng/ml) and (interferon gamma) IFNγ (5 ng/ml) was used to stimulate BV2 microglia in all neuroinflammation associated experiments. LPS was derived from *Salmonella enterica* serotype Typhimurium SL118, purchased from Sigma. IFNγ was derived from *Escherichia coli*, obtained from R & D systems. A working stock of 100 µg/ml was prepared from the primary stock of LPS (1 mg/ml) using sterile deionised double distilled water and aliquots were stored in 4ºC. These were discarded after one month of use. Sterile PBS was used to prepare aliquots of 10 µg/ml of IFNγ from the provided primary stock and

stored in -80°C. Before the addition of LPS and IFNγ, aliquots were vortexed for at least 15 minutes.

2.1.2 XTT assay

The XTT assay (Invitrogen) was used to determine the sensitivity of tiliroside in BV2 microglia. There are several inherent advantages of this assay over other cell viability methods such as MTT assay. For example, exposure to the large quantities of dimethyl sulphide (DMSO) while performing MTT assay was considered as a safety hazard. Also, the deleterious effects of DMSO on laboratory equipment prompted to look for other alternative methods (Scudiero et al. 1988). Unlike other dyes, XTT reagent 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) cannot diffuse into the cell. However, the reduction reaction of XTT dye occurs at the cell surface facilitated by plasma membrane electron transporters and mediated by mitochondrial oxidoreductases (Figure 2.1). An intermediate electron acceptor such as N-methyl dibenzopyrazine methyl sulphate (PMS) was used to improve the XTT results. PMS is an activation reagent that has been shown to mediate XTT reduction by picking up electrons from the cell surface and then further reduces XTT to brightly orange formazan derivative. Resulting colorimetric change can be measured using plate reader.

BV2 microglia were seeded at a density of 2×10^5 cells/ml in a 96-well cell culture plate and incubated at 37°C. At confluence, cells were stimulated with various concentrations of LPS and IFNγ and incubated for 24 hours. Stimulation was terminated by adding 25 µl of XTT/PMS solution. The plate was then further incubated for 2 hours at 37°C and resulting colour change was read at 450 nm with a plate reader (Infinite F50, Tecan). Later, BV2 cells were incubated with tiliroside (6 µM) for various time points followed by stimulation with the combination of LPS (100 ng/ml) and IFNγ (5 ng/ml). XTT/PMS solution was added and the orange colour was read at 450 nm with a plate reader.

BV2 microglia were seeded at a density of 2×10^5 cells/ml in a 96-well cell culture plate and incubated at 37°C. At confluence, cells were treated with tiliroside (2-6 µM) for 30 minutes followed by stimulation with the combination of LPS and IFNγ for a further 24 hours. Stimulation was terminated by adding 25 µl of XTT/PMS solution. The plate was incubated for 2 hours at 37°C, followed by gentle shaking for few

seconds to distribute the orange colour before absorbance was read at 450 nm with a plate reader (Infinite F50, Tecan).

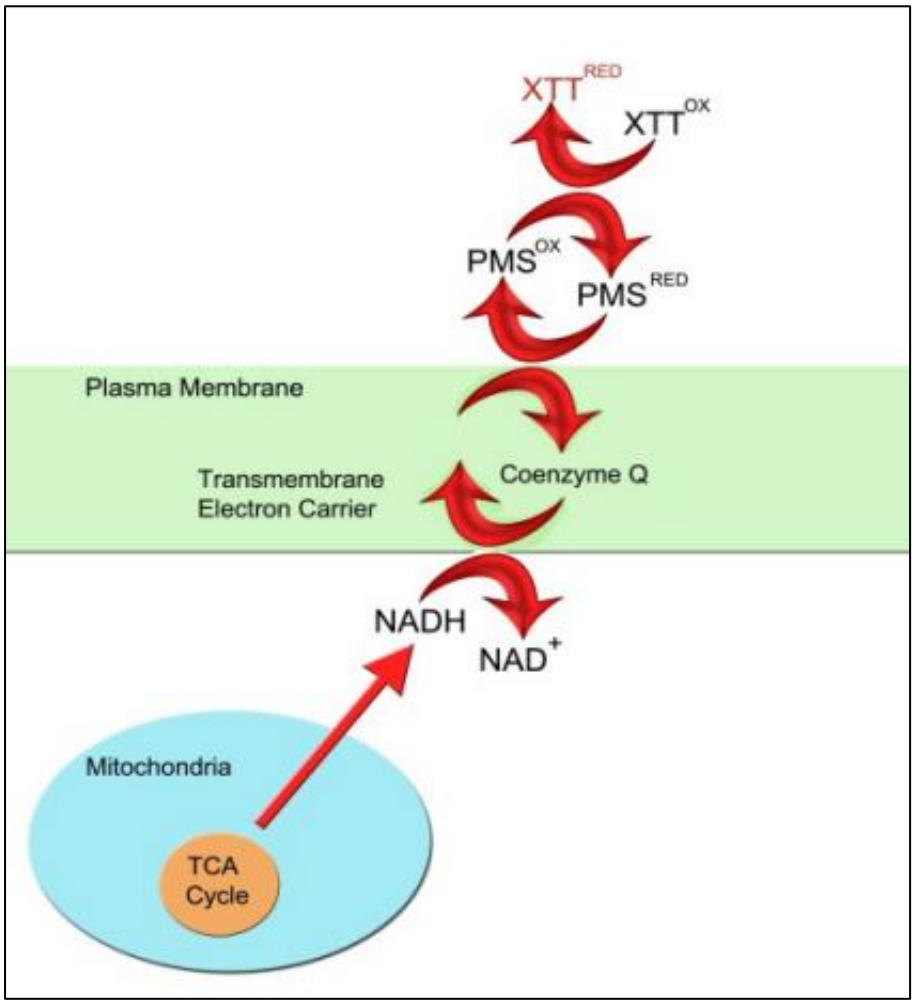

Figure 2.1 Colorimetric reduction of XTT dye by cellular enzymes in the presence of N-methyl dibenzopyrazine methyl sulphate (PMS).

Source:- (Scudiero et al. 1988)

2.1.3 Nitrite production

Nitric oxide (NO) is an important signalling molecule that plays a vital role in the pathogenesis of neuroinflammation. It serves as anti-inflammatory under normal physiological conditions, while large amounts are shown to be neurotoxic in the brain (Dheen, Kaur, & Ling, 2007). Since NO is volatile in nature, levels of nitrite (NO_2-), a stable and non-volatile breakdown product of nitric oxide can be measured using Griess assay (Griess 1879). The main principle of this assay relies on a diazotisation reaction that is formed when nitrite in the cell culture supernatant reacts with sulphanilamide in the dark. The resulting diazonium salt reacts with N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions to produce a pink colour (Figure 2.2). The absorbance of the colour change is then measured using a plate reader.

BV2 cells were seeded at a density of 2×10^5 cells/ml and cultured until confluent. After that, cells were pre-treated with tiliroside (2-6 µM) for 30 minutes before stimulation with LPS (100 ng/ml) and IFNγ (5 ng/ml) for 24 hours. Levels of nitrite in culture media were measured using a Griess reagent kit (Promega, Southampton). Supernatants were collected and centrifuged for 5 minutes at 2500 rpm to remove cell debris. Sulphanilamide solution was added to 50 µl of culture supernatants in a microplate and incubated in the dark for 10 minutes. Thereafter, N-1-napthylethylenediaminedihydrochloride (NED) was added followed by incubation in the dark for further 10 minutes. Absorbance was measured within 30 minutes at 540 nm in a microplate reader (Infinite F50, Tecan).

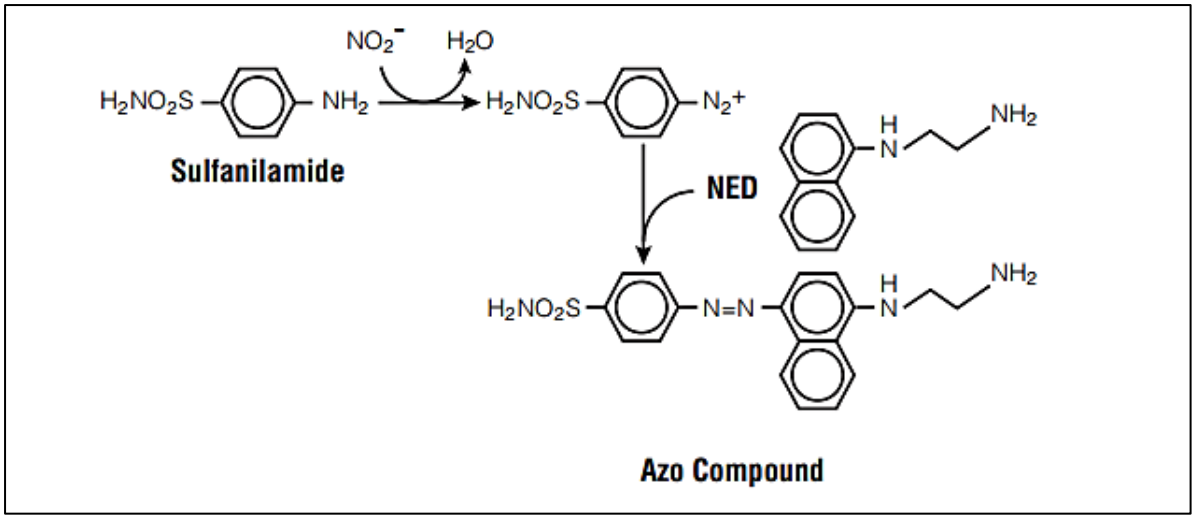

Figure 2.2 Chemical reactions that are involved in the measurement of NO_2- using the Griess reagent system.

Source:- (Griess 1879)

2.1.4 Prostaglandin E2 (PGE_2) production

Prostaglandin E_2 (PGE_2) is an arachidonic acid derivative released by activated microglia through the enzymatic action of cyclooxygenase-2 (COX-2), a rate-limiting pro-inflammatory enzyme. Studies have revealed that high levels of PGE_2 modulate the production of inflammatory cytokines during neuroinflammation (Kim et al. 2015) and (Loane et al. 2014). This observation, in turn, suggests that inhibition of PGE_2 production in the activated microglia may be beneficial in the treatment of neuroinflammation and its associated neurodegeneration.

Enzyme immune assay (EIA) is a reliable method for measuring the levels of PGE_2 in culture supernatants. EIA uses basic concept of an antigen binding to its specific antibody, which allows detection of tiny quantities of antigens (PGE_2) in the culture supernatants. Antigens are allowed to bind with specific pre-coated antibody that is subsequently detected by an enzyme-coupled secondary antibody. A chromogenic substrate then binds to the enzyme and yields a visible colour change that can be measured using plate reader.

BV2 cells were seeded in a 24-well plate (2×10^5 cells /ml) and incubated at 37°C until confluence. Later, cells were pre-treated with tiliroside (2-6 µM) for 30 minutes followed by stimulation with LPS and IFNγ and incubated at 37°C for further 24 hours. Levels of PGE_2 secretion in culture supernatants was measured with commercially available PGE_2 EIA kit (Arbor Assays, Michigan). Firstly, contents of the kit were brought to room temperature from -20°C. In the first step, standards were prepared and added to the 96-well strip plate that has been pre-coated with monoclonal capture antibody. Later, 100 µl of centrifuged culture supernatants were added, followed by 25 µl of PGE_2 conjugate solution (prostaglandin E_2-peroxidase conjugate in stabilising solution) and 25 µl of mouse detection monoclonal antibody. The plate was covered with a sealer and shaken at room temperature for 2 hours at 300 rpm. Then, contents of the plate were discarded and washed with freshly prepared washing buffer to remove non-specifically bound proteins or antibodies. Thereafter, 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature for 30 minutes without shaking. The reaction was stopped by adding stop solution (0.16M sulphuric acid), and absorbance was read at 450 nm using microplate reader (Infinite F50, Tecan). Concentrations of PGE_2 were calculated from standards (6.25-1000 pg/ml).

2.1.5 Enzyme-linked immunosorbent assay (ELISAs)

In neuroinflammation, pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β are considered as significant neurotoxic factors released by activated microglia. Studies have shown that these cytokines further activate chemokines and other additional inflammatory mediators that induce neuronal apoptosis signalling cascades in the hippocampus regions of the brain (Bernhardi et al. 2015) and (Spencer et al. 2012). On the other hand, the progression of neuroinflammation was blocked by anti-inflammatory cytokine production like IL-10 by microglia (Park et al. 2007). Therefore, cytokine production in the microglia remains an important target in reducing neuroinflammation.

The ELISA is a specific analytical assay technique used to quantify inflammatory cytokines in cell supernatants. The critical step in the assay is the direct detection of antigen in the sample by immobilising antigen-specific capture antibody directly onto the 96-well surface. This is followed by addition of a specific primary antibody. The antigen is thus “sandwiched” between such capture antibody and a detection primary antibody. Wells are thoroughly washed to remove unbound antibody. In the next step, enzyme-linked secondary antibody is applied, which then binds to the primary antibody, followed by addition of enzymatic substrate solution. The resulting colour change (reflecting the antigen concentration in the sample) is then quantified.

BV2 microglia cells were seeded in a 24-well plate (2×10^5 cells /ml) and incubated at 37°C. Once confluent, cells were pre-treated with tiliroside (2-6 µM) for 30 minutes followed by stimulation with LPS and IFNγ and incubated at 37°C for further 24 hours. Stimulation was terminated, and culture supernatants were collected and centrifuged for 5 minutes at 2500 rpm. Concentrations of TNFα, IL-6, IL-1β and IL-10 were measured with commercially available ELISA kits (Biolegend). This method uses a 96-well strip plate that is pre-coated with specific target capture monoclonal antibody. Firstly, 50 µl of assay buffer was added to each well. Later, 50 µl of freshly prepared standard dilutions were added, followed by 50 µl of culture supernatants. The plate was then sealed and incubated at room temperature with shaking at 200 rpm. After 2 hours, the contents of the wells were discarded, and the plate was washed four times with at least 300µl of wash buffer and dried by tapping firmly on absorbent paper. Specific detection antibody solution was added and incubated for 60 minutes at room temperature while shaking at 200 rpm. The washing process

was repeated, followed by addition of 100µl avidin-horseradish peroxidase enzyme (HRP) solution to the wells and incubated for 30 minutes at room temperature while shaking at 200 rpm. Contents in the plate were discarded, washed and substrate solution was added to the each well and incubated for 15 minutes in the dark. Subsequently, the reaction was terminated by adding 100 µl of stop solution (0.16M sulphuric acid). The resulting colour change was read at a wavelength of 450 nm using plate reader (Infinite F50, Tecan). The concentration of cytokines present in the samples was calculated from the absorbance compared to the relevant standard curve.

2.1.6 Isolation of cytoplasmic lysates

One way to study the extent of protein expression inside the cell is to prepare lysates by disrupting and extracting the cytoplasmic cellular contents. This was achieved by washing cells with cold PBS, followed by addition of ~25 µl of radioimmunoprecipitation assay buffer (RIPA) lysis buffer (Cell signalling) that contains 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) and incubated for 15 minutes. Subsequently, cells were scraped, and lysates were further centrifuged at 4°C for 15 minutes at 13500 rpm. The resulting cytoplasmic lysates in the supernatants were collected, quantified and stored away at -80°C.

2.1.7 Isolation of nuclear lysates

Nuclear lysates were prepared to study the behaviour of some transcription factors (e.g., NF-κB and Nrf2) inside the nucleus of the cell. This was performed using EpiSeeker Nuclear Extraction Kit (Abcam). Cells were washed with cold PBS, followed by addition of 20 µl of pre-extraction buffer (ENE1 buffer) and incubated on ice for 10 minutes. After that, cells were centrifuged at 12000 rpm for 1 minute. Supernatants were discarded, and 10 µl of nuclear extraction buffer (ENE2 buffer) was added to the pellet and incubated on ice for further 15 minutes with occasional vortexing (every 5 minutes). This was followed by centrifugation at 13500 rpm for 15 minutes at 4°C. The resulting nuclear lysates in the supernatants were collected, quantified and stored away at -80°C.

2.1.8 Protein quantification

Protein concentrations from cytoplasmic and nuclear lysates were quantified using Coomassie (Bradford) protein assay kit (Thermo Fisher). The main principle of this assay relies on the calorimetric reaction between the proteins in lysates with

Coomassie dye in acidic medium. The resulting colour change (brown to blue) was measured at 540 nm using a plate reader. In this assay, bovine serum albumin (BSA) was used as standard (125-2000 µg/ml). Lysates and specific standards were diluted with de-ionized double distilled water, 5 µl of both samples and standards were pipetted into a 96-well microplate. Later, 250 µl of Coomassie reagent was added, and the plate was incubated for 10 minutes at room temperature. Absorbance was measured at 540 nm using microplate reader (Infinite F50, Tecan). Later, protein concentrations of the samples are calculated from the standard curve.

2.1.9 Western blot analysis

Western blotting is an important technique used to separate and identify cellular proteins based on their molecular weights. Following protein quantification, 25 µg of protein was denatured by heating with 5 µl of lithium dodecyl sulphate (LDS) (Invitrogen) and 2 µl sample reducing agent (500 mM dithiothreitol (DTT)), (Invitrogen) at 70°C for 10 minutes. Electrophoresis was performed using Bolt™ 4-12% Bis-Tris Plus pre-cast polyacrylamide gels (Invitrogen) with running buffer (Invitrogen) at a constant voltage of 200 V for 35 minutes. Running buffer contains a mixture of Tris base, glycine, SDS in H_2O and pH was adjusted to 8.3. Later, separated proteins were transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane (Millipore) using transfer buffer (Invitrogen) for 2 hours at 25 V. Transfer buffer contains a mixture of 25 mM Tris, 190 mM glycine, 20% methanol and finally pH was adjusted to 8.3. Membranes were then washed three times (5 minutes each wash) with Tris-buffered saline (TBS-T) and blocked for 60 minutes at room temperature using non-fat dry milk. Later, membranes were washed and incubated with primary antibodies overnight at 4°C. Primary antibodies used in this research are listed in Table 1. The next day, membranes were washed three times with TBS-T (5 minutes each wash) and then incubated with anti-rabbit Alexa Fluor 680 goat secondary antibody (Invitrogen) for 60 minutes in the dark (fluorophore attached to the secondary antibody are light sensitive). Later, membranes were washed briefly, and band detection was done using Licor Odyssey image station. Molecular weight determination was done by comparing bands to the Precision Plus Protein™ unstained standards (Bio-Rad). The relative density of the protein expressions was measured using Image J (National Institutes of Health, USA) and was normalised by comparing to anti-actin (cytoplasmic proteins) or Lamin B

(nuclear proteins) antibodies. All antibodies and buffers are prepared in TBS-T to reduce background signal.

Antibodies	Supplier	Host	Type	Dilution
iNOS	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
COX-2	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
p-IκB-α	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
Total-IκB-α	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
p-IKK-α	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
Total-IKK-α	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
p-NF-κB-p65	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
Total-NF-κB-p65	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
p-p38	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
Total-p38	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
p-MK2	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
Total-MK2	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
p-MKK3/6	Cell signalling	Rabbit	Polyclonal	1:1000
TRAF6	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
TLR4	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
HO-1	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
NQO1	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
Nrf2	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
SIRT1	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
Acetyl-NF-κB-p65	Cell signalling	Rabbit	Polyclonal	1:1000
Beta-actin	Sigma Aldrich	Rabbit	Polyclonal	1:1000
Lamin B	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500
MAP2	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:500

Table 2 Primary antibodies used in the western blot analysis.

2.1.10 Transient transfection and luciferase reporter gene assays

Over the past few years, research has focused on studying the role of various transcription factors such as NF-κB and Nrf2 in neuroinflammatory conditions. It is imperative to explore the roles of these transcription factors in neuroinflammation due to their crucial role in controlling the transcription of various inflammatory mediators (Didonato et al. 2012), (Lee & Johnson 2004) and (Kim et al. 2010b). In general, genetic reporters are used in studying gene expressions and other intracellular events coupled to gene expression at transcriptional level. Typically, in luciferase-based reporter assays, luciferase reporter gene is cloned with a DNA sequence of interest into an expression vector which is then transferred into cells using a transfection reagent. Once cells are activated, reporter protein (luciferin) is converted to oxyluciferin in the presence of firefly luciferase enzyme along with high efficient light which is further quantified using luminometer (Figure 2.3). The efficiency of the gene reporters typically depends on the type of vector used during transfection. Luciferase reporter assay system uses pGL4.32 vectors to deliver luciferase reporter gene [luc2P] into cells. The pGL4 Vectors are engineered in a way to facilitate high-end gene expression and reduced anomalous transcription. The pGL4.32 [luc2P/ NF-κB-RE/Hygro] (Promega) vector was used to study the NF-κB-mediated gene expression at the transcriptional level in the microglia. This vector contains five copies of NF-κB response elements (NF-κB-RE) that drive transcription of luciferase reporter gene luc2P. Also, to understand antioxidant response element (ARE)-mediated gene expression in microglia, pGL4.37 [luc2P/ARE/Hygro] (Promega) vector was used, which contains four copies of ARE that drive transcription of the luciferase reporter gene luc2P.

The day before the transfection BV2 cells were subcultured at a ratio of 1:3. The next day, cells were harvested and seeded at a density of 4 x 10^5 cells/ml in a solid white 96-well plate using Opti-MEM® (modified Eagle's Minimum Essential Media) (Invitrogen) mixed with 5% FBS. The pGL4.32 [luc2P/ NF-κB -RE/Hygro] vector was used for NF-κB at a concentration of 1 ng DNA/µl, mixed with Fugene 6 (Promega) transfection reagent. This mixture was later added to the cells in the 96-well plate and incubated for a further 16 hours at 37°C in 5% CO_2 incubator. After that, media was changed to Opti-MEM® (without 5% FBS) and incubated for a further 8 hours. To determine NF-κB-mediated gene expression, transfected cells were pre-treated

with tiliroside for 30 minutes followed by stimulation with LPS and IFNγ for 60 minutes at 37°C. At the end of the stimulation, 100 µl of luciferase assay buffer containing luminescence substrate was added to each well and luminescence was read with FLUOstar OPTIM reader (BMG LABTECH).

A different vector was used to investigate the effects of tiliroside on ARE-associated gene expression in BV2 microglia. Cells were seeded at a density of 4 x 10^5 cells/ml in a white 96-well plate using Opti-MEM® that contains 5% FBS. Later, the pGL4.37 [luc2P/ARE/Hygro] vector (1 ng DNA/µl) was mixed with Fugene 6 and added to the cells followed by incubation for 16 hours at 37°C. Thereafter, media was changed to Opti-MEM® (without 5% FBS) and incubated for a further 8 hours. Transfected cells were treated with tiliroside (2-6 µM) alone for 24 hours (for time point experiments refer to the section 3.2.11, 5.2.1 and 5.2.2) to investigate ARE-associated gene expression. At the end of the stimulation, 100 µl of luciferase assay buffer containing luminescence substrate was added to each well and luminescence was read with FLUOstar OPTIM reader (BMG LABTECH).

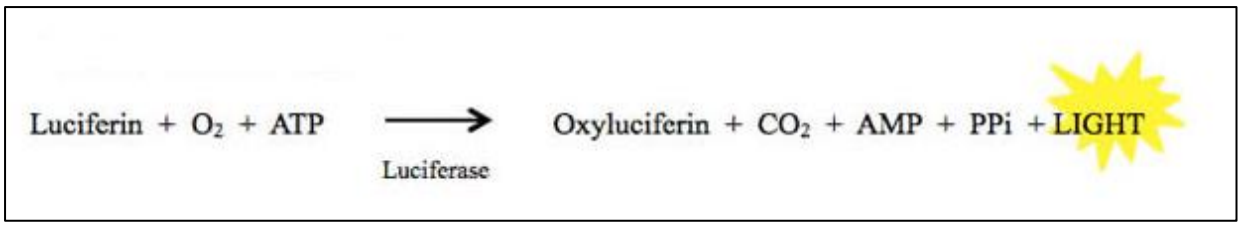

Figure 2.3 Chemical reaction involved in the luciferase reporter assay

Source:- (Alam & Cook 1990)

2.1.11 Nrf2 and SIRT1 siRNA transfections

Small interfering RNAs (siRNAs) are short non-coding double-stranded RNAs with high efficacy and excellent specificity to inhibit or shut down target gene expression prior to translation. These siRNAs are widely used to investigate the individual contributions of specific genes of interest to an assortment of cellular processes. siRNAs mainly consist of two RNA strands, an antisense or guide strand and a sense or passenger strand. This small double-stranded siRNAs are transfected into cells with the help of polymer based transfection reagents, where the guide strand is loaded into RNA-induced Silencing Complex (RISC) (Figure 2.4). This activated protein, and nucleic acid complex can then elicit gene silencing by binding, through perfect complementarity, to a single target mRNA sequence, thereby targeting it for cleavage and degradation (Reynolds et al. 2004).

Several studies have highlighted the roles of transcription factors Nrf2 and SIRT1 in neuroinflammation (Lee & Johnson 2004) and (Shen et al. 2009). Compounds that have shown to activate these transcription factors blocked LPS-induced neuroinflammation in the microglia (Y. Li et al. 2015) and (Onasanwo et al. 2016). Therefore, to further determine the neuroinflammation inhibitory activity of tiliroside in the absence of Nrf2, siRNA technique was used. Nrf2 gene was knocked out in BV2 microglia using Nrf2 siRNA (Santa Cruz Biotechnology). BV2 cells were cultured and seeded in a 6-well plate at a density of 2×10^5 cells/ml using antibiotic-free (penicillin and streptomycin) RPMI 1640 growth medium and incubated at 37°C in a 5% CO_2 incubator until 50% confluent. In tube A, 2 µl of Nrf2 siRNA duplex (Santa Cruz Biotechnology) was diluted into 100 µl of siRNA transfection medium (Santa Cruz Biotechnology). In tube B, 2 µl of transfection reagent (Santa Cruz Biotechnology) was diluted into 100 µl of siRNA transfection medium. The contents of tube A were transferred gently to tube B to prepare transfection cocktail and incubated for 45 minutes at room temperature. Next, 200 µl of Nrf2 siRNA transfection cocktail was added to the cells in the plate and further incubated for 6 hours at 37°C. Control BV2 microglia were transfected with control siRNA. Following transfection, media was changed to RPMI 1640 growth media and incubated for a further 18 hours at 37°C.

Effects of tiliroside (6 µM) on nitrite, PGE_2, TNFα and IL-6 production in LPS (100 ng/ml)/IFNγ (5 ng/ml)-stimulated control siRNA and Nrf2-siRNA-transfected BV2 cells were then determined. Also, iNOS and COX2 protein expressions were

evaluated using western blots. NF-κB DNA binding assays were conducted for both control siRNA and Nrf2 siRNA-transfected BV2 cells treated with tiliroside (6 μM). Transfection efficiency was determined by subjecting nuclear lysates from both control siRNA and Nrf2 siRNA-transfected BV2 microglia to western blotting to detect levels of Nrf2 protein.

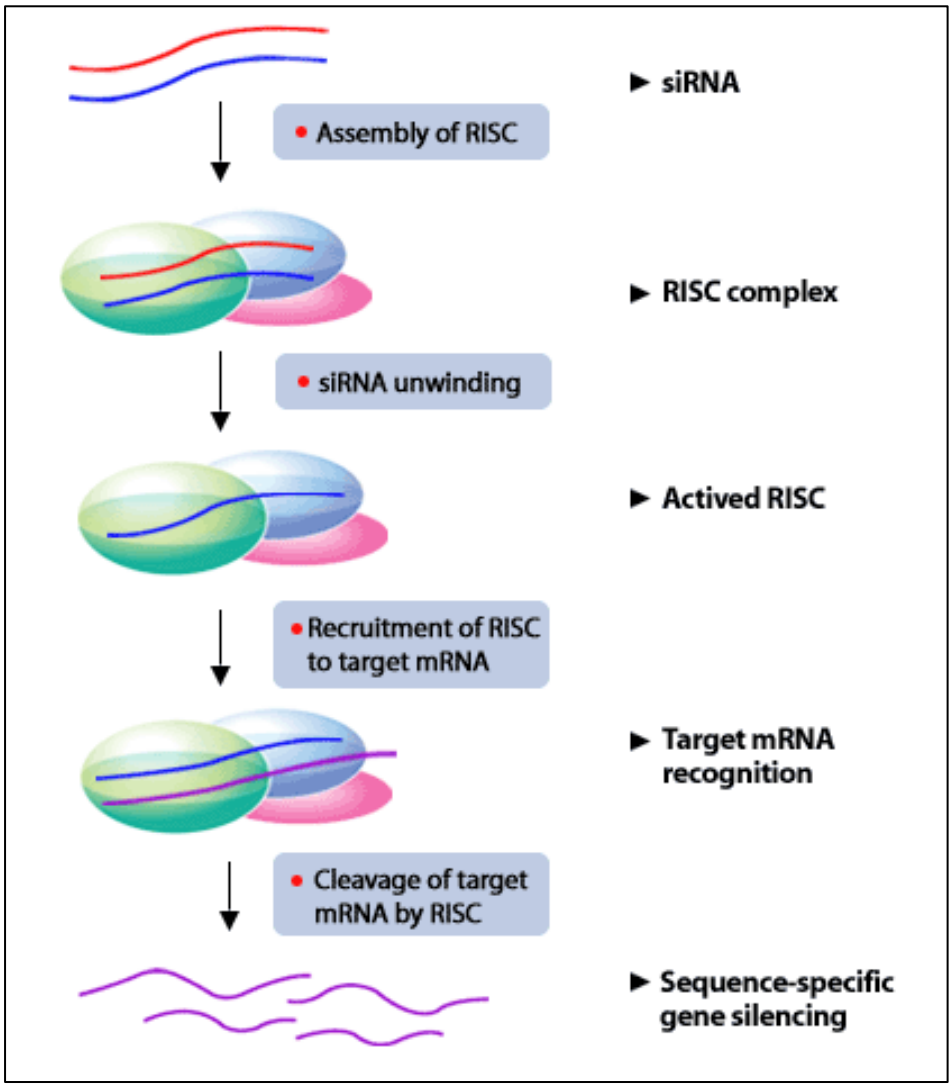

Figure 2.4 The mechanism of RNA interference using siRNA.

Source:- (Agrawal et al. 2003)

To further determine the neuroinflammation inhibitory activity of tiliroside in the absence of SIRT1, siRNA technique was used. SIRT1 gene was knocked out in BV2 microglia using SIRT1 siRNA (Santa Cruz Biotechnology). BV2 cells were cultured and seeded in a 6-well plate at a density of 2×10^5 cells/ml using antibiotic-free (penicillin and streptomycin) RPMI 1640 growth medium and incubated at 37°C in a 5% CO_2 incubator until 50% confluent. In tube A, 2 µl of SIRT1 siRNA duplex (Santa Cruz Biotechnology) was diluted into 100 µl of siRNA transfection medium (Santa Cruz Biotechnology). In tube B, 2 µl of transfection reagent (Santa Cruz Biotechnology) was diluted into 100 µl of siRNA transfection medium. The contents of tube A were transferred gently to tube B to prepare transfection cocktail and incubated for 45 minutes at room temperature. Next, 200 µl of SIRT1 siRNA transfection cocktail was added to the cells in the plate and further incubated for 6 hours at 37°C. Control BV2 microglia were transfected with control siRNA. Following transfection, media was changed to RPMI 1640 growth media and incubated for a further 18 hours at 37°C. Effects of tiliroside (6 µM) on nitrite, PGE_2, TNFα and IL-6 production in LPS/IFNγ-stimulated control siRNA and SIRT1-siRNA-transfected BV2 cells were then determined. Transfection efficiency was determined by subjecting nuclear lysates from both control siRNA and SIRT1 siRNA-transfected BV2 microglia to western blotting to detect levels of SIRT1 protein.

2.1.12 Immunofluorescence

Immunofluorescence is an imaging technique that is widely accepted as a robust method to assess localisation and expression of proteins of interest in cells. The principle mainly relies on the direct or indirect antigen-antibody reaction, which allows the end user to observe the changes under fluorescence or confocal microscope. BV2 microglia were seeded at a density of 2×10^5 cells /ml in 24-well plates. At confluence, cells were pre-treated with tiliroside (2-6 µM) for 30 minutes followed by stimulation with the combination of LPS (100 ng/ml) and IFNγ (5 ng/ml) for various time points (see chapters 3, 4 and 5 for details of time points). Cells were fixed with ice-cold methanol (100%) for 15 minutes at -20°C and later washed three times for 5 minutes with phosphate buffer saline (PBS). Non-specific binding sites were blocked by incubating cells with 5% bovine serum albumin (BSA) blocking solution (containing 10% horse serum) in 1X TBS-T) for 60 minutes at room temperature followed by washing with PBS. Thereafter, cells were incubated with

primary antibodies (Table 3) for overnight at 4°C. Following overnight incubation, cells were washed 3 times with PBS and incubated for 2 hours in the dark with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Life Technologies) secondary antibody (1:500). Later, cells were washed with PBS and counterstained with 4', 6 diamidino-2-phenylindole dihydrochloride (50 nM, DAPI; Invitrogen) for 5 minutes. After rinsing cells with PBS, the excess buffer was removed, and gold antifade reagent (Invitrogen) was added to prevent the signal from fading. All staining procedures were performed at room temperature. Representative fluorescence images were obtained using EVOS® FLoid® cell imaging station (Invitrogen).

Antibodies	Supplier	Host	Type	Dilution
p-NF-κB-p65	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:100
Total-NF-κB-p65	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:100
TLR4	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:100
HO-1	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:100
Nrf2	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:100
SIRT1	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:100
Acetyl-NF-κB-p65	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:100
MAP2	Santa Cruz Biotechnology	Rabbit	Polyclonal	1:100

Table 3 Primary antibodies used in immunofluorescence experiments.

2.1.13 Electrophoretic mobility shift assays (EMSA)

In neuroinflammation, transcription factors play a crucial role in regulating several pro-inflammatory genes by controlling their transcription. However, these gene expressions are tightly controlled by the transcriptional protein-DNA interactions inside the nucleus. EMSA is an important technique used to study these protein-DNA interactions in mammalian tissues and nuclear cell lysates.

ELISA-based EMSA

Activation of NF-κB transcription factor has been shown to increase the production of pro-inflammatory cytokines and other mediators involved in the neuroinflammation. Studies have demonstrated that LPS alone can predominantly induce transcription of several inflammatory genes via nuclear NF-κB-DNA interactions in the microglia (Hanamsagar et al. 2012), (Zhou et al. 2014), (Xu et al. 2014) and (Takeda & Akira 2004). Therefore, it is important to study the interactions between NF-κB-DNA in the activated microglia during neuroinflammatory conditions. Commercially available non-radioactive TransAM® NF-κB transcription factor EMSA kit, (Active Motif, Belgium) was used to determine the effects of tiliroside on DNA binding of NF-κB in BV2 microglia. This kit contains a 96-stripwell plate in which a specific double-stranded oligonucleotide that contains multiple copies of the consensus-binding site has been immobilised on the well surface. The transcription factor that is present in the nuclear extract binds specifically to this oligonucleotide, which is further quantified by adding a specific primary antibody, secondary antibody and a developing solution. The resulting colorimetric outcome can be easily quantified using a plate reader at a wavelength of 450 nm (Figure 2.5). Gel based EMSA or conventional EMSA involves several steps such as preparation of DNA complexes using various buffers, pre-annealed nucleotides and most importantly the binding reaction. In addition, only a limited number of samples can be subjected to electrophoresis, as these issues prompted to look for other alternative methods. In ELISA based EMSA is a straightforward assay and does not require any additional steps or reaction mixtures. Moreover, 96-stripwell format enables to run a high number of samples at a time.

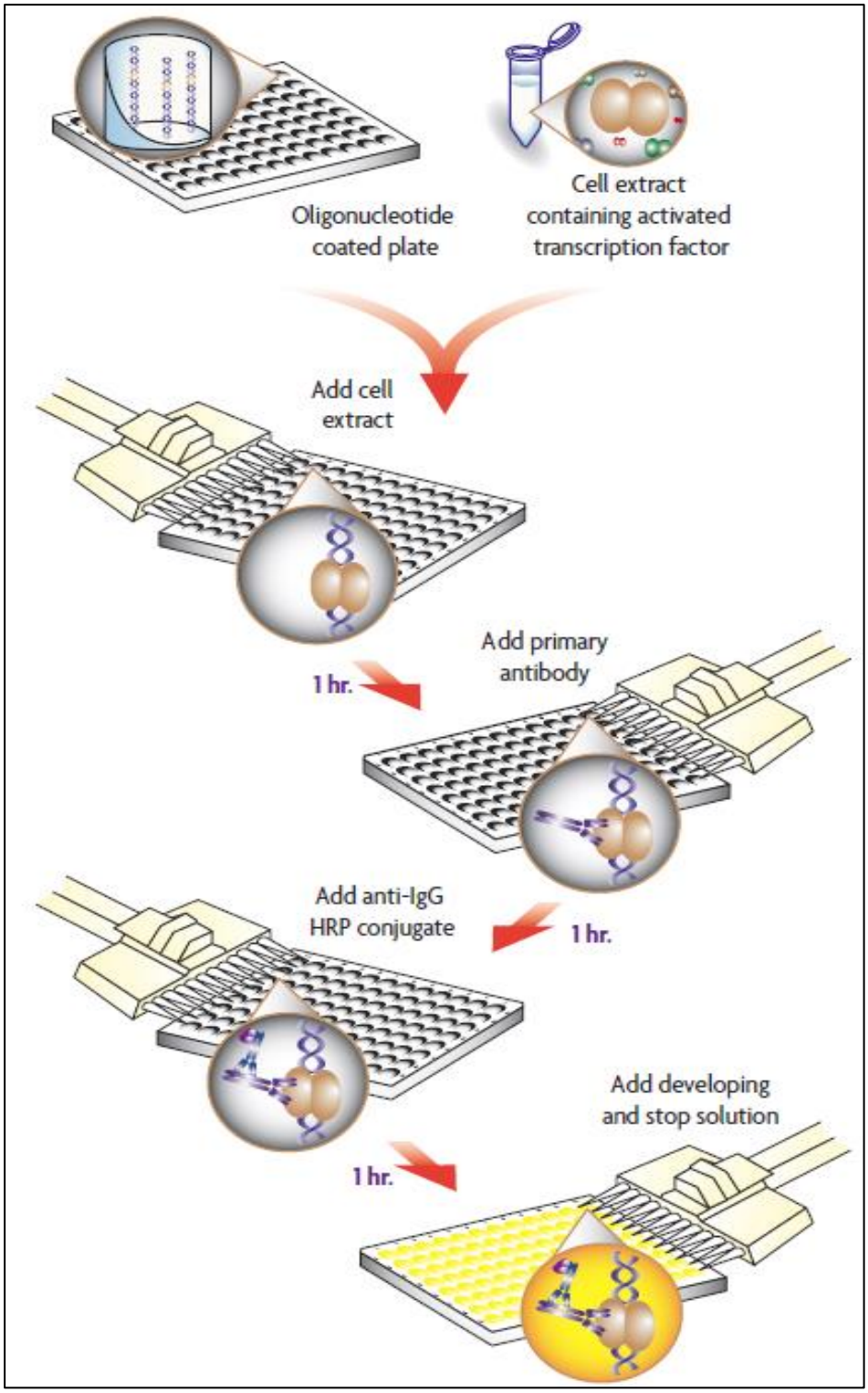

Figure 2.5 Flow chart for the TransAM® NF-κB transcription factor ELISA based EMSA kit.

BV2 microglia were pre-treated with increasing concentrations of the compound for 30 minutes and later, cells were stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml). After 60 minutes, nuclear lysates were prepared using EpiSeeker Nuclear Extraction Kit (Abcam), as explained previously in section 2.1.7. The EMSA kit employs a 96-well plate to which an oligonucleotide containing the NF-κB consensus site (5' GGGACTTTCC-3') has been immobilised. Later, 30 µl of complete binding buffer was added to each well, followed by 20 µg nuclear extract samples. The plate was covered and shook (100 rpm) for 60 minutes at room temperature. This was followed by discarding contents of the plate and washing it for three times with 200 µl/well of wash buffer, followed by addition of 100µl of NF-κB antibody (1:1000). The plate was sealed and incubated for further 60 minutes at room temperature while shaking, followed by washing three times. Later, 100 µl of HRP-conjugated antibody (1:1000) was added and incubated for 60 minutes at room temperature followed by washing. In the end, 100 µl of developing solution was added and incubated in the dark for 15 minutes, and the reaction was stopped by adding 100 µl of stop solution. Absorbance was read on a Tecan F50 microplate reader at 450 nm.

To investigate DNA binding of Nrf2, BV2 microglia were treated with tiliroside (2-6 µM) for 24 hours and nuclear lysates were prepared using EpiSeeker Nuclear Extraction Kit (Abcam), as explained previously in section 2.1.7. Later, 20 µg of nuclear lysates were added to 96-well plates on which oligonucleotide containing the ARE consensus binding site (5' GTCACAGTGACTCAGCAGAATCTG-3') has been immobilised. After that, 30 µl of complete binding buffer was added to each well and incubated for 60 minutes at room temperature while shaking. This was followed by discarding contents of the plate and washing it for three times with 200 µl/well of wash buffer, followed by addition of 100µl of Nrf2 antibody (1:1000). Cover the plate and incubate it for further 60 minutes at room temperature while shaking, followed by three times washing. Later, 100 µl of HRP-conjugated antibody (1:1000) was added and incubated for further 60 minutes at room temperature followed by washing. In the end, 100 µl of developing solution was added and incubated in the dark for 15 minutes and the reaction was terminated by adding 100 µl of stop solution. Absorbance was read on a Tecan F50 microplate reader at 450 nm.

2.1.14 Measurement of intracellular reactive oxygen species (ROS)

A series of studies has revealed the crucial role of ROS in neuroinflammatory processes and its mediated neurodegeneration in the CNS (Ray & Lahiri 2009). Microglia was shown to produce ROS in response to the inflammatory stimuli. This intracellular accumulation of ROS triggers the release of various inflammatory mediators through activation of downstream signalling pathways like MAPKs and NF-κB in microglia (Rojo et al. 2014). Several lines of evidence show that antioxidants inhibit NF-κB activation and block inflammatory cytokines production by modulating the generation of ROS (Zhang et al. 2016). Intracellular ROS production was measured using 2', 7'-dichlorofluorescein diacetate DCFDA-cellular reactive oxygen species detection assay kit (Abcam). This assay system uses the cell permeant reagent 2', 7' –dichlorofluorescein diacetate (DCFDA), a fluorogenic dye that measures the activity of ROS within the cell. DCFDA dye diffuses into the cell and gets deacetylated into to a non-fluorescent compound by cellular esterases, which is later oxidised by intracellular ROS into 2', 7' –dichlorofluorescein (DCF). As DCF is a highly fluorescent compound that can be detected by fluorescence with maximum excitation of 495 nm and emission of 529 nm respectively (Figure 2.6).

BV2 microglia were seeded in non-transparent white bottomed 96-well plates at a density of 2×10^5 cells/ml. Once confluent, the cells were washed with PBS and stained with 20 μM DCFDA for 30 minutes at 37°C. After incubation, the cells were washed with PBS and further incubated for 30 minutes with or without tiliroside (2-6 μM) prior to stimulation with LPS (100 ng/ml) and IFNγ (5 ng/ml) for 24 hours. Intracellular production of ROS was measured by the fluorescence detection of dichlorofluorescein (DCF) as the deacetylated product of DCFDA in a Polar Star Optima microplate reader (BMG LABTECH) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

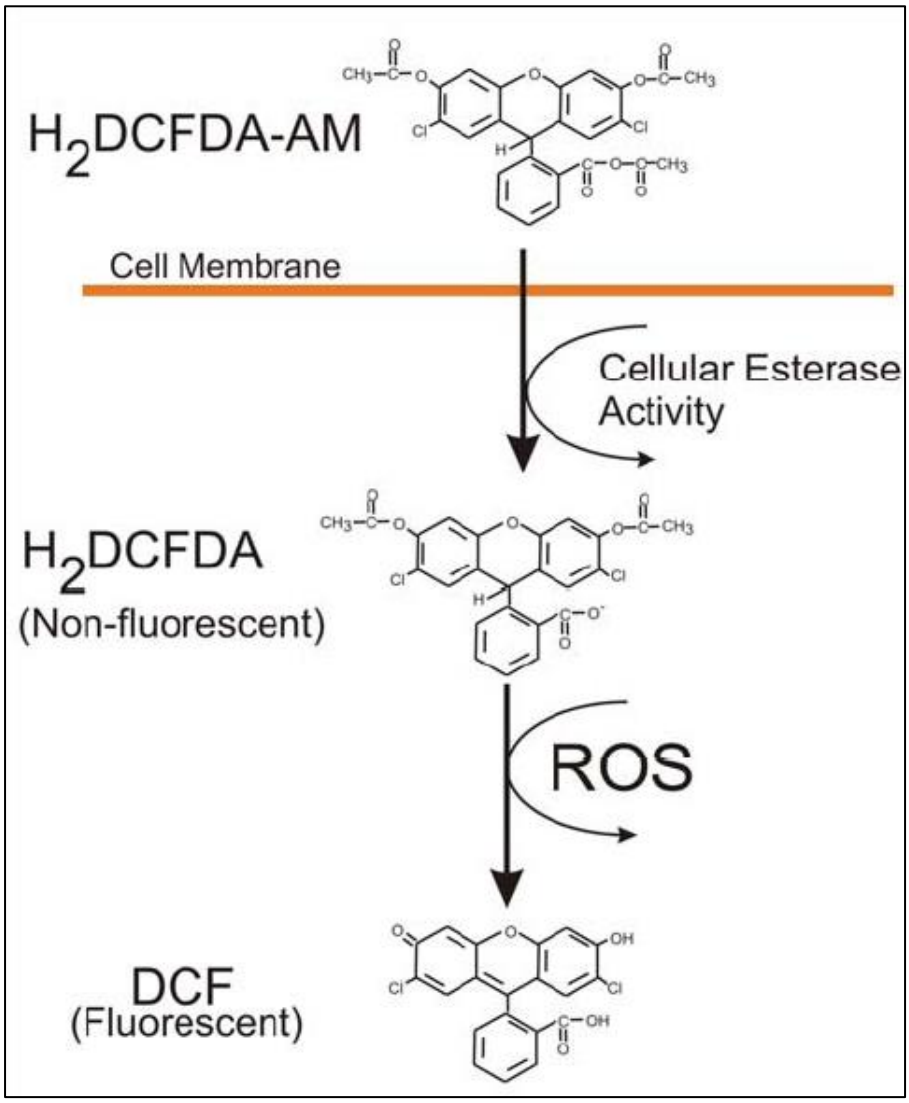

Figure 2.6 Formation of fluorescent compound DCF from DCFDA dye by ROS.

Source:- (Held 2008)

2.1.15 Measurement of glutathione (GSH)

Depletion of glutathione (GSH) levels has been identified as an early event in the progression of neuroinflammation and its mediated neurodegenerative diseases. Reduced GSH levels are shown to promote oxidative stress and potentially leading to the death of microglia in the brain (Lee et al. 2010). Concentrations of GSH in the BV2 cell culture supernatants were measured using GSH-Glo™ Glutathione assay kit (Promega). This luminescence assay is based on the conversion of a luciferin derivative into luciferin in the presence of glutathione, catalysed by glutathione S-transferase enzyme (GST). The stable luminesce signal generated during this coupled reaction in the presence of firefly luciferase (Figure 2.7) was measured using luminometer and is proportional to the amount of glutathione present in the supernatants.

BV2 microglia were harvested in a 96-well plate (2×10^5 cells/ml). When cells reached confluence, the culture medium was changed to serum-free medium, pre-treated with tiliroside (2-6 µM) for 30 minutes and later stimulated with a combination of LPS (100 ng/ml) and IFNγ (5 ng/ml) for 24 hours. At the end of the experiment, the culture medium was removed, and 100 µl of 1X GSH-Glo™ reagent was added to each well and incubated with shaking at room temperature for 30 minutes. Thereafter, 100µl of luciferin detection reagent was added to each well and incubated with shaking at room temperature for 15 minutes. Luminescence was then read with FLUOstar OPTIM reader (BMG LABTECH).

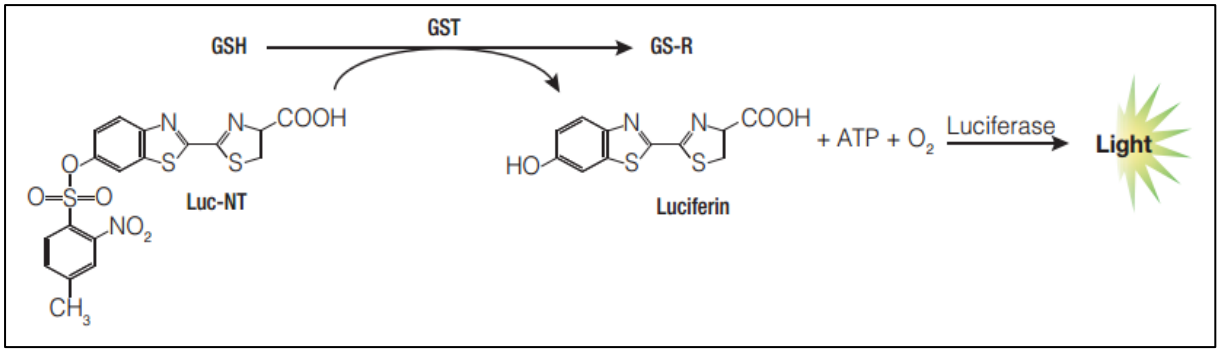

Figure 2.7 Chemical reactions that are involved in the measurement of GSH using GSH-Glo assay kit.

Source:- (Sies 1999)

2.2 HT22 mouse hippocampal neurons

HT22 neuronal cells were a kind gift from Dr Jeff Davis. These are immortalised mouse hippocampal neuronal cell lines that were derived from HT4 cells (Davis & Maher, 1994). HT22 neuronal cells were cultured in DMEM supplemented with 10 % FBS, 100 mM sodium pyruvate (Sigma), 100 U/ml penicillin and 100 mg/ml streptomycin in a 5 % CO_2 incubator at 37 °C. Cells were cultured in 75 cm^2 flasks. Confluent monolayers were washed with PBS and trypsinised with 2 ml of 0.25% trypsin-EDTA solution. Trypsinisation was terminated by adding 8 ml of complete DMEM medium, followed by centrifugation at 1200 rpm for 5 minutes. Once cells were pelleted, the medium was aspirated and resuspended in fresh complete medium. Cell count was performed using a haemocytometer and seeded at a concentration of 2×10^5 cells /ml in various cell culture plates respectively.

2.2.1 Microglial conditioned medium

BV2 cells were seeded at a density of 2×10^5 cells/ml in a 6-well plate and incubated at 37°C. Once confluent, cells were pre-treated with tiliroside (2-6 µM) for 30 minutes and stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml) for 24 hours. Stimulation was terminated by removing supernatants (conditioned medium) from the cells and centrifuged at 2500 rpm for 5 minutes to remove cellular debris, aliquoted and then stored at -80°C.

2.2.2 Determination of HT22 cell viability using conditioned medium

The effect of conditioned medium obtained from microglia on the viability of HT22 cells was measured using the XTT assay (Invitrogen). BV2 cells were pre-treated with tiliroside (2-6 µM) for 30 minutes and stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml) for 24 hours. Stimulation was terminated by collecting conditioned medium from the cells and centrifuged and stored at -80°C. HT22 hippocampal neurons were seeded at a density of 2×10^5 cells /ml in 96-well cell culture plates and incubated at 37°C. When cells reach confluence, the culture medium was removed and replaced with 100 µl of conditioned medium and further incubated for 24 hours. Stimulation was terminated by adding 25 µl of XTT/PMS solution and incubated for 2 hours at 37°C, followed by gentle shaking for few seconds to distribute the orange colour before absorbance was read at 450 nm with a plate reader (Infinite F50, Tecan).

2.2.3 Measurement of ROS in neuronal cells

The effect of conditioned medium on levels of intracellular ROS in HT22 neuronal cells was investigated using DCFDA-cellular reactive oxygen species detection assay kit, (Abcam) that uses a cell permeable fluorogenic dye DCFDA. Conditioned medium was obtained from BV2 cells that were pre-treated with tiliroside (2-6 µM) for 30 minutes and stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml) for 24 hours. HT22 hippocampal neurons were seeded at a density of 2×10^5 cells /ml in 96-well cell culture plates and incubated at 37°C. After 48 hours, cells were washed with PBS and stained with 20 µM DCFDA followed by further incubation at 37°C for 30 minutes. After incubation, the cells were washed with PBS and thereafter treated with 200 µl of microglia conditioned medium and incubated for further 24 hours at 37°C. Intracellular production of ROS was measured by the fluorescence detection of dichlorofluorescein (DCF) using Polar star Optima microplate reader (BMG LABTECH) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.2.4 Immunofluorescence in HT22 neuronal cells

HT22 neuronal cells were seeded at a density of 2×10^5 cells /ml in 24 well plates. Once confluent, cells were treated with conditioned medium for 24 hours (for MAP2). Cells were then fixed with ice-cold methanol (100%) for 15 minutes at -20°C and later washed 3 times for 5 minutes with PBS. Non-specific binding sites were blocked by incubating cells with 5% BSA blocking solution (containing 10% horse serum) in 1X TBS-T) for 60 minutes at room temperature followed by washing with PBS. Thereafter, the cells were incubated with 1:100 dilution of rabbit anti-mouse MAP2 (Santa Cruz) antibody for overnight at 4°C. Following overnight incubation, cells were washed 3 times with PBS and incubated for 2 hours in dark with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Life Technologies) secondary antibody (1:500). Later, cells were washed with PBS and counterstained with 4', 6 diamidino-2-phenylindole dihydrochloride (50 nm, DAPI; Invitrogen) for 5 minutes. After rinsing cells with PBS, the excess buffer was removed and gold antifade reagent (Invitrogen) was added. All staining procedures were performed at room temperature. Representative fluorescence images were obtained using EVOS® FLoid® Cell imaging station (Invitrogen).

2.2.5 DNA fragmentation assay in HT22 neuronal cells

In neurodegeneration, inflammation-induced DNA fragmentation is considered as one of the important biomarkers that feature neuronal apoptosis (Park et al. 2000) and (Koh et al. 2008). Modulations of the chromatin DNA fragmentation in neuronal cells by means of compounds or treatment strategies have the potential to eliminate the neuroinflammation-associated toxicity. An ELISA based non-radioactive cellular DNA fragmentation assay kit (Roche Diagnostics) was used to measure the DNA fragmentation of HT22 neuronal cells treated with conditioned medium. The principle of this assay is based on a simple enzymatic reaction between the pre-coated immobilised anti-DNA antibody and 5-bromo-2'-deoxyuridine (BrdU) labelled DNA fragments in the supernatants. Firstly, cells are labelled with BrdU dye and later stimulated with conditioned medium. The cell-mediated cytotoxicity is mainly characterised by cleavage of the DNA into fragments because DNA cleavage is an important hallmark of apoptosis. Subsequently, cells that are pre-labelled with BrdU are lysed and resulting BrdU-complexed DNA fragments were analysed by ELISA. HT22 neurons were seeded at a density 2 x 10^5 cells /ml and allowed to become confluent. Thereafter, cells were labelled with 5-bromo-2'-deoxyuridine (BrdU) for 12 hours. Later, the BrdU-containing culture medium was removed and conditioned medium was added and incubated for 24 hours. Next day, cells were collected and centrifuged at 2500 rpm for 10 minutes and supernatants were discarded. Later, cells were lysed by adding 200 µl of incubation buffer (a non-ionic detergent) and incubated for 30 minutes at room temperature, followed by centrifugation at 2500 rpm for 10 minutes. 100 µl of the supernatants were pipetted into anti-DNA pre-coated 96-well plate and incubated for 90 minutes at room temperature. Contents of the plate were removed and washed three times with 250 µl of wash buffer. Later, 100 µl/well of exonuclease III solution was added and further incubated at 37°C for 30 minutes. The solution in the plate was discarded and washed for three times with 250 µl of wash buffer, followed by addition of anti-BrdU peroxidase conjugate solution and incubated for 90 minutes at room temperature. Contents of the plate were emptied and washed. Later, 100 µl of substrate solution was added and incubated in the dark for 30 minutes. The reaction was terminated by adding 25 µl of stop solution and absorbance was measured at 450nm using microplate reader (Infinite F50, Tecan).

2.2.6 Calcium quantification in HT22 neuronal cells

Calcium is an essential intracellular messenger that mediates several signalling processes in all cell types of the CNS. High intracellular levels of calcium were observed in adjacent neurons during neuroinflammatory conditions which could damage DNA, cell membranes, enzyme dysfunctions and could lead to neuronal death (Ceulemans et al. 2010). Levels of calcium in the cell cytoplasm were measured using Calcium Detection assay kit (Abcam). Calcium ions in the sample react with the o-cresolphthalein reagent to form a chromogenic complex with a colour change that can be measured at a wavelength of 575 nm. HT22 neuronal cells were seeded into 24-well at a density of 2 x 10^5cells/ml and allowed to settle. At confluence, the medium was replaced with conditioned medium as explained previously and incubated for 24 hours at 37°C. Later, lysates were collected using RIPA lysis buffer followed by centrifugation at 4°C, 13500 rpm for 15 minutes. Fifty microliters of the lysates were pipetted into 96-well plate followed by 90 µl of chromogenic reagent along with 60 µl of calcium assay buffer, incubated in the dark for 15 minutes at room temperature. Output was measured using Tecan F50 microplate reader at a wavelength of 575 nm. Concentrations of calcium in the cytoplasmic lysates were calculated by comparing to the standard curve (0-2.0 µg/ml).

2.2.7 Statistical analysis

Statistical analysis was done by one-way analysis of variance (ANOVA) with post-hoc Student-Newman-Keuls test (multiple comparisons). The ANOVA compares the mean between the groups and will determine whether those means are significantly different from each other. To determine the specific groups that are significantly different from each other were evaluated by using post-hoc Student-Newman-Keuls test with multiple comparisons. Calculations for statistical analysis were performed with Graph Pad Prism software version 5 (San Diego, US). Differences were considered significant at $p < 0.05$. For neuroinflammation experiments designations include: $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 in comparison with LPS/IFNγ control.

For siRNA experiments: $^{\theta}p< 0.05$, $^{\theta\theta}p<0.01$, $^{\theta\theta\theta}p<0.001$ in comparison within the groups of the untreated control. $^{\$}p< 0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ as compared within the groups stimulated with LPS/IFNγ and $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ as compared

within the groups pre-treated with tiliroside (6 µM). At least three independent experiments were performed for analysis. Where necessary original data were converted into % values of control or LPS/IFNγ and mean ± S.E.M. was calculated.

Chapter 3. Tiliroside inhibited LPS/IFNγ-induced neuroinflammation in BV2 microglia

3.1 Introduction

Neuroinflammation is a defence mechanism aimed at protecting against pathogens and infections in the central nervous system (CNS) through activation of microglia. Excessive neuroinflammatory responses in the microglia have been shown to cause neuronal damage in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) (Lehnardt 2010). Activated microglia secretes a variety of pro-inflammatory cytokines, neurotoxic factors, reactive oxygen mediators and arachidonic acid derivatives, which are considered to be a major hallmark of brain inflammation, and plays a key role in regulating neuroinflammatory responses (Ray & Lahiri 2009) and (Lyman et al. 2013).

In general, brain inflammation may be divided into three phases, (1) acute phase (2) chronic and (3) resolution phase. The acute phase is an initial stage where ramified microglia is found at resting state with a small static cell body and processed branches that actively monitor their microenvironment for pathogens or infections in the brain. Cell wall components, DNA/RNA of the bacteria or pathogens, damaged host cells and heat shock proteins are recognised by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), NOD-like receptors (NLRs) on microglia. Once these receptors are activated, microglia produces several pro-inflammatory cytokines such as IL-1β, TNF α and IL-6 via different intracellular signalling cascades. This activation is also accompanied by partial rounding up and mobility of adjacent glial cells and astrocytes that ultimately creates a vicious cycle by releasing various neurotoxic factors. Microglia at this phase is termed as chronically active. The resolution phase, where microglia exhibit amoeboid, highly phagocytic, and produce anti-inflammatory cytokines such as IL-4, IL-6, IL-10, IL-11, IL-13 and TGFβ, in order to resolve the local inflammation and clear up the bacteria or pathogens (Morales et al. 2014), (Opal & DePalo 2000) and (Halliday et al. 2000). However, in neuroinflammatory conditions, microglia become hyperactive and release excess neurotoxic factors in the brain. For instance, increased microglial activation was observed in early stages of AD and PD (Vehmas et al. 2003) and (Krause & Muller

2010). Therefore, therapies that target microglial activation and its mediated neuroinflammatory factors gained a decent attention in recent years.

Hyperactive microglia facilitates the activation of several intracellular signalling pathways; however in-depth understanding of these pathways is very limited. Prostaglandin E2 (PGE_2) is an arachidonic acid derivative, the production of which is catalysed by cyclooxygenase enzymes in activated microglia. Cyclooxygenases are two subtypes, cyclooxygenase-1 (COX-1) and cycloxygenase-2 (COX-2). COX-1 is constitutively expressed in most cell types, whereas expression of COX-2 is induced by various factors including inflammatory cytokines, and is mainly responsible for the production of PGE_2 (Lima et al. 2012). Sustained upregulation of COX-2 and its mediated PGE_2 in microglia contributes to the progressive damage in neurodegenerative diseases. In cultured rat brain microglia, bacterial lipopolysaccharide (LPS) has been shown to induce COX-2 expression, and this is prevented in the presence of inhibitors of NF-κB (Zhou et al. 2014). Also, COX-2 and PGE_2 are highly expressed in glial cells of substantia nigra of post-mortem AD patients, and specific inhibitors of COX-2 has been found to exert neuroprotective effects (Peterson & Toborek 2014) and (Knott et al. 2000). Recent epidemiological studies clearly suggest that COX inhibitors reduce the risk of AD and PD (Krause & Muller 2010). Therefore, inhibition of COX-2 and its mediated PGE_2 production would be an effective therapeutic approach in alleviating the progression of neuroinflammation.

In addition to PGE_2, the cellular messenger, nitric oxide (NO) has been widely implicated in neuroinflammation and neurodegenerative processes. Elevated levels of NO have been reported in both microglia and astrocytes, which are activated during the neuroinflammatory response (Morales et al. 2014). A significant body of evidence also suggests that the release of large amounts of NO from activated astrocytes and microglia, mediated by the inducible nitric oxide synthase (iNOS) enzyme, is important in the pathogenesis of neurodegenerative disorders. Research evidence suggests that iNOS is not commonly expressed in brain cells. However, inflammatory mediators such as LPS and cytokines can induce its expression (Brown & Neher 2010) and (Ghasemi & Fatemi 2014). High levels of NO have been shown to induce neuronal toxicity by causing mitochondrial depolarisation by targeting cytochrome oxidase in neurons. This further facilitates glutamate release

from neuronal cells and causes excitotoxicity in adjacent astrocytes via calcium release from intracellular stores, thus inducing toxicity in both neurons and astrocytes in the brain (McGeer & McGeer 2015) and (Spencer et al. 2012). Also, this is evident in the induction of neuronal death through both necrotic and apoptotic pathways (Doherty 2011). Consequently, both COX-2-mediated PGE_2 and iNOS-mediated NO production in the microglia remain important targets for reducing neuroinflammation in the microglia.

Along with COX-2 and PGE_2, activated microglia release a variety of proinflammatory cytokines such as IL-1β, TNF α, IL-6 and low levels of anti-inflammatory cytokines like IL-10, TGF-β and IL-1 receptor antagonist (IL-1Ra) during chronic inflammatory conditions in neurodegenerative diseases. Pathogen components such as LPS can readily induce IL-1β, TNF α and IL-6 in both rodent models and cultured microglial cells (Kim & Joh 2006), (Morales et al. 2014) and (Olajide et al. 2013). In particular, IL-1β and TNFα have been shown to be involved in the development of CNS inflammation through the disruption of BBB, which further facilitates the infiltration of peripheral immune cells such as leukocytes and other foreign bodies into CNS. Also, recent studies on TNFα overexpressing mice demonstrated that TNFα might directly initiate brains tissue destruction via autoimmune inflammation (Akassoglou et al. 1998) and (Park & Bowers 2010). Together these cytokines play a critical role in neuronal damage in various neurodegenerative disorders. Therefore, the inhibition of these inflammatory mediators is essential in preventing escalation of the inflammatory process in microglia. On the other hand, anti-inflammatory cytokine IL-10 inhibits microglia activation through modulation of pro-inflammatory cytokines, chemokines and oxidative species in microglia. Thus substances activating IL-10 would be beneficial in targeting neuroinflammatory conditions (Heyen et al. 2000).

During neuroinflammation, the transcription factor, nuclear factor-κB (NF-κB) has been shown to regulate cytokines production, COX-2 and iNOS-mediated NO and PGE_2 production, respectively. A diverse spectrum of activators such as LPS, ROS and heat shock proteins, has been shown to activate this pleiotropic transcription factor, which further facilitates the transcription of inflammatory genes in neuroinflammation (Bozic et al. 2015), (Cuaz-Pérolin et al. 2008) and (Olajide et al. 2013). In general, mammalian NF-κB consists of five subunits, which can form homo

and heterodimers in any combination; RelA (p65), RelB, c-Rel, p50 and p52. As each of these proteins consists of a prototypical amino-terminal sequence known as Rel homology domain (RHD), that facilitates cytoplasmic retention by IκB, DNA-binding and nuclear localisation. In neuroinflammation, specific phosphorylation and degradation of IκB are critical in regulating NF-κB DNA-binding activity in brain cells. The cytosomal IκB binds to the nuclear localisation signal (NLS) of NF-κB to form an inactive complex (Ghosh & Hayden 2012). However, upon stimulation, intracellular signalling cascades phosphorylates IKK complex, which mediates phosphorylation of IκB at specific amino acid residues. For instance, Serine-32 and Serine-36 in IκB. This phosphorylation of IκB further liberates NF-κB for nuclear translocation and bind to related sites in inflammatory target genes (Didonato et al. 2012). In the nucleus, NF-κB has to undergo several post-translational modifications to regulate the transcription of pro-inflammatory genes to become fully active. One such modification is reversible acetylation of NF-κB-p65 subunit. Studies have shown that compounds that inhibit acetylation of NF-κ-p65 blocked the production of various inflammatory cytokines that are controlled by NF-κB signalling (Chen & Greene 2005) and (Zhu et al. 2011). Therefore, it is crucial to investigate for the compounds that target NF-κB signalling pathway, which is essential in controlling the escalation of the inflammatory process during neuroinflammation in microglia.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases which are critical in regulating the production of inflammatory mediators (Fyhrquist et al. 2010). Pro-inflammatory cytokine-driven neuroinflammation is a persistent pathological hallmark in several neurodegenerative diseases. The MAPKs have been shown to be critical regulators of various inflammatory cytokines in the neurodegenerative process. Four types of MAPKs are identified in mammalian cells: the extracellular signal-related kinases (ERKs), the c-jun N-terminal kinases (JNKs), the atypical MAPKs (ERK3, ERK5, and ERK8), and the p38 MAPKs.(Cuadrado et al. 2010) and (Neininger et al. 2002). Among these, the p38 MAPK has been shown to play a central role in mediating the production of the pro-inflammatory cytokines and other neurotoxic factors (Culbert et al. 2006). In the light of neuroinflammation, the general role of p38 MAPK was extensively studied in external stimuli-treated glia and blocking of this kinase expression has shown to resolve microglial activation (Xing et al. 2011a) and (Kim et al. 2004). Studies demonstrate that, in brain cells, p38 MAPK

has to undergo dual phosphorylation at Thr-180 and Tyr-182 residues by MAP kinase kinase 3/6 (MKK 3/6) (Herlaar & Brown 1999). In the cultures of microglia, astrocytes and neurons, activation of p38 MAPK were induced through upstream activation of MKK3/6, which further produced cytokines and other neurotoxic factors respectively (Kim et al. 2004). Activation of p38 MAPK signalling and production of its mediated IL-1β and other cytokines was observed in Aβ-stimulated microglia and neuroblastoma cell cultures. Similar observations were noted in other animal models of neuroinflammation when treated with LPS (Savage et al. 2002), (Haddad et al. 2001), (Olajide et al. 2014) and (Corrêa et al. 2012).

Also, in response to extracellular stress, phosphorylated p38 shown to activate mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK-2) in the microglia (Duraisamy et al. 2008). MK-2 is one of the several kinases that are regulated by p38 MAPK, which makes it an important target in neuroinflammatory and neurodegenerative pathologies. The activation of MK-2 was increased in microglial cells when stimulated with LPS and gamma interferon, resulting in the expression and production of inflammatory mediators such as IL-1β, TNF α, IL-6, NO, iNOS and PGE_2 (Ramanan et al. 2008) and (Culbert et al. 2006). Mice lacking MK-2 gene showed low levels of TNFα and other cytokines when exposed to endotoxic shock, supports that MK-2 is involved in the production of cytokines during inflammation (Gaestel et al. 1999) and (Fyhrquist et al. 2010). Thus, there is a desperate need to investigate for compounds that inhibit p38 MAPK signalling and its associated inflammatory cytokines in neuroinflammation.

Toll-like receptor 4 (TLR4) signalling has been well established in the pathologies of neuroinflammation. During innate immunity, TLR4 receptor modification has been identified to play a central role in the activation of various intracellular signalling pathways (Hanamsagar et al. 2012). The stimulation of TLR4 by external stimuli, such as LPS, activates NF-κB and p38 MAPK signalling pathways, leading to the production of critical pro-inflammatory cytokines that are necessary to induce potent immune responses (Nguyen et al. 2013) and (Park et al. 2011). Also, studies from the researchers confirmed that, blocking LPS-induced TLR4 activation in the glial cells resulted in the significant inhibition of neuroinflammation (Nguyen et al. 2013),(Lei et al. 2015) and (Takeda & Akira 2004). Also, evidence from the literature demonstrates the involvement of tumour necrosis factor-receptor-associated factor 6

(TRAF 6) in the induction of pro-inflammatory cytokines triggered by TLR4 activation. TRAF 6 has been shown to mediate the activation of p38 MAPK and NF-κB signalling via TGF-β-activated kinase 1 (TAK1) (Maeda 2010). For this reason, inhibiting the activation of TLR signalling is thought to be an effective strategy in neuroinflammation and its associated neurological disorders.

In activated microglia, NF-κB signalling has to undergo several post-translational modifications to regulate the transcription of pro-inflammatory genes. One such modification is reversible acetylation of NF-κB-p65 subunit. Studies have shown that sirtuins negatively regulate NF-κB signalling via deacetylation of Lys310 residue of RelA/p65 subunit (Chen et al. 2005) and (Jieming Ye et al. 2013). Sirtuins belong to class III histone deacetylases (HDACs), which plays a significant role in regulating ageing, inflammatory diseases, and senescence by targeting histones, coregulators and inflammatory transcription factors like NF-κB, p53 and Nrf2 in brain cells (Haigis & Sinclair 2011) and (Kim et al. 2007). Among seven sirtuins, SIRT1 has been reported to be involved in promoting longevity in various species. Thus activation of SIRT1 inhibits NF-κB signalling by promoting deacetylation of the p65 subunit. Also, reports have shown that inhibition of SIRT1 protein expression by LPS in endothelial cells significantly increased the expression of acetylated-NF-κB-p65 and further enhanced NF-κB transcriptional activity (Yang et al. 2012). Moreover, microglia treated with LPS has significantly increased the production of inflammatory cytokines like IL-1β and TNFα, coinciding with the inhibition of SIRT1, suggesting that SIRT1 is involved in regulating pro-inflammatory cytokines (S. H. Cho et al. 2015) and (J Ye et al. 2013). Thus, there is an urgent need to investigate for compounds that activate SIRT1 expression in neuroinflammation driven neurological diseases.

Reactive oxygen species (ROS) are produced for the maintenance of many physiological functions and act as second messengers. Accumulating evidence has suggested that the pathogenesis of neurodegenerative disorders, including AD, is related to excessive production of ROS and the resultant increased oxidative stress (Zhang et al. 2016). However, the exact role of redox signalling cascade and its associated crosstalk with inflammatory mediators during neuroinflammation is yet to be understood. Increased ROS production has been shown to control the expression of several critical inflammatory mediators such as COX-2, iNOS and the adhesion

molecules induced by cytokines, infections and neurotoxic substances during neuroinflammation (Lee & Yang 2012), (Hsieh & Yang 2013) and (Gu et al. 1998). Alternatively, there is increasing evidence that ROS triggers the activation of redox-sensitive transcription factors such as NF-κB and AP-1 in the microglia (Zhang et al. 2016) and (Lee et al. 2015). Recently, Kim and his team demonstrated that apocynin, a known NOX inhibitor showed potent antioxidant activities and inhibitory effects on TNFα-induced NF-κB and AP-1 signalling in animal models of asthma (Kim et al. 2012). In addition, several other researchers showed that blocking ROS production in LPS-induced *in vitro* models will reverse detrimental pathologies involved in neuroinflammation (Lee & Yang 2012), (Wang et al. 2011), (Lee et al. 2015) and (Zeng et al. 2012). Depletion of endogenous levels of GSH was also observed during oxidative stress which is implicated in the pathogenesis of neuroinflammation and associated neurotoxicity (Lee et al. 2010). Animal studies showed the unique capacity of GSH to preserve, protect and rescue human microglial and neuronal cells from cytokines induced neurotoxicity (Zhu et al. 2014). Therefore, targeting ROS production and GSH activation in microglia would be an efficient therapeutic strategy for the treatment of neuroinflammation and its associated neurotoxicity.

Tiliroside is a glycosidic flavonoid found in several medicinal and dietary plants, such as linden, rose hip and strawberry (Matsuda et al. 2002). This compound has been shown to exhibit anti-inflammatory, antioxidant, anticarcinogenic, and hepatoprotective activities (Tsukamoto et al. 2004). However, the anti-neuroinflammatory effects of the compound are not yet studied; therefore the effects of tiliroside are investigated in LPS/IFNγ-induced neuroinflammation in BV2 microglia. Further experiments were done to assess the effects of the compound on SIRT1 activation in BV2 microglia.

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3.2 Results

3.2.1 Tiliroside reduced production of pro-inflammatory cytokines in LPS/IFNγ-activated BV2 microglia without affecting the BV2 cell viability at the concentrations used in pharmacological experiments

The cytotoxic effect of various concentrations of tiliroside (2-6 μM) was determined using XTT assay. BV2 microglia were pre-treated with the compound for 30 minutes before stimulation with a combination of LPS (100 ng/ml) and IFNγ (5 ng/ml) for 24 hours. Results showed that there was no significant difference in the viability of cells treated with various concentrations of tiliroside when compared with control (untreated) cells (Figure 3.1).

Hyperactive microglia are known to release pro-inflammatory cytokines such as IL-1β, TNF α and IL-6, which are considered as most significant neurotoxic factors that contributes to neuroinflammation (MacEwan 2002). Therefore, cytokines production in the activated microglia remains an important target in reducing neuroinflammation. The levels of IL-1β, TNF α and IL-6 in the supernatants were analysed using ELISA. Upon stimulation with LPS (100 ng/ml)/IFNγ (5 ng/ml), a marked increase ($p<0.001$) in the release of IL-1β (~7-fold increase, ~900 pg/mL), TNF α (~45-fold increase, ~ 950pg/mL) and IL-6 (~6-fold increase, ~890 pg/mL) was observed in microglia when compared to untreated cells. However, tiliroside (2 μM, ~1.3-fold [~800 pg/mL]; 4 μM, ~ 1.4-fold [~750 pg/mL]; 6 μM, ~ 1.8-fold [~450 pg/mL] reduction) significantly ($p<0.01$) inhibited IL-1β production in the activated microglia. In addition, the compound reduced the release of TNF α (4 μM, ~ 1.4-fold [~760 pg/mL]; 6 μM, ~ 1.7-fold [~600 pg/mL] reduction) ($p<0.001$) in the microglia (Figure 3.2 a and b). However, at 2 μM, the inhibitory effect of tiliroside on TNF α production was not significant. As expected, tiliroside exhibited a similar effect on IL-6 levels when microglia were pre-incubated with the compound (2 μM, ~1.3-fold [~850 pg/mL]; 4 μM, ~ 1.4-fold [~780 pg/mL]; 6 μM~1.6-fold [~600 pg/mL] reduction $p<0.01$) (Figure 3.2c). These results indicate that selected concentrations of tiliroside reduced LPS/IFNγ-induced proinflammatory cytokines released by microglia.

Several reports have shown that the combination of LPS and IFNγ is capable of inducing pro-inflammatory cytokines along with other chemokines via activating toll-like receptors in microglia (TLRs) (Xu et al. 2014). However, the precise effective

concentrations that can induce pro-inflammatory parameters in the microglia are yet to be evaluated. In the current research, the effective concentrations of LPS and IFNγ were determined using XTT cell viability assay. BV2 microglia were stimulated with various concentrations of LPS and IFNγ and incubated for 24 hours. Results revealed that the combination of LPS at 100 ng.ml and IFNγ 5ng.ml exhibited maximal cytotoxic effects compared to other concentrations (Figure 3.3a). The incubation time of the tiliroside with BV2 cells was assessed using XTT assay. Figure 3.3b shows that tiliroside (6µM) at 30 minutes in the presence of LPS (100 ng/ml) and IFNγ (5 ng/ml) was less cytotoxic compared to other time points. Therefore, the incubation time of 30 minutes was adopted in all neuroinflammation experiments.

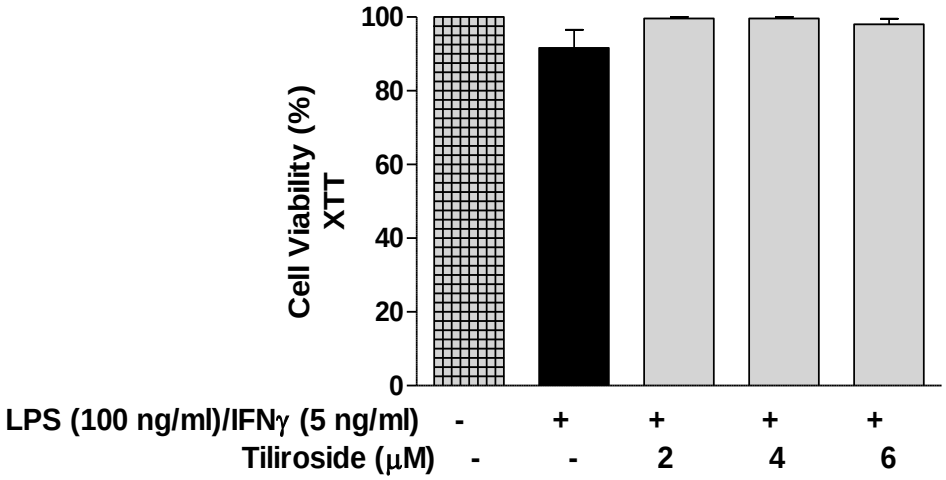
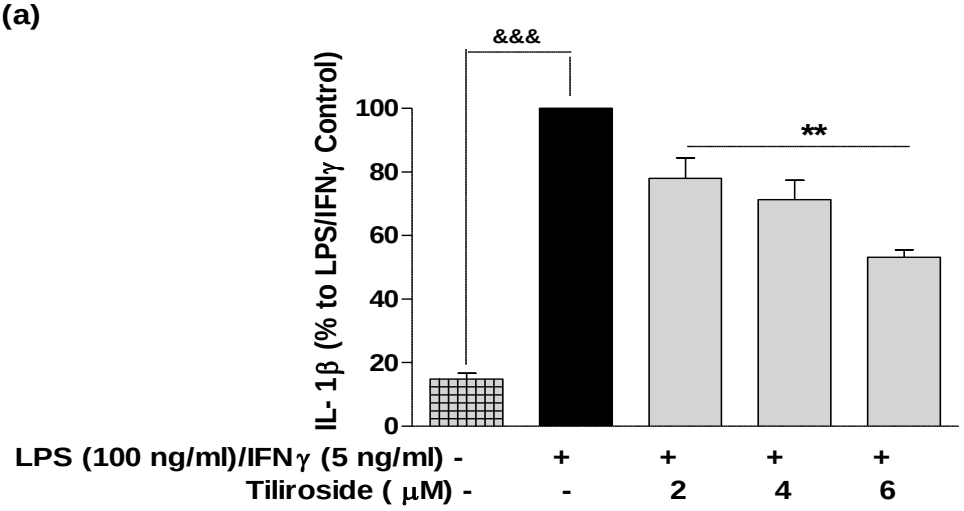


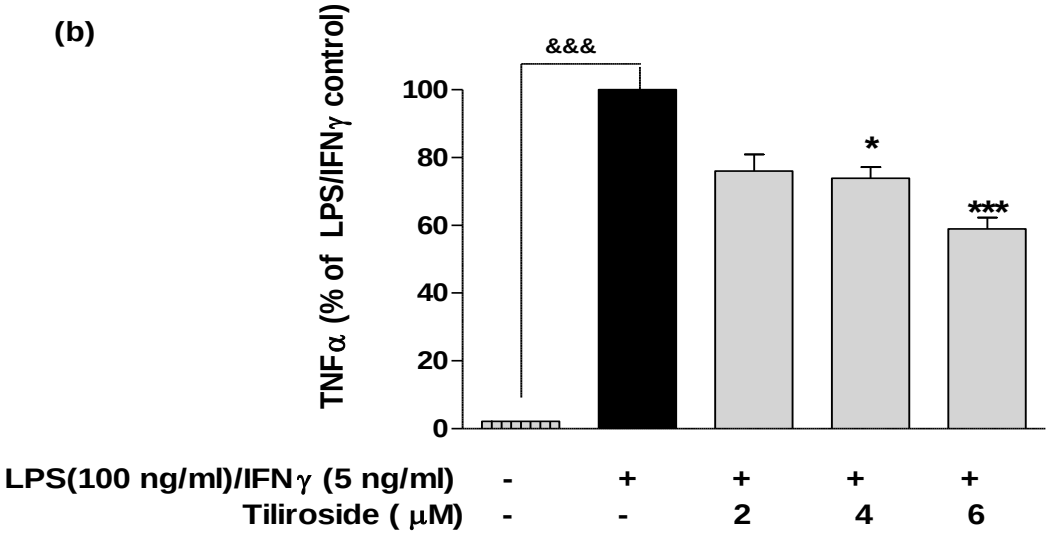
Figure 3.1 Pre-treatment with tiliroside did not affect the viability of BV2 microglia

Cells were stimulated LPS/IFNγ in the presence or absence of tiliroside (2-6 μM) for 24 hours. At the end of the incubation period, XTT assay was carried out on cells. Results show that tiliroside did not affect the viability of microglia cells. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p < 0.05, **p<0.01, ***p<0.001 compared with LPS/IFNγ control.

(a)


&&&
**
IL- 1β (% to LPS/IFNγ Control)
100
80
60
40
20
0
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

(b)


&&&
*
***
TNFα (% of LPS/IFNγ control)
100
80
60
40
20
0
LPS(100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

(c)

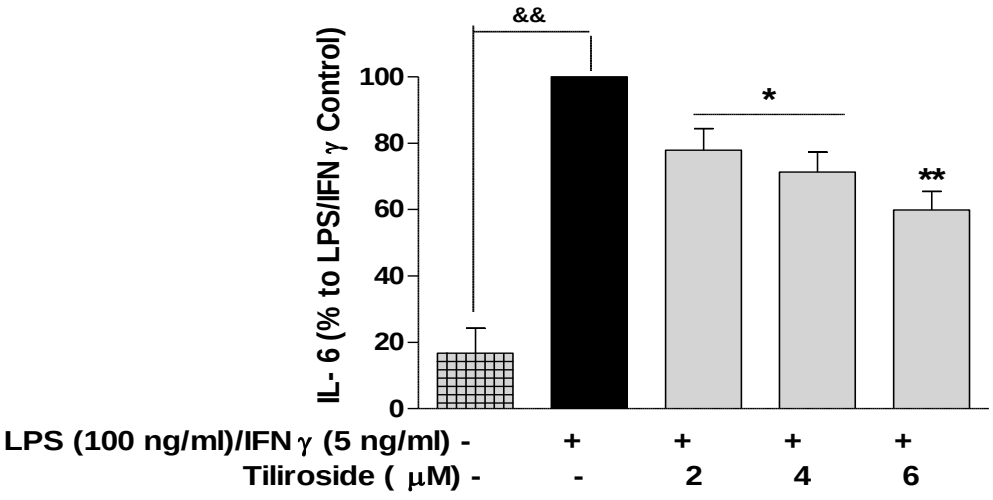
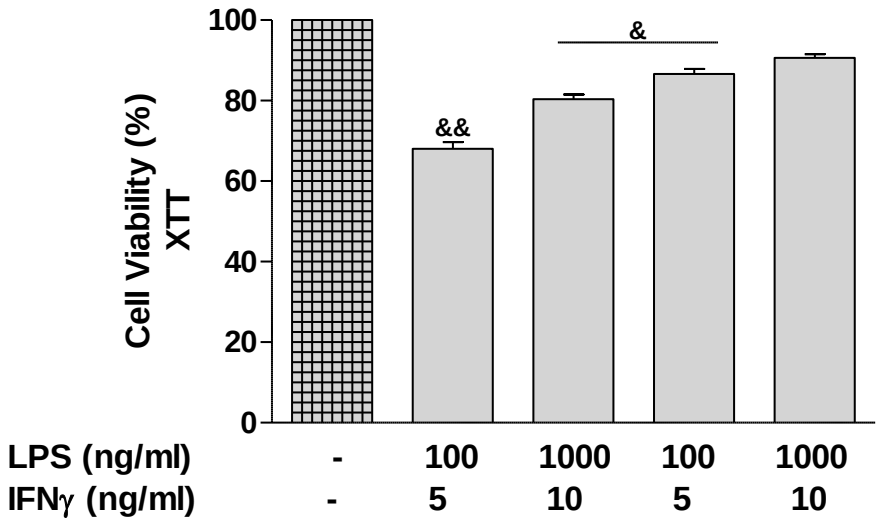

&&
*
**
IL- 6 (% to LPS/IFNγ Control)
100
80
60
40
20
0
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

Figure 3.2 Tiliroside reduced IL-1β, TNFα and IL-6 production in LPS/IFNγ-activated BV2 microglia.

Cells were stimulated with LPS/IFNγ in the presence or absence of tiliroside (2–6 μM) for 24 hours. Stimulation was terminated, and supernatants were collected and levels of (a) IL-1β, (b) TNF-α and (c) IL-6 were measured using ELISA. Tiliroisde inhibited the production of IL-1β ,TNF-α and IL-6 in LPS/IFNγ-stimulated BV2 cells. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to LPS/IFNγ.

(a)



(b)

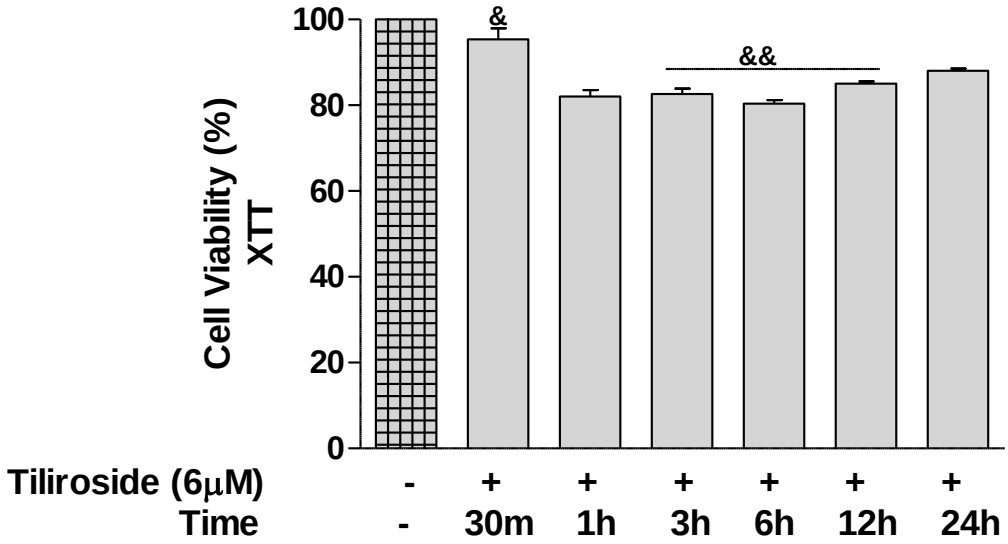


Figure 3.3 Pre-tretament with tilrioside (6μM) did not effect the viability of BV2 cells at 30 min when stimulated with LPS and IFNγ.

(a) BV2 cells were stimulated with various concentrations of LPS and IFNγ and incubated for 24 hours. Stimulation was terminated by adding 25 µl of XTT/PMS solution. Results show that the combination of LPS (100 ng/ml) and IFNγ (5 ng/ml) were cytotoxic. (b) BV2 microglia were incubated with tiliroside (6 µM) for various time points followed by stimulation with the combination of LPS (100 ng/ml) and IFNγ (5 ng/ml). Cell vibility assay revelaed that tilroside at

30 min did not effect the vaibility of Bv2 cells compared to other time points. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and $*p<0.05$, $**p<0.01$, $***p<0.001$ compared to LPS/IFNγ.

3.2.2 Tiliroside increased the production of IL-10 in LPS/IFNγ-activated BV2 microglia

In chronic neuroinflammation, prolonged exposure to inflammatory stimuli leads to increased inflammatory cytokines and inhibition in the production of anti-inflammatory cytokines like IL-10 and TGFβ was observed (Ha et al. 2012). Since tiliroside has blocked the production of cytokines, the effect of the compound on IL-10 production was further evaluated. Cells were pre-incubated for 30 minutes with tiliroside (2-6 μM) and then stimulated with LPS/IFNγ for 24 hours. Thereafter, IL-10 production was determined using ELISA. Stimulation with a combination of LPS and IFNγ significantly ($p<0.001$) reduced IL-10 levels (~3.4-fold reduction, ~200 pg/mL) compared to unstimulated microglia. Interestingly, tiliroside (2 μM, ~1.3-fold [~300 pg/mL], 4 μM, ~2-fold [~450 pg/mL] and 6 μM, ~3.3-fold [~800 pg/mL] increase) significantly ($p<0.001$) and dose-dependently reversed LPS/IFNγ-mediated IL-10 reduction in the microglia (Figure 3.4).

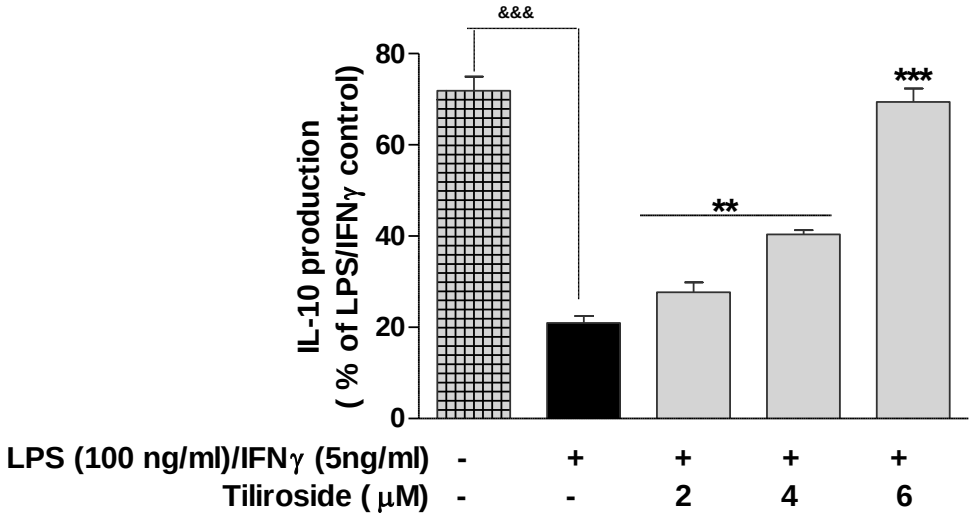


Figure 3.4 Tiliroside increased IL-10 production in LPS/IFNγ-activated BV2 microglia

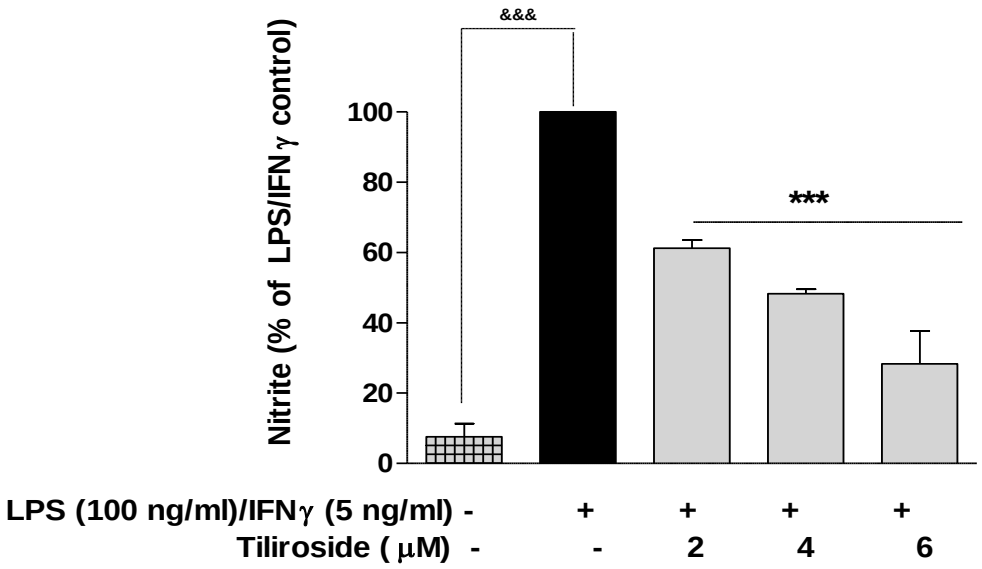
Cells were stimulated with a combination LPS/IFNγ in the presence or absence of tiliroside for 24 hours. At the end of the incubation, supernatants were collected, and levels of IL-10 were measured using ELISA. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

3.2.3 Suppression of iNOS protein expression and its mediated nitrite production by tiliroside in LPS/IFNγ-activated BV2 microglia

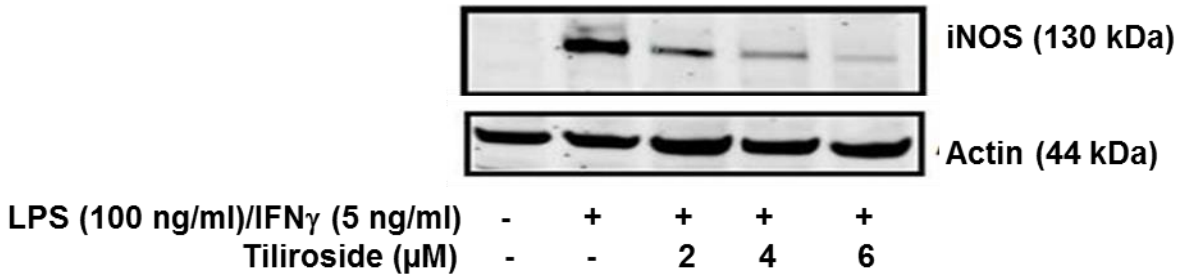
This experiment was conducted to investigate the effects of the compound on iNOS protein expression and it's mediated NO release in LPS/IFNγ-stimulated microglia. Nitric oxide (NO) is an important mediator for regulating chronic inflammation in microglia and high concentrations of NO has been shown to be neurotoxic to adjacent neurons (Cao et al. 2010) and (Spencer et al. 2012). Therefore, the effect of tiliroside (2-6 μM) on nitrites, a breakdown product of NO, was assessed using Griess assay. Results show that there was a marked increase (~13-fold, ~90 μM]) in nitrite production ($p<0.001$) when cells were stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml) alone, compared with unstimulated cells. However, pre-treatment with tiliroside (2 μM, ~1.6-fold [~60 μM], 4 μM, ~2.1-fold [~55 μM] and 6 μM, ~3.6-fold [~30 μM] reduction) significantly inhibited nitrite production (Figure 3.5a).

In activated microglia, NO production is directly proportional to the expression of iNOS protein. Encouraged by the nitrite results, further investigation was done to assess the effect of the compound on iNOS protein expression. BV2 microglia were pre-incubated with tiliroside for 30 minutes, followed by stimulation with LPS/IFNγ for 24 hours. Cytoplasmic lysates were collected and subjected to western blotting. Results showed that tiliroside (4 μM~1.5-fold and 6 μM~1.8-fold reduction) produced significant ($p<0.001$) suppression of iNOS protein expression (Figure 3.5b), however, at 2 μM, compound exhibited a sharp inhibition of iNOS (2 μM~1.1-fold reduction), which was not significant. These results indicate that tiliroside regulated NO production in LPS/IFNγ-stimulated BV2 cells through inhibition of iNOS protein expression.

(a)


&&&
***
Nitrite (% of LPS/IFNγ control)
100
80
60
40
20
0
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

(b)


iNOS (130 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

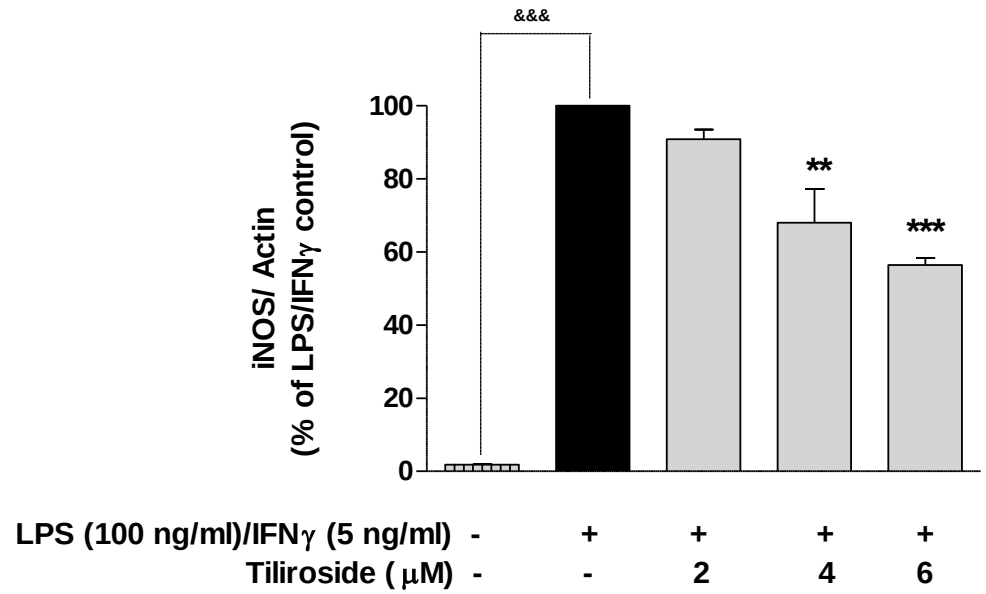


Figure 3.5 Tiliroside inhibited nitrite production and iNOS protein expression in LPS/IFNγ-activated microglia.

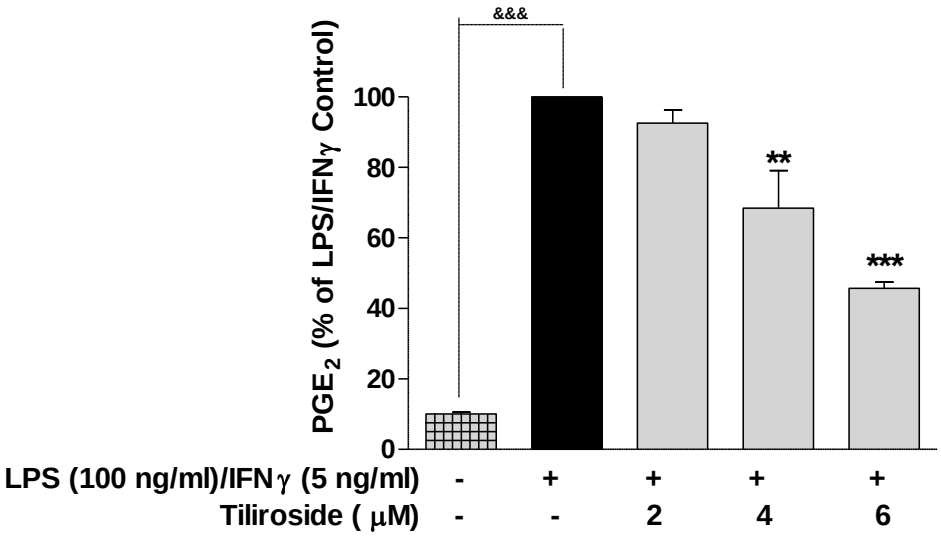
Cells were stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml) in the presence or absence of the compound for 24 hours. Subsequently, culture supernatants and cytoplasmic lysates were collected and analysed for nitrite production and iNOS protein expression. (a) Tiliroside reduced NO production in LPS/IFNγ-activated BV2 cells. (b) Tiliroside inhibited levels of iNOS protein in LPS/IFNγ-activated BV2 cells. Cytoplasmic lysates were analysed for iNOS protein expression using western blot. Actin was used as a loading control. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

3.2.4 Tiliroside suppressed PGE_2 production by inhibiting COX-2 protein expression in LPS/IFNγ-activated BV2 microglia

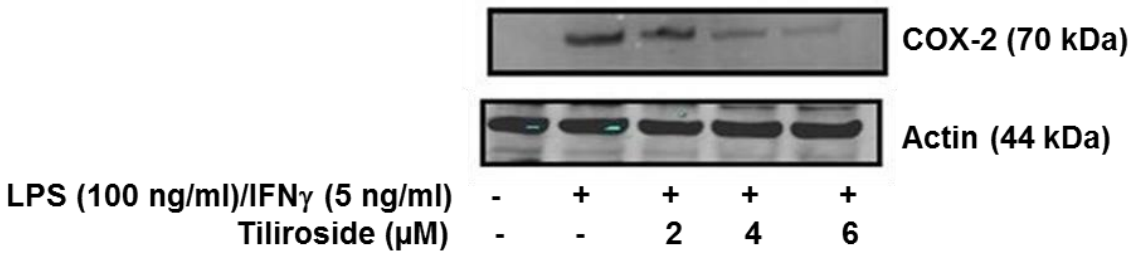
Encouraged by results showing the effects of tiliroside on iNOS expression and it's associated NO production, its effects on PGE_2 and COX-2 expression in the activated microglia were further assessed. In neuroinflammation, uncontrolled activation of microglia are shown to produce high levels of neurotoxic factors like COX-2-mediated PGE_2 (Dargahi et al. 2011). As shown in Figure 3.6a, LPS/IFNγ-treated BV2 cells produced detectable levels of PGE_2 (~10-fold increase, ~950 pg/mL) compared to the untreated microglial cells. Tiliroside produced a significant ($p<0.001$) reduction of PGE_2 production by ~1.5-fold (~690 pg/mL) at 4 µM and at 6 µM by ~ 2.2-fold (~400 pg/mL) in a concentration-dependent manner. However, at 2 µM (~ 1.1-fold [~850 pg/mL] reduction) the inhibition was not significant.

To understand the mechanism further, the effect of tiliroside on COX-2 expression was investigated. Results in Figure 3.6b show marked expression of the COX-2 protein (~63.1-fold increase) in LPS/IFNγ stimulated cells compared to untreated microglia. This effect was significantly ($p<0.001$) counteracted through pre-incubation with compound (2 µM, ~1.3-fold, 4 µM, ~2-fold and 6 µM, 2.7-fold reduction). These results suggest that tiliroside inhibits LPS/IFNγ-induced PGE_2 production by inhibiting COX-2 protein expression in BV2 microglia.

(a)


&&&
PGE2 (% of LPS/IFNγ Control)
100
80
60
40
20
0
**
***
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

(b)


COX-2 (70 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

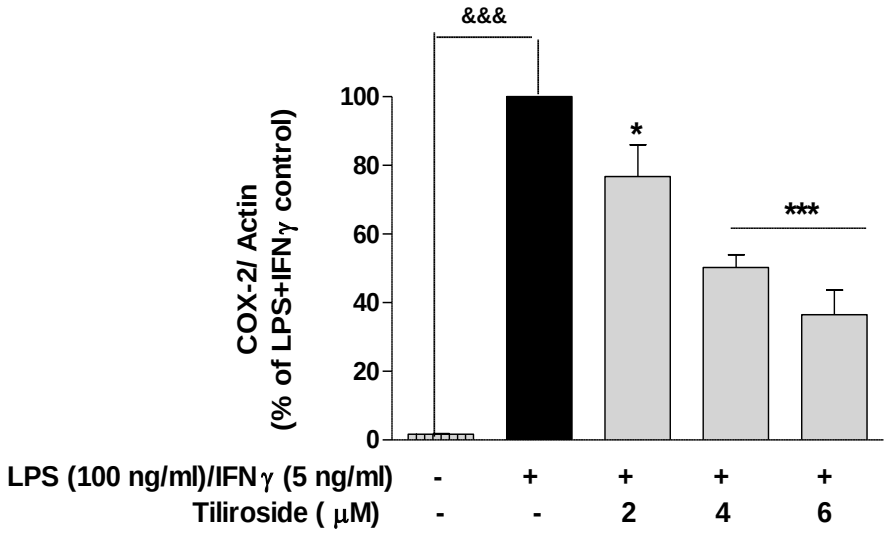

&&&
COX-2/ Actin
(% of LPS+IFNγ control)
100
80
60
40
20
0
*
***
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

Figure 3.6 Tiliroside inhibited PGE_2 production and COX-2 protein expression in LPS/IFNγ-activated microglia.

Cells were stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml) in the presence or absence of the compound for 24 hours. Subsequently, culture supernatants and cytoplasmic lysates were collected and analysed. (a) Tiliroside reduced PGE_2 production in LPS/IFNγ-activated BV2 cells. (b) Tiliroside inhibited levels of COX-2 protein in LPS/IFNγ-activated BV2 cells. Cytoplasmic lysates were analysed for COX-2 protein expression using western blot. Actin was used as a loading control. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and $*p<0.05$, $**p<0.01$, $***p<0.001$ compared to LPS/IFNγ.

3.2.5 Tiliroside inhibited LPS/IFNγ-induced neuroinflammation through NF-κB signalling in BV2 microglia

The transcription factor NF-κB, plays a central role in regulating the production of proinflammatory cytokines and the expression of genes like COX-2 which mediates PGE_2 production and iNOS which control nitrite production in stimulated microglia (Ghosh & Hayden 2012). Since tiliroside inhibited the release of pro-inflammatory cytokines and levels of iNOS and COX-2 proteins, its effects on NF-κB signalling was examined in BV2 microglia. Firstly, a luciferase reporter gene assay was used to evaluate the effect of the compound on nuclear transactivation of NF-κB in microglia. BV2 cells were transfected with a vector containing NF-κB regulated luciferase reporter construct. The experiment revealed that tiliroside (2 μM, ~1.1-fold, 4 μM, ~1.7-fold and 6 μM ~3.8-fold reduction) significantly ($p<0.001$) inhibited NF-κB regulated luciferase reporter gene expression following stimulation with LPS/IFNγ (Figure 3.7a). Once it was confirmed that tiliroside inhibited transactivation of NF-κB, its effects on NF-κB-DNA binding activity was further investigated using ELISA based EMSA. Figure 3.7b show that LPS/IFNγ induced (~9.1-fold increase) the DNA binding of NF-κB compared to untreated cells, following 60-minute incubation. However, this nuclear DNA binding of NF-κB was significantly suppressed when pre-treated with tiliroside (2 μM~1.3-fold, 4 μM~1.8-fold and 6 μM ~3.9-fold reduction) in the microglia.

Based on the above results, the effect of tiliroside on the nuclear-phosphorylated p65 subunit, which is critical in the activation of NF-κB pathway, was further examined. BV2 microglia were pre-treated with tiliroside for 30 minutes and later incubated with LPS and IFNγ for 60 minutes. Western blotting results show that treatment with LPS/IFNγ remarkably increased phosphorylation of p65. In contrast, levels of p-p65 was significantly ($p<0.01$) inhibited (4 μM, ~1.7-fold and 6 μM, ~3-fold reduction) upon pre-treatment with the compound, (Figure 3.8a) while at 2 μM (~1.4-fold-reduction) there was no significant inhibition. In addition, immunofluorescence experiments were done to further examine the effects of the compound on phosphorylation of p65 in microglia. Figure 3.8b show that p-p65 was highly expressed in the microglia when challenged with LPS and IFNγ compared to untreated cells. However, pre-treatment with tiliroside resulted in suppression of p-

p65 activation. Collectively, these results together suggest that tiliroside alleviates LPS/IFNγ-induced neuroinflammation through inhibition of NF-κB signalling in the microglia.

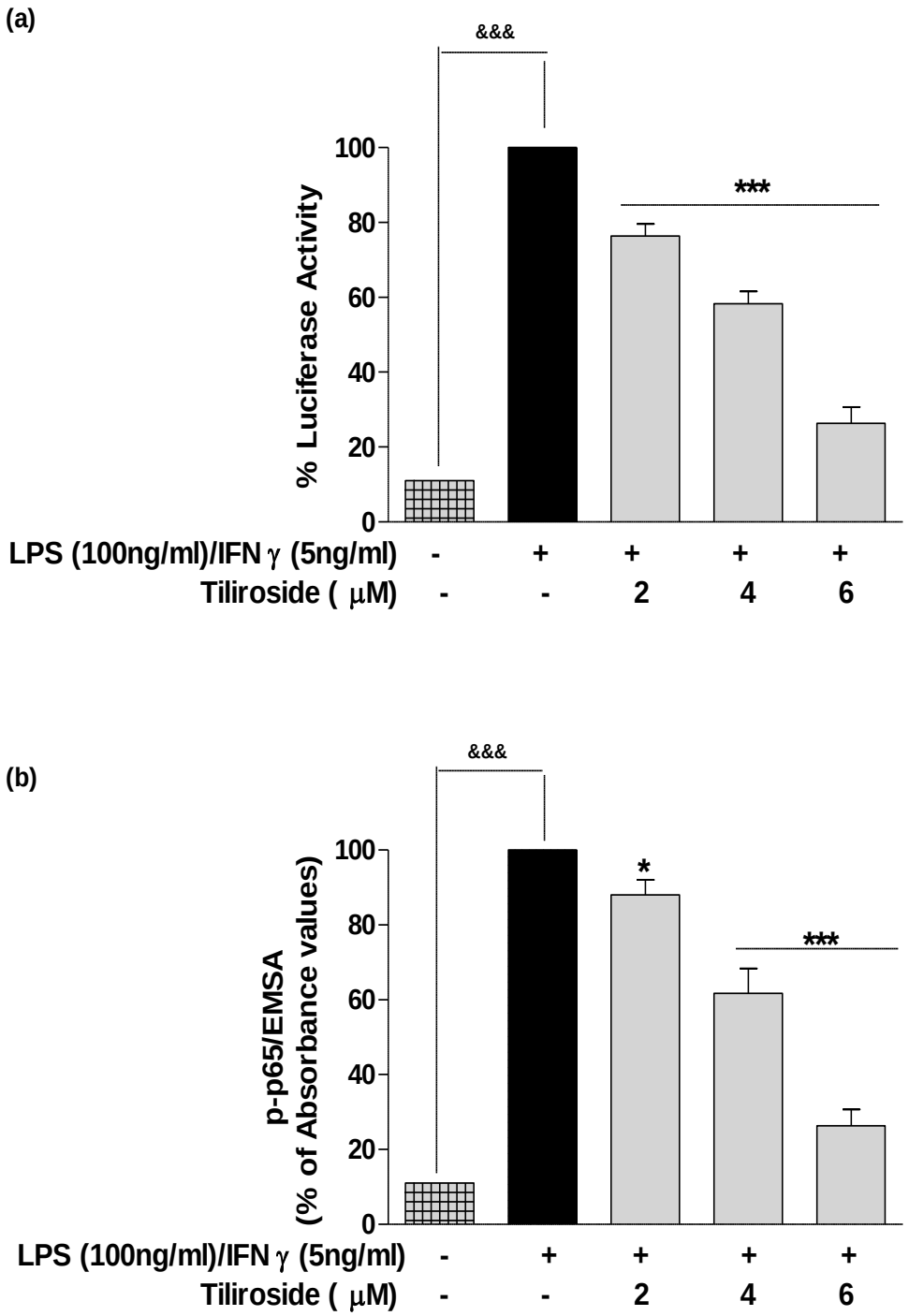
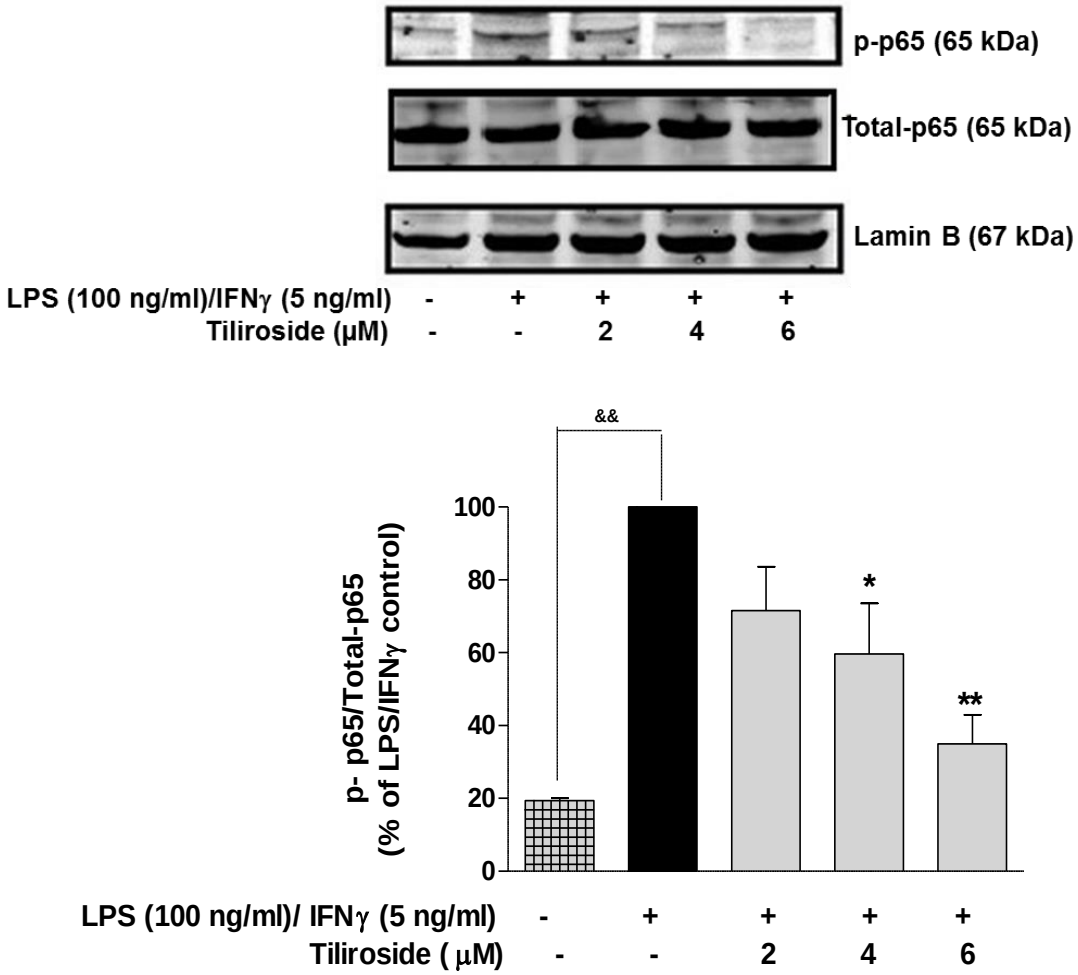


Figure 3.7 Tiliroside inhibited neuroinflammation by targeting NF-κB signalling in activated BV2 microglia.

(a) Transfected BV2 cells were treated with LPS/IFNγ in the presence or absence of the tiliroside (2-6 μM) for 6 hours. At the end of the stimulation, luciferase assay buffer containing luminescence substrate was added, and luminescence was read. Tiliroside suppressed NF-κB activity in transfected BV2 cells stimulated with LPS/IFNγ. (b) Increasing concentrations of

tiliroside inhibited DNA binding of NF-κB in LPS (100 ng/ml)/IFNγ (5 ng/ml)-stimulated BV2 microglial cells. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to LPS/IFNγ.

(a)


p-p65 (65 kDa)
Total-p65 (65 kDa)
Lamin B (67 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6
&&
*
**
p- p65/Total-p65
(% of LPS/IFNγ control)
100
80
60
40
20
0
LPS (100 ng/ml)/ IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

(b)

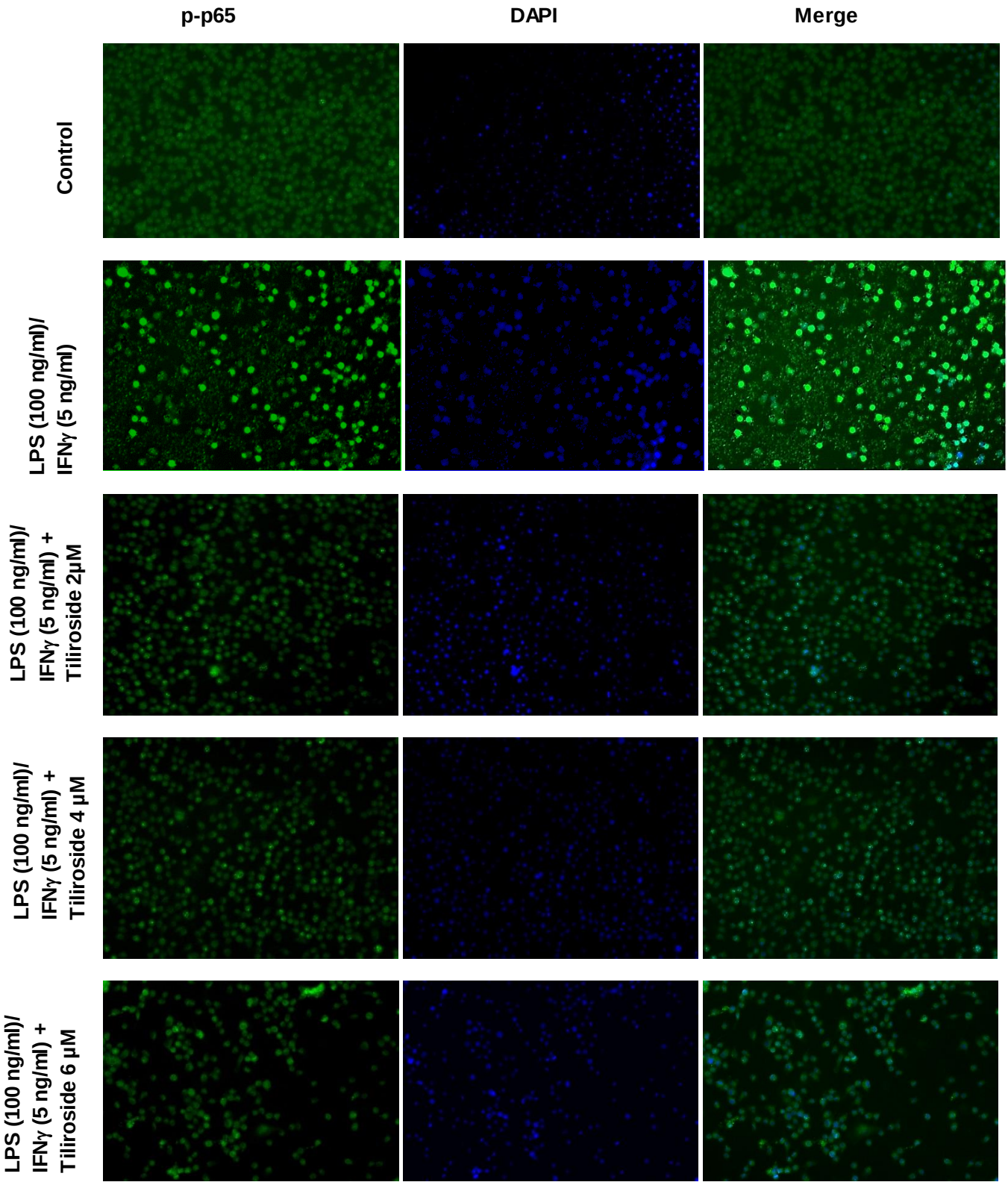

p-p65
DAPI
Merge
Control
LPS (100 ng/ml)/ IFNγ (5 ng/ml)
LPS (100 ng/ml)/ IFNγ (5 ng/ml) + Tiliroside 2µM
LPS (100 ng/ml)/ IFNγ (5 ng/ml) + Tiliroside 4 µM
LPS (100 ng/ml)/ IFNγ (5 ng/ml) + Tiliroside 6 µM

Figure 3.8 Tiliroside inhibited neuroinflammation by targeting NF-κB signalling pathway in BV2 microglia

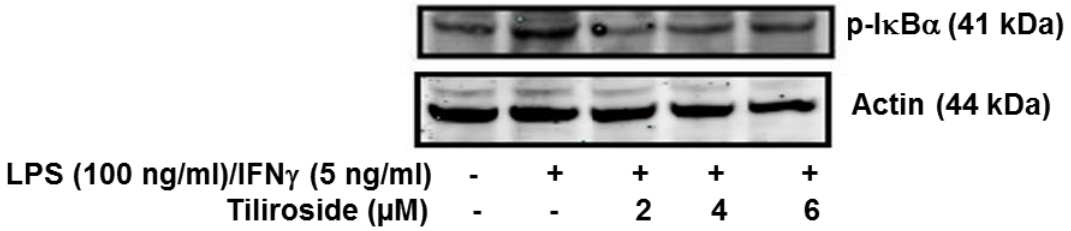
(a) Cells were treated with LPS (100 ng/ml)/IFNγ (5 ng/ml) in the presence or absence of the tiliroside for 60 minutes. Later, nuclear lysates were collected, and western blot was done for p-p65 and Total-p65 protein detection. Tiliroside inhibited p-p65 translocation in LPS/IFNγ-activated microglia compared to total-p65. Lamin B was used as a loading control. (b) Immunofluorescence experiments were done to investigate the effects of various concentrations of tiliroside on p-p65 protein expression in activated microglia at 60 minutes. Cells were labelled with p-p65 antibody and counterstained with DAPI and fluorescence images acquired with an EVOS® FLoid® cell imaging station (scale bar=100 µm) and processed using image J. Tiliroside treatment decreased the LPS/IFNγ-induced p-p65 expression in microglia. Experiments were repeated at least three times All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\theta}$ p< 0.05, $^{\theta\theta}$ p<0.01, $^{\theta\theta\theta}$ p<0.001 in comparison with untreated control. using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}$p<0.05, $^{\&\&}$p<0.01, $^{\&\&\&}$p<0.001 compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

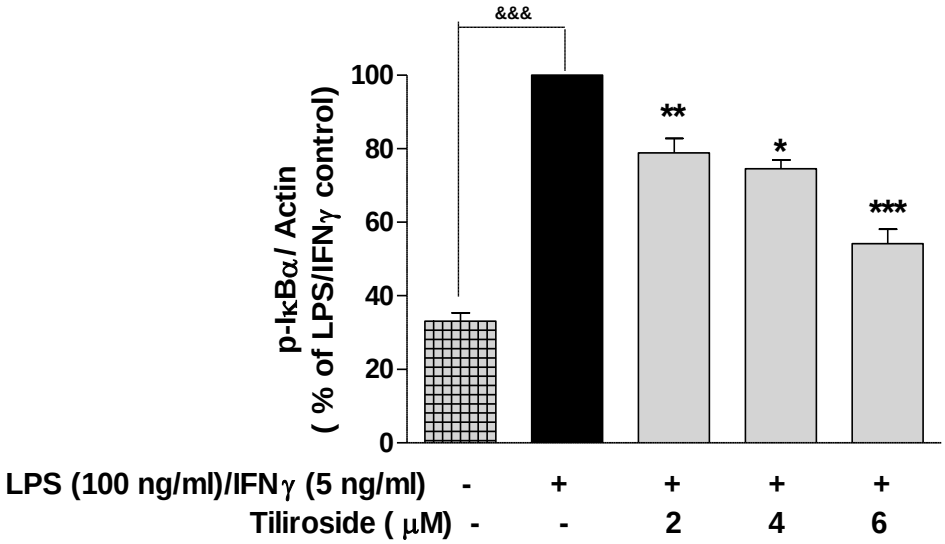
3.2.7 Tiliroside inhibited phosphorylation of IKKα and IκBα in LPS/IFNγ-induced neuroinflammation in microglia

In response to an inflammatory stimulus, IκBα/NF-κB complex become phosphorylated by IKKα. As a result, active NF-κB-p65 subunit is translocated to the nucleus thereby regulating the expression of target genes. Cytoplasmic proteasomes further degrade free IκBα. Based on the outcome of the experiments on NF-κB, an attempt was made to further understand the effect of tiliroside on upstream proteins in LPS/IFNγ-stimulated microglia. BV2 cells were pre-treated with tiliroside for 30 minutes, followed by LPS/IFNγ stimulation for 60 minutes. Western blotting results show that the combination of LPS and IFNγ significantly upregulated (~3.1-fold increase) the expression of p-IκBα in the microglia, compared to control cells. However, this inhibition was significantly ($p<0.05$ and $p<0.001$) counteracted by the tiliroside (2 μM, ~1.2-fold, 4 μM, ~1.3-fold and 6 μM, ~2-fold reduction) (Figure 3.9a). A western blot experiment was performed to detect the levels of total-IκBα in LPS/IFNγ-stimulated microglia. High levels of total-IκBα protein (~2.6-fold increase) was observed in the untreated cells compared to LPS/IFNγ treated. Interestingly, tiliroside (2 μM, ~1.3-fold, 4 μM ~2-fold and 6 μM, ~3.3-fold increase) inhibited the proteasomal degradation of IκBα (Figure 3.9b).

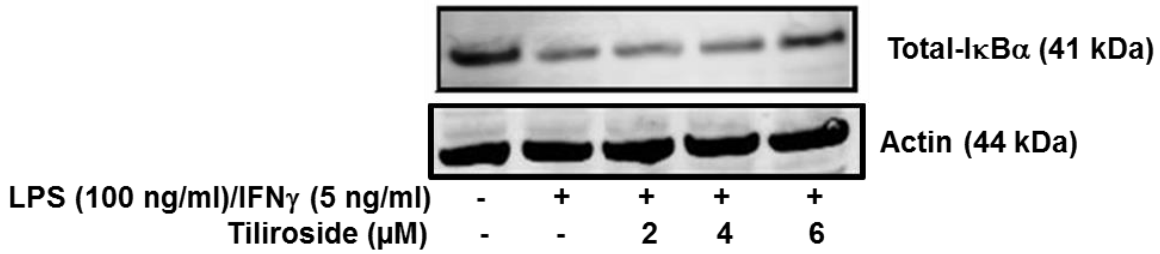
Later, the effect of the compound on the phosphorylation of IKKα was investigated in LPS/IFNγ-activated microglia. As shown in Figure 3.9c, LPS (100 ng/ml)/IFNγ (5 ng/ml) combination have induced IKKα phosphorylation compared to control cells. Interestingly, compound (6 μM, ~2-fold decrease, $p<0.01$) suppressed IKKα phosphorylation in the microglia, while 2 μM and 4 μM of the compound had no significant inhibitory effect. These results seem to suggest that tiliroside prevents LPS/IFNγ-induced neuroinflammation by interfering with IKKα/IκBα/NF-κB signalling in BV2 microglia.

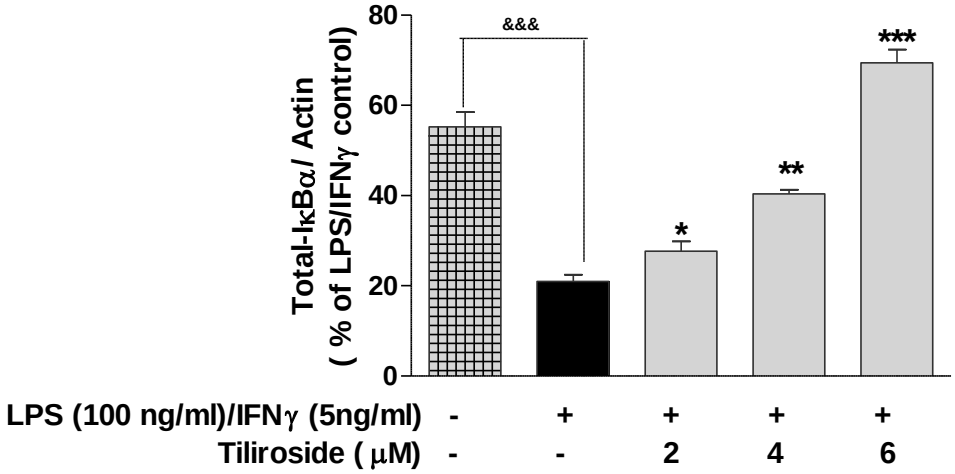
(a)


p-IκBα (41 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6


&&&
**
*
***
p-IκBα/ Actin
(% of LPS/IFNγ control)
100
80
60
40
20
0
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

(b)


Total-IκBα (41 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6


Total-IκBα/ Actin
(% of LPS/IFNγ control)
80
60
40
20
0
&&&
*
**
***
LPS (100 ng/ml)/IFNγ (5ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

(c)

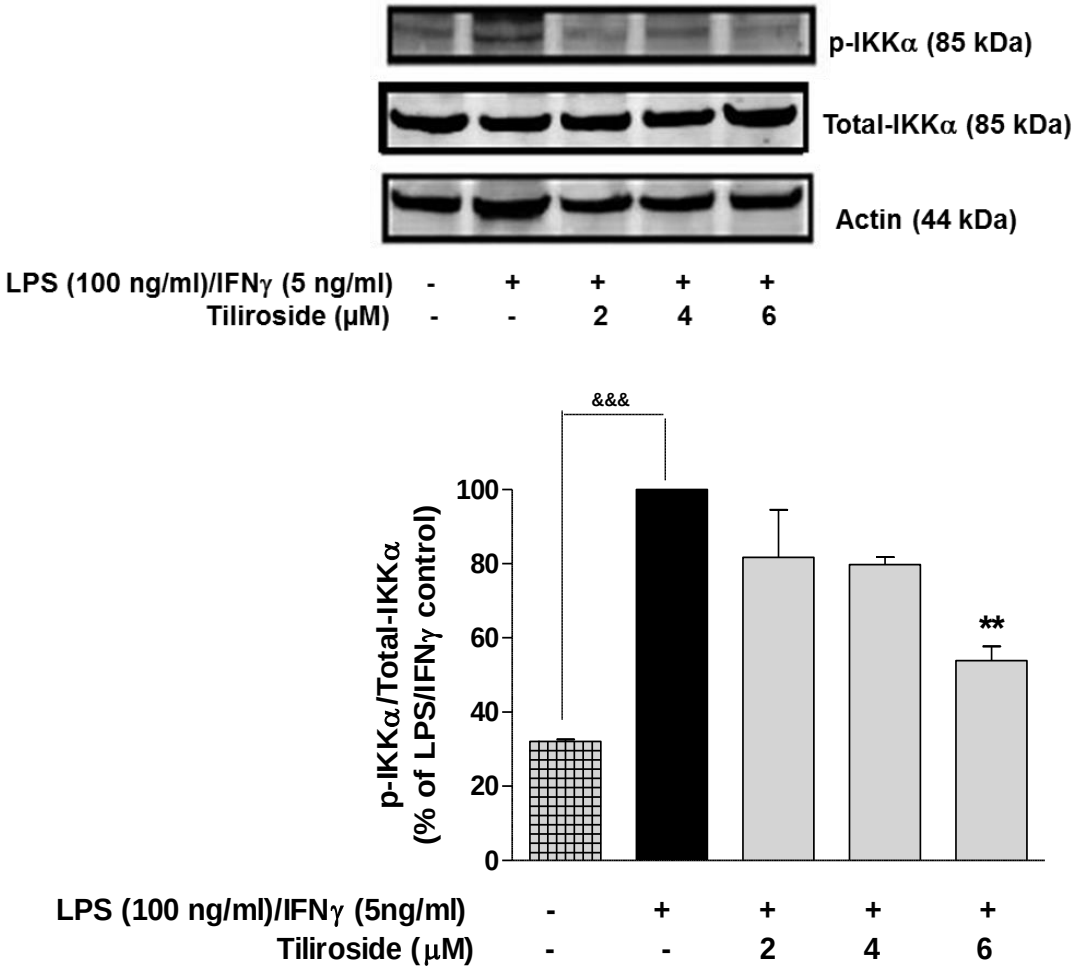


Figure 3.9 Tiliroside inhibited phosphorylation of IκBα and IKKα in LPS/IFNγ-stimulated BV2 microglia

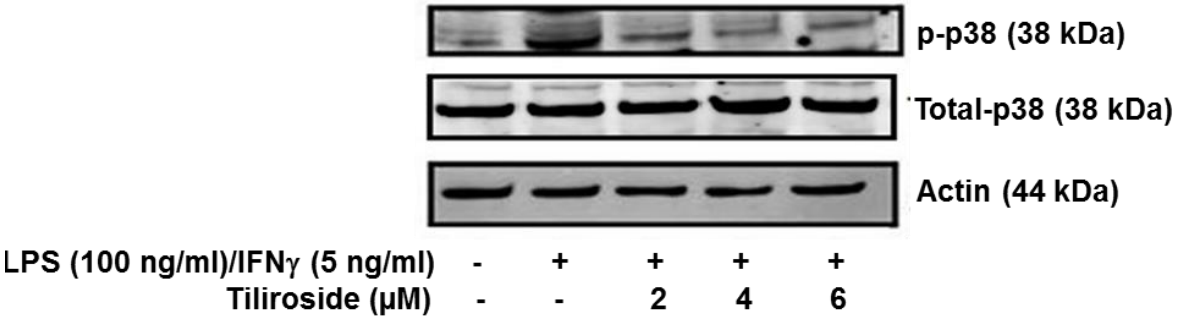
Cells were stimulated with a combination of LPS/IFNγ in the presence or absence of the tiliroside for 60 minutes. Subsequently, cytoplasmic lysates were collected and analysed for phosphorylation of IκBα, as well as IKKα and degradation of IκBα protein using western blot. (a) Tiliroside abrogated IκBα phosphorylation in LPS/IFNγ-activated BV2 cells. (b) Tiliroside inhibited degradation of IκBα in LPS/IFNγ-activated BV2 microglia. (c) Tiliroside inhibited IKKα phosphorylation in LPS/IFNγ-activated BV2 cells compared to Total-IKKα expression. Actin was used as a loading control. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

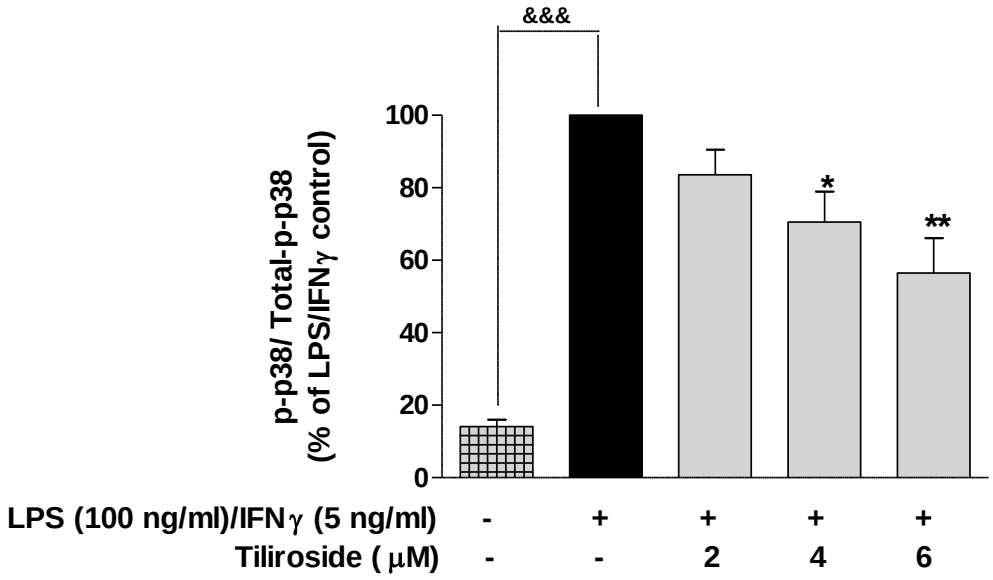
3.2.8 Tiliroside inhibited LPS/IFNγ-induced p38 MAPK signalling pathway

Increasing evidence suggests that p38 mitogen-activated protein (MAP) kinase regulates NF-κB-dependent transcription and controls the production of inflammatory mediators such as IL-1β, TNF α, IL-6, NO, iNOS, PGE_2 and COX-2 (Cuadrado et al. 2010) and (Didonato et al. 2012). Consequently, an attempt was made to investigate whether compound interferes with p38 signalling in LPS/IFNγ-activated BV2 microglia. Firstly, the effect of tiliroside on phosphorylation of p38 following activation of BV2 microglia with a combination of LPS and IFNγ was evaluated. This was achieved by pre-incubating microglia with tiliroside (2-6 μM) for 30 minutes, followed by stimulation with LPS (100 ng/ml)/IFNγ (5 ng/ml) for 60 minutes. Upregulation of p-p38 protein expression (~2-fold increase) was observed in LPS and IFNγ-treated microglia compared to control microglia. Interestingly, pre-incubation with compound significantly suppressed phosphorylation of p38 (4 μM, ~1.3-fold decrease, $p<0.05$ and 6 μM, ~1.8-fold decrease, $p<0.01$), while at 2 μM the inhibition was insignificant (Figure 3.10a).

Further investigations on the downstream substrate of p38, MK2 showed an inhibition of its phosphorylation by tiliroside (4 and 6 μM), following activation of BV2 microglia with LPS/IFNγ (Figure 3.10b). Tiliroside followed a similar pattern in the inhibition of LPS/IFNγ-induced MK2 compared to p-p38 results. At 4 and 6 μM (4 μM, ~1.4-fold decrease, $p<0.05$ and 6 μM, ~1.9-fold decrease, $p<0.001$) the inhibition was efficient and at 2 μM the effect of tiliroside was minimal. After observing the inhibitory effects of the compound on phosphorylation of p38 and its downstream target MK2 its effect on MKK3/6, an upstream kinase of p38 MAPK signalling was investigated. Results show that MKK3/6 was activated at 60 minutes in the microglia (Figure 3.11a). From Figure 3.11b, it appears that LPS/IFNγ strongly activated MKK 3/6 in microglia. However, tiliroside at 4 μM (~1.3-fold decrease, $p<0.05$) and 6 μM (~2-fold decrease, $p<0.001$) significantly inhibited MKK 3/6 phosphorylation, suggesting that compound inhibits LPS/IFNγ-induced neuroinflammation via p38 MAPK signalling pathway.

(a)


p-p38 (38 kDa)
Total-p38 (38 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6


&&&
p-p38/ Total-p38
(% of LPS/IFNγ control)
100
80
60
40
20
0
*
**
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

(b)

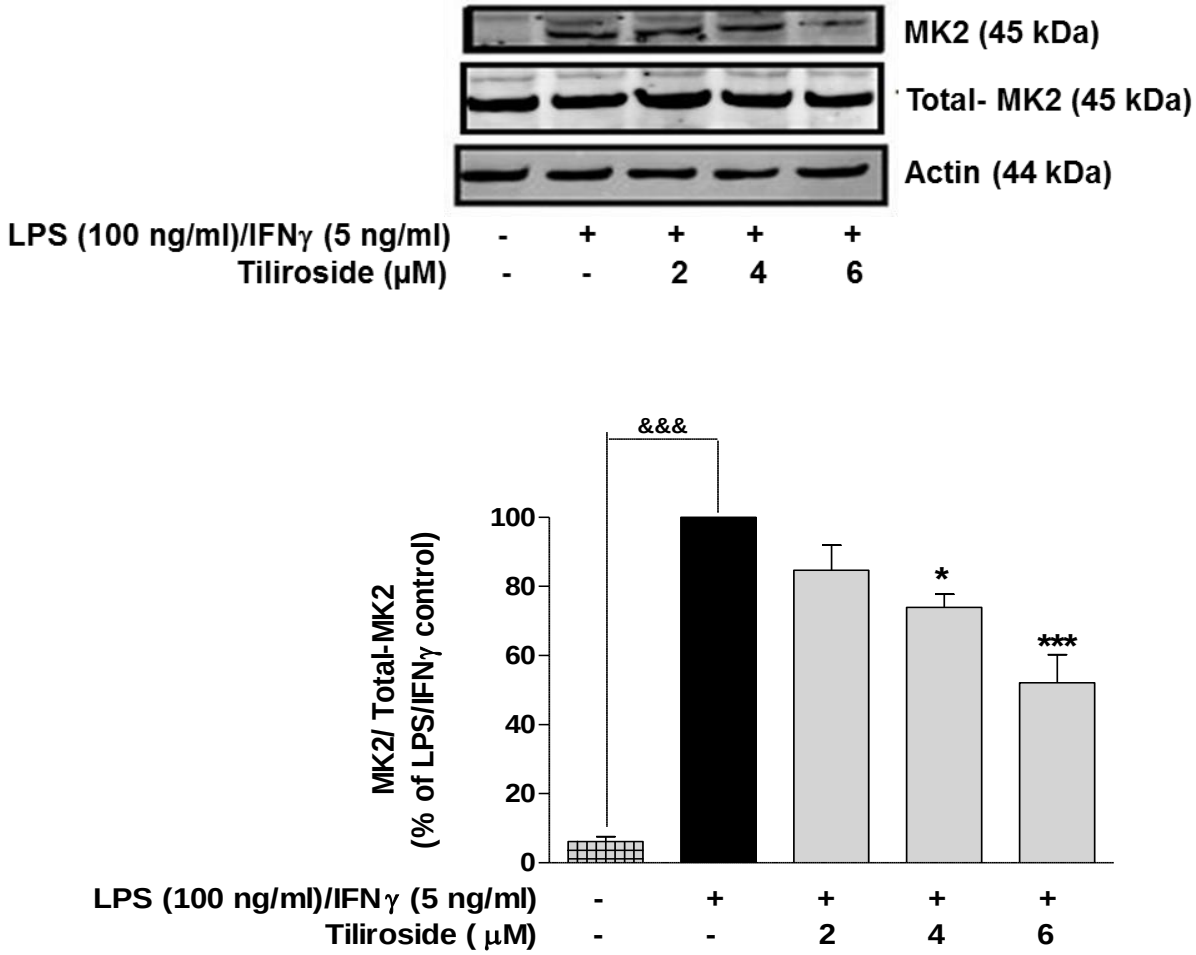
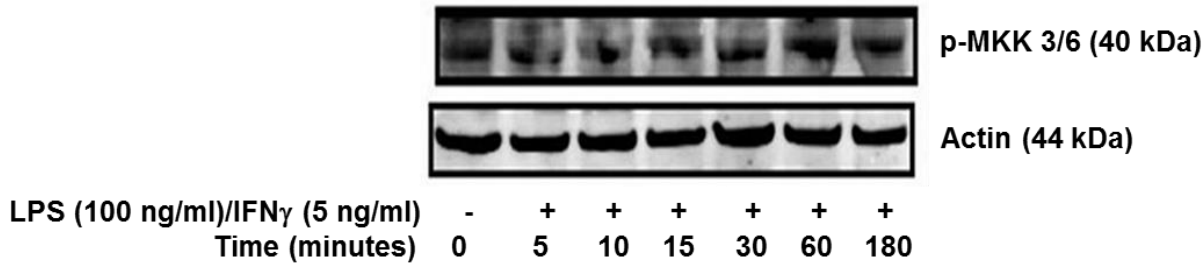
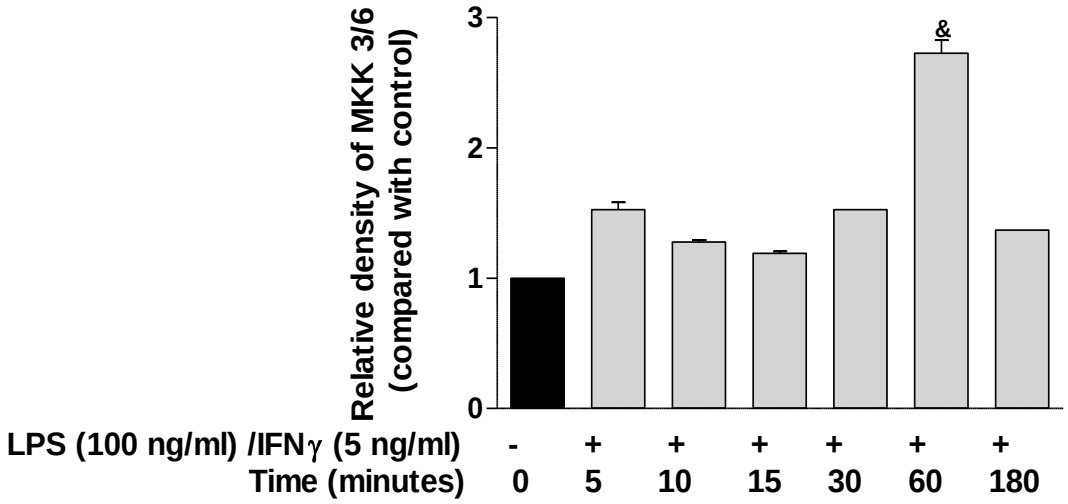


Figure 3.10 Tiliroside inhibited phosphorylation of p38 and MK2 in LPS/IFNγ-activated microglia

Microglia were treated with LPS and IFNγ for 60 minutes in the presence or absence of tiliroside. Subsequently, cytoplasmic lysates were collected and western blot was done for p38α and MK2 protein detection. (a) Tiliroside inhibited p38α phosphorylation compared to total-p38 in LPS/IFNγ-activated BV2 cells. (b) The compound also reduced MK2 phosphorylation compared to total-MK2 in activated BV2 cells. Actin was used as a loading control. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

(a)


p-MKK 3/6 (40 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + + + +
Time (minutes) 0 5 10 15 30 60 180


Relative density of MKK 3/6 (compared with control)
3
2
1
0
&
LPS (100 ng/ml) /IFNγ (5 ng/ml) - + + + + + +
Time (minutes) 0 5 10 15 30 60 180

(b)

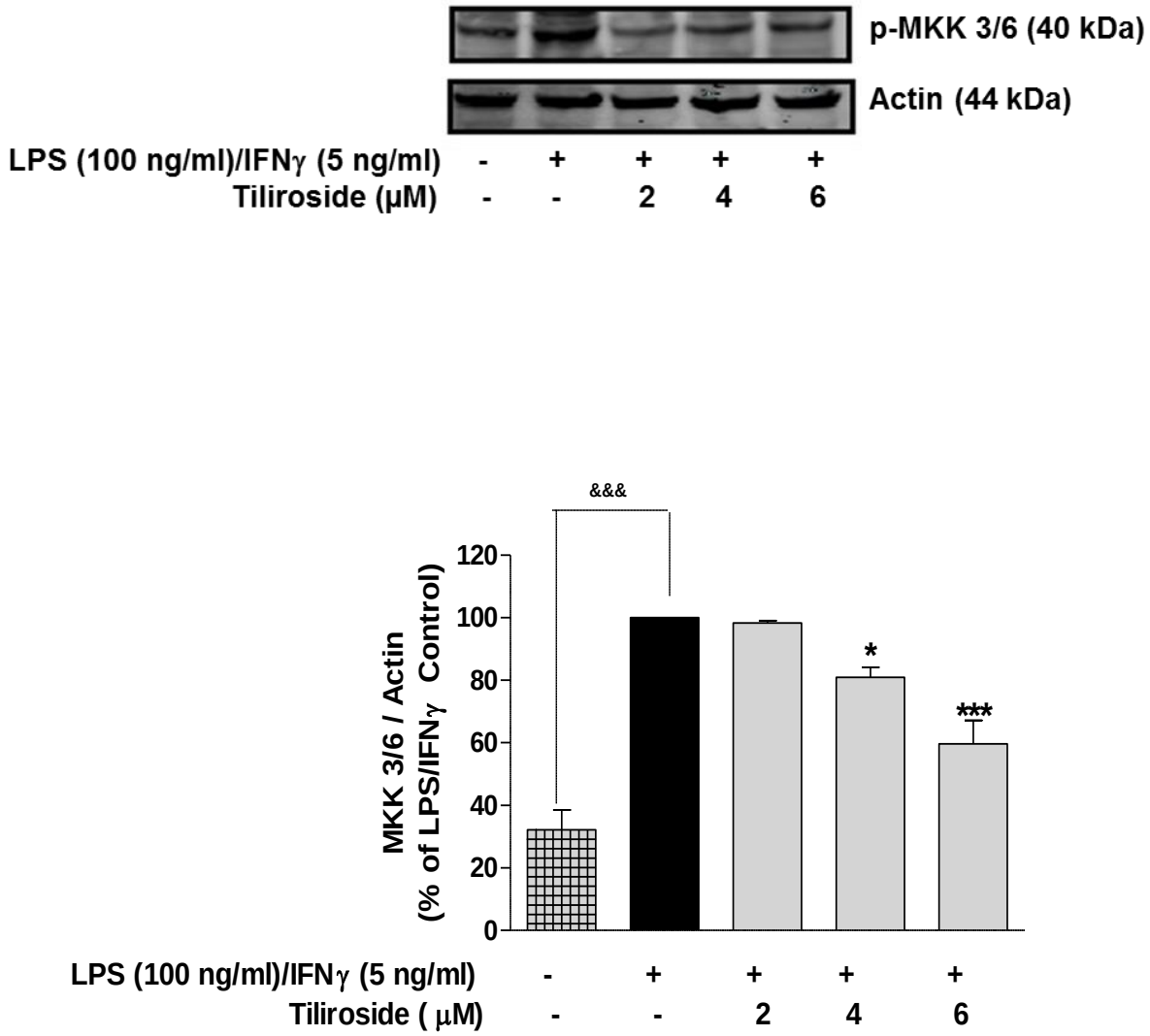


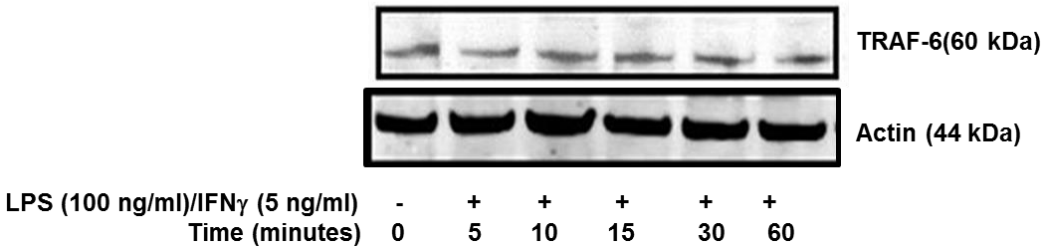
Figure 3.11 Tiliroside inhibited phosphorylation of MKK3/6 in response to LPS/IFNγ

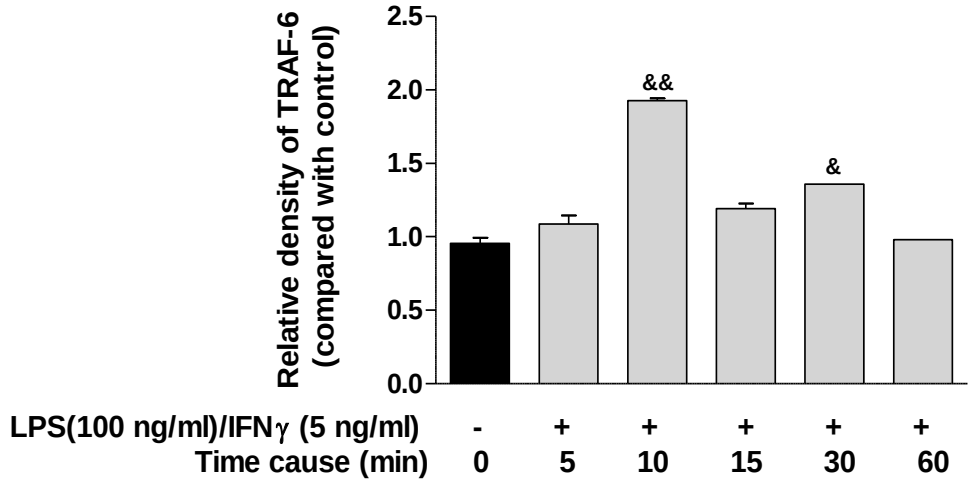
(a) Cells were treated with LPS/IFNγ for several time points. Later, cytoplasmic lysates were collected and subjected to western blot to determine the phosphorylation of MKK 3/6. This experiment showed that MKK3/6 was phosphorylated at 60 minutes in LPS/IFNγ-activated microglia. (b) Microglia were challenged with LPS and IFNγ in the presence or absence of tiliroside for 60 minutes. Subsequently, cytoplasmic lysates were collected and western blot was done for MKK 3/6 protein detection. Tiliroside inhibited MKK3/6 phosphorylation in LPS/IFNγ-treated BV2 cells. Actin was used as a loading control. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

3.2.9 Tiliroside inhibited LPS/IFNγ-induced neuroinflammation by targeting TLR4 and TRAF6 in BV2 microglia

Studies have shown that microglia upon stimulation with LPS, can predominantly induce NF-κB and MAPK activation through TLR4 receptor by activatingTRAF-6 protein (Hanamsagar et al. 2012), (Zhou et al. 2014), (Xu et al. 2014) and (Takeda & Akira 2004).Western blotting was used to show that TRAF6 protein was maximally expressed within 10 minutes of LPS (100 ng/ml)/IFNγ (5 ng/ml) stimulation and its expression was significantly inhibited by tiliroside 4 μM (~1.3-fold decrease, $p<0.001$) and 6 μM (~1.9-fold decrease, $p<0.001$) (Figure 3.12a and b). The effect of the compound was further investigated against LPS/IFNγ-induced TLR4 activation in microglia. As shown in the Figure 3.13a, when microglia was exposed to LPS (100 ng/ml)/IFNγ (5 ng/ml) for 10 minutes, the TLR4 expression was upregulated (~3.4-fold increase) compared to untreated cells. Notably, this activation was abrogated by tiliroside (6 μM, ~2-fold decrease, $p<0.01$) treatment, while 2 μM and 4 μM has minimal effect. In addition, immunofluorescence was used to examine whether TLR4 activation is involved in LPS/IFNγ-induced neuroinflammation. Figure 3.13b shows that TLR4 was highly expressed in the microglia when incubated with LPS/IFNγ compared to untreated cells. However, pre-treatment with tiliroside resulted in suppression of TLR4 activation, suggesting that TLR4 pathway is primarily involved in the inhibitory effects of the compound in activated microglia.

(a)


TRAF-6(60 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + + +
Time (minutes) 0 5 10 15 30 60


Relative density of TRAF-6
(compared with control)
2.5
2.0
1.5
1.0
0.5
0.0
&&
&
LPS(100 ng/ml)/IFNγ (5 ng/ml) - + + + + +
Time cause (min) 0 5 10 15 30 60

(b)

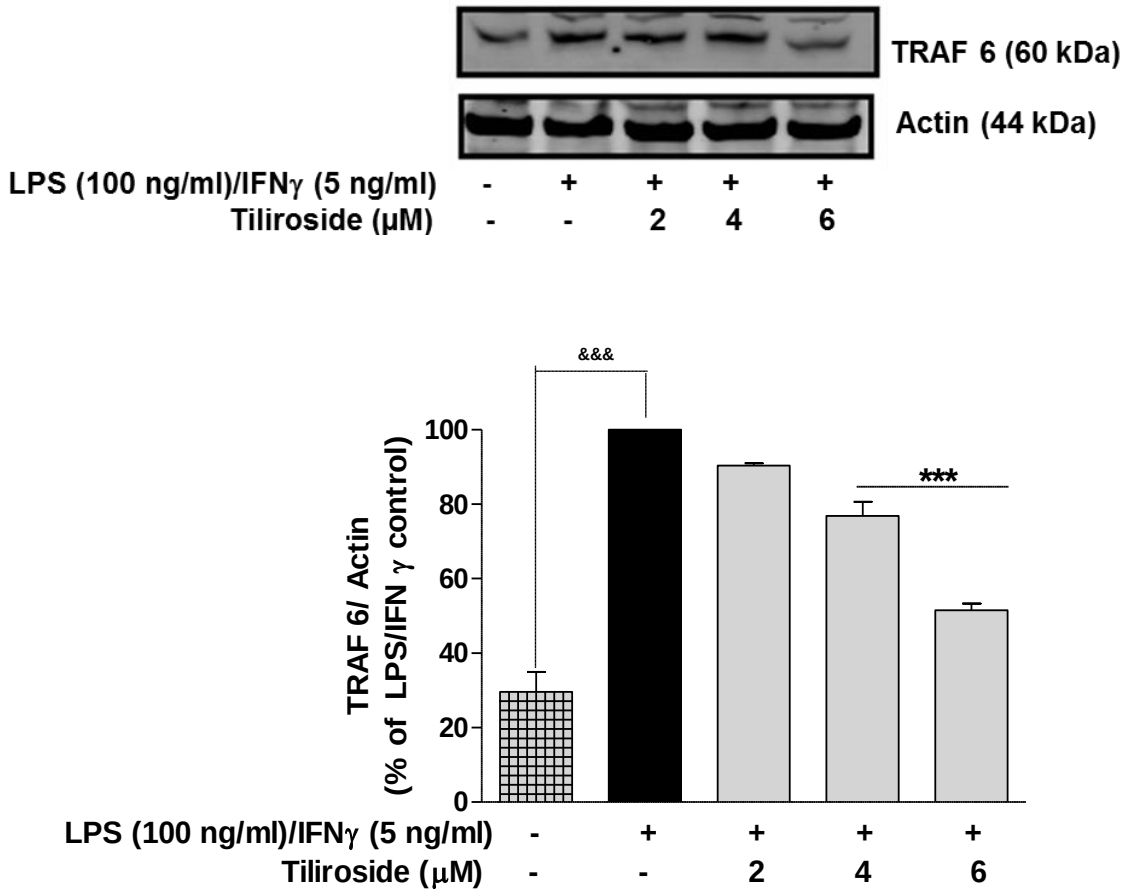
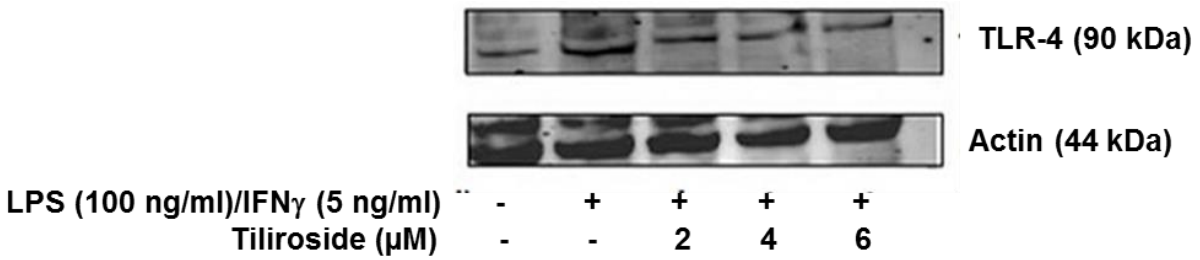
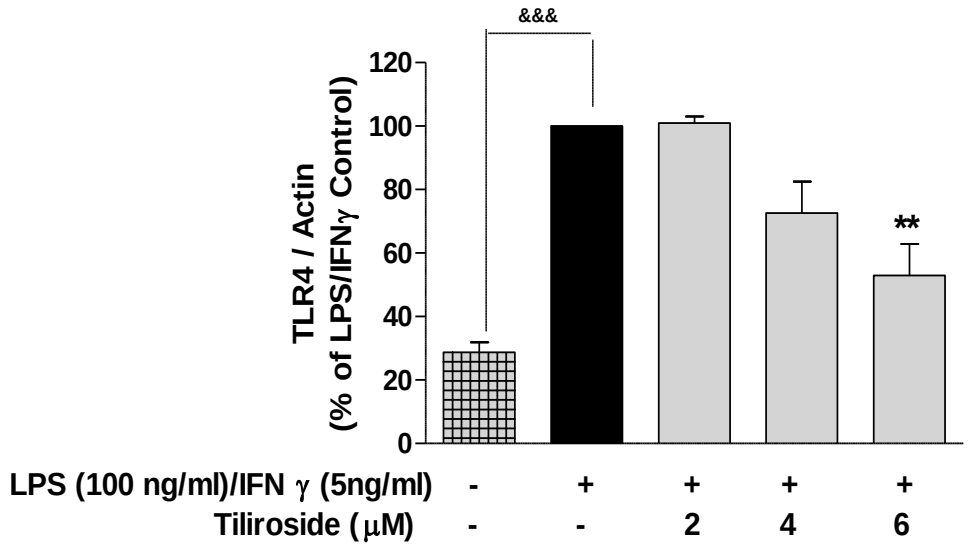


Figure 3.12 Tiliroside inhibited activation of TRAF6 in LPS/IFNγ-induced microglia

(a) Cells were treated with a combination of LPS and IFNγ for several time points. Later, cytoplasmic lysates were collected and subjected to western blot to determine the expression of TRAF6. This experiment showed that TRAF6 was activated at 10 minutes in activated microglia. (b) Microglia were treated with LPS and IFNγ in the presence or absence of the tiliroside (2-6 μM) for 10 minutes. Subsequently, cytoplasmic lysates were collected and western blot was done for TRAF6 protein detection. Tiliroside inhibited TRAF6 activation in LPS/IFNγ-treated BV2 cells. Actin was used as a loading control. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

(a)


TLR-4 (90 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6


&&&
TLR4 / Actin
(% of LPS/IFNγ Control)
120
100
80
60
40
20
0
**
LPS (100 ng/ml)/IFN γ (5ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6

(b)

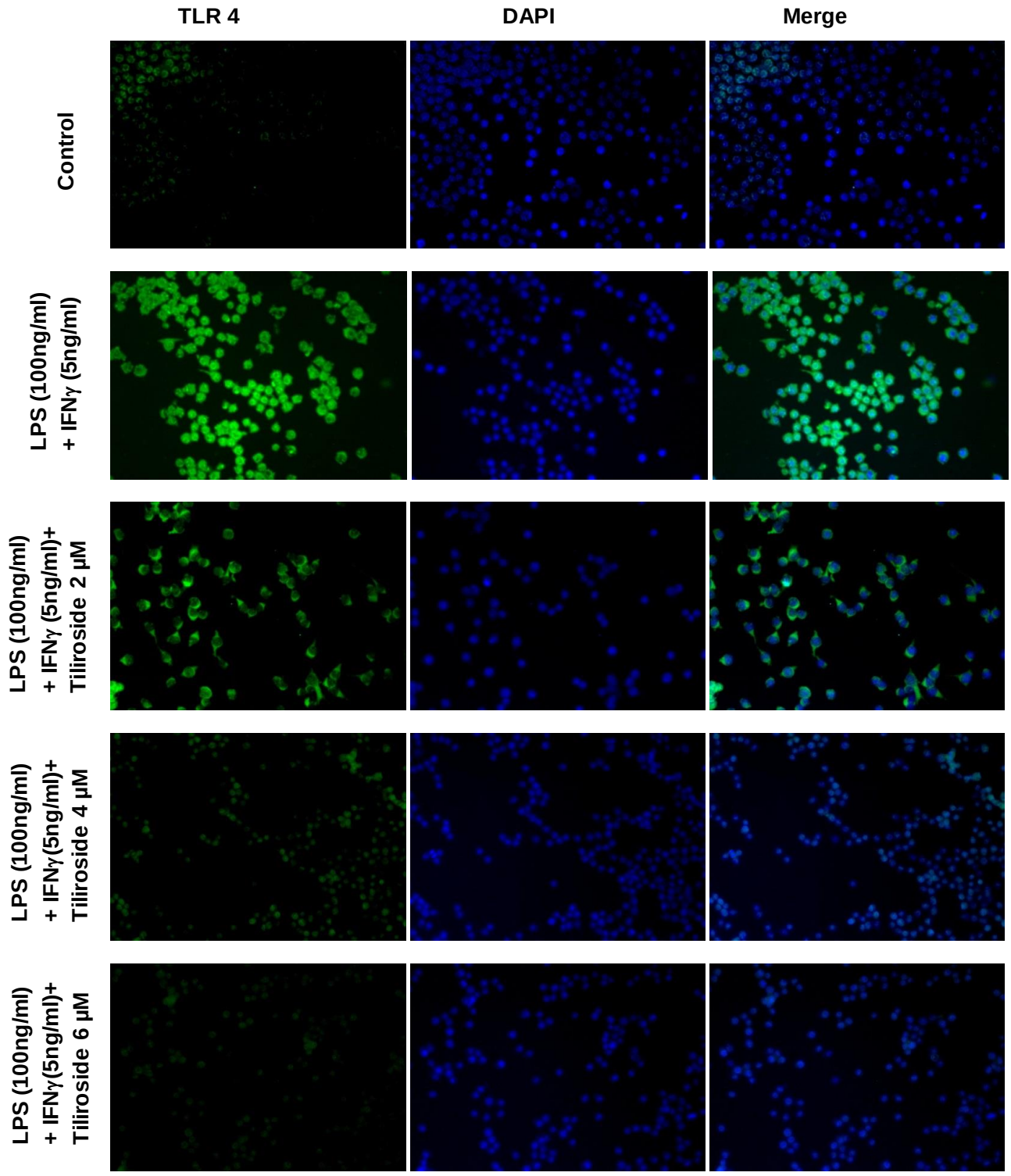


Figure 3.13 Tiliroside inhibited TLR4 activation in LPS/IFNγ-activated microglia

(a) Cells were treated with the combination of LPS and IFNγ in the presence or absence of the tiliroside for 10 minutes. Subsequently, cytoplasmic lysates were collected and western blot was done for TLR4 protein detection. Tiliroside inhibited TLR4 activation in LPS/IFNγ-activated BV2 cells. Actin was used as a loading control. (b) BV2 microglia was pre-treated with tiliroside

for 30 minutes and later stimulated with LPS/IFNγ for 10 minutes. Subsequently, cells were labelled with TLR4 antibody and counterstained with DAPI and fluorescence images acquired with an EVOS® FLoid® cell imaging station (scale bar=100 µm) and processed using image J. Tiliroside treatment decreased the LPS/IFNγ-induced TLR4 activation in microglia. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. &p<0.05, &&p<0.01, &&&p<0.001 compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

3.2.10 Tiliroside suppresses acetylation of NF-κB-p65 in LPS/IFNγ-activated microglia

Compounds that inhibit the activation of NF-κB signalling pathway in hyperactive microglia have been shown to block the expression of acetylated-NF-κB-p65 (Chen & Greene 2005) and (Cao et al. 2010). Since tiliroside blocked the neuroinflammation via NF-κB signalling, its effect on LPS/IFNγ-induced acetyl-NF-κB-p65 was investigated in microglia. The combination of LPS and IFNγ markedly increased the expression of acetyl-NF-κB-p65 (~52-fold increase, $p<0.001$) in microglia when compared to unstimulated cells. Interestingly, this upregulation of acetyl-NF-κB-p65 was significantly attenuated by tiliroside (4 μM, ~1.4-fold, $p<0.01$ and 6 μM, ~2.1-fold reduction, $p<0.001$) treatment, while at 2 μM compound did not show any effect (Figure 3.14).

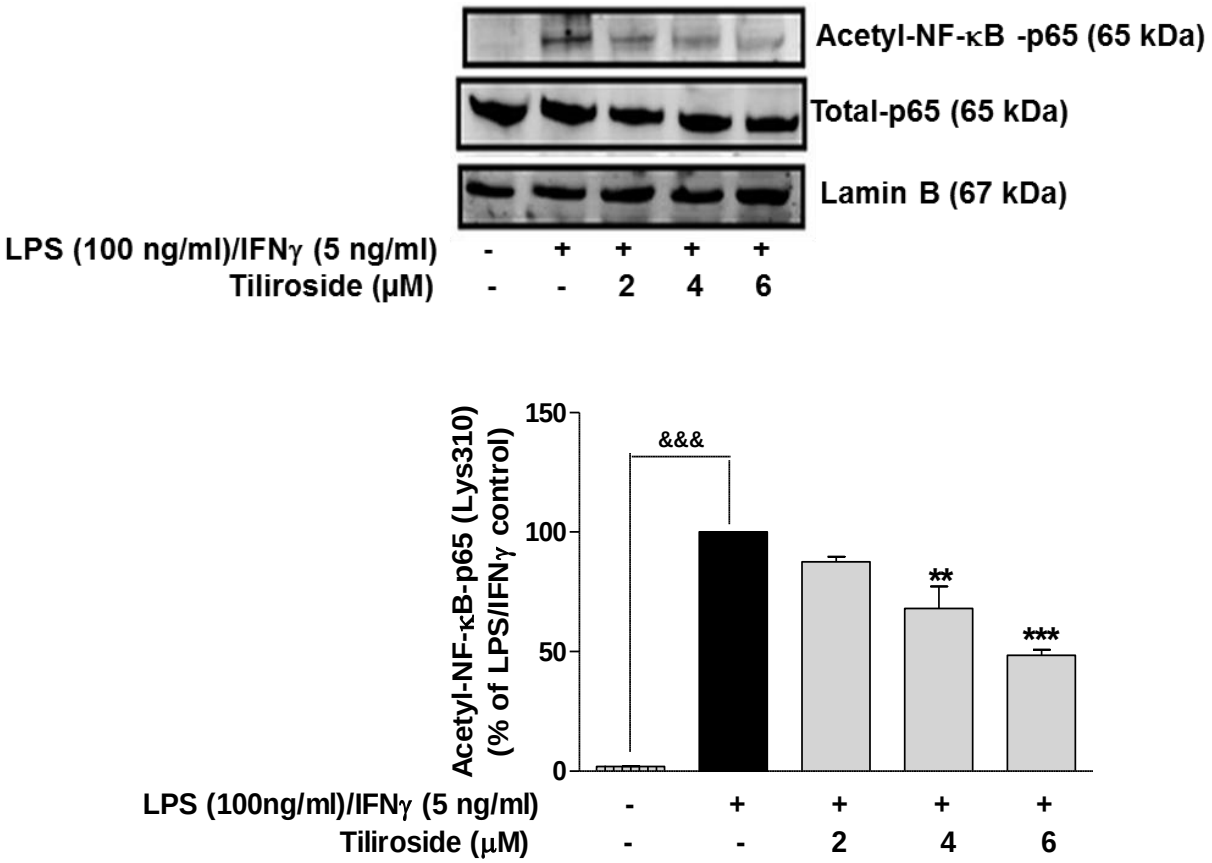


Figure 3.14 Tiliroside inhibited acetylation of NF-κB-p65 in LPS/IFNγ-activated BV2 microglia.

BV2 Cells were treated with LPS/IFNγ in the presence or absence of the tiliroside for 60 minutes. Later, nuclear lysates were collected and western blotting was done for Acetyl- NF-κB-p65 and Total-NF-κB-p65 protein detection. Tiliroside inhibited acetylation of NF-κB-p65 in LPS/IFNγ-activated microglia compared to Total-p65. Lamin B was used as a loading control. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

3.2.11 Tiliroside activates SIRT1 signalling in BV2 microglia

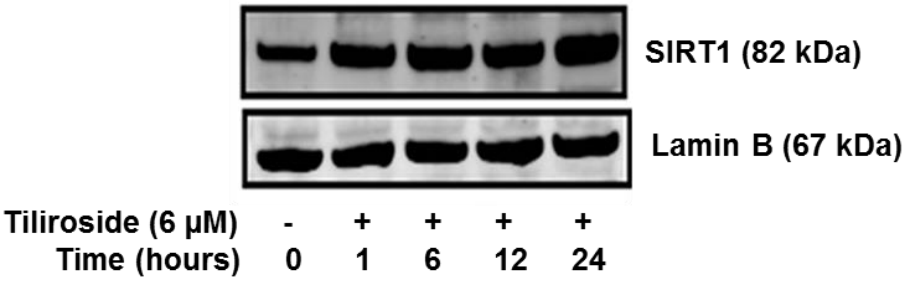
SIRT1 is a class III histone deacetylase enzyme that has been shown to inhibit NF-κB signalling by deacetylation of NF-κB-p65 subunit at Lysine 310 (Salminen et al. 2013) and (Yeung et al. 2004). Activation of SIRT1 protein could resolve neuroinflammation by downregulating the expression of NF-κB-mediated inflammatory genes (Jieming Ye et al. 2013). Tiliroside was shown to block the accumulation of LPS/IFNγ-induced acetyl-NF-κB-p65 in microglia. Therefore, it was thought to investigate the effects of the compound on SIRT1 activation in microglia. BV2 cells were pre-incubated with a most effective concentration of tiliroside (6 μM) and the levels of SIRT1 were measured at various time points using western blotting. Figure 3.15a shows time-dependent activation of SIRT1 in the presence of tiliroside (6 μM), however, highest expression (~3.1-fold increase, $p<0.001$) was observed at 24 hours, compared to untreated cells.

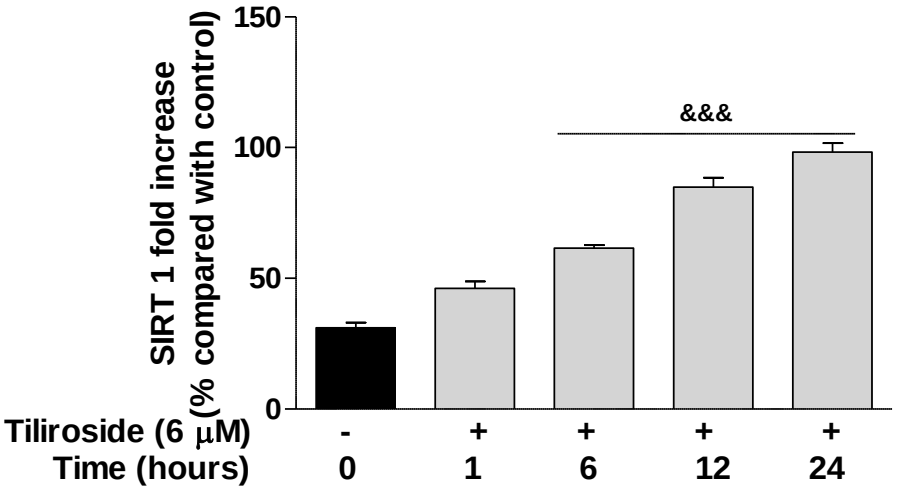
Based on the above results, the expression of SIRT1 was further investigated using various concentrations of tiliroside. Consequently, microglia was incubated with the compound (2-6 μM) for 24 hours. Western blotting results show that SIRT1 was significantly ($p<0.001$) and dose-dependently activated by tiliroside in the microglia (Figure 3.15b). Immunofluorescence experiments were done to investigate the nuclear accumulation of SIRT1 in microglia. As shown in Figure 3.15c, there was no fluorescence observed in untreated cells, which shows no expression of SIRT1. However, when BV2 cells were incubated with tiliroside (2-6 μM), there was a dose-dependent increase in the expression of SIRT1, suggests that tiliroside activated SIRT1 in microglia.

There is an abundant literature indicating that the functional dysregulation of SIRT1 during neuroinflammation is associated with many age-related neurodegenerative diseases (Haigis & Sinclair 2011) and (Guarente 2011). Consequently, the effect of the tiliroside was investigated against SIRT1 expression in LPS/IFNγ-treated microglia. Stimulation of BV2 microglia with the combination of LPS and IFNγ resulted in marked decrease in the expression of SIRT1 (Figure 3.16). With 2 μM of tiliroside, there was no significant effect on SIRT1 expression. However, pre-treatment with tiliroside (4 μM and 6 μM) resulted in a significant activation (~2.4-fold

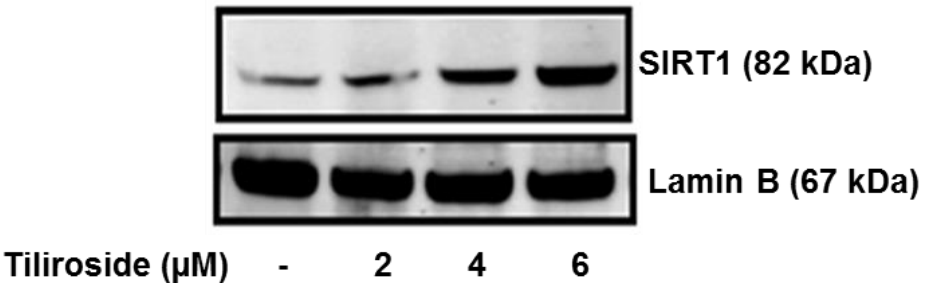
increase, $p<0.01$ and ~3.6-fold increase, $p<0.001$) of SIRT1 protein was observed when compared with LPS/IFNγ control.

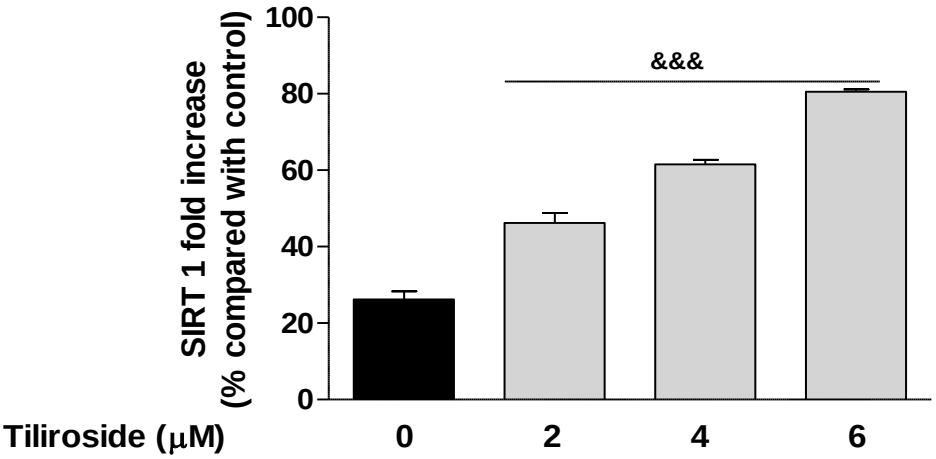
(a)


SIRT1 (82 kDa)
Lamin B (67 kDa)
Tiliroside (6 µM) - + + + +
Time (hours) 0 1 6 12 24


SIRT 1 fold increase (% compared with control)
150
100
50
0
&&&
Tiliroside (6 µM) - + + + +
Time (hours) 0 1 6 12 24

(b)


SIRT1 (82 kDa)
Lamin B (67 kDa)
Tiliroside (μM) - 2 4 6


SIRT 1 fold increase
(% compared with control)
100
80
60
40
20
0
&&&
Tiliroside (μM)
0
2
4
6

(c)

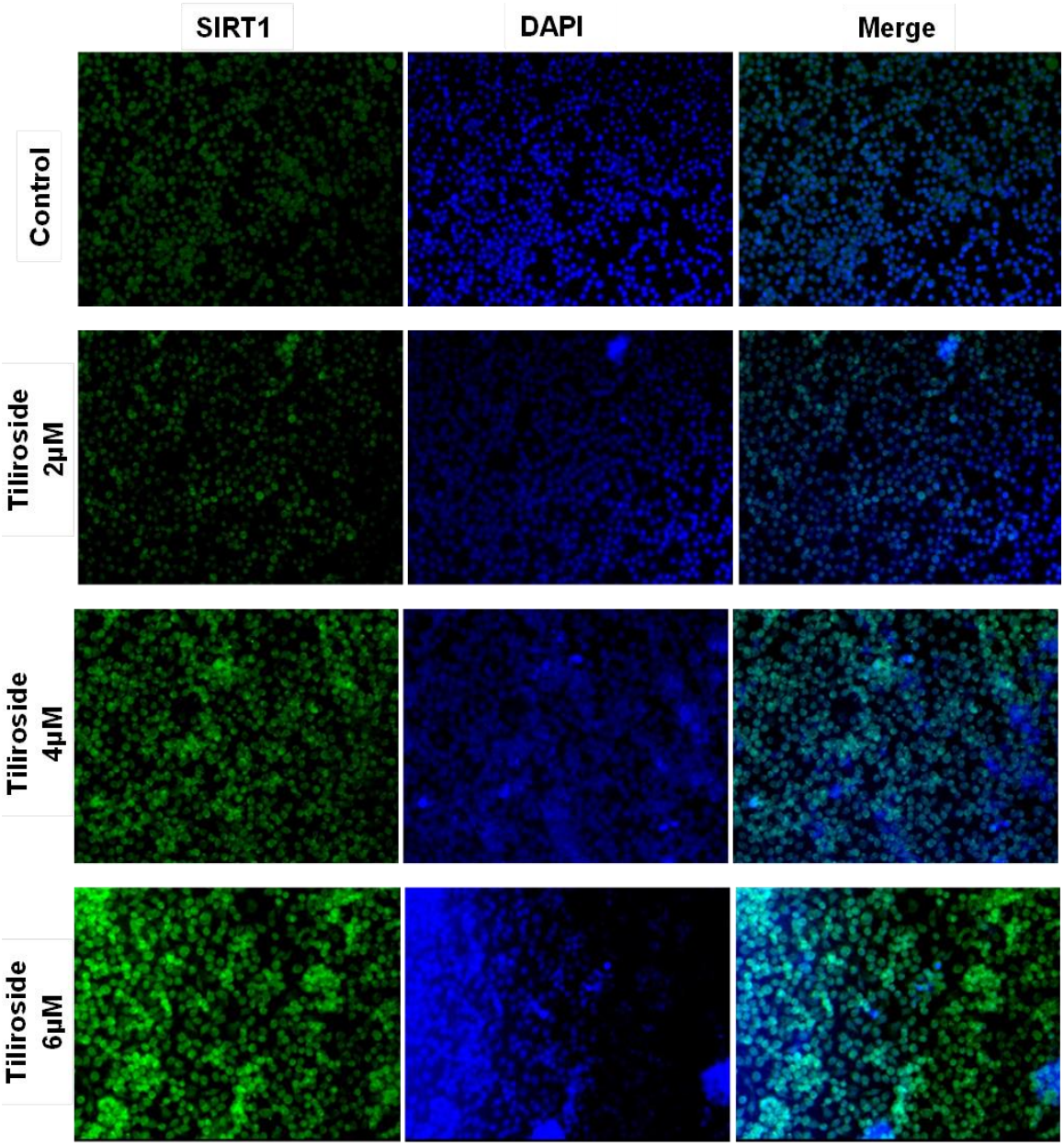


Figure 3.15 Tiliroside upregulated SIRT1 expression in BV2 microglia

Time point experiments was done by treating BV2 microglia with tiliroside 6 µM and incubated for different time points. Later, nuclear lysates were collected and subjected to western blotting.(a) Tiliroside upregulated SIRT1 expression in microglia. (b) Also, western blot results showed that tiliroside dose-dependently increased the expression of SIRT1 in microglia. Lamin B was used as a loading control. (c) BV2 cells were treated with increasing concentrations of tiliroside for 24 hours. Later, cells were fixed, blocked and stained with SIRT1 antibody for overnight. Following day, cells were washed and incubated with secondary antibody for 2 hours in the dark. Cells were counterstained with DAPI and fluorescence images acquired with an EVOS® FLoid® cell imaging station (scale bar=100 µm) and processed using image J. All

values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to LPS/IFNγ.

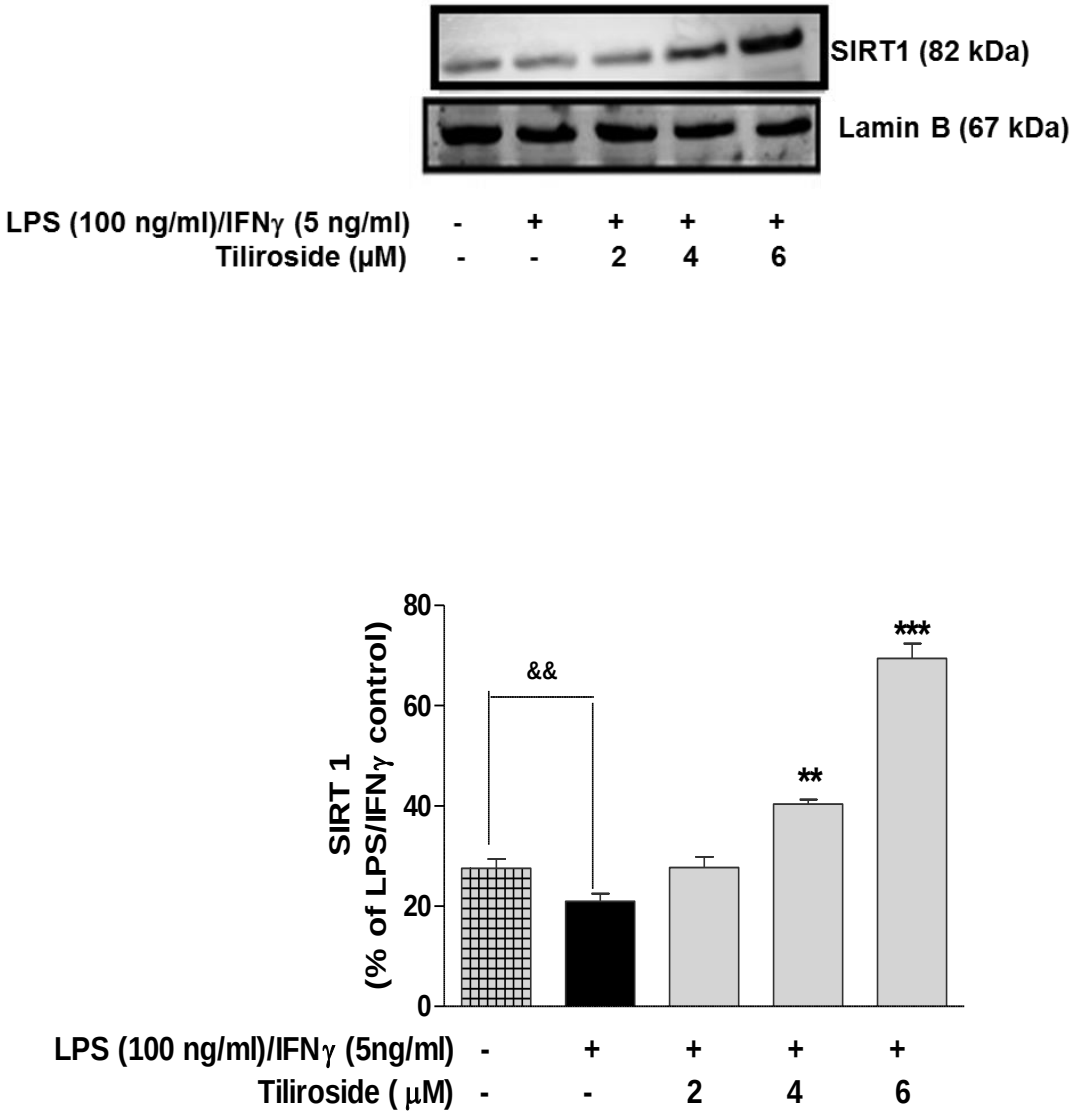


Figure 3.16 Tiliroside activated SIRT1 protein expression in LPS/IFNγ-treated BV2 microglia.

BV2 cells were pre-incubated with tiliroside (2-6 μM) for 30 minutes, following stimulation with LPS/IFNγ for 24 hours. Nuclear lysates were collected and subjected to western blotting to detect SIRT1 expression. Results show that the SIRT1 expression was downregulated in neuorinflammation, where tiliroside significantly reversed this inhibition. Lamin B was used as a loading controlAll values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

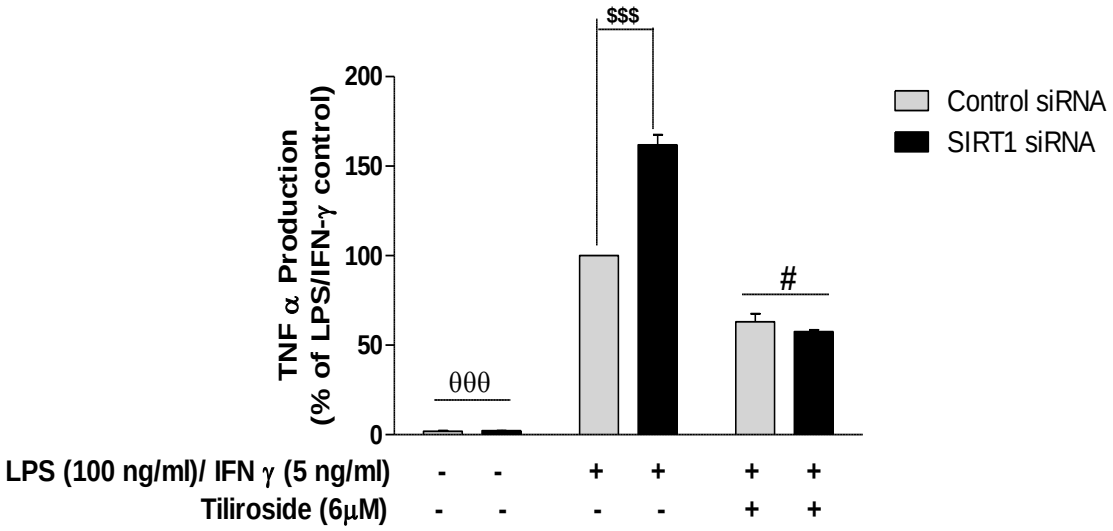
3.2.12 Anti-neuroinflammatory effects of tiliroside are independent of SIRT1 activity in LPS/IFNγ-activated BV2 microglia

To further confirm the contributions of nuclear SIRT1 to the suppressive effects of tiliroside on LPS/IFNγ-activated inflammatory responses, BV2 cells were transiently transfected with control siRNA and SIRT1 siRNA. Following transfection, cells were pre-treated with tiliroside (6 μM) and stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml) for 24 hours. Later, supernatants were collected and levels of cytokines were measured using ELISA. Figure 3.17 shows that in control siRNA cells, the combination of LPS and IFN produced a marked increase in TNF α (a), IL-6 (b) and IL-1β (c) production, while in SIRT1 silenced cells a further increase in the production of the cytokines was observed. As expected, tiliroside significantly inhibited the production of cytokines in control siRNA cells, interestingly, in SIRT1 silenced cells, the inhibitory activity of the compound was not reversed suggesting that the activity of the compound is independent of SIRT1 in microglia.

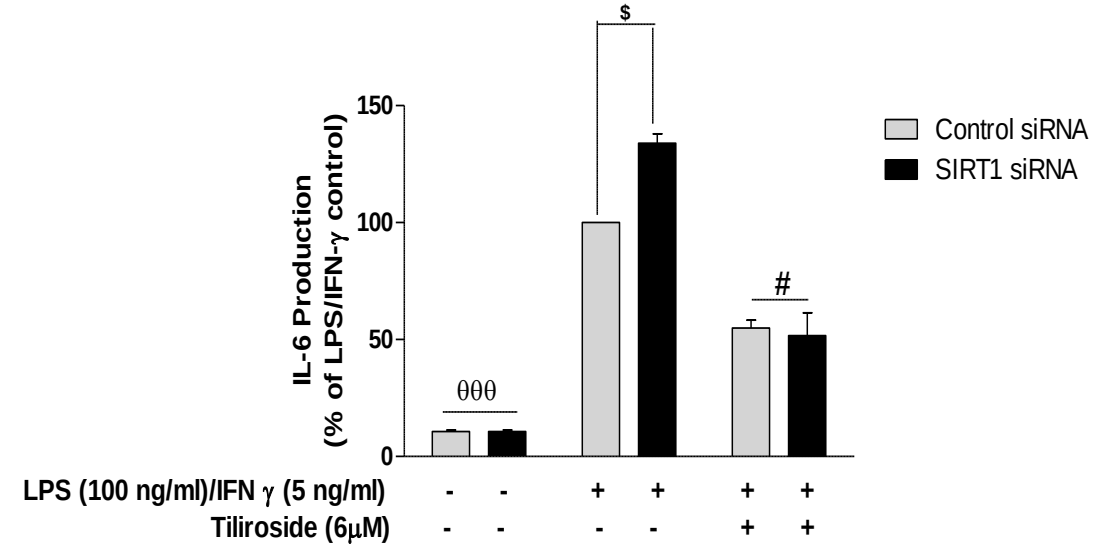
Encouraged by this outcome, the effect of the compound on the production of NO and PGE_2 is further investigated in SIRT1 silenced LPS/IFNγ-treated BV2 microglia. Cells were transfected with control siRNA and SIRT1 siRNA and pre-incubated with tiliroside (6 μM) followed by stimulation with LPS and IFNγ for 24 hours. Microglial SIRT1 deficiency elevated nitrites (a by-product of NO) and PGE_2 production, however, no reversal of this upregulation was observed in the presence of tiliroside. These results seem to be consistent with data on cytokines suggesting that anti-neuroinflammatory activities of the compound are not SIRT1 mediated (Figure 3.18).

The efficiency of SIRT1 gene knockdown in BV2 cells was assessed using western blotting experiments. Results show that cells that are transfected with control siRNA significantly expressed SIRT1. However, following transfection of microglia with SIRT1 siRNA, there was a marked deletion of nuclear SIRT1 protein in the cells (Figure 3.19). Tiliroside (6 μM) has significantly upregulated SIRT1 expression in control siRNA transfected cells compared to SIRT1 gene silenced microglia, which shows that SIRT1 gene was knocked out efficiently in microglia.

(a)


$$$
200
150
100
50
0
TNF α Production
(% of LPS/IFN-γ control)
θθθ
#
Control siRNA
SIRT1 siRNA
LPS (100 ng/ml)/ IFN γ (5 ng/ml) - - + + + +
Tiliroside (6μM) - - - - + +

(b)


$
150
100
50
0
IL-6 Production
(% of LPS/IFN-γ control)
θθθ
#
Control siRNA
SIRT1 siRNA
LPS (100 ng/ml)/IFN γ (5 ng/ml) - - + + + +
Tiliroside (6μM) - - - - + +

(c)

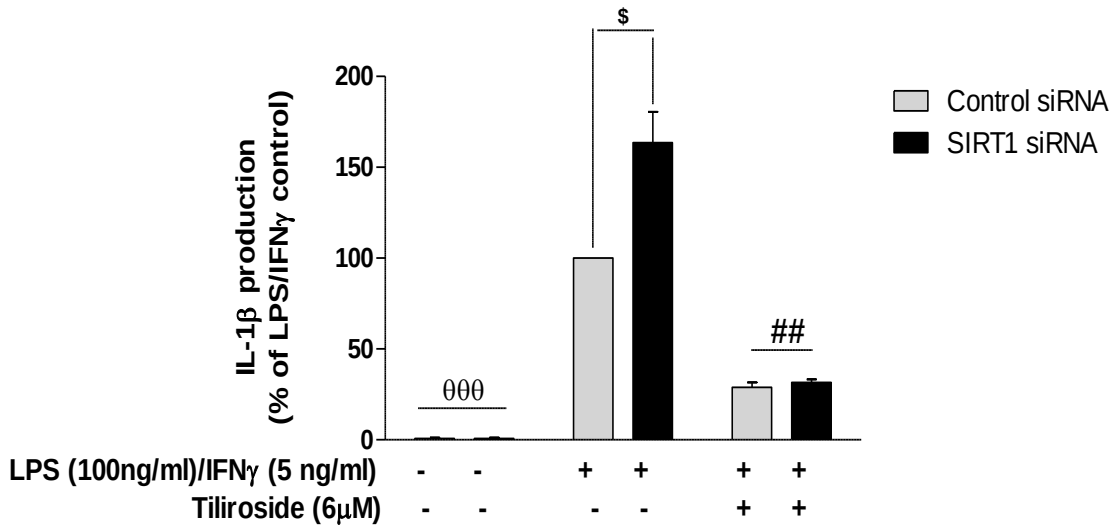
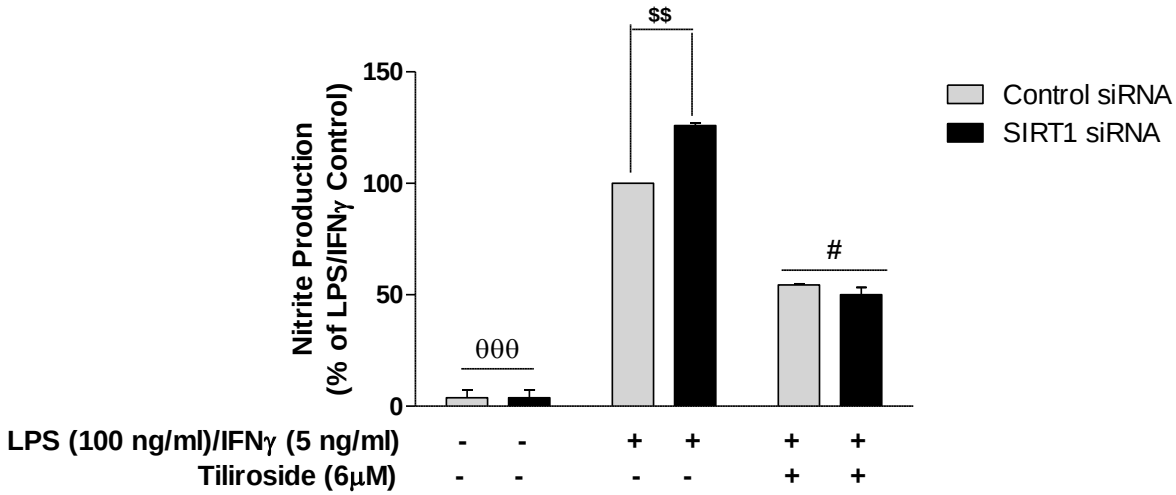


Figure 3.17 SIRT1 gene knockdown in BV2 microglia did not affect neuroinflammation inhibitory effects of tiliroside

SIRT1 siRNA and control siRNA transfected BV2 cells were pre-treated with tiliroside for 30 minutes prior to stimulation with LPS/IFNγ for 24 hours. Subsequently, culture supernatants were analysed for TNF α (a), IL-6 (b) and IL-1β (c) production using ELISA. Results revealed that tiliroside significantly inhibited the production of pro-inflammatory cytokines in control siRNA cells. However, the inhibitory effects of the compound are not reversed in SIRT1 siRNA BV2 cells. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\theta}p< 0.05$, $^{\theta\theta}p<0.01$, $^{\theta\theta\theta}p<0.001$ as compared within the groups of the untreated control. $p< 0.05, $$p<0.01, $$$p<0.001 as compared within the groups stimulated with LPS/IFNγ and #p<0.05, ##p<0.01, ###p<0.001 as compared within the groups pre-treated with tiliroside (6 μM).

(a)



(b)

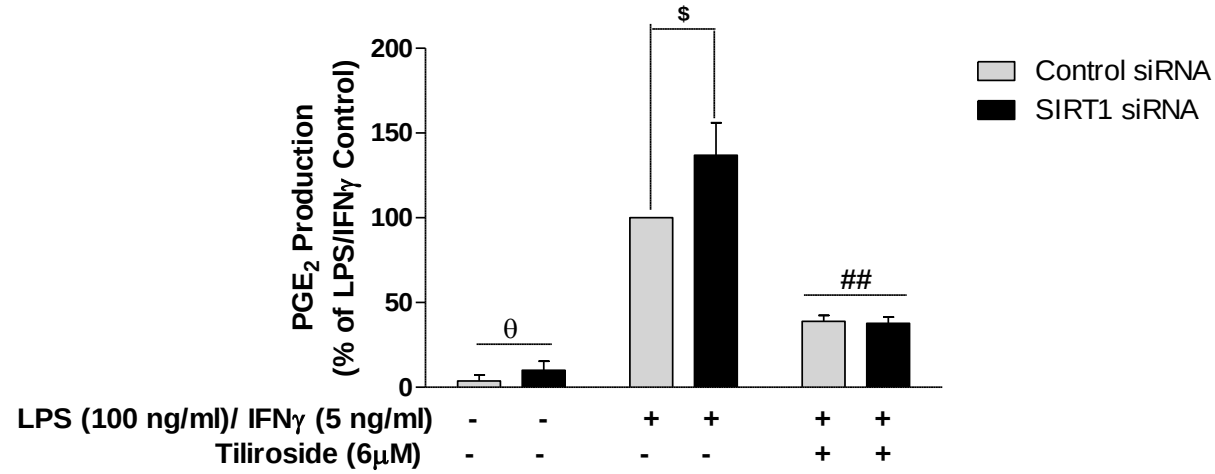


Figure 3.18 Neuroinflammation inhibitory effects of tiliroside are independent of SIRT1 activation in BV2 microglia

SIRT1 siRNA and control siRNA transfected BV2 cells were pre-treated with tiliroside (6 μM) for 30 minutes prior to stimulation with LPS/IFNγ for 24 hours. Subsequently, culture supernatants were analysed for nitrites using Griess assay and PGE2 by EIA assay. Results revealed that tiliroside significantly inhibited nitrite and PGE2 production in control siRNA cells. However, the inhibitory effects of the compound are not reversed in SIRT1 siRNA BV2 cells. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\theta}p< 0.05$, $^{\theta\theta}p<0.01$, $^{\theta\theta\theta}p<0.001$ as compared within the groups of the untreated control. $p< 0.05, $$p<0.01, $$$p<0.001 as compared within the groups stimulated with LPS/IFNγ and #p<0.05, ##p<0.01, ###p<0.001 as compared within the groups pre-treated with tiliroside (6 μM).

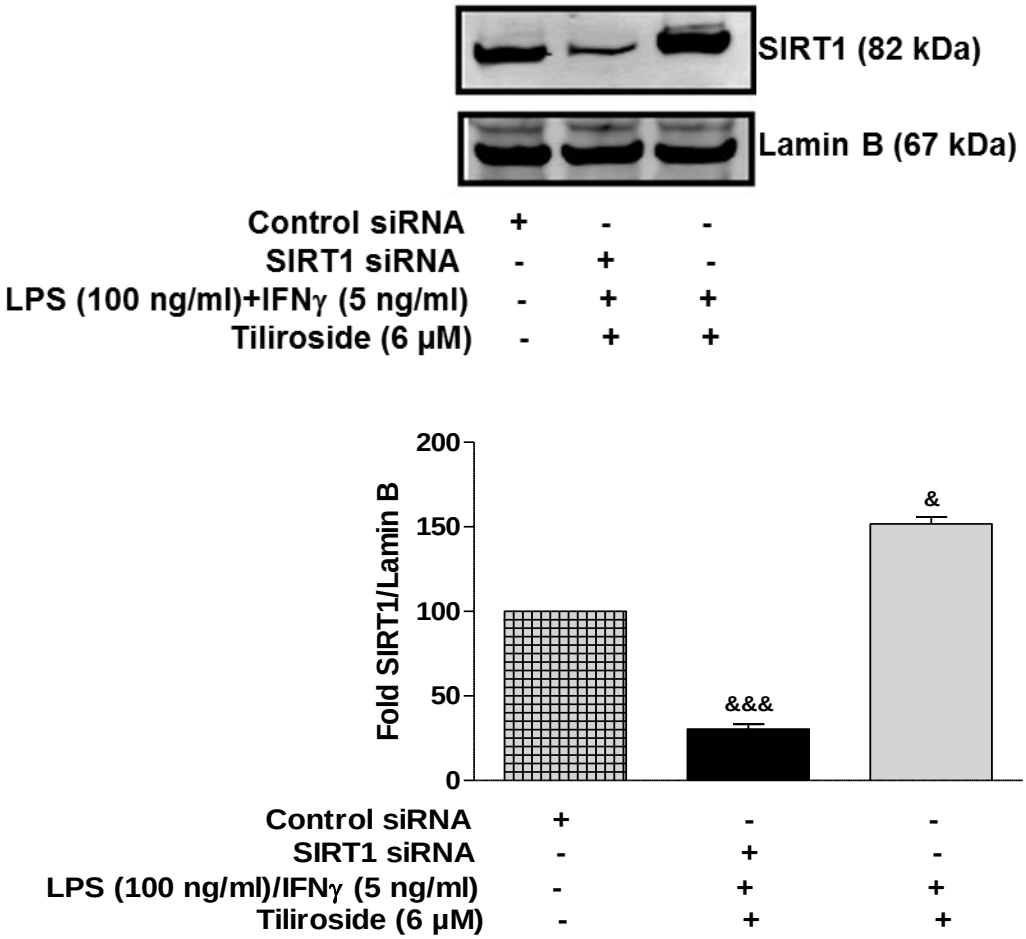


Figure 3.19 SIRT1 protein was knocked out efficientley in SIRT1 siRNA transfected BV2 microglia

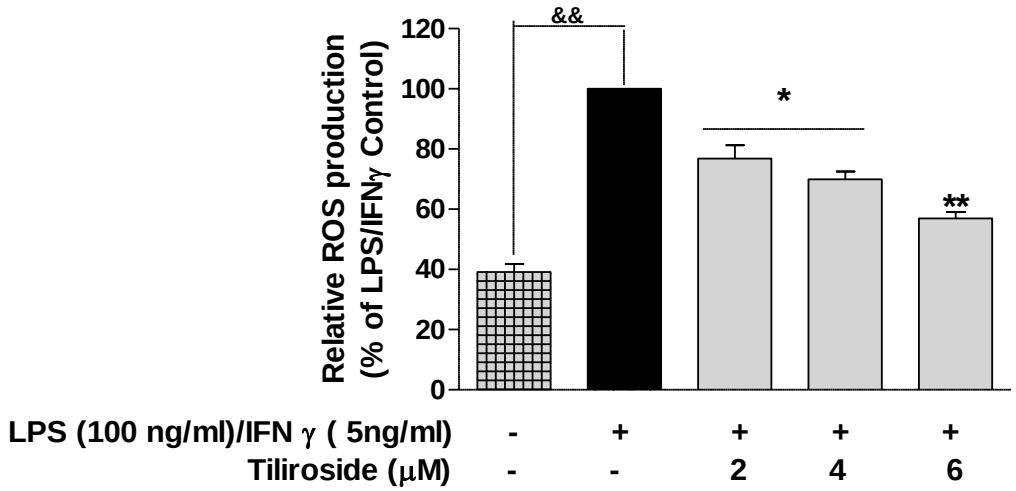
Control siRNA and SIRT1 siRNA-transfected BV2 microglia were treated with tiliroside (6 µM) for 30 minutes prior to stimulation with LPS/IFNγ for 24 hours. Nuclear lysates were collected and assessed for SIRT1 expression using western blot. SIRT1 protein was successfully knocked out compared to control siRNA in the microglia. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared to untreated control.

3.2.13 Tiliroside prevents LPS/IFNγ-induced ROS production and GSH inhibition in BV2 microglia

Chronic activation of microglia generates excess cellular ROS, which ultimately damages adjacent neurons. This intracellular accumulation of ROS in microglia triggers the release of various inflammatory mediators through activation of downstream signalling pathways like MAPKs and NF-κB (Zhang et al. 2016). The effect of tiliroside on LPS/IFNγ-activated ROS production in BV2 microglia was investigated. BV2 cells were challenged with the combination of LPS and IFNγ for 24 hours in the presence and absence of the compound. Results show that tiliroside (2-6 μM) produced significant ($p<0.01$) reduction of ROS following activation by LPS/IFNγ in microglia. Compound at 2 μM significantly inhibited ROS generation by ~1.3-fold, 4 μM ~1.4-fold and at 6 μM by ~1.8-fold in the activated microglia (Figure 3.20a).

Glutathione (GSH) is a major cellular protectant against ROS and has been shown to be dysregulated during neuroinflammatory conditions (Roychowdhury et al. 2003) (Ray et al. 2011), (Barger et al. 2007) and (Cho et al. 2012). Consequently, tiliroside was investigated for its effect on the levels of GSH in LPS/IFNγ-activated microglia. Stimulation with LPS and IFNγ resulted in a significant decrease in GSH levels compared to untreated cells (Figure 3.20b). However, when pre-treated with tiliroside (2-6 μM), there was a significant ($p<0.001$) and dose-dependent increase (~1.7-fold increase, ~2.2-fold increase and ~2.5-fold increase) in the levels of GSH.

(a)



(b)

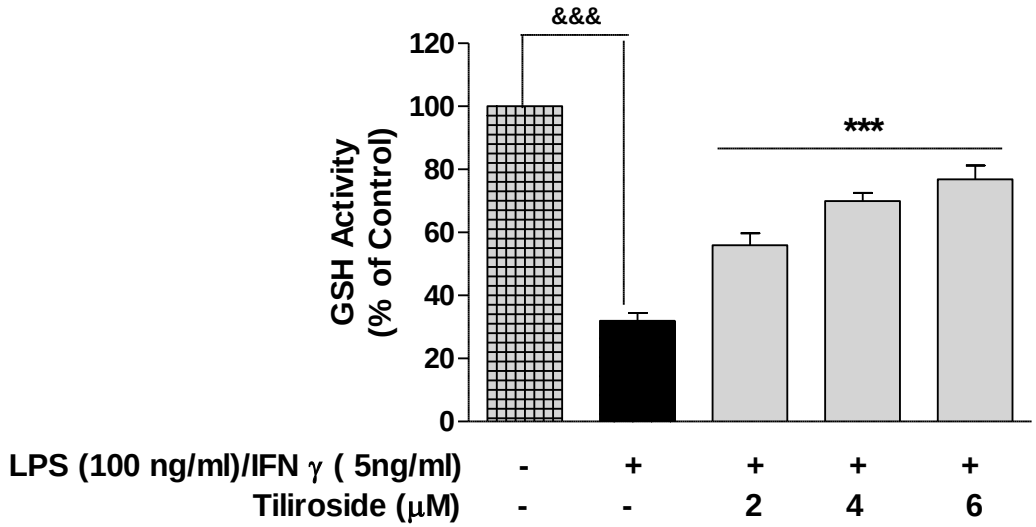


Figure 3.20 Tiliroside inhibited ROS generation and increased GSH levels in LPS/IFNγ-stimulated microglia

(a) BV2 microglia were pre-treated with tiliroside (2-6 μM) for 30 minutes and later stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml)-incubated for 24 hours. (a) Cells were washed with PBS and stained with 20 μM DCFDA for 30 minutes at 37°C, and then stimulated with LPS /IFNγ for 24 hours in the presence or absence of the compound. Fluorescence detection of ROS production was done using Polar star Optima plate reader. Tiliroside inhibited ROS production in the microglia. (b) Cells were pre-treated with compound for 30 minutes and later stimulated with LPS/IFNγ-incubated for 24 hours. Subsequently, the culture medium was replaced with GSH-Glo™ reagent and incubated at room temperature for 30 minutes. Thereafter, luciferin

detection reagent was added to each well and luminescence was then read. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ in comparison with untreated control and $*p<0.05$, $**p<0.01$, $***p<0.001$ in comparison with LPS/IFNγ.

3.3 Discussion

Neuroinflammation is a critical component of a diverse range of neurodegenerative diseases. Furthermore, increasing evidence suggests that hyperactive microglia is a key causative factor in this process. In this research, neuroinflammation inhibitory actions of the dietary flavonoid tiliroside were investigated in LPS/IFNγ-activated BV2 microglia.

As expected, stimulation of microglia with a combination of bacterial lipopolysaccharide (LPS) and gamma interferon (IFNγ) resulted in an increase in the production of pro-inflammatory cytokines like TNFα, IL-1β and IL-6. Several reports have shown that LPS is capable of inducing pro-inflammatory cytokines along with other chemokines via activating toll-like receptors (TLRs) (Xu et al. 2014). Further studies revealed that IFNγ can be used to prime macrophages derived from bone marrow to enhance the inflammatory response to TLRs (Schroder et al. 2006) and (Ye et al. 2001). Interestingly, when these macrophages were challenged with IFNγ alone, there were no effects on the induction of pro-inflammatory cytokines like TNFα, IL-6 and IL-1β. However, when combined with LPS high levels of pro-inflammatory cytokines were observed (Southworth et al. 2012). In other studies, this combination of LPS and IFNγ has been shown to increase the expression of MYD88 and TRAF6 in macrophages (Mita et al. 2001) and (Southworth et al. 2012). In the current research, tiliroside suppress the production of TNFα in LPS/IFNγ-activated BV2 microglia, suggesting that the compound is capable of inhibiting neuroinflammation. Studies have demonstrated that TNFα released from activated microglia during neuroinflammation binds to the TNFR superfamilies' on adjacent microglia and neurons (Didonato et al. 2012) and (Opal & DePalo 2000). This further triggers the release of excess TNFα into the brain environment and in turn exacerbates neuroinflammatory conditions leading to neuronal toxicity. In addition to TNFα, excess levels of IL-1β and IL-6 released from activated microglia are shown to induce neuronal death via activating several intrinsic pathways in neurons (Aïd & Bosetti 2011) and (Chatterjee et al. 2014). Tiliroside suppressed IL-1β release from LPS/IFNγ-stimulated microglia. Furthermore, the effect of tiliroside was investigated against LPS/IFNγ-induced IL-6 release in microglia. Results show that the compound significantly blocked the production of IL-6 in microglia, suggesting that tiliroside is

capable of inhibiting interleukins in neuroinflammation. Several lines of studies have shown that anti-inflammatory cytokines like IL-10 and TGF-β are capable of blocking the binding of TNFα to its receptors on the microglia and could resolve neuroinflammation (Park et al. 2007) and (Zhou et al. 2012). Tiliroside in this study reversed the inhibition of IL-10 production in activated microglia. All these results seem to suggest that tiliroside blocked the progression of neuroinflammation by inhibiting the production of pro-inflammatory and activating anti-inflammatory cytokines in microglia.

The combination of LPS and IFNγ increased the production of nitric oxide and the expression of iNOS protein in the microglia. Tiliroside reduced these events suggesting another mechanism for inhibiting neuroinflammation in activated BV2 cells. Stimulation of glial cells has been proposed to lead to the generation of large quantities of nitric oxide through iNOS protein expression and can contribute to neurodegeneration (Knott et al. 2000), (Calvello et al. 2012) and (Liu et al. 2008). The role of iNOS and its mediated nitric oxide in neuroinflammation is well documented. Excess nitric oxide has shown to induce ROS production in microglia via targeting mitochondrial electron transport chain function (Doherty 2011). Furthermore, excessive nitric oxide production has proved to be detrimental, as it is capable of inducing protein misfolding during post-translational modifications such as S-nitrosylation in the microglia. Dysregulated S-nitrosylation will hyperactivate transcription factors like NF-κB and further enhances inflammatory conditions in the brain (Chattopadhyay et al. 2010) and (Vauzour et al. 2015). Studies have shown that blocking iNOS protein expression and its mediated nitric oxide production will inhibit neuroinflammation in glial cells (Xing et al. 2008), (Choi & Park 2012) and (Bozic et al. 2015).

Tiliroside significantly suppressed LPS/IFNγ-induced PGE_2 production in activated microglia. Also, compound reduced COX-2 protein expression at all the concentrations investigated in microglia following stimulation with LPS and IFNγ. These results indicate that the activity of tiliroside is more pronounced at the level of COX-2 protein expression. Activated microglia are shown to generate eicosanoids like prostaglandins that are under control of cyclooxygenase (COX-2) enzyme pathway (Morales et al. 2014). Prostaglandin E2 (PGE_2) is a major eicosanoid found

in many CNS related inflammatory diseases and is a primary target for non-steroidal anti-inflammatory drugs (Doherty 2011) and (Wang et al. 2015).

The transcription factor NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells), plays a major role in regulating immune responses, inflammation and cell survival in the microglia. Out of five members of NF-κB family, heterodimers of the p50 and p65 subunits are widely studied in neuroinflammation, as they are considered as potent activators of inflammatory gene transcription (Hayden & Ghosh 2012). Several lines of evidence suggest that binding of translocated NF-κB to the promoter region of chemokines, pro-inflammatory cytokines (TNFα, IL-1β and IL-6), COX-2 and iNOS expressing gene is required for the target gene transcription (Lyman et al. 2013), (Didonato et al. 2012) and (Yamamoto et al. 1995). Moreover, hyper-activation of NF-κB signalling and its associated inflammatory mediators has been linked to the broad range of brain immune diseases such as AD and PD (Kawai & Akira 2007). Consequently, targeting NF-κB signalling in neuroinflammation could ablate the transcription and release of inflammatory mediators in the brain. NF-κB is coupled to IκB to form an inactive complex in the cytoplasm. However, when challenged with inflammatory stimuli or during endogenous inflammatory conditions, phosphorylation by active IKKα results in further phosphorylation and subsequent degradation of IκBα by proteasomes, followed by NF-κB translocation into the nucleus. In the nucleus, NF-κB undergo phosphorylation and binds to the promoter regions of inflammatory genes to initiate the transcription of inflammatory genes (Didonato et al. 2012). Therefore, the effect of tiliroside on phosphorylated-p65 in LPS/IFNγ-activated microglia was studied. Results revealed that the p-p65 suppressive effect of tiliroside was not efficient at 2 µM. However, as the concentration was increased to 4 and 6 µM, a dose-dependent inhibition of p65 phosphorylation was observed. To induce transcription, p-p65 has to bind to the DNA of the inflammatory genes. Therefore, further investigation was carried out to know the effect of the tiliroside on DNA binding of NF-κB. Surprisingly, tiliroside significantly blocked LPS/IFNγ-induced NF-κB-DNA binding in the microglia. These NF-κB-DNA binding inhibitory actions further support the anti-neuroinflammatory effects of tiliroside on LPS and IFNγ-induced TNFα, iNOS, COX-2 and IL-1β production in microglia. During neuroinflammation, transactivated NF-κB will be

translocated to the nucleus and will bind to the respective genes to drive the transcription. The compound was shown to produce a significant inhibition of NF-κB-dependent gene expression, through suppression of LPS/IFNγ-induced activation of the NF-κB-driven luciferase expression in microglia. These inhibitory effects further suggest that tiliroside can inhibit transactivation of NF-κB in activated microglia. From all these results, it appears that tiliroside inhibit neuroinflammation by targeting NF-κB signalling in BV2 microglia.

After confirming that tiliroside inhibited NF-κB signalling events in activated microglia, its effect on upstream kinases were investigated next. Results showed that tiliroside inhibited phosphorylation and subsequent degradation of IκBα in LPS/IFNγ-activated microglia. In addition to its inhibitory effects on IκBα phosphorylation, tiliroside also blocked phosphorylation of IKKα following stimulation with a combination of LPS and IFNγ in the microglia. Blocking these events during neuroinflammation could inhibit nuclear translocation of NF-κB and further transcription of inflammatory mediators (Xu et al. 2014), (Kang, Choi, et al. 2013) and (Hayden & Ghosh 2012). It appears from the above results that the NF-κB signalling pathway is associated with the neuroinflammation inhibitory activity of tiliroside in microglia.

The p38 MAPK signalling play a critical role in controlling signalling events during neuroinflammation and contribute to the production of inflammatory mediators such as TNFα, IL-1β, iNOS and NO (Kim et al. 2004), (Jeong et al. 2013) and (Herlaar & Brown 1999). Therefore, small molecules that are capable of down-regulating MAP kinase signalling could potentially inhibit neuroinflammation (Xing et al. 2008) and (Okorji et al. 2015). Further experiments showed that tiliroside attenuated the phosphorylation of p38 MAPK in LPS/IFNγ-stimulated BV2 microglia. Also, tiliroside blocked the activation of p38 downstream substrate MK2 and upstream kinase MAPK kinase 3/6 (MKK3/6). Several studies have demonstrated that, compounds that inhibit LPS-activated MKK3/6 and MK2 proteins blocks the production of TNFα, IL-6 and IL-8 and other cytokines in the microglia (Duraisamy et al. 2008), (Corrêa et al. 2012), (Fyhrquist et al. 2010) and (Neininger et al. 2002). Collectively, these results seem to indicate that p38 MAPK signalling pathway is involved in the

inhibitory effect of tiliroside in the production of inflammatory mediators when stimulated with a combination of LPS and IFNγ in microglia.

Studies have shown that inflammatory stimuli such as LPS initiate expression of pro-inflammatory cytokines by binding to the membrane-bound toll-like receptor 4 (TLR4). Results show that the combination of LPS and IFNγ significantly activated TLR4 in BV2 microglia. Furthermore, activated TLR4 culminates TRAF6 protein complex in the microglia. Activated TRAF6 complex has been shown to trigger the induction and nuclear translocation of NF-κB via rapid phosphorylation of specific serine residues of IκB by IKK complex along with p38 MAPK signalling (Karin & Gallagher 2009). Several studies have revealed that blocking TLR4 activation could culminate the production of NF-κB and p38 MAPK associated inflammatory mediators such as iNOS, COX-2, IL-1β and NO (Lehnardt et al. 2003), (Takeda & Akira 2004) and (Xu et al. 2014). This study revealed that tiliroside at highest concentration blocked the activation of TLR4 in activated microglia. Production of various pro-inflammatory factors during neuroinflammation are shown to be mediated by activated TLR4 via induction of TRAF6 complex (Karin & Gallagher 2009) and (Zhu et al. 2014). In addition to its TLR4 inhibitory activity, tiliroside also inhibited LPS and IFNγ-induced upregulation of TRAF6 expression in microglia showing that its activity is more pronounced at the level of TLR4/TRAF6. All these results seem to suggest that tiliroside alleviates LPS/IFNγ-induced neuroinflammation by inhibiting TLR4 mediated NF-κB and p38 MAPK signalling in microglia.

In the resting microglia, sirtuins especially SIRT1 were shown to inhibit NF-κB transcriptional activity by deacetylation of p65 subunit in the nucleus (Herskovits & Guarente 2014) and (Donmez & Donmez 2012). In this study, the combination of LPS and IFNγ increased the levels of acetylated-NF-κB-p65 in the microglia. However, tiliroside suppressed the expression of acetylated-NF-κB-p65 in a concentration–dependant manner suggesting that the compound is capable of inhibiting neuroinflammation via promoting deacetylation of NF-κB-p65 in microglia. Studies in the past have demonstrated that the binding of acetylated-NF-κB to the promoter regions of COX-2, iNOS, TNFα, IL-1β and IL-6 expressing gene is required for the target gene transcription in activated microglia (Chen et al. 2001), (Kawai et

al. 2011) and (Guarente 2011). Compounds that target NF-κB signalling during inflammation also exhibited inhibitory effects on acetylated-NF-κB-p65 subunit in microglia (Kumar & Sharma 2010) and (Zhu et al. 2011).

After confirming that tiliroside is capable of reducing the levels of acetylated-NF-κB-p65, further studies were conducted to investigate the effect of the compound on SIRT1 activation in microglia. Results show that tiliroside dose-dependently activated SIRT1 protein in the microglia. These data suggest that activation of SIRT1 by tiliroside probably results in the inhibition of acetylated-NF-κB-p65 in the microglia. Neuroinflammation is known to influence the expression of SIRT1 in the microglia. Emerging evidence demonstrates that there is a strong link between acetylated-NF-κB-p65 and SIRT1 (Cho et al. 2015). Therefore, the effect of the compound against LPS/IFNγ-induced SIRT1 nuclear accumulation was investigated in microglia. Data revealed a significant decrease in the level of SIRT1 protein in LPS/IFNγ-treated microglia, which explains the high levels of acetylated NF-κB-p65 during neuroinflammatory conditions. Studies have reported that inhibition of SIRT1 by LPS significantly increased the acetylation of RelA/p65 subunit and further enhanced NF-κB transcriptional activity in endothelial cells (Yang et al. 2012). Results from the current research suggest that the activation of LPS/IFNγ-induced SIRT1 protein by tiliroside probably results in the inhibition of NF-κB signalling in the microglia.

Tiliroside was shown to block LPS/IFNγ-induced neuroinflammation in the microglia, as well as supressed the expression of acetylated-NF-κB-p65 and also activated SIRT1. It should be kind of interesting to investigate whether SIRT1 activation contributes to the neuroinflammation inhibitory effects of tiliroside in LPS/IFNγ-treated microglia. This was achieved by using control and SIRT1 siRNA-transfected cells. Results show that in control siRNA-transfected cells, tiliroside inhibited the production of LPS/IFNγ-induced PGE_2, nitric oxide and pro-inflammatory cytokines such as TNFα, IL-1β and IL-6. However, these inhibitory activities of the compound were not affected in SIRT1 siRNA-transfected cells. These results further suggest that LPS/IFNγ-induced neuroinflammation inhibitory actions of tiliroside are independent of SIRT1 activation. The efficiency of SIRT1 gene knockdown in BV2 cells was assessed using western blotting experiments Results revealed that SIRT1 gene was successfully knocked out in all these experiments.

Glial cells have been shown to produce different ROS in response to various inflammatory stimuli (Lee & Yang 2012) and (Zhang et al. 2016). This intracellular accumulation of ROS triggers the release of various inflammatory mediators through activation of downstream signalling pathways like MAPKs and NF-κB in microglia (Rojo et al. 2014). Small molecules that exhibit inhibitory actions on intracellular signalling cascades involving NF-κB and p38 MAPK, have also been shown to block ROS generation in LPS-treated microglia (Zeng et al. 2012) and (Lee et al. 2015). Since tiliroside has produced a remarkable inhibition of NF-κB and p38 MAPK signalling, its effect on intracellular ROS production was assessed in LPS/IFNγ-stimulated microglia. Results demonstrate that a combination of LPS and IFNγ significantly increased ROS production in BV2 microglia. Tiliroside dose-dependently blocked this intracellular ROS accumulation, suggesting that the compound possess inhibitory actions against ROS-mediated neuroinflammation in microglia. Glutathione (GSH) is a critical cellular antioxidant and has been shown to be involved in scavenging ROS and peroxynitrite produced during neuroinflammation (Roychowdhury et al. 2003) and (Barger et al. 2007). In this study, the combination of LPS and IFNγ inhibited the endogenous level of GSH in the microglia. This observation further confirms that glutathione levels will be affected in neuroinflammation. However, tiliroside dose-dependently increased the production of GSH in activated microglia suggesting that compound possesses strong anti-inflammatory effects.

Tiliroside inhibited neuroinflammation in BV2 microglia through a mechanism involving TLR4 receptor-mediated activation of NF-κB and p38 MAPK signalling pathways. Also, the compound was shown to activate SIRT1 and inhibited the expression of acetylated- NF-κB-p65 in LPS/IFNγ-treated microglia. However, further RNAi experiments revealed that neuroinflammation inhibitory activities of the compound are not SIRT1 mediated.

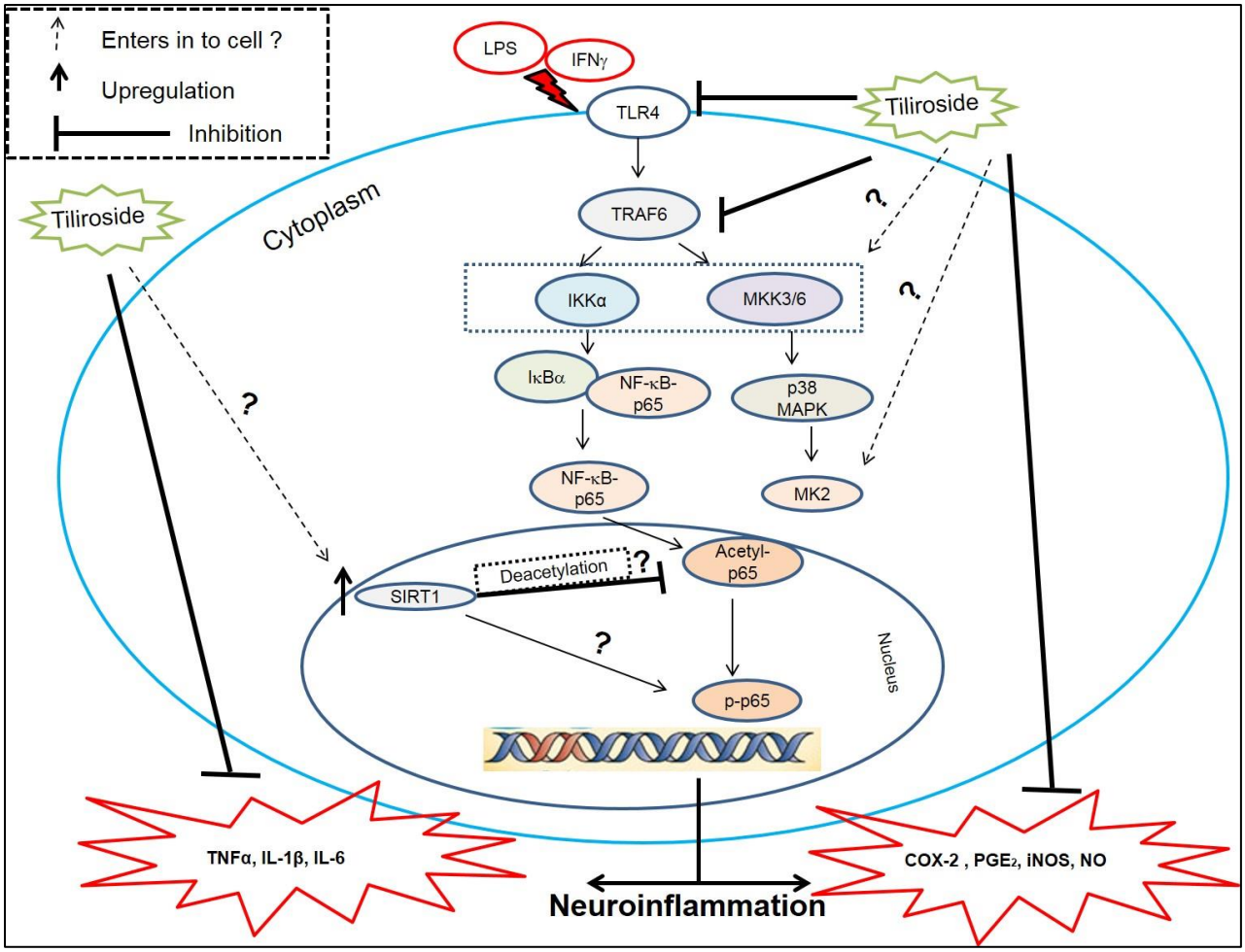


Figure 3.21 Proposed neuroinflammation inhibitory effects of tiliroside in microglia challenged with LPS and IFNγ. LPS: Lipopolysaccharide, IFNγ: Interferon gamma, TLR4: Toll-like receptor 4, TRAF6: TNF receptor associated factor 6, IKK: IκB kinase, MKK3/6: Mitogen-activated protein kinase kinase 3/6, MK2: MAP kinase-activated protein kinase 2, TNFα: Tumor necrosis factor alpha, IL-1β: Interleukin 1 beta, IL-6: Interleukin 6, COX: Cyclooxygenase, PGE_2: Prostaglandin E2, iNOS: inducible Nitric oxide synthase, NO: Nitric oxide.

Chapter 4. Tiliroside inhibited neuroinflammation-induced neuronal toxicity

4.1 Introduction

In response to local injury, infection or harmful toxins, microglial cells become hyperactivated and exhibit chronic inflammatory responses by secreting inflammatory mediators like nitric oxide, PGE_2, ROS/RNS and pro-inflammatory cytokines including IL-6 and TNFα (Peterson & Toborek 2014), (Spencer et al. 2012) and (Huang et al. 2015). Sustained activation of microglia consistently produces powerful neurotoxic mediators which can cause neuronal injury and thereby contribute to the progressive death of neurons in the CNS. Microglia-derived excessive cytotoxic factors and its associated chronic neuroinflammation have been recognised as important pathological events in several neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis, and dementia (Zecca et al. 2004) and (Ray & Lahiri 2009). However, how these cytotoxic factors become more detrimental to neurons is yet to be investigated.

In adverse neurological diseases, progressive loss of structure and function of neurons was observed, leading it to profound brain atrophy and cognitive deficit (Cobourne-Duval et al. 2016). Studies have shown that elevated levels of pro-inflammatory cytokines and ROS-mediated oxidative stress play a vital role in inducing these adverse effects in neurons (Hamadi et al. 2016) and (Hwang et al. 2009). Limited production of NO by active microglia is turned to be beneficial to adjacent neurons. However, recent research has shown that high levels of NO during chronic inflammation can induce neuronal death by causing selective inhibition of mitochondrial cytochrome C oxidase in neurons releasing high levels of ROS into the cytoplasm (Uttara et al. 2009) and (Watanabe et al. 2000). In addition to these effects, NO in excess reacts with superoxide from NADPH oxidase leads to the generation of peroxynitrite ($ONOO^-$), which is a key regulator in driving the neurotoxic responses (Brown & Neher 2010). Compounds that are shown to inhibit neuroinflammation exhibited neuroprotective potential by blocking ROS-mediated neuronal death (Zeng et al. 2010).

Interestingly, ROS accumulation within the neuronal cells has been linked to the disruption of calcium homoeostasis and its mediated induction and activation of pro-apoptotic proteins such as p53, caspases, Bax and Bcl2 leading to a programmed

neuronal death resulting in neuronal toxicity (Mattson 2007) and (Pfeiffer et al. 2014). LPS-induced neuroinflammation has been shown to evoke calcium influx in the neuronal cells of the animal brain, while inhibition of excessive calcium appeared to be beneficial to the neurons (Hoffmann et al. 2013). This observation gives a hint that blocking neuroinflammation-induced ROS production and calcium increase in neuronal cells would be a useful therapeutic approach in the treatment of various neurodegenerative diseases.

In addition to ROS, active microglia are shown to release various pro-inflammatory cytokines which have been reported to initiate morphological changes in the adjacent neurons (Lull & Block 2010). It has been demonstrated that inflammatory cytokines such as TNFα and IL-1β from hyperactive active microglia are capable of inducing redox imbalance and DNA strand breakage in neurons. These were considered as a common feature of many neurodegenerative conditions which cause neuronal death in hippocampal regions of the brain (Vauzour et al. 2015) and (Michelle L. Block et al. 2007). From this perspective, targeting neuroinflammation-mediated intracellular ROS accumulation and its associated DNA damage with suitable pharmacologically-active compounds may help to reduce neuronal toxicity.

Neurotoxic factors released during neuroinflammation can be studied *in vitro* by treating neuronal cells with conditioned culture medium collected from BV2 microglia that are stimulated using external stimuli such as LPS (Bevinahal et al. 2014) and (Gresa-Arribas et al. 2012). Tiliroside was shown to have neuroinflammation inhibitory effects; however, its neuroprotective potentials were not yet studied. In the current research, culture medium collected from BV2 microglia that were pre-treated with tiliroside (2-6 µM) before stimulation with LPS and IFNγ was investigated for its neuroprotective effects against HT22 neuronal toxicity induced by neuroinflammation.

4.2 Results

4.2.1 Tiliroside inhibited neuroinflammation-mediated neurotoxicity in HT22 cells

It is now well established that microglia-induced neuroinflammation is involved in neuronal damage in the brain causing neurodegenerative diseases like AD and PD (Michelle L. Block et al. 2007). The neurotoxic effects of conditioned medium collected from microglia that were stimulated with LPS and IFNγ was tested on HT22 neuronal cells. Results of XTT assay showed that there was a marked reduction in the viability of HT22 neurons when exposed to conditioned medium from the microglia that were incubated with LPS and IFNγ for 24 hours. However, culture medium collected from BV2 cells that were pre-treated for 30 minutes with increasing concentrations of tiliroside (2-6 μM) before the stimulation with LPS/IFNγ significantly ($p < 0.01$) inhibited neuronal toxicity induced by neuroinflammation. Microglia culture medium pre-treated with 2 μM of the tiliroside increased the viability of HT22 neurons by ~1.5-fold, 4 μM by ~ 1.9-fold and 6 μM by ~2-fold (Figure 4.1).

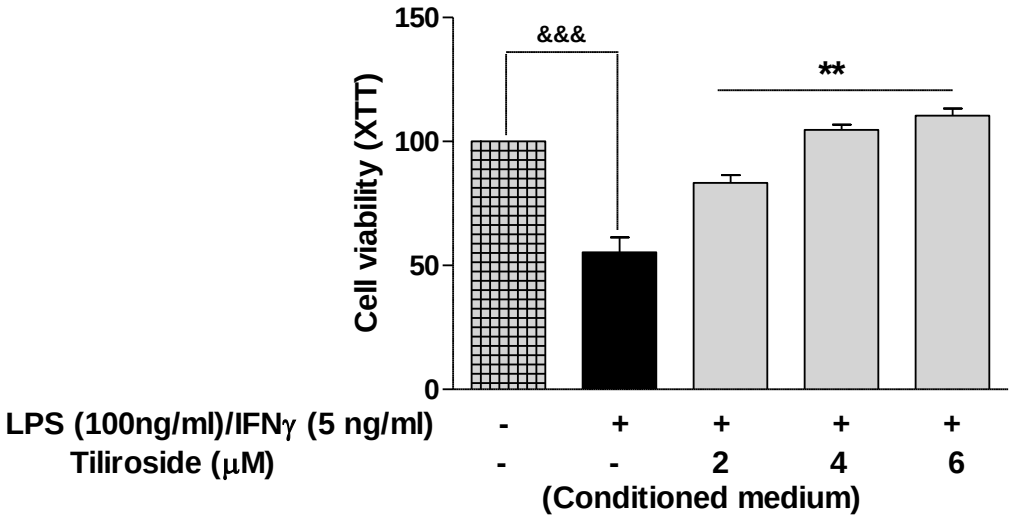


Figure 4.1 Tiliroside inhibited microglial conditioned medium-induced neuronal death in HT22 neurons

HT22 neurons were incubated with conditioned culture medium obtained from BV2 microglia that were stimulated with LPS and IFNγ. Subsequently, other wells containing HTT22 neurons were exposed to conditioned medium obtained from BV2 cells that were pre-treated with increasing concentrations of tiliroside (2-6 μM) prior to stimulation with LPS/IFNγ for 24 hours. XTT assay was carried out to assess the viability of HT22 neurons. Results showed that tiliroside reversed the neuroinflammation-induced HT22 neuronal death. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

4.2.2 Tiliroside inhibited neuroinflammation-induced calcium accumulation in HT-22 neuronal cells

Studies have shown that calcium ions are universal regulators that control various cellular processes such as cell death (Tran & Watanabe 2006). High levels of calcium were observed in neuronal cells during neuroinflammatory conditions (Hoffmann et al. 2013). Moreover, several lines of evidence reported that excess generation of ROS in neurons tend to induce calcium influx resulting in the death of neurons (Ye et al. 2013) and (McCoy & Tansey 2008). The addition of conditioned culture medium from BV2 cells that were stimulated with LPS/IFNγ to HT22 neurons induced high levels of calcium (~10-fold increase, $p<0.001$). However, incubation of HT22 cells with conditioned medium collected from BV2 cells that are pre-treated with tiliroside showed a minimal effect at concentrations of 2 and 4 µM. Surprisingly culture medium from BV2 cells pre-treated with 6 µM tiliroside significantly reduced (~1.8-fold reduction, $p<0.01$) neuroinflammation-induced calcium accumulation in HT22 neurons (Figure 4.2).

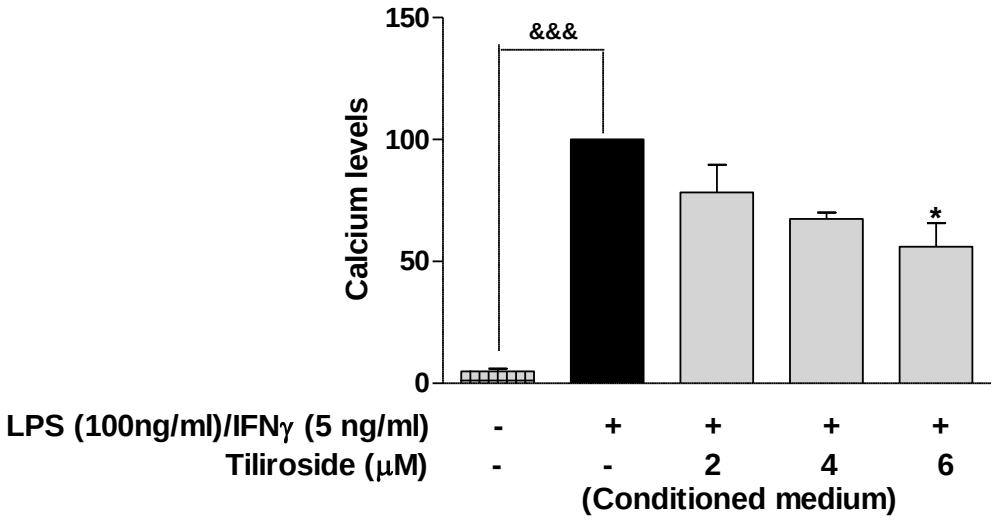


Figure 4.2 Tiliroside inhibited microglial conditioned medium-induced calcium accumulation in HT22 neuronal cells

HT22 neurons were incubated with conditioned culture medium obtained from BV2 microglia that were stimulated with LPS and IFNγ. Subsequently, HT22 neurons in other wells were incubated with conditioned medium obtained from BV2 cells that were pre-treated with increasing concentrations of tiliroside (2-6 μM) and stimulated with LPS/IFNγ for 24 hours. After that, calcium accumulation assay was carried out in HT22 cells. Results show that microglia-induced neuroinflammation increased calcium levels in HT22 neurons. Culture medium from BV2 cells pre-treated with compound was shown to reduce calcium accumulation in HT22 neurons. All values are expressed as mean ± SEM for at least three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with the untreated control and *p<0.05, **p<0.01, ***p<0.001 in compared to LPS/IFNγ.

4.2.3 Tiliroside inhibited ROS production in HT-22 neuronal cells

Reactive oxygen species from hyperactive microglia causes oxidative damage, induction of apoptosis and DNA fragmentation in adjacent neurons (Hsieh & Yang 2013). Since conditioned culture medium from microglia treated with tiliroside and LPS/IFNγ was shown to inhibit calcium accumulation in HT22 neuronal cells, its effect on ROS generation was further investigated. Incubation of HT22 cells with conditioned culture medium collected from LPS and IFNγ stimulated BV2 cells significantly increased (~5-fold increase, $p<0.001$) intracellular ROS production compared to the cells that were incubated with culture medium collected from control BV2 microglia. Generation of ROS was significantly inhibited ($p<0.01$) when HTT22 neuronal cells were incubated with conditioned culture medium obtained from microglia cells that were pre-treated with tiliroside 2 µM (~1.8-fold), 4 µM (~2.3-fold) and 6 µM (~1.8-fold) in a concentration-dependent manner (Figure 4.3).

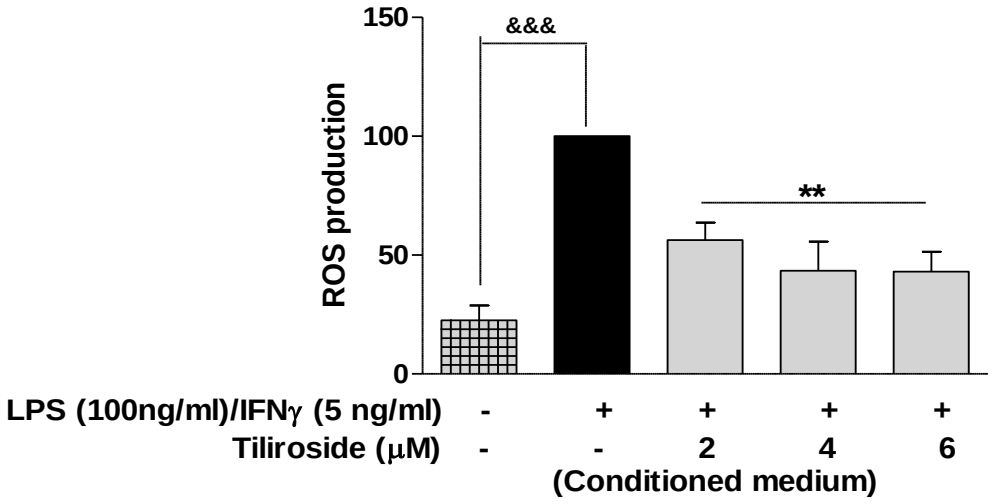


Figure 4.3 Tiliroside inhibited microglial conditioned medium-induced ROS production in HT22 neurons

HT22 neurons were exposed to conditioned culture medium obtained from BV2 microglia that were stimulated with LPS and IFNγ or to the medium from untreated microglia. At the same time, HT22 neurons were incubated with conditioned medium obtained from BV2 cells that were pre-treated with increasing concentrations of tiliroside (2-6 μM) before stimulation with LPS and IFNγ for 24 hours. Later, ROS production in HT22 neurons was determined by using DCFDA-assay kit. Results showed that tiliroside dose-dependently reduced ROS generation in HT22 neurons. All values are expressed as mean ± SEM for at least three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ as compared with untreated control and *p<0.05, **p<0.01, ***p<0.001 in compared to LPS/IFNγ..

4.2.4 Tiliroside inhibited neuroinflammation-mediated DNA fragmentation of HT22 neuronal cells

Inflammatory cytokines released by activated microglia were shown to induce apoptosis that leads to the damage and death of adjacent neurons (Thornton et al. 2006). In general, cleavage of chromatin DNA into fragments is considered as an essential hallmark of neuronal apoptosis (Koh et al. 2008). The effect of conditioned medium obtained from BV2 microglia that were stimulated with the combination of LPS and IFNγ on HT22 neuronal DNA fragmentation was determined. Results in Figure 4.4 show that conditioned medium from BV2 cells that were stimulated with LPS and IFNγ significantly increased ($p<0.001$) DNA fragmentation in HT22 neurons. Neuroinflammation increased DNA fragmentation of HT22 neurons by ~2.9-fold compared to neurons treated with culture medium collected from control BV2 cells. Interestingly, conditioned medium from microglial cells that were pre-treated with tiliroside 2 μM has no significant inhibitory effect on DNA fragmentation, however at 4 and 6 μM showed a dose-dependent reduction (~1.2-fold, $p< 0.001$ and ~1.6-fold, $p< 0.001$) in the DNA fragmentation of HT22 neuronal cells.

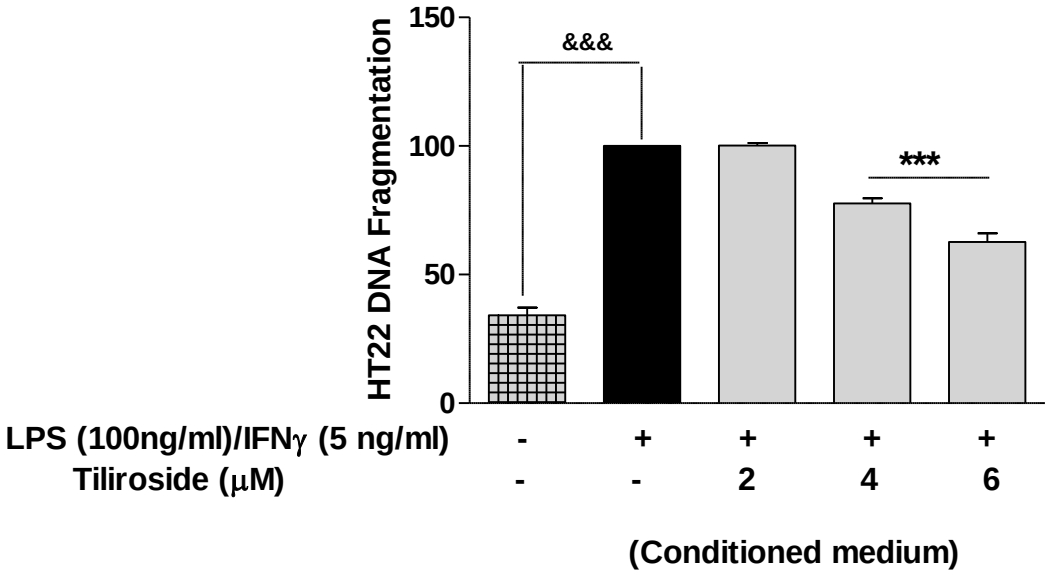


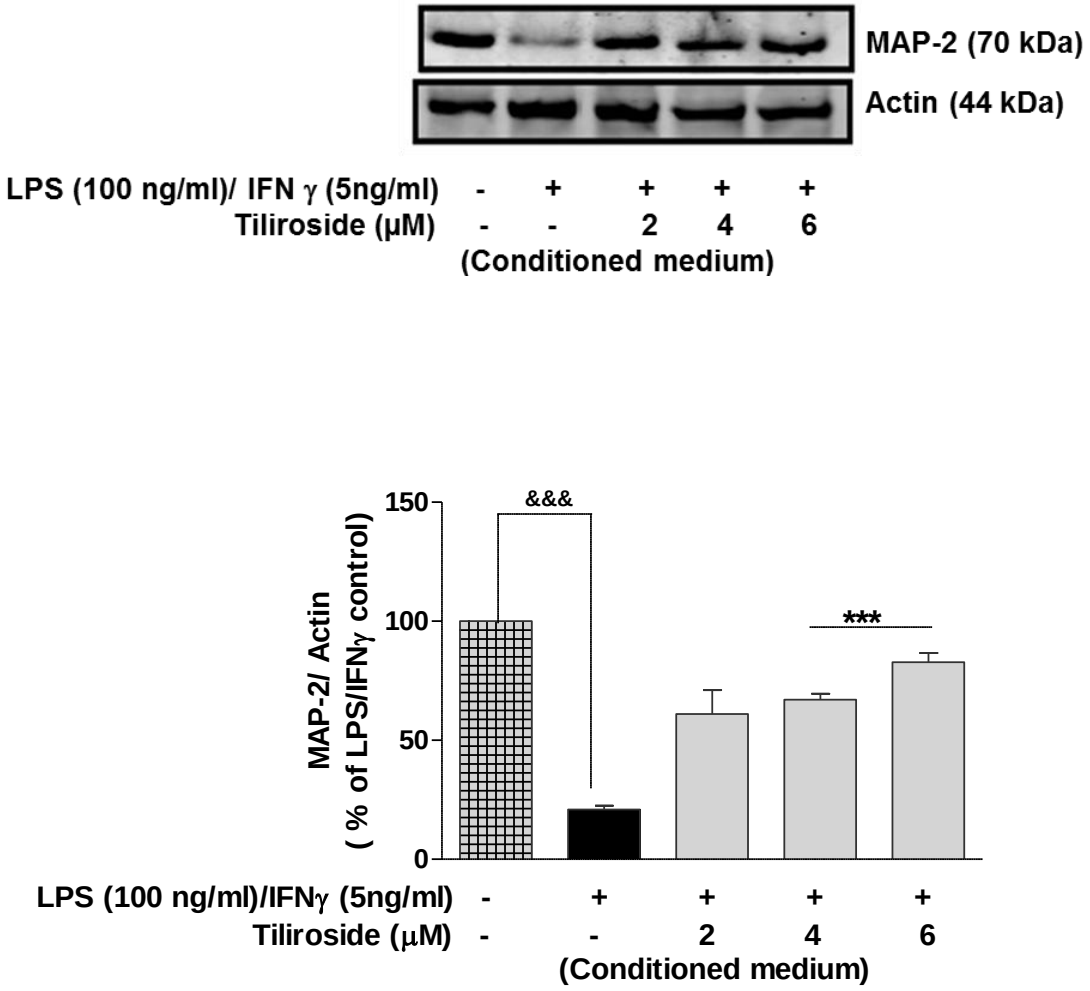
Figure 4.4 Tiliroside inhibited neuroinflammation-induced DNA fragmentation of HT22 neuronal cells

HT22 neurons were exposed to conditioned culture medium obtained from BV2 microglia that were stimulated with LPS and IFNγ. Subsequently, other wells with HT22 neurons were incubated with conditioned medium obtained from BV2 cells that were pre-treated with increasing concentrations of tiliroside (2-6 μM) prior to treat with LPS and IFNγ for 24 hours. Later, DNA fragmentation assay was performed using ELISA based DNA fragmentation assay kit. Results showed that tiliroside (2-6 μM) reduced neuroinflammation-induced DNA fragmentation of HT22 neurons. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ in comparison with untreated control and *p<0.05, **p<0.01, ***p<0.001 compared to LPS/IFNγ.

4.2.5 Tiliroside reduced MAP2 expression in microglial conditioned medium-induced HT22 neuronal death

In the brain, active neuronal cells have been shown to express high levels of microtubule-associated protein 2 (MAP2) (Strauss et al. 2000). Studies have shown that neurotoxic mediators along with pro-inflammatory cytokines influence the structure and synaptic plasticity of neurons in the CNS (Brown & Neher 2010). Notably, microglia-mediated neuroinflammation was shown induce neuronal toxicity, where the expression of MAP2 is typically reduced (Hwang et al. 2009). The conditioned medium obtained from BV2 microglia that were stimulated with LPS/ IFNγ was tested against MAP2 protein expression in HT22 neurons. Western blotting results show that when HT22 neurons were incubated with culture medium from microglia that were stimulated with LPS and IFNγ significantly reduced (~4.1-fold reduction, $p<0.001$) the expression of MAP2 protein compared to neurons that were treated with culture medium collected from control BV2 cells. Surprisingly, at 2 μM the effect of the compound was minimal on the neuroinflammation-induced MAP2 protein expression (Figure 4.5a). However, conditioned medium from the microglia that were pre-treated with tiliroside significantly increased (4 μM, ~3.1-fold, $p<0.001$ and 6 μM, ~3.9-fold, $p<0.001$) MAP2 protein expression. Immunofluorescence experiment was done to further confirm the effect of microglia-induced neuroinflammation on the expression of MAP2 protein in HT22 neurons. Figure 4.5b shows that MAP2 protein expression was reduced in neurons incubated with culture medium obtained from BV2 cells that were stimulated with LPS and IFNγ compared to neurons that were incubated with culture medium collected from control BV2 microglia. Interestingly, conditioned culture medium from BV2 cells that were pre-incubated with tiliroside (2-6 μM) was shown to increase the expression of MAP2 protein in HT22 neurons.

(a)


MAP-2 (70 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/ IFN γ (5ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6
(Conditioned medium)
MAP-2/ Actin
(% of LPS/IFNγ control)
150
100
50
0
&&&
***
LPS (100 ng/ml)/IFNγ (5ng/ml) - + + + +
Tiliroside (μM) - - 2 4 6
(Conditioned medium)

(b)

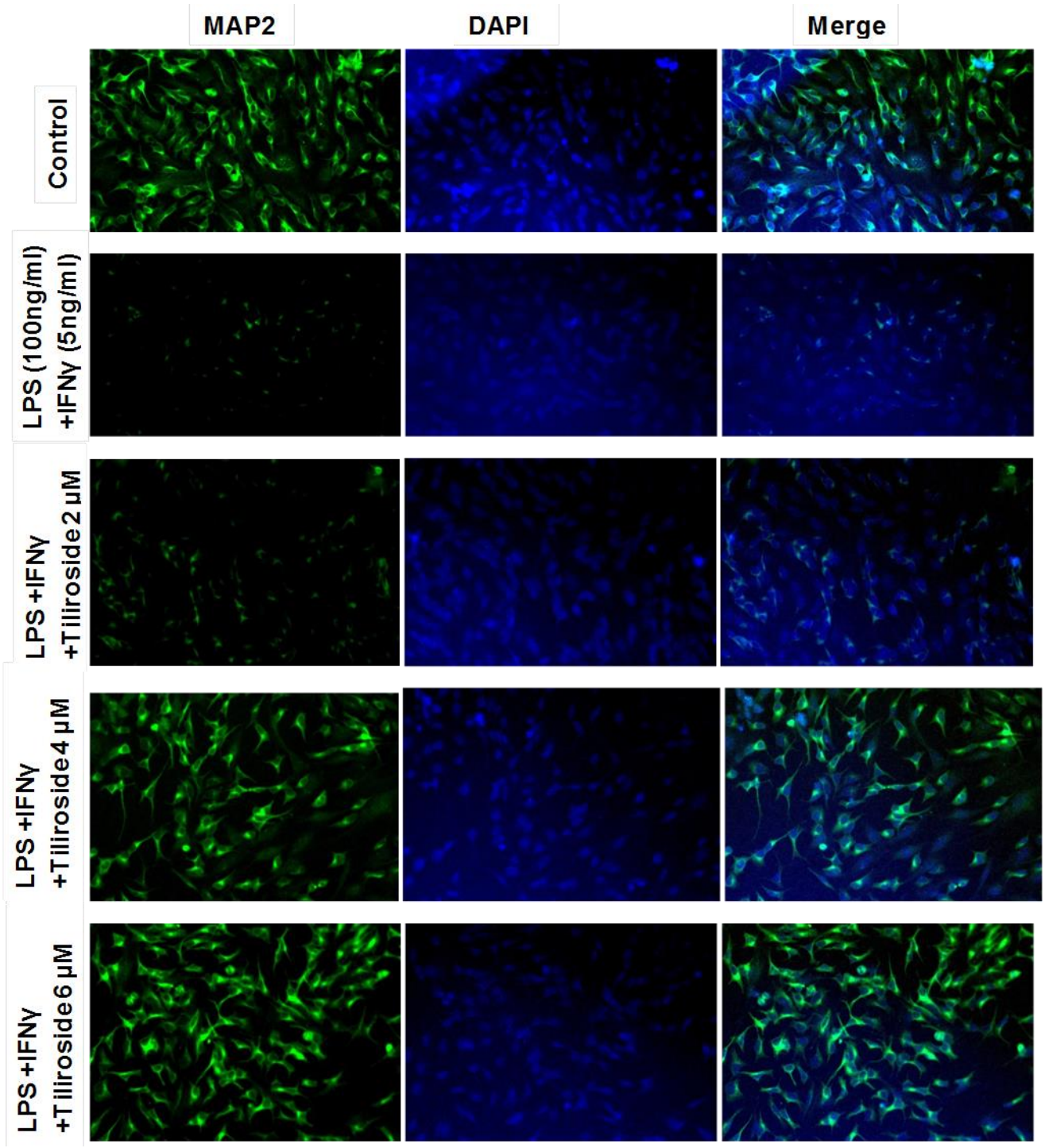


Figure 4.5 Tiliroside reversed neuroinflammation-induced MAP2 expression in HT22 cells

HT22 neurons were incubated with conditioned culture medium obtained from BV2 microglia that were stimulated with LPS and IFNγ. Subsequently, HT22 neurons in other wells were incubated with conditioned medium obtained from BV2 cells that were pre-treated with increasing concentrations of tiliroside (2-6 µM) and stimulated with LPS/IFNγ for 24 hours. (a)

Cytoplasmic lysates were collected and subjected to western blotting to assess the expression of MAP2 protein. Results showed that tiliroside dose-dependently increased MAP2 protein expression in HT22 neurons. (b) Immunofluorescence experiments were done to investigate the effects of microglia-induced neuroinflammation on the expression of MAP2 protein. HTT22 neuronal cells were treated with microglial conditioned medium for 24 hours. Later, cells were fixed, blocked and stained with MAP2 antibody for overnight. The following day, cells were washed and incubated with secondary antibody for 2 hours in the dark. Cells were counterstained with DAPI and fluorescence images acquired with an EVOS® FLoid® cell imaging station (scale bar=100 µm) and processed using image J. Results revealed that tiliroside increased the expression of MAP2 protein in HT22 neurons. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control and $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to LPS/IFNγ.

4.3 Discussion

During neuroinflammation, microglia becomes hyperactivated and release high amounts of neurotoxic factors that are detrimental to adjacent neurons. Studies have shown that pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-18 and TNFα released by microglia are critically involved in promoting neurotoxicity (Ye et al. 2013). The primary purpose of this study was to evaluate HT22 neuronal toxicity when treated with microglial conditioned medium that was previously collected from microglia that were stimulated with the combination of LPS and IFNγ. Also, to further investigate the neuroprotective effects of the culture medium obtained from BV2 microglia that were pre-treated with various concentrations of tiliroside before treatment with LPS/IFNγ.

In the CNS, microglia promotes inflammatory responses following bacterial invasion or local injury by consistently producing pro-inflammatory cytokines, interleukins and chemokines. However, in neuroinflammation, high levels of these neurotoxic factors released by microglia have been shown to influence surrounding neurons (Michelle L. Block et al. 2007). Cell viability results revealed that microglia-mediated neuroinflammation induced toxicity in HT22 neurons when treated with conditioned culture medium from BV2 cells that were stimulated with LPS/IFNγ. However, culture medium from microglia that were pre-incubated with tiliroside protected neurons from neuroinflammation-induced toxicity. Results from several other animal models revealed that neuroinflammation is closely associated with the risk of inducing neuronal death via releasing excess pro-inflammatory cytokines in neurodegenerative diseases like AD and PD (Kumar & Sharma 2010) and (Lee et al. 2010). These results seem to suggest that neurotoxic mediators that are released into microglial conditioned media may contribute to HT22 neuronal death. While culture medium collected from microglia that were pre-treated with tiliroside was shown to block neuroinflammation-mediated neuronal death, suggesting the neuroprotective potential of the compound.

Relatively high levels of intrinsic calcium accumulation was observed in adjacent neurons during neuroinflammation (Pfeiffer et al. 2014) and (Mattson 2007). HT22 neuronal calcium levels were significantly increased when exposed to conditioned culture medium obtained from BV2 cells that were stimulated with LPS/IFNγ.

Surprisingly, culture medium collected from BV2 cells pre-incubated with increasing concentrations of tiliroside was shown to reduce calcium accumulation in the HT22 neurons. Studies have shown that microglial TNFα during neuroinflammation induces calcium influx in neurons by binding to TNFR1 receptor (Olmos & Llad 2014). Possibly, this leads to the excessive accumulation of Ca^{2+} in neurons which will further induce neuronal death by increasing the excitatory synaptic strength (Yu et al. 2002). In addition to TNFα, IL-1β secreted by hyperactive microglia was shown to induce neurotoxicity through high glutamate production in neurons (Ye et al. 2013). A different set of studies revealed that excess calcium in the neurons also tends to release high levels of free radicals and ROS, which will further damage DNA by inducing apoptosis cascades (McCoy & Tansey 2008).

Excessive generation of ROS in neurons is considered as an important hallmark in neuroinflammation-induced neurotoxicity (Mitra et al. 2015) and (Mattson 2007). Therefore, production of ROS in HT22 neuronal cells was measured after incubated with conditioned culture medium obtained from BV2 microglia that were challenged with LPS and IFNγ. Results revealed that conditioned culture medium collected from BV2 cells that are challenged with the combination of LPS and IFNγ increased ROS production in HT22 neuronal cells. Interestingly, neuroinflammation-induced intracellular ROS accumulation was prevented when HT22 neurons were incubated with culture medium collected from microglia that were pre-treated with tiliroside before stimulation with LPS/IFNγ. In previous studies, compounds that possess neuroprotective effects were shown to block neuroinflammation-mediated calcium and ROS accumulation in neuronal cells (Zeng et al. 2010), (Bisht et al. 2010) and (Ishige et al. 2001). In SH-SY5Y neuronal cells, dietary flavonoid kaempferol inhibited rotenone-induced ROS production at a concentration of 30 µM and further blocked neurodegeneration (Filomeni et al. 2012). In the current research, tiliroside, a derivative of kaempferol blocks neuroinflammation-induced ROS generation at a concentration of 6 µM. These overall results tend to suggest that conditioned culture medium collected from microglia that were pre-incubated with tiliroside and stimulated with LPS/IFNγ was shown to inhibit HT22 neuronal toxicity by blocking calcium and ROS accumulation.

Activated microglia produces pro-inflammatory cytokines and chemokines along with ROS have been implicated in neuronal apoptosis and also shown to be linked to the

pathogenesis of neurodegenerative diseases (Ishige et al. 2001) and (Hamadi et al. 2016). Also, it is evident from several studies that when neurons were stimulated with inflammatory cytokines such as TNFα or IL-1β, they undergo apoptosis, which suggests that neuroinflammation will induce neuronal apoptosis (Bevinahal et al. 2014) and (Wiese et al. 2006). Neurons that undergo apoptosis release DNA fragments into the cytoplasm (Zhou et al. 2014) and (Koh et al. 2008). When the conditioned culture medium from microglia stimulated with LPS/IFNγ incubated with HT22 neuronal cells, an increase in the DNA fragmentation was observed. This observation further suggests that conditioned culture medium collected from BV2 microglia that were stimulated with combination of LPS and IFNγ was neurotoxic. However, data from the results show that the conditioned medium from BV2 cells that were pre-treated with various concentrations of tiliroside significantly inhibited neuroinflammation-mediated HT22 neuronal DNA fragmentation.

After observing the effects of conditioned medium on neuronal DNA fragmentation, its effect on MAP2 protein expression was next investigated. MAP2 belongs to microtubule-associated family and are thought to be involved in the assembly of the neuronal microtubules (Strauss et al. 2000). It is a neuronal marker and was shown to highly express in healthy neuronal cells. Results show that conditioned medium that contained neurotoxic factors was shown to reduce the expression of MAP2 protein. However, conditioned medium collected from BV2 cells that were pre-treated with tiliroside significantly increased the expression of MAP2 protein in HT22 neurons. It appears from the results that neuroprotective potentials of culture medium obtained from BV2 microglia that were pre-treated with tiliroside inhibited neuroinflammation-induced HT22 neuronal death via blocking neurotoxic cellular events such as DNA fragmentation, ROS production, calcium accumulation and partly by increasing MAP2 protein expression.

Chapter 5. Anti-neuroinflammatory effects of tiliroside are Nrf2 mediated

5.1 Introduction

Several studies indicate that neuroinflammation is a double-edged sword that executes both beneficial and detrimental effects on the adjacent neurons in the brain (Tang & Le 2014). This execution depends on the functional phenotype of the microglia, which ranges from pro-inflammatory M1 phenotype to immunosuppressive M2 phenotype. Hyperactive microglia that induces inflammatory cytokines such as TNF-α, IL-1β, IL-6, as well as superoxide, ROS, NO and NF-κB pathways are termed as M1 microglia. On the other hand, M2-microglia have been shown to inhibit inflammatory responses by releasing anti-inflammatory cytokines such as IL-4, IL-13, IL-10, and TGF-β (Tang & Le 2016) and (Zhou et al. 2012). Studies have also shown that M2-microglia could suppress the production of pro-inflammatory cytokines, neurotoxic factors and reduce NO release, which collectively blocks LPS-induced neuroinflammation (Ledeboer et al. 2000) and (Park et al. 2005). Further research on M2-microglia revealed that several antioxidant and cytoprotective genes, which are under control of transcription factor nuclear factor erythroid 2 related factor 2 (Nrf2) are activated to inhibit neuroinflammation in the brain (Jazwa et al. 2011). Novel findings also indicate that Nrf2-deficient mice showed increased levels of neuroinflammatory M1 markers (COX-2, iNOS, IL-6, and TNF) and reduced levels of M2 markers (ARG1, and IL-4) in response to neurotoxin (Rojo et al. 2010) and (Jazwa et al. 2011). Also, other studies have suggested that NF-κB is considered as a master regulator of M1 phenotype, while Nrf2 may be a regulator of the M2 phenotype (Rojo et al. 2014).

In this regard, activating intracellular anti-inflammatory mechanisms such as Nrf2 signalling could be a popular strategy to prevent inflammation-mediated neuronal toxicity. Nrf2 is a critical regulator of endogenous inducible defence systems in the brain and is actively produced by microglia in response to oxidative stress. Under normal conditions, Nrf2 is bound to cysteine residues of repressor protein Kelch-like ECH-associated protein 1 (Keap-1), in the cytoplasm. When countered with oxidative or nitrosative stimuli, these cysteine residues get modified enabling translocation of Nrf2 into the nucleus, where it will bind to *cis*-acting antioxidant responsive element (ARE). And further initiate the activation of ARE-dependent detoxifying genes such

as, heme oxygenase-1 (HO-1), NADPH quinine oxidoreductase (NQO1), γ-glutamyl synthase , γ-glutamylcysteine synthase (GCS), glutathione reductase (GR), superoxide dismutase's (SODs), UDP-glucuronosyl transferases (UGTs) and glutathione S-transferase (GST) (Johnson et al. 2008) and (Buendia et al. 2016). Recently, several studies have suggested the relevance of these antioxidant proteins in the immunomodulation of microglia (Pan et al. 2012) and (Kim et al. 2012). Notably, HO-1 and NQO1 are considered to be critical cytoprotective mediators in the cellular response to nitrosative and oxidative stress during neuroinflammation. Two types of heme oxygenase isoforms (HO-1 and HO-2) are ubiquitously expressed in microglia. HO-1 is considered as rapidly inducible protein which is under control of transcription factor Nrf2 and HO-2 is a constitutive protein that is expressed under normal conditions. Out of two isoforms, HO-1 has been shown to degrade heme to generate iron, carbon monoxide (CO) and bilirubin, which exert an important implication in limiting neuroinflammation (Sawle et al. 2005) and (Syapin 2008). Notably, HO-1 induced iron is involved in maintaining the structure of the glial cells, which was inhibited during inflammation in the brain (Gu et al. 1998). (Gu et al. 1998). Also, heme generated iron plays a critical role in DNA synthesis, mitochondrial respiration, oxygen transportation, myelin synthesis, and neurotransmitter synthesis, in contrast, dysregulation of iron homoeostasis can cause neurotoxicity (Zecca et al. 2004). Surprisingly, deprived HO1 expression and its generated final by-products were observed in neurodegenerative conditions like AD, PD and ALS (Lee & Suk 2007). On the other hand, its activation has been shown to suppress LPS-induced inflammation in mouse peritoneal macrophages and microglial cells in the brain (Li et al. 2008), (Kang, Kim, et al. 2013) and (Y. Li et al. 2015). Therefore, activation of HO-1 could be a better therapeutic strategy for the treatment of neuroinflammatory conditions.

Under conditions of neuroinflammation, activation of NQO1 was shown to protect hyperactive microglia from highly reactive quinone by catalysing them to redox-stable hydroquinone (Nioi & Hayes 2004). Also, increase in ROS are detrimental to normal cell function as this condition can facilitate the damage of DNA, however, the presence of high levels of NQO1 has been shown to modulate this DNA damaging stimuli by stabilising the endogenous p53 (Nioi & Hayes 2004) and (Wakabayashi et al. 2010). From this perspective, targeting Nrf2/HO-1/NQO1-antioxidant axis with

suitable pharmacologically-active compounds may help to reduce neuroinflammation and its associated neurodegeneration.

NF-κB has been shown to attenuate Nrf2 signalling and its mediated antioxidant genes (Liu et al. 2008). It appears that NF-κB communicate with Nrf2 at the transcriptional level in neuroinflammation. These two transcription factors have been shown to compete against co-activator CREB binding protein (CBP/p300) at DNA level. Transcription factor NF-κB binds to CBP/p300 and facilitate the transcription of inflammatory genes such as c-Fos, c-jun, Arc, Fosb and brain-derived neurotrophic factors (BDNF), which has shown to induce neuroinflammation in microglia (A. Cuadrado et al. 2014) and (Krasnova et al. 2016). Previous studies have suggested that pharmacological activation of Nrf2 might inhibit NF-κB binding capacity to CBP/p300 and thus block neuroinflammation (Beaumont et al. 2012) and (Didonato et al. 2012). Studies in the past showed that during neuroinflammation, high levels of ROS, nitrosative stress and upstream kinases like MAPKs, initiate the activation of Nrf2 leading it to translocate to the nucleus and compete with NF-κB at the transcriptional level (Surh & Na 2008) and (Kawai et al. 2011). However, activation of NF-κB has been shown to attenuate the transcription of antioxidant genes like HO-1 and NQO1 that are under the control of Nrf2 (Cuadrado et al. 2014). Also, studies in various animal models had illustrated that when Nrf2 gene was knocked out, increase in the NF-κB-mediated neuroinflammation was observed (Boyanapalli et al. 2014) and (Ichimura et al. 2013). Interestingly, dietary flavonoids that activate Nrf2 signalling were shown to inhibit neuroinflammation in various cell cultures and animal models (Lee et al. 2011), (Y. Li et al. 2015), (Onasanwo et al. 2016) and (Foresti et al. 2013). Taken together, there appears to be a crosstalk between two transcription factors NF-κB and Nrf2 in neuroinflammation which needs to be further investigated.

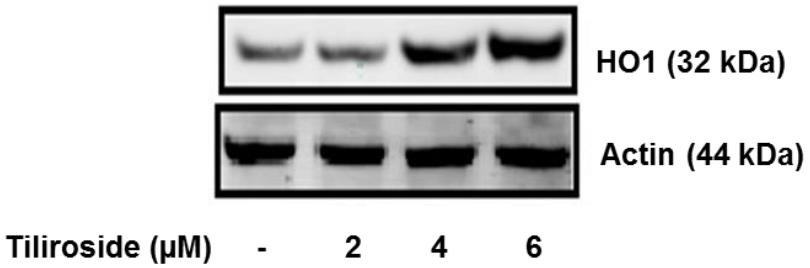
Tiliroside has been shown to have neuroinflammation inhibitory effects in LPS/IFNγ-induced BV2 microglia, however, the effect of the compound on Nrf2 signalling is not known. Therefore, the effect of tiliroside on Nrf2/HO-1/NQO1-antioxidant axis was assessed in BV2 microglia.

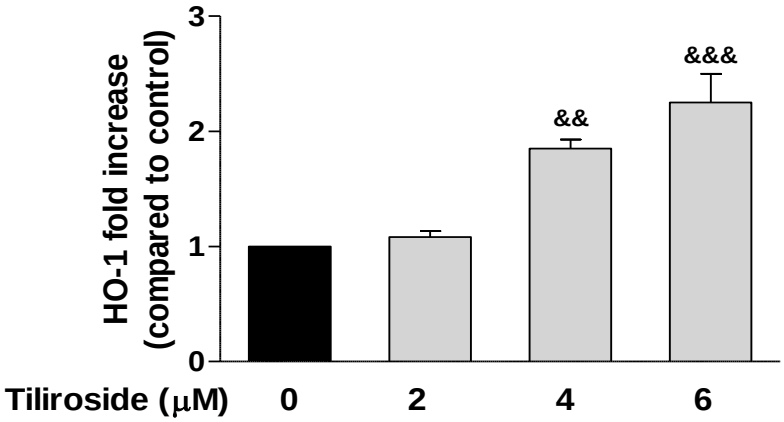
5.2 Results

5.2.1 Tiliroside increased the levels of HO-1 and NQO-1 proteins in BV2 microglia

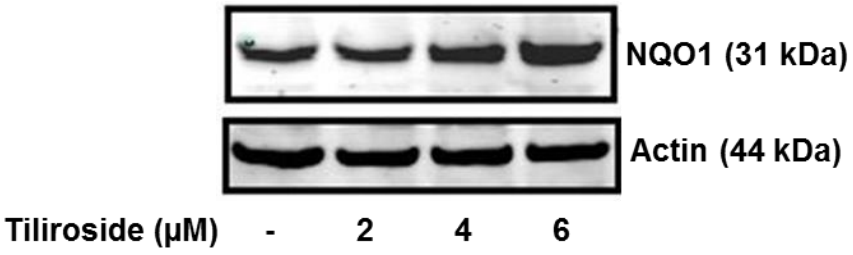
Microglia has been shown to trigger the activation of repair mechanisms that are involved in the protection of microglia during sustained neuroinflammation by inducing Nrf2 expression and its mediated inducible detoxifying antioxidant proteins such as HO-1 and NQO1 (Foresti et al. 2013). These two antioxidant proteins have been reported to be involved in the resolution of neuroinflammation (Gill et al. 2014) and (Lee et al. 2015). As a result, the ability of tiliroside to activate these antioxidant proteins was investigated in BV2 microglia. Western blot experiments revealed that increasing concentrations of tiliroside produced a significant increase in the expression of HO-1. Activation of HO-1 by the compound was not effective at 2 μM, however at 4 μM tiliroside increased the expression by ~1.8-fold ($p<0.01$) compared to control BV2 cells. Tiliroside at 6 μM increased the expression of HO-1 protein by ~2.3-fold ($p<0.001$) compared to untreated control microglia. Besides, tiliroside was shown to activate NQO1 protein expression (4 μM, ~1.4-fold increase, $p<0.01$ and 6 μM, ~1.8-fold increase, $p<0.01$) at 24 hours compared to untreated microglial cells (Figure 5.1).

(a)


HO1 (32 kDa)
Actin (44 kDa)
Tiliroside (μM) - 2 4 6


HO-1 fold increase
(compared to control)
3
2
1
0
&&
&&&
Tiliroside (μM) 0 2 4 6

(b)



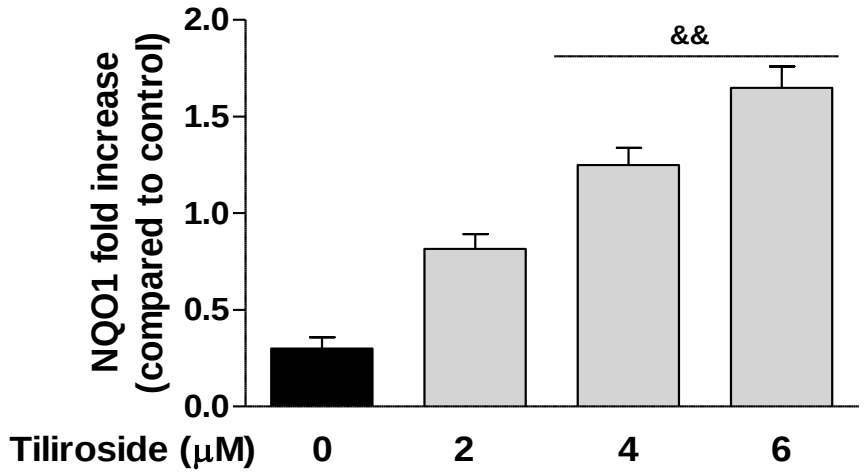


Figure 5.1 Tiliroside increased levels of HO-1 and NQO1 proteins in BV2 microglia cells

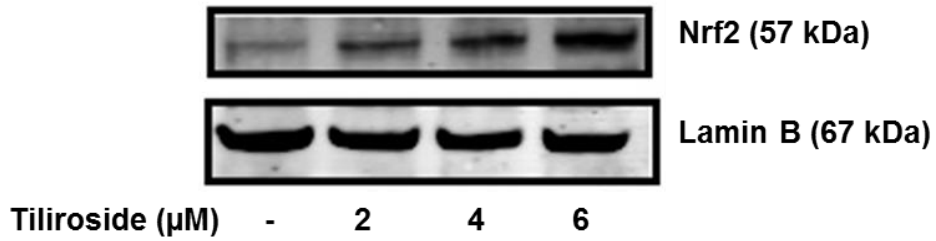
BV2 microglia were treated with various concentrations of tiliroside for 24 hours. Cytoplasmic lysates were collected and analysed for (a) HO-1 and (b) NQO1 protein expression using western blot. Tiliroside significantly increased protein levels of HO-1 and NQO1 in the microglia. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control.

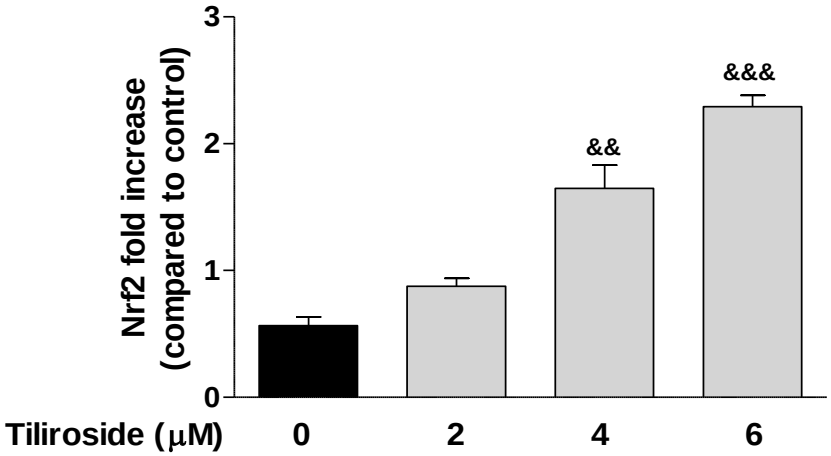
5.2.2 Tiliroside activated Nrf2/ARE antioxidant protective mechanism in BV2 microglia

After confirming that both HO-1 and NQO1 were strongly activated by tiliroside, its effect on Nrf2 activation was next examined. Firstly, Nrf2-DNA binding activity of tiliroside in the microglia was done using EMSA assay. Results show that the compound (2-6 µM) produced a dose-related increase (2 µM, ~2.4-fold, $p<0.05$, 4 µM, ~3.8-fold, $p<0.01$ and 6 µM, ~6-fold, $p<0.001$) in the DNA binding of Nrf2 compared to untreated cells (Figure 5.2b). Later, a luciferase reporter gene assay was performed to verify whether the effects of the drug were mediated through activation of the antioxidant responsive elements, which is under the control of a promoter containing the ARE consensus. As shown in Figure 5.2c, tiliroside (2-6 µM) produced a significant and dose-dependent increase in the ARE-luciferase activity in BV2 microglia. Notably, at 6 µM, tiliroside significantly increased ($p<0.001$) ARE-luciferase activity by ~6.6-fold compared to untreated cells, while at 2 µM ~5.6-fold and at 4 µM by ~3.3-fold increase was observed.

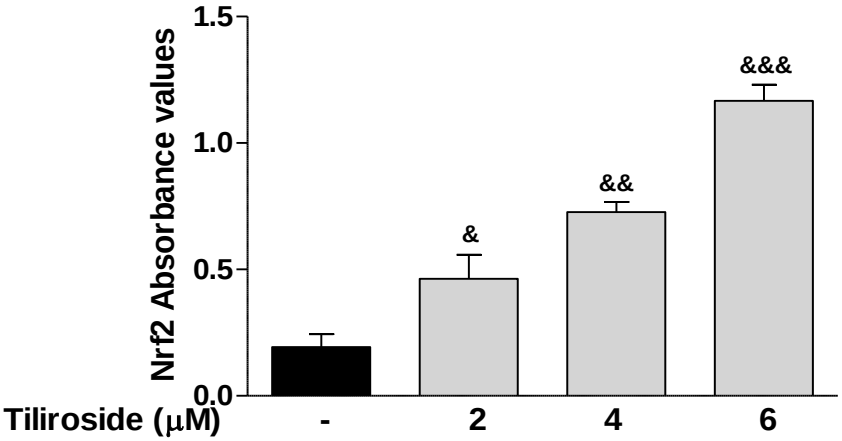
Based on the tiliroside effect on ARE-luciferase activity and Nrf2-DNA binding further experiments were carried out to determine whether tiliroside could activate nuclear Nrf2 in BV2 microglia. Western blot results showed that treatment of BV2 microglia with increasing concentrations of tiliroside resulted in a significant and dose-dependent increase (4 µM, ~2.3-fold, $p<0.01$ and 6 µM, ~2.6-fold, $p<0.01$) in the accumulation of Nrf2 protein in the nucleus (Figure 5.2a). Immunofluorescence experiments were conducted to further assess the effect of tiliroside in microglia cells. As shown in Figure 5.2d, relatively low levels of Nrf2 were detected in the untreated cells. The expression of Nrf2 was upregulated after treatment with increasing concentrations of tiliroside (2-6 µM), suggesting that the compound is capable of activating Nrf2 signalling in microglia.

(a)

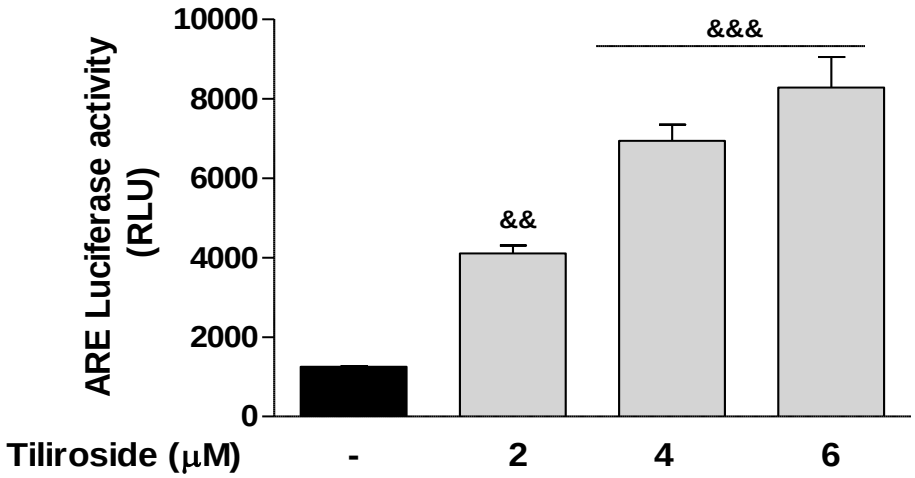

Nrf2 (57 kDa)
Lamin B (67 kDa)
Tiliroside (μM) - 2 4 6


Nrf2 fold increase (compared to control)
3
2
1
0
&&
&&&
Tiliroside (μM) 0 2 4 6

(b)


Nrf2 Absorbance values
1.5
1.0
0.5
0.0
&
&&
&&&
Tiliroside (μM)
-
2
4
6

(c)


ARE Luciferase activity (RLU)
10000
8000
6000
4000
2000
0
&&
&&&
Tiliroside (μM)
-
2
4
6

(d)

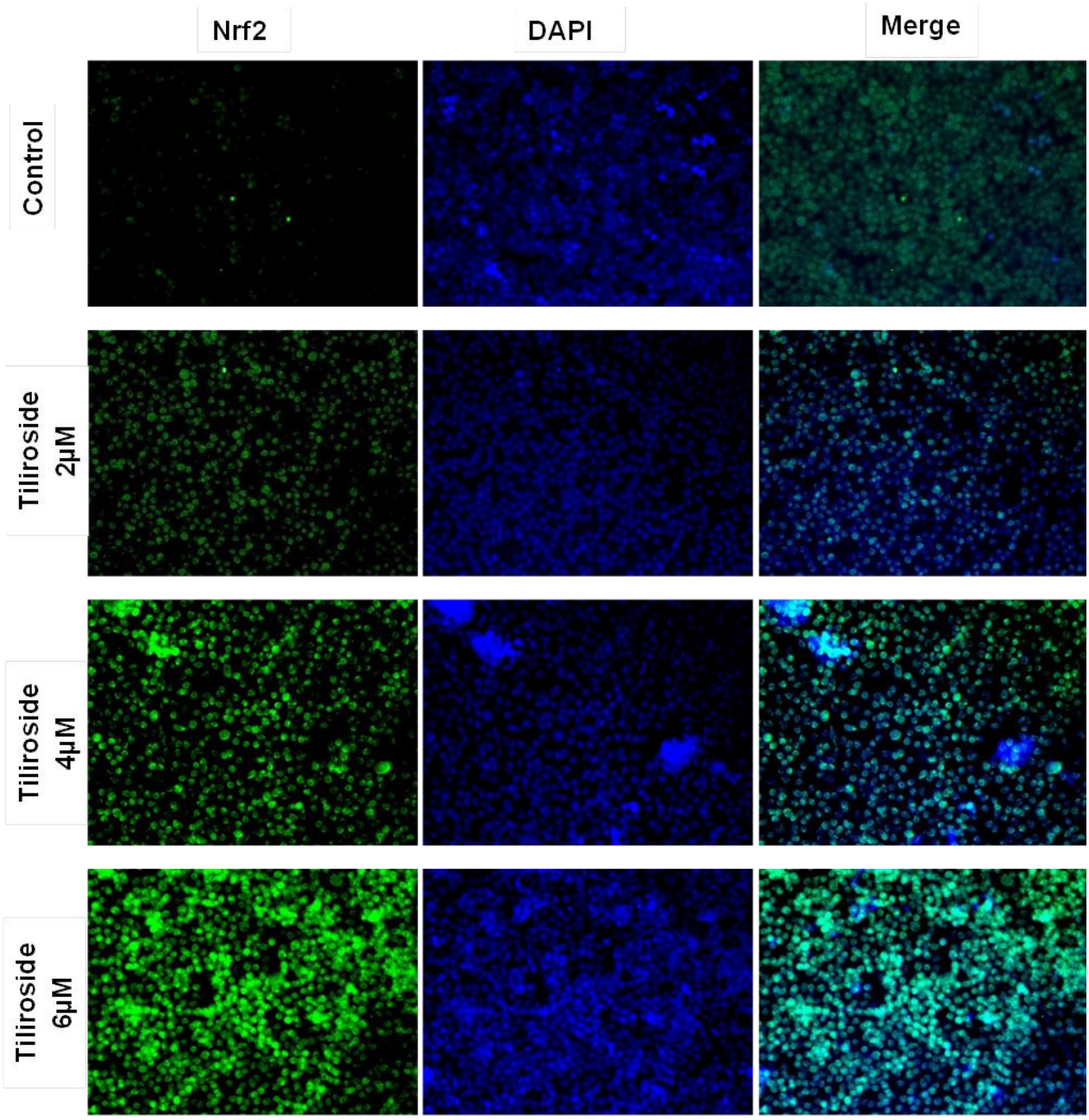


Figure 5.2 Tiliroside increased Nrf2 protein expression in BV2 microglia.

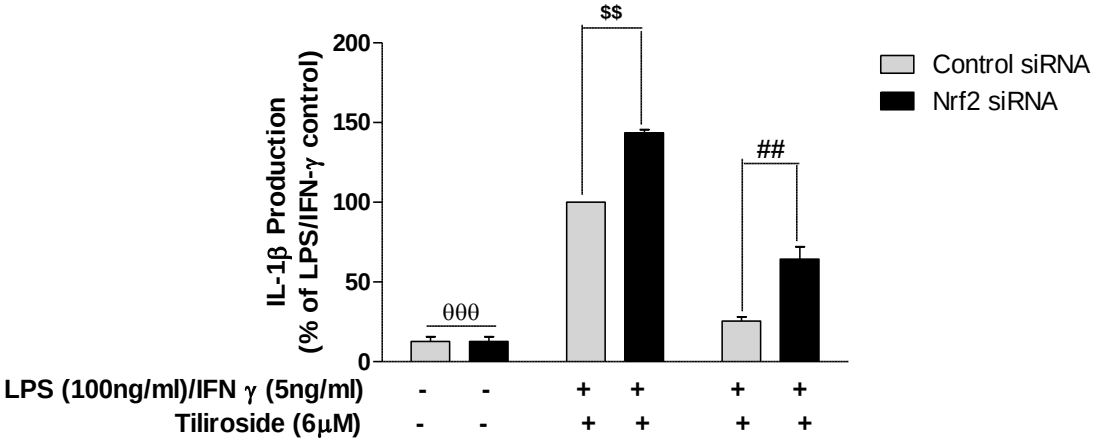
Microglia were treated with tiliroside for 24 hours. After that, nuclear lysates were collected and analysed for the activation of Nrf2 using western blot and DNA binding assay. (a) Tiliroside upregulated the protein expression of Nrf2 when incubated for 24 hours in microglia. (b) Tiliroside produced a dose-related increase in DNA binding of Nrf2 to immobilised ARE consensus binding site in microglia. (c) Tiliroside activated ARE-luciferase activities transfected with ARE construct in BV2 cells using luciferase reporter gene assay. Microglia cells were transfected with ARE-reporter construct for 18 hours. Thereafter, cells were treated with tiliroside for 8 hours and luciferase activity was further measured. (d) Immunofluorescence experiments were carried out to detect Nrf2

activation by tiliroside. Nrf2 protein was not detected in untreated control cells; however, increasing concentrations of tiliroside activated Nrf2 protein. Cells were counterstained with DAPI and fluorescence images acquired with an EVOS® FLoid® cell imaging station (scale bar=100 µm) and processed using image J. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared with untreated control.

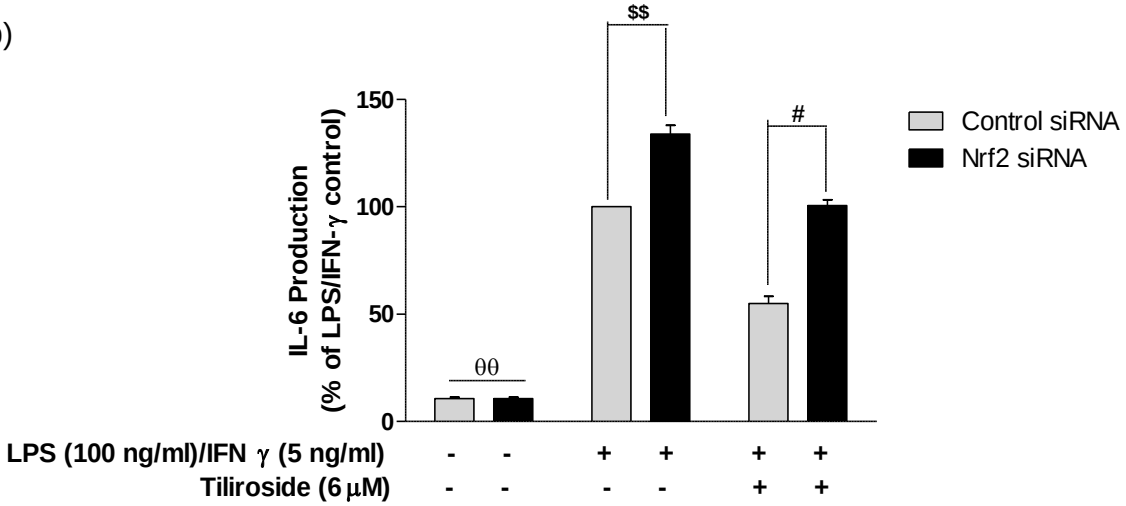
5.2.3 Anti-neuroinflammatory effects of tiliroside are dependent on Nrf2 activity in LPS/IFNγ-activated BV2 microglia

In earlier experiments, tiliroside inhibited LPS/IFNγ-induced neuroinflammation by blocking the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα in microglia. It is possible that Nrf2 antioxidant protective mechanisms may partly mediate these suppressive effects. Therefore, experiments were conducted to investigate whether the anti-neuroinflammatory effects of the tiliroside were dependent on Nrf2 activation in the microglia. To achieve this, control siRNA and Nrf2 siRNA-transfected BV2 microglia were pretreated with an effecient concentration of tiliroside (6 µM) and stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml) for 24 hours. As shown in Figure 5.3a LPS/ IFNγ produced high levels of IL-1β, IL-6 and TNFα in control siRNA cells, while in Nrf2 siRNA cells a significant increase in the cytokines production was observed, which proves that absence of Nrf2 gene increases cytokine induction in LPS/IFNγ-treated microglia. However, tiliroside (6 µM) produced a significant reduction in LPS/IFNγ-induced IL-1β production in control siRNA-transfected cells. In contrast, the IL-1β inhibitory effects of the compound were significantly reversed in Nrf2 siRNA-transfected cells. Also, tiliroside inhibited LPS/IFNγ-induced IL-6 and TNFα production in control siRNA cells, when compared with Nrf2 siRNA-transfected BV2 cells, these inhibitory effects were significantly abolished (Figure 5.3b and c).

(a)


$$
##
θθθ
IL-1β Production
(% of LPS/IFN-γ control)
200
150
100
50
0
Control siRNA
Nrf2 siRNA
LPS (100ng/ml)/IFN γ (5ng/ml) - - + + + +
Tiliroside (6μM) - - + + + +

(b)


$$
#
θθ
IL-6 Production
(% of LPS/IFN-γ control)
150
100
50
0
Control siRNA
Nrf2 siRNA
LPS (100 ng/ml)/IFN γ (5 ng/ml) - - + + + +
Tiliroside (6 μM) - - - - + +

(c)

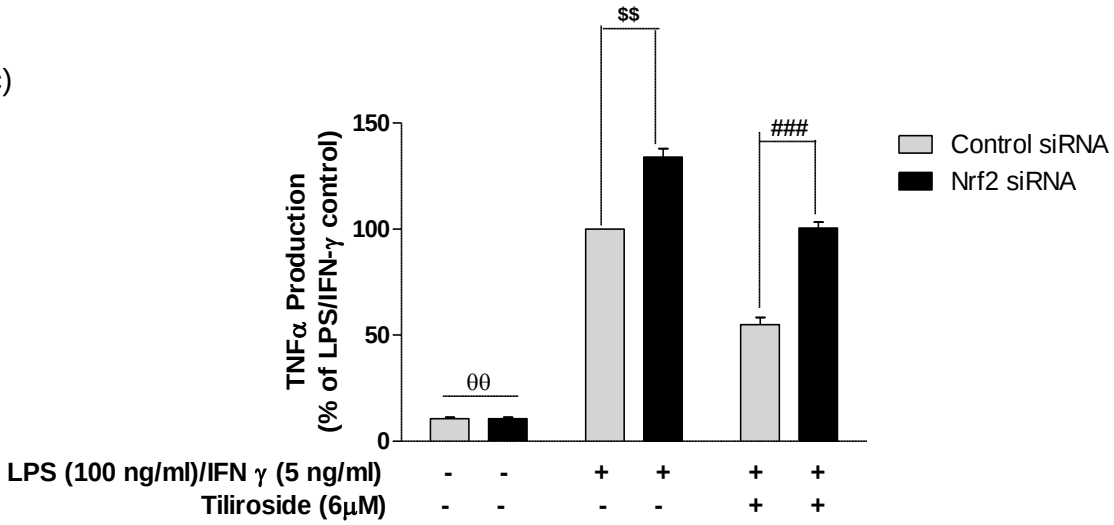

$$
###
θθ
TNFα Production
(% of LPS/IFN-γ control)
150
100
50
0
Control siRNA
Nrf2 siRNA
LPS (100 ng/ml)/IFN γ (5 ng/ml) - - + + + +
Tiliroside (6μM) - - - - + +

Figure 5.3 Inhibitory actions of tiliroside on pro-inflammatory cytokine are dependent on Nrf2 activity

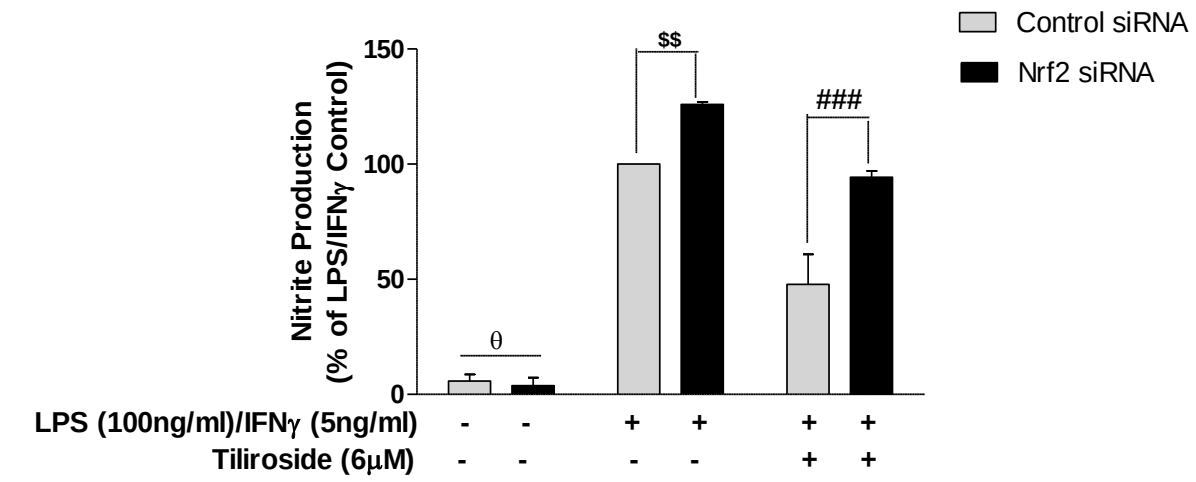
Both Nrf2 siRNA and control siRNA transfected BV2 cells were incubated with tiliroside (6 µM) prior to stimulation with LPS and IFNγ for 24 hours. Subsequently, culture supernatants were analysed to detect the levels of IL-1β (a), IL-6 (b) and TNFα (c) using ELISA. Inhibition of IL-1β, IL-6 and TNFα was observed in control siRNA transfected microglia; however, these inhibitory actions were disappeared in Nrf2 knockout cells. All values are expressed as mean ± SEM for at least three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\theta}p< 0.05$, $^{\theta\theta}p<0.01$, $^{\theta\theta\theta}p<0.001$ as compared within the groups of the untreated control. $p< 0.05, $$p<0.01, $$$p<0.001 as compared within the groups stimulated with LPS/IFNγ and #p<0.05, ##p<0.01, ###p<0.001 as compared within the groups pre-treated with tiliroside (6 µM).

5.2.4 Inhibitory actions of tiliroside on iNOS and COX-2 protein expressions and its mediated NO and PGE2 production was dependent on Nrf2 activation in BV2 microglia

Encouraged by the outcome of the effects on tiliroside in Nrf2 silenced LPS/IFNγ-induced cytokines, its effect on nitrites and iNOS protein expression in Nrf2 knockout microglia were investigated. This was achieved by transfecting BV2 cells with control siRNA and Nrf2 siRNA, followed by pre-incubation with tiliroside (6 μM) for 30 minutes and stimulated with the combination of LPS (100 ng/ml) and IFNγ (5 ng/ml) for 24 hours. Results show that tiliroside significantly inhibited nitrite production in LPS/IFNγ-stimulated control siRNA-transfected cells. Interestingly, in Nrf2 silenced BV2 cells, these inhibitory effects of the compound were significantly reversed in comparison with control siRNA cells (Figure 5.4a). Similar results were observed from the western blot experiments conducted to assess iNOS protein expression. Tiliroside inhibited LPS/IFNγ-induced iNOS protein in control siRNA-transfected cells, however, these inhibitory actions of the compound were significantly reversed in Nrf2 siRNA-transfected microglia (Figure 5.4b).

Tiliroside has been shown to inhibit COX-2 protein expression and PGE_2 production in LPS/IFNγ-activated microglia. Further investigation was done to find out whether inhibitory effects of the compound on PGE_2 production and COX-2 protein expression have depended on Nrf2. This was achieved by transfecting cells with control siRNA and Nrf2 siRNA, followed by pre-incubation with tiliroside (6 μM) for 30 minutes and stimulated with LPS (100 ng/ml)/IFNγ (5 ng/ml) for 24 hours. As expected, PGE_2 production was abrogated by tiliroside in LPS/IFNγ-treated control siRNA cells, while in Nrf2 siRNA cells this inhibitory effect was significantly reversed (Figure 5.5a). Western blot experiments for COX-2 protein expression show that, in control siRNA cells, tiliroside (6 μM) significantly inhibited the upregulation of COX-2 protein expression. However, in the absence of Nrf2 protein, this inhibitory effect was significantly reversed (Figure 5.5b).

(a)


Control siRNA
Nrf2 siRNA
Nitrite Production
(% of LPS/IFNγ Control)
150
100
50
0
$$
###
θ
LPS (100ng/ml)/IFNγ (5ng/ml) - - + + + +
Tiliroside (6μM) - - - - + +

(b)

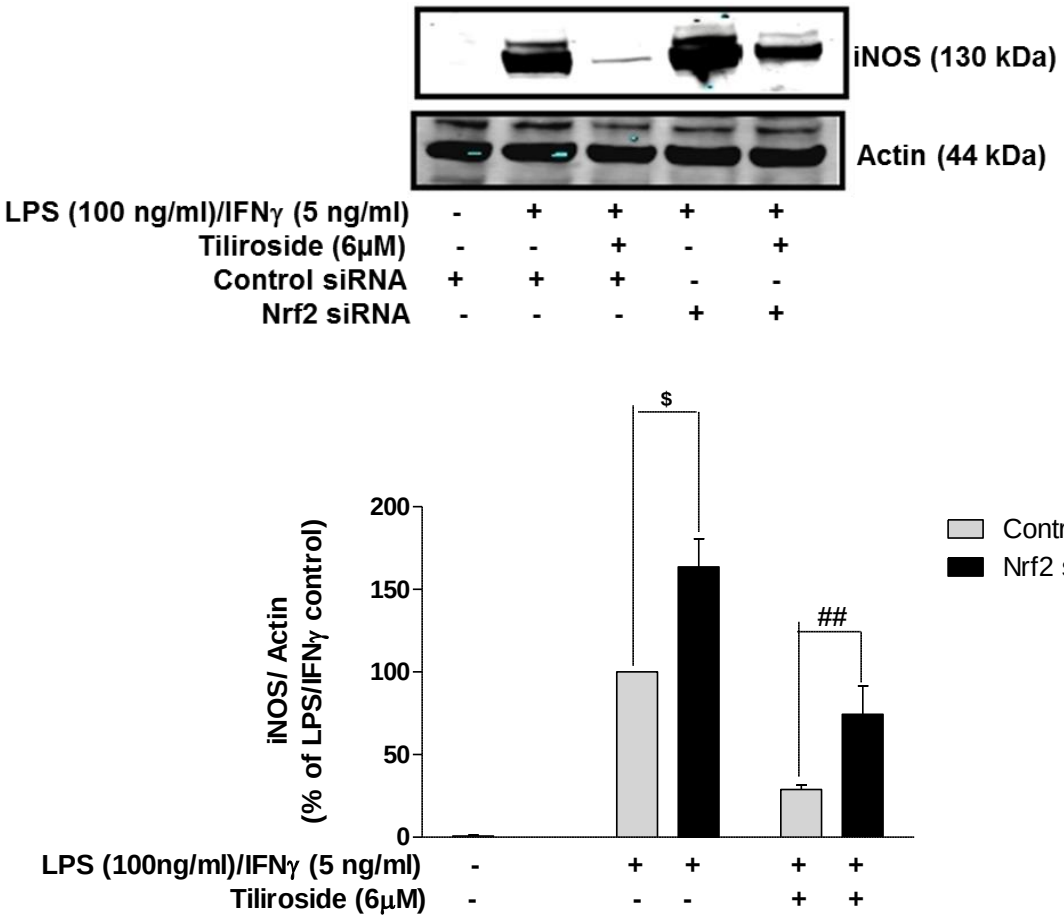
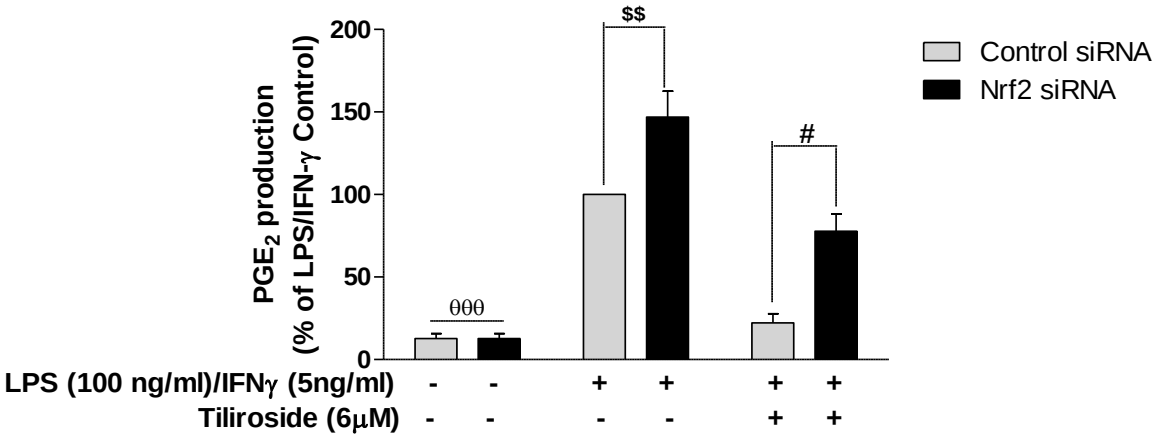

iNOS (130 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (6μM) - - + - +
Control siRNA + + + - -
Nrf2 siRNA - - - + +
$
##
iNOS/ Actin
(% of LPS/IFNγ control)
200
150
100
50
0
Control siRNA
Nrf2 siRNA
LPS (100ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (6μM) - - - + +

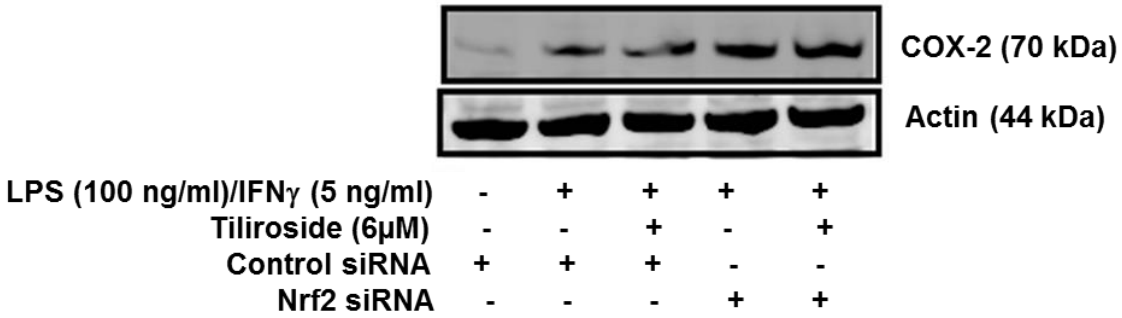
Figure 5.4 Nrf2 knockout in BV2 microglia reversed suppressive effects of tiliroside on LPS/IFNγ-induced iNOS and nitrite production

BV2 microglia were transfected with Nrf2 siRNA and control siRNA. After that, cells were treated with tiliroside (6 µM) prior to LPS and IFNγ stimulation for 24 hours. Subsequently, supernatants and cytoplasmic lysates were analysed for nitrite production and iNOS protein expression. (a) Inhibition of nitrite production and (b) iNOS protein expression was observed in control siRNA transfected microglia; however, these inhibitory actions were disappeared in Nrf2 knockout cells. All values are expressed as mean ± SEM for at least three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\theta}p< 0.05$, $^{\theta\theta}p<0.01$, $^{\theta\theta\theta}p<0.001$ as compared within the groups of the untreated control. $p< 0.05, $$p<0.01, $$$p<0.001 as compared within the groups stimulated with LPS/IFNγ and #p<0.05, ##p<0.01, ###p<0.001 as compared within the groups pre-treated with tiliroside (6 µM).

(a)


PGE_2 production (% of LPS/IFN-γ Control)
200
150
100
50
0
$$
#
θθθ
Control siRNA
Nrf2 siRNA
LPS (100 ng/ml)/IFNγ (5ng/ml) - - + + + +
Tiliroside (6μM) - - - - + +

(b)


COX-2 (70 kDa)
Actin (44 kDa)
LPS (100 ng/ml)/IFNγ (5 ng/ml) - + + + +
Tiliroside (6μM) - - + - +
Control siRNA + + + - -
Nrf2 siRNA - - - + +

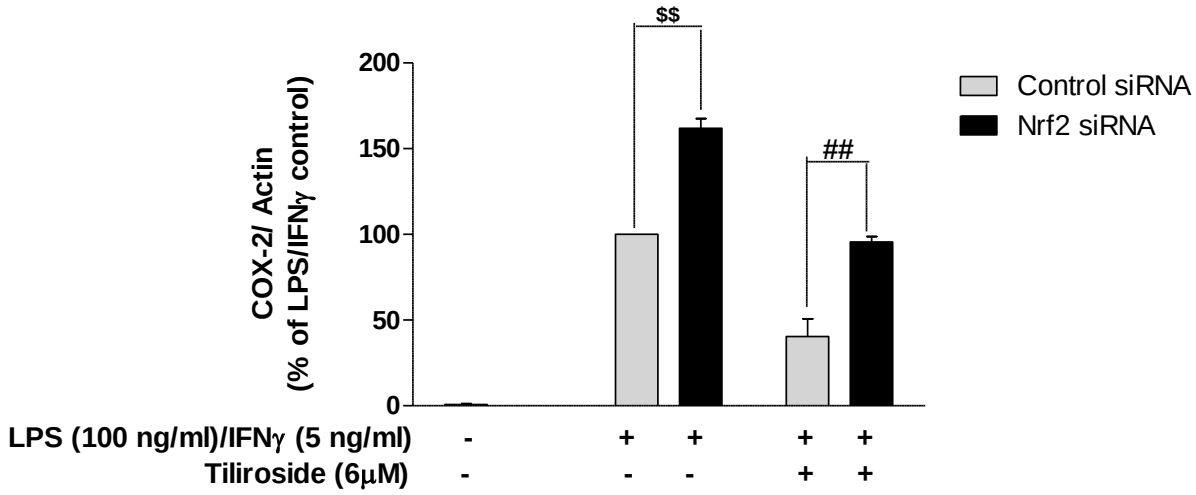


Figure 5.5 Nrf2 mediates the inhibitory effects of tiliroside on COX-2 protein expression and PGE_2 prodcution in LPS/IFNγ-treated microglia

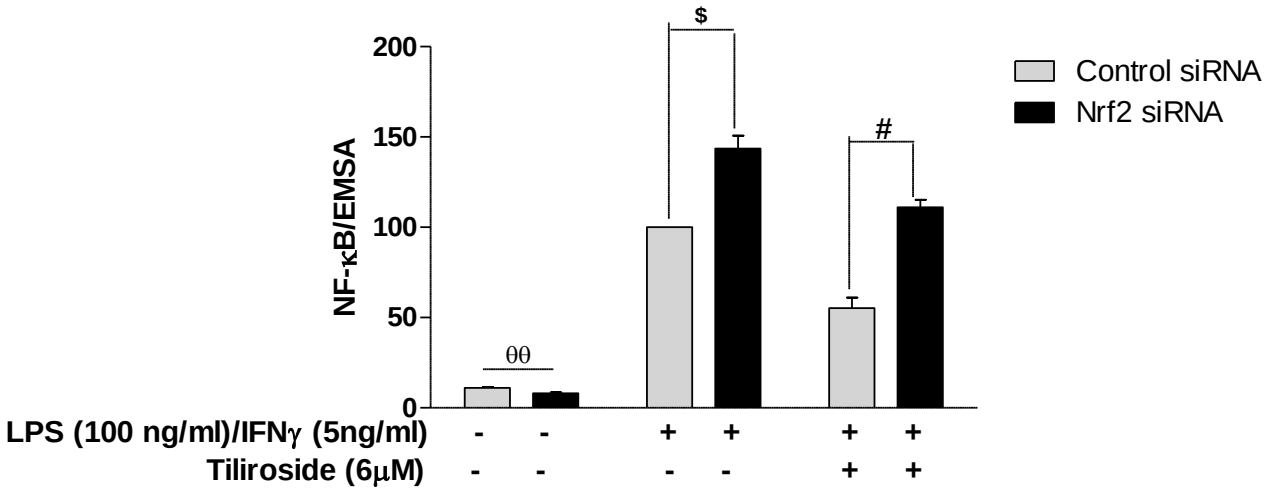
Nrf2 siRNA and control siRNA transfected BV2 cells were pre-treated with tiliroside (6 μM) before stimulation with LPS and IFNγ for 24 hours. Later, supernatants and cytoplasmic lysates were analysed for PGE2 production and COX-2 protein expression. (a) Tiliroside inhibited PGE2 production and (b) COX-2 protein expression in control siRNA transfected microglia; however, these inhibitory actions were disappeared in Nrf2 knockout cells. All values are expressed as mean ± SEM for at least three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\theta}p< 0.05$, $^{\theta\theta}p<0.01$, $^{\theta\theta\theta}p<0.001$ as compared within the groups of the untreated control. $p< 0.05, $$p<0.01, $$$p<0.001 as compared within the groups stimulated with LPS/IFNγ and #p<0.05, ##p<0.01, ###p<0.001 as compared within the groups pre-treated with tiliroside (6 μM).

5.2.5 NF-κB inhibitory actions of tiliroside are Nrf2 mediated in LPS/IFNγ-stimulated microglia

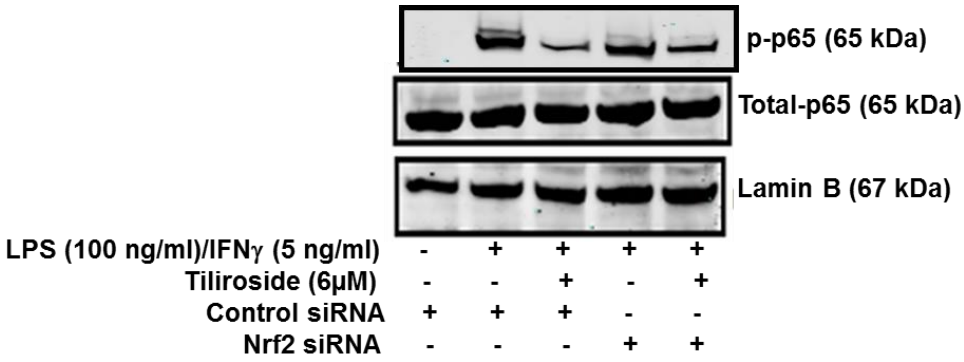
After observing that inhibitory effects of tiliroside on cytokines, NO, PGE_2, iNOS and COX-2 were reversed in the absence of Nrf2 gene in microglia, the inhibitory activity of the compound on NF-κB signalling in Nrf2-silenced BV2 microglia was further investigated. Cells were transfected with control siRNA and Nrf2 siRNA, followed by pre-incubation with tiliroside (6 μM) for 30 minutes and stimulated with LPS/IFNγ for 60 minutes. Later, nuclear lysates were collected and subjected to ELISA based EMSA. As shown in Figure 5.6a, in control siRNA cells, tiliroside significantly inhibited DNA binding of NF-κB. Interestingly, this DNA binding inhibitory activity of the compound was reversed in Nrf2 siRNA-transfected cells. To assess the transfection efficiency of Nrf2 siRNA, western blot experiments were done using nuclear extractions. Results show that, in control siRNA cells, a combination of LPS and IFNγ upregulated phosphorylation of p65 which was significantly inhibited by tiliroside. However, in Nrf2 siRNA-transfected cells, inhibitory actions of the compound were completely abolished (Figure 5.6b).

To determine whether the anti-neuroinflammatory effect of tiliroside was dependent on Nrf2 activity, control siRNA and Nrf2 siRNA-transfected BV2 cells were pre-treated with tiliroside (6 μM) and incubated with LPS/IFNγ for 24 hours. Later, nuclear lysates were collected and subjected to western blotting. Results showed that BV2 microglia transfected with control siRNA expressed nuclear Nrf2 protein. However, following transfection of BV2 microglia with Nrf2 siRNA, there was a marked deletion of nuclear Nrf2 protein in the cells (Figure 5.7). Tiliroside (6 μM) has upregulated Nrf2 expression in control cells, while there was a marked reduction of Nrf2 protein was observed in Nrf2 gene silenced cells. These experiments show that Nrf2 gene was significantly knocked down at the nuclear level when treated with Nrf2 siRNA compared to control siRNA in microglia.

(a)



(b)



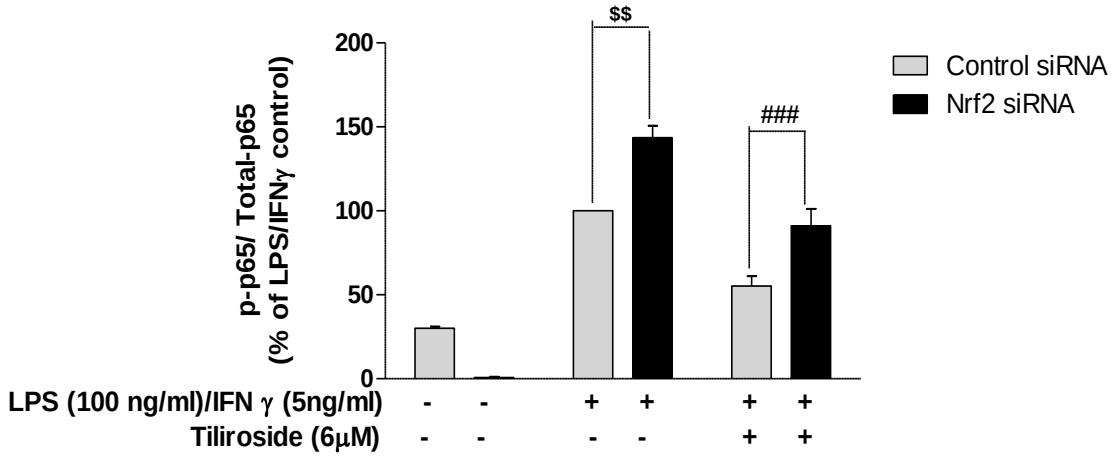


Figure 5.6 Inhibitory actions of tiliroside on NF-κB were dependent on Nrf2 activity in activated

BV2 microglia.

Nrf2-silenced BV2 cells were pre-treated with tiliroside prior to stimulation with LPS/IFNγ for 24 hours. Subsequently, nuclear and cytoplasmic lysates were collected and subjected to (a) ELISA based EMSA and (b) western blot. Tiliroside inhibited NF-κB DNA-binding and phosphorylation of p65 in BV2 microglia that were transfected with control-siRNA. However, these inhibitory effects of the compound were disappeared in the absence of Nrf2. All values are expressed as mean ± SEM for at least three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\theta}p< 0.05$, $^{\theta\theta}p<0.01$, $^{\theta\theta\theta}p<0.001$ as compared within the groups of the untreated control. $p< 0.05, $$p<0.01, $$$p<0.001 as compared within the groups stimulated with LPS/IFNγ and #p<0.05, ##p<0.01, ###p<0.001 as compared within the groups pre-treated with tiliroside (6 μM).

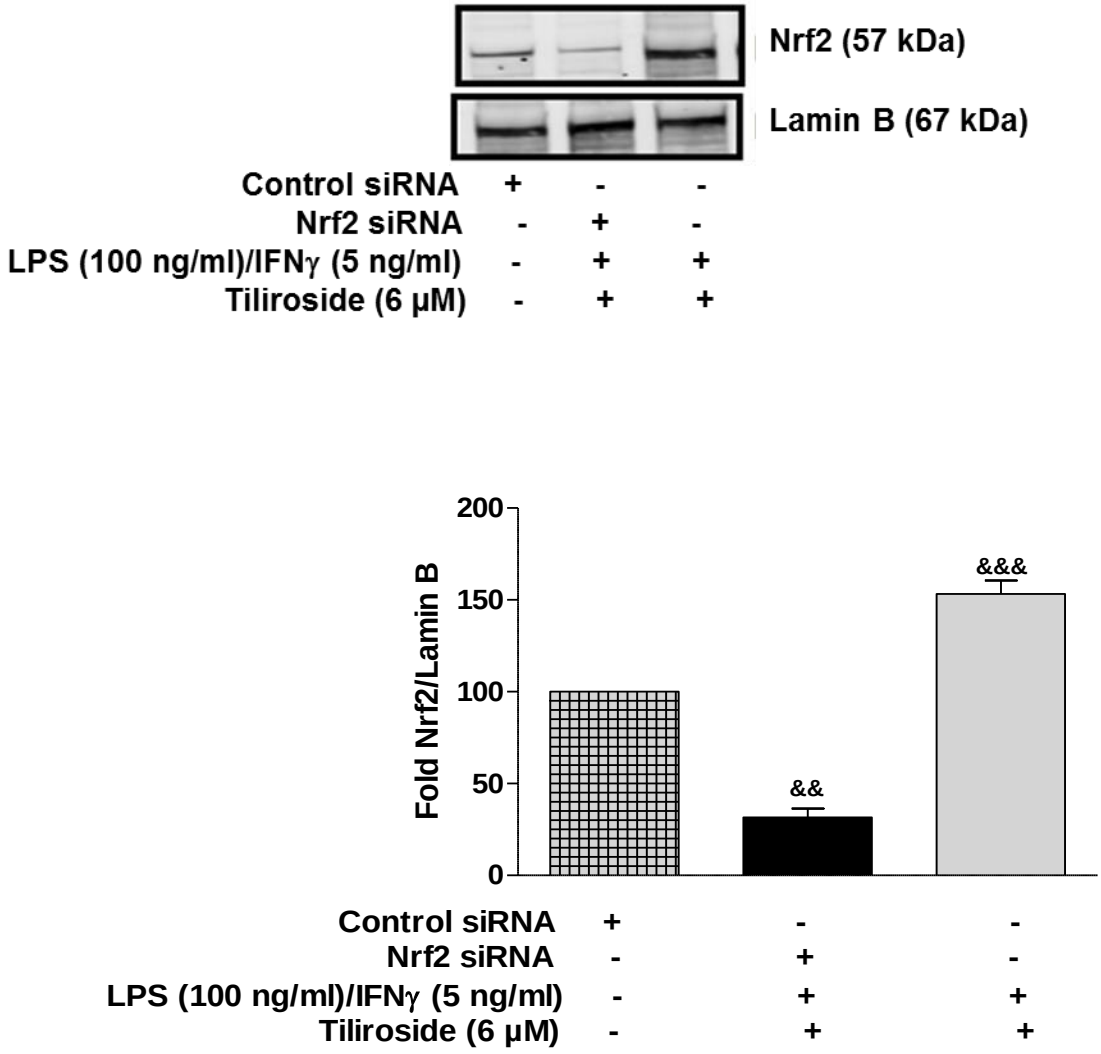


Figure 5.7 Western blot experiment show Nrf2 knockout efficiency in BV2 microglia

Control siRNA and Nrf2 siRNA-transfected BV2 microglia were treated with tiliroside 6µM before stimulation with a combination of LPS and IFNγ for 24 hours. Nuclear lysates were collected and assessed for Nrf2 protein expression using western blot. Nrf2 protein expression was significantly knocked down compared to control siRNA in the microglia. All values are expressed as mean ± SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ in comparison with untreated control.

5.3 Discussion

The transcription factor Nrf2 protects microglia from cellular stress by activating cytoprotective proteins such as HO-1 and NQO1. Moreover, several *in vivo* and *in vitro* disease models revealed that activation of Nrf2 antioxidant mechanism provides protection against neuroinflammation and oxidative stress (Gan & Johnson 2014), (Na et al. 2008) and (Kim et al. 2012). Consequently, activation of microglial Nrf2 was thought to be a right approach in resolving neuroinflammatory conditions. Increased consumption of dietary supplements and fruits are associated with the prevention of neuroinflammatory conditions and its mediated neurodegenerative diseases. Tiliroside, a dietary compound inhibits LPS/IFNγ-mediated neuroinflammation and ROS production in microglia. However, its effect against Nrf2 signalling in BV2 microglia is yet to be investigated.

The role of microglial Nrf2 signalling in neuroinflammation is well documented (Turpaev 2013), (Luo et al. 2011) and (W. Li et al. 2008). Hyperactive microglia tends to release excess pro-inflammatory cytokines, interleukins, as well as ROS and nitrogen species, that leads to demyelination of neuronal axons and neuronal death (Pan et al. 2012). However, activating natural antioxidant mechanisms like Nrf2 signalling could prevent this hyper activation of microglia. Antioxidant proteins such as HO-1 and NQO1 are known to play a crucial role in inhibiting neuroinflammation and its mediated neuronal loss (Gill et al. 2014). Results show that tiliroside (6 µM) significantly increased endogenous levels of antioxidant proteins HO-1 and NQO1 in the microglia. These results suggest that the compound is capable of activating Nrf2 signalling in microglia. In several *in vitro* studies, activation of HO-1 was shown to be beneficial against neuroinflammation by inhibiting NADPH oxidase, the primary enzyme responsible for microglial ROS release, and TLR 4 signalling activation (Nakahira et al. 2006) and (Sandberg et al. 2014). Furthermore, HO-1 is involved in the metabolism of heme into Fe^{2+}, biliverdin and carbon monoxide that eventually get converted to bilirubin (Sawle et al. 2005) and (Zecca et al. 2004). These by-products of HO-1 were shown to limit neuroinflammation in microglia (Lee & Suk 2007). In addition to HO-1, tiliroside was shown to activate the expression of NQO1. Excessive NQO1 production was shown to protect hyperactive microglia from reactive quinones by reducing them to stable hydroquinone (Nioi & Hayes 2004) and (Wakabayashi et al. 2010).

There have been some studies investigating the effects of natural dietary flavonoids on Nrf2 activation in neuroinflammation that exhibited some promising results (Na et al. 2008), (Kim et al. 2012), (Wakabayashi et al. 2010) and (Liu et al. 2008). Also, studies in the past showed that the compounds activate Nrf2 signalling in microglia blocked the production of pro-inflammatory cytokines and other neurotoxic mediators associated in neuroinflammation (Lastres-Becker et al. 2014), (Onasanwo et al. 2016) and (Pan et al. 2012). Expression of HO-1 and NQO1 are predominantly regulated by the transcription factor Nrf2 (Kim et al. 2012) and (Huang et al. 2013). After observing the effects of tiliroside on HO-1 and NQO1 protein expression, its activity on Nrf2 activation was investigated next. Results show that tiliroside at 6μM increased nuclear accumulation of Nrf2. Further experiments revealed that tiliroside significantly enhanced Nrf2 DNA-binding activity. Also, the compound was shown to increase the transcriptional activity of the *cis*-acting antioxidant responsive element (ARE), which means that tiliroside is acting at the transcriptional level of Nrf2 to induce expression of antioxidant proteins HO-1 and NQO1 in the microglia. These results further encouraged to investigate the effects of the compound on Nrf2 transactivation in microglia. Results show that tiliroside significantly increased Nrf2-driven luciferase expression, suggesting that compound activates Nrf2 signalling in microglia. It, therefore, appears from the above results that tiliroside inhibit neuroinflammation probably by activating Nrf2/HO-1/NQO1 axis in BV2 microglia.

Tiliroside inhibited neuroinflammation that was induced by the combination of LPS and IFNγ in microglia. Studies have revealed that Nrf2 activation might inhibit neuroinflammation via modulating NF-κB-mediated inflammatory mediators in microglia (Lee et al. 2015) and (Jazwa et al. 2011). Also, compounds that inhibit NF-κB activation have been shown to activate Nrf2 (Surh & Na 2008). For instance, the antimalarial drug artemether inhibited LPS-induced neuroinflammation via Nrf2 signalling and those inhibitory effects were lost in the absence of Nrf2 in microglia (Okorji et al. 2015). Moreover, Nrf2 gene knockout experiments showed that microglia tends to generate excess amounts of inflammatory mediators when stimulated with LPS compared to control, highlights the protective role of Nrf2 (Na et al. 2008), (Rojo et al. 2010) and (Lee et al. 2011). Results of Nrf2 knockout experiments show that in control siRNA-transfected cells, tiliroside inhibited pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα production in LPS/IFNγ-

stimulated microglia. However, these inhibitory activities of tiliroside were significantly abolished in Nrf2 siRNA-silenced cells, suggesting that neuroinflammation inhibitory effects of the compound are Nrf2 mediated in microglia. Western blot results revealed that Nrf2 gene was successfully knocked down in all Nrf2 siRNA experiments.

Similar observations were made in other mechanistic studies conducted by other researchers. For example, kolaviron, a biflavonoid was shown to inhibit LPS-induced NF-κB signalling in BV2 microglia and also activated Nrf2. Further experiments demonstrated that Nrf2 was required for kolaviron to exhibit neuroinflammation inhibitory effects in microglia (Onasanwo et al. 2016). Also, Licochalcone E attenuated LPS-induced inflammatory responses in BV2 microglia by a mechanism involving Nrf2 activation and upregulation of its downstream antioxidant proteins, and its effects were reversed in Nrf2 siRNA cells (Kim et al. 2012). In addition to the inhibitory effects of tiliroside on pro-inflammatory mediators, it was observed that compound inhibited iNOS mediated NO production and COX-2 induced PGE_2 production in control siRNA cells. The inhibitory effects of tiliroside on iNOS, nitric oxide, COX-2 and PGE_2 were reversed in the absence of Nrf2 protein in the microglia, highlighting that transcription factor Nrf2 plays a critical role in mediating the anti-inflammatory action of tiliroside.

Studies in various experimental models revealed that several redox-sensitive factors regulate Nrf2 and NF-κB and lack of Nrf2 shown to increase nitrosative and oxidative stress leading to upregulation of cytokine production (Yerra et al. 2013) and (Cuadrado et al. 2014). To further understand the relation between Nrf2 and NF-κB in microglia, Nrf2 gene was knocked out using siRNA-transfection and stimulated with a combination of LPS and IFNγ. Results show that tiliroside significantly inhibited LPS/IFNγ-induced NF-κB DNA-binding and protein expression in control siRNA-transfected microglia and as expected these inhibitory effects of the compound were significantly reversed in Nrf2 silenced cells. These results suggest that transcription factor Nrf2 mediates the neuroinflammation inhibitory actions of tiliroside in LPS/IFNγ-stimulated microglia. Interestingly, p-NF-κB-p65 was highly expressed in Nrf2 siRNA transfected cells when treated with LPS and IFNγ compared to control cells. This observation explains the excessive generation of pro-inflammatory cytokines in the absence of Nrf2 protein. Studies on $Nrf2^{-/-}$ deficient

mice showed that IKKβ activity was upregulated suggesting that this step will further enhance phosphorylation of IκBα and releasing NF-κB into the nucleus (Thimmulappa et al. 2006). In contrary to these observations, studies conducted by Yu et al. revealed that NF-κB activity could negatively regulate Nrf2-mediated ARE expression. However, this modulatory action is cell dependent (Yu et al. 2011). Collectively, all these results seem to suggest that tiliroside attenuated LPS/IFNγ-induced neuroinflammation in BV2 microglia and these inhibitory actions of the compound are reversed in the absence of Nrf2.

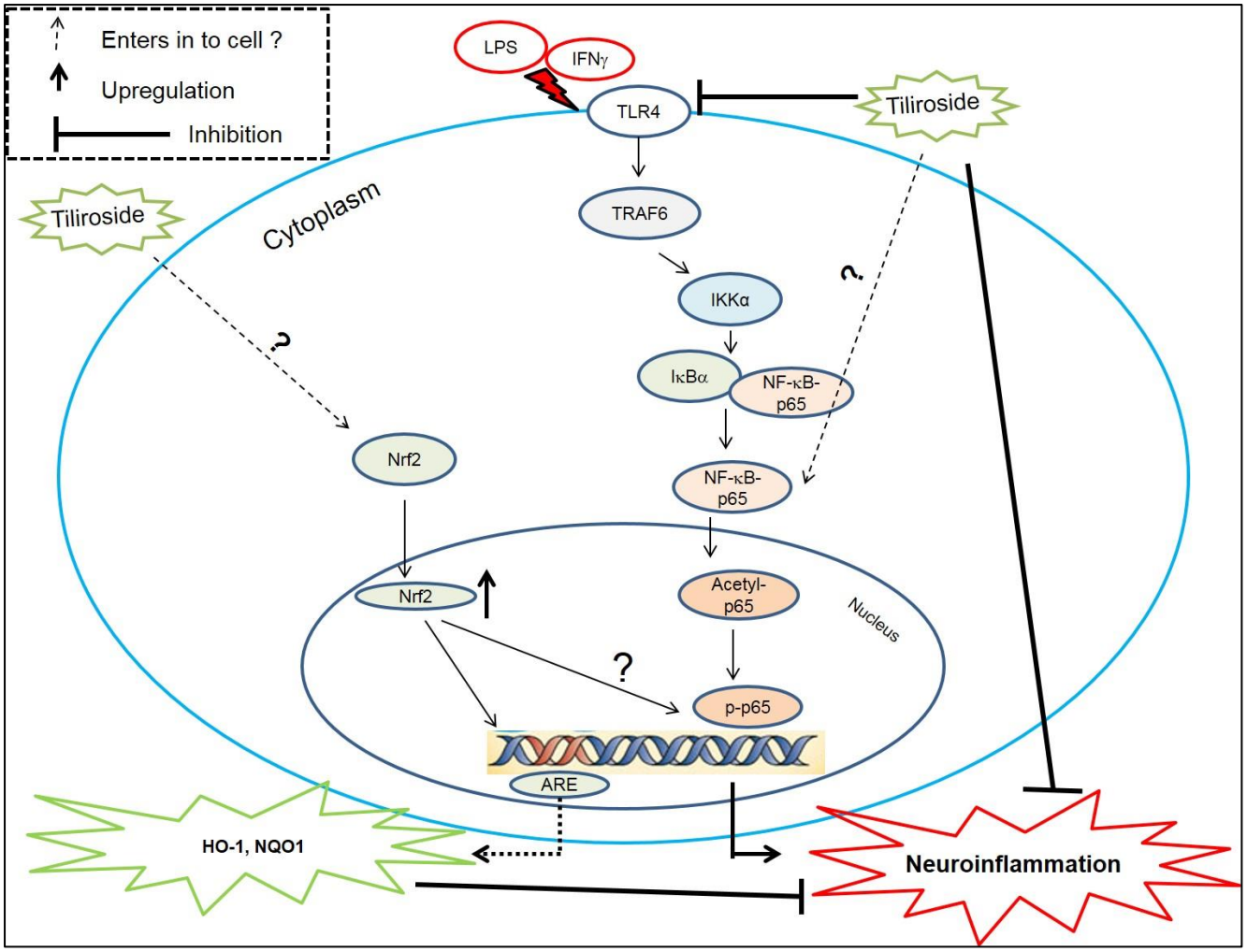


Figure 5.8 The neuroinflammation inhibitory activity of tilrioside is Nrf2 mediated in activated microglia . LPS: Lipopolysaccharide, IFNγ: Interferon gamma, TLR4: Toll-like receptor 4, TRAF6: TNF receptor associated factor 6, IKK: IκB kinase, Nrf2: nuclear factor erythroid 2 related factor 2, HO-1: Heme oxygenase 1, NQO1: NAD(P)H dehydrogenase [quinone] 1, ARE: antioxidant response element.

Chapter 6. General discussion and conclusion

Tiliroside is a natural polyphenolic glycoside found in various dietary supplements. Several studies have demonstrated anti-inflammatory activity of tiliroside in different models of inflammation (Goto et al. 2012), (Rao et al. 2005), (Sala et al. 2003) and (Qiao et al. 2011). However, there is no report to date demonstrating inhibition of neuroinflammation by the compound. This study evaluated the anti-neuroinflammatory effects of tiliroside in BV2 microglia which were stimulated with a combination of LPS and IFNγ.

Inflammatory mediators released by activated microglia are believed to be a major target for anti-inflammatory compounds. This study established that tiliroside inhibits the production of TNFα, IL-1β and IL-6 in BV2 microglia stimulated with LPS/IFNγ. On the other hand, tiliroside increased the levels of IL-10 in the activated microglia. Tiliroside also inhibited neuroinflammation by blocking iNOS protein expression and nitric oxide production in the microglia. In addition, tiliroside reduced the expression of COX-2 and its mediated PGE_2 production in LPS/IFNγ-stimulated microglia, suggesting that the compound possesses strong neuroinflammation inhibitory activities. All these observations demonstrate that tiliroside produces a marked inhibition of neuroinflammation in activated BV2 microglia. Previously, tiliroside was reported to exhibit anti-inflammatory activity through the inhibition of phospholipase A2-induced paw oedema, TPA-induced ear inflammation, and leukocyte migration in mice (Sala et al. 2003). In addition, a study conducted by Rao et al. showed that tiliroside inhibits LPS/IFNγ-induced TNFα, NO production and iNOS protein expression in mouse macrophages (Rao et al. 2005).Results from the current study appears to be consistent with the previous observations made by Rao and co-workers.

Tiliroside is a 3-O-β-D-(6″-O-coumaroyl) glucopyranoside derivative of kaempferol. Previous studies have reported that tiliroside was synthesised by condensation of a coumaroyl residue at the (6″) carbon of the kaempferol glucose moiety (Vermes et al. 1981). Kaempferol was reported to block LPS-induced TNFα, IL-1β and IL-6 production in BV2 microglia and J774.2 murine macrophages. In addition, kaempferol reduced the expression of iNOS and COX-2 proteins in activated microglia and J774.2 murine macrophages (Park et al. 2011) and (Kowalski et al.

2005). In the current research, tiliroside exhibited neuroinflammation inhibitory effects in LPS/IFNγ-treated BV2 microglia at much lower concentrations compared to kaempferol.

The activation of NF-κB signalling in microglia is a pre-requisite for the transcription of pro-inflammatory genes. Pharmacological inhibition of NF-κB signalling has been shown to reduce neuroinflammation (Shih et al. 2015), (Xu et al. 2014) and (Jeong et al. 2013). In the microglia, inflammatory stimuli like LPS induce phosphorylation of IKKα. This results in phosphorylation of IκBα followed by NF-κB-p65 translocation into the nucleus. NF-κBp-65 subunit in the nucleus undergoes phosphorylation and acetylation, thereby binding to the promoter sequences of the target DNA. This binding results in the expression of target genes including pro-inflammatory cytokines such as TNFα, IL-1β IL-6, iNOS and COX-2. This study demonstrated that tiliroside inhibits LPS/IFNγ-induced phosphorylation, nuclear translocation and binding of NF-κB to the consensus DNA sequence. In addition, tiliroside blocked the phosphorylation of IKKα and IκBα in the activated microglia. From these observations, it appears tiliroside directly targets NF-κB signalling to prevent LPS/IFNγ-induced neuroinflammation in microglia. However, there is a possibility that the compound might be blocking upstream signalling pathways that activate NF-κB signalling. Studies by Jin et al. showed that tiliroside extracted from *Agrimonia pilosa Ledeb* (Rosacea) exhibited strong anti-inflammatory effects in LPS-treated RAW 264.7 macrophages (Jin et al. 2016). However, inflammation inhibitory effects of tiliroside were not mediated by NF-κB signalling. The differences in the effect of tiliroside on these cells might be related to differences in the molecular signatures of macrophages and microglia, which are brain-resident macrophages.

Acetylation of nuclear NF-κB-p65 subunit regulates various functions of NF-κB such as DNA binding affinity and transcriptional activation of pro-inflammatory genes. In the nucleus, SIRT1 deacetylates NF-κB subunit and blocks the binding of acetylated-NF-κB-p65 to the promotor regions of the DNA. Previous studies demonstrated that compounds which activate SIRT1 will reduce the acetylation of NF-κB-p65 and further inhibit the transcription of inflammatory mediators in neuroinflammation (Cho et al. 2015), (Haigis & Sinclair 2011) and (Ye et al. 2013). In this research, tiliroside increased the expression of SIRT1 in the microglia. In addition, tiliroside inhibited the

acetylation of NF-κB-p65 in LPS/IFNγ-stimulated microglia, suggesting that there is a possibility that this compound inhibits NF-κB signalling via accelerating deacetylation of NF-κB-p65. However, further research in this study demonstrated that neuroinflammation inhibitory actions of tiliroside are not SIRT1 mediated. This observation suggests that SIRT1 activation may not be involved in the inhibition of acetylated-NF-κB-p65 by tiliroside in the microglia. A study conducted by Zhu et al. revealed that resveratrol, a potent SIRT1 activator inhibits NF-κB-mediated inflammation in TNF α-induced NIH/3T3 fibroblast cell line (Zhu et al. 2011). However, knockdown of SIRT1 gene reduced anti-inflammatory effects of resveratrol. This observation is in contrast to the current outcome, suggesting that there might be an involvement of other signalling pathways in SIRT1 activation to block neuroinflammation in the microglia.

The p38 MAPK signalling has been shown to be critical for the expression and production of proinflammatory cytokines in the CNS (Cuadrado et al. 2010) and (Kaminska 2005). Several studies have demonstrated that pharmacological modulation of p38 MAPK signalling in LPS-stimulated microglia decreases the production of inflammatory cytokines and attenuates neuroinflammation (Zeng et al. 2010) and (Herlaar & Brown 1999). LPS is known to trigger the activation of p38 MAPK signalling in microglia through activation of MKK3/6. This results in the phosphorylation of p38α and further activates mitogen-activated protein kinase (MAPK)-activated protein kinases 2 (MK2). Activated MK2 is involved in regulating the production of TNFα, IL-6 and IL-1β and other cytokines in microglia. This study established that tiliroside inhibits LPS/IFNγ-induced MKK3/6 and p38α in the microglia. In addition, tiliroside reduced phosphorylation of MK2 in activated microglia. These observations suggest that tiliroside targets p38 MAPK signalling to inhibit neuroinflammation in LPS/IFNγ-stimulated microglia. Tiliroside isolated from *Agrimonia pilosa Ledeb* inhibits LPS-induced inflammation by blocking p38 MAPK signalling in RAW 264.7 macrophages (Jin et al. 2016). These results are consistent with observations from the current study showing that tiliroside reduced p38 signalling in LPS/IFNγ-treated BV2 microglia.

The toll-like receptor 4 (TLR4) plays a critical role in the induction of neuroinflammatory responses in the microglia (Takeda & Akira 2004). Excessive

activation of TLR4 leads to the accumulation of cytotoxic factors and pro-inflammatory cytokines. Membrane-bound TLR4 could be activated by inflammatory stimuli such as bacterial lipopolysaccharide (LPS). Following TLR4 activation, the TRAF6 complex becomes activated. This activated TRAF6 further culminates in the activation of the p38 MAPK and NF-κB signalling pathways. This study established that tiliroside prevents the activation of TLR4, suggesting that tiliroside interferes with TLR4-mediated neuroinflammatory signalling in the microglia. This study also showed that tiliroside reduced the activation of TRAF6 in LPS/IFNγ-stimulated BV2 microglia. Earlier, kaempferol was reported to exhibit anti-neuroinflammatory activity through the inhibition of LPS-induced TLR4/NF-κB and p38 MAPK signalling in microglia (Park et al. 2011).

Activation of the intrinsic antioxidant and anti-inflammatory mechanisms in the microglia is crucial in the downregulation of neuroinflammation (Foresti et al. 2013) and (Wakabayashi et al. 2010). The Nrf2 signalling is one of the important antioxidant pathways in the microglia (Innamorato et al. 2008) and (Pan et al. 2012). In normal conditions, Nrf2 is bound to cysteine residues of Keap-1 protein in the cytoplasm. In response to oxidative stress, these cysteine residues get modified enabling translocation of Nrf2 into the nucleus, where it will bind to *cis*-acting antioxidant responsive element (ARE). This initiates the activation of ARE-dependent detoxifying genes such as HO-1 and NQO1. In this study, tiliroside increased endogenous expression of HO-1and NQO1 in the microglia. In addition, tiliroside increased nuclear translocation and DNA binding of Nrf2 to the ARE-dependent genes in BV2 microglia. This study also showed that Nrf2 activation is required for tiliroside to inhibit pro-inflammatory cytokines and NF-κB activity in activated microglia. These observations suggest that tiliroside might be directly activating Nrf2 protein or may be targeting Nrf2/Keap1 interaction in the cytoplasm to inhibit neuroinflammation. This is the first report linking the neuroinflammation inhibitory property of tiliroside in LPS/IFNγ-activated microglia to Nrf2 activity.

Neuroinflammation-related neurotoxicity contributes to the development of neurodegenerative diseases like AD, PD, HD and ALS (Block et al. 2007). Pro-inflammatory mediators such as TNFα, IL-1β and IL-6 released by activated microglia are shown to induce damage to adjacent neurons. TNFα released by activated microglia promotes neuronal damage by inhibiting cell survival

mechanisms such as PI3K/AKT pathway and further activates Bcl-2 family members Bad, Bax, and caspase-9 (Takeuchi et al. 2006). Also, treatment with IL-1β induced activation of caspases and other apoptosis signalling cascades leading to the death of neurons via increasing the generation of ROS and DNA fragmentation (Thornton et al. 2006) and (Barzilai & Yamamoto 2004). These observations further indicate that inflammatory mediators from activated microglia lead to neuronal death. In theory, compounds that are shown to inhibit the production of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 would be expected to confer protection against neurotoxicity. This study demonstrated that inhibition of pro-inflammatory mediator release by tiliroside in the activated microglia increased the viability of HT22 neurons by reducing calcium accumulation, ROS generation and DNA fragmentation. From this observation, it appears that tiliroside indirectly protects HT22 neurons by blocking the inflammatory responses in the activated microglia. Similar results were obtained in studies conducted by Lee et al., which revealed that tiliroside isolated from *Agrimonia eupatoria* (Rosaceae) exhibits neuroprotective activity against glutamate-induced toxicity in HT22 neurons.

In the current research, pharmacologically relevant doses were used to assess the neuroinflammation inhibitory actions of tiliroside against activated BV2 microglia. However, these doses may not be physiologically achievable in *in vivo* experiments due to various reasons. For example, the concentrations of a compound that cells are exposed to *in vivo* depends on diverse factors including the pharmacodynamic and pharmacokinetic properties of that compound. Also, the efficacy of the compound in *in vivo* depends on the bioavailability, first pass effect and how well the compound crosses the blood-brain barrier. On the other hand, it is merely impossible to convert pharmacological doses (ex: micro molar) to physiologically achievable doses (ex: mg/kg). The only way to investigate the activity of the compound in *in vivo* is to perform a wide range of efficacy dose selection experiments such as rodent toxicity studies by following some strict guidelines.

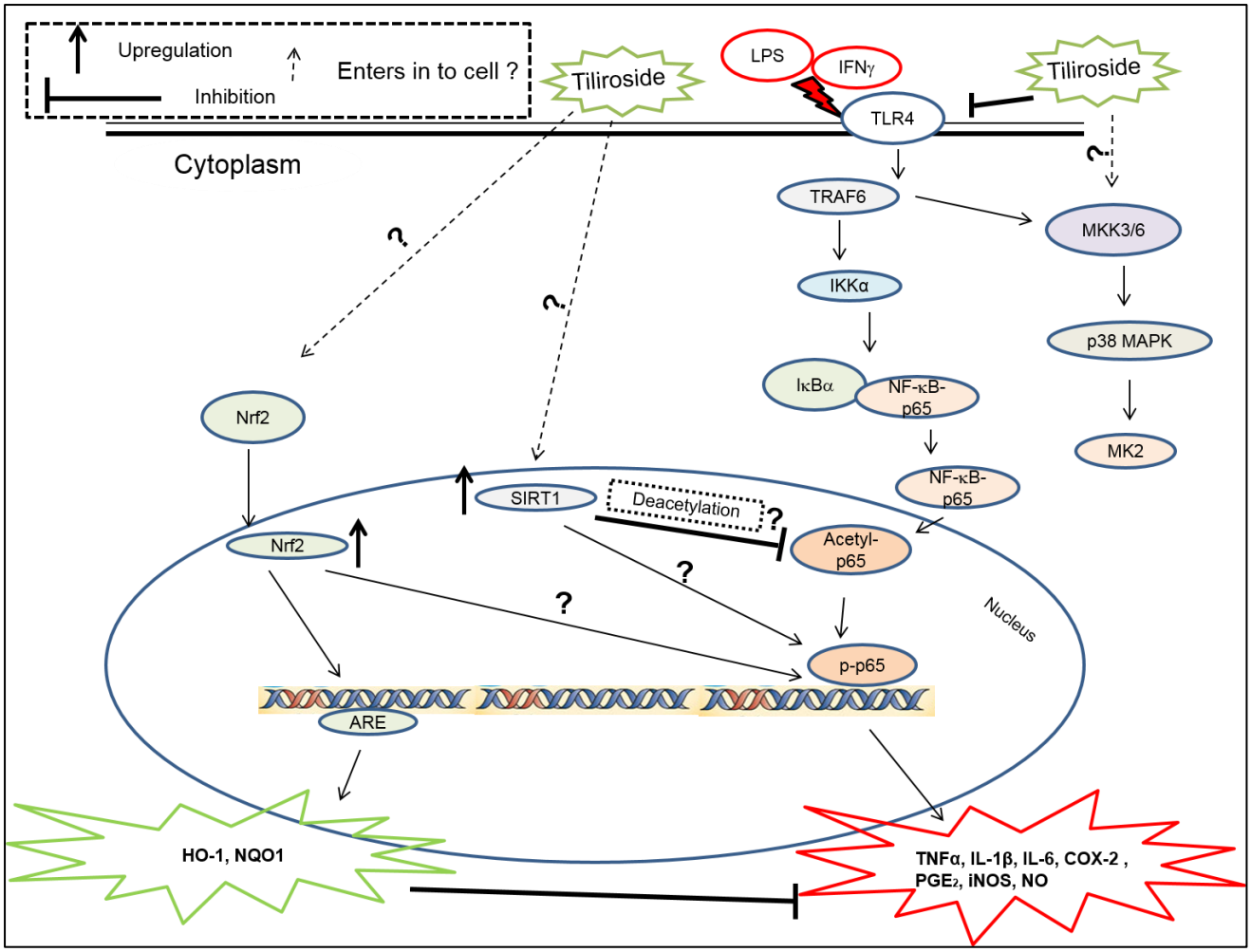


Figure 6.1 Proposed mechanism of actions of tiliroside in BV2 microglia that were stimulated with the combination of LPS and IFNγ. LPS: Lipopolysaccharide, IFNγ: Interferon gamma, TLR4: Toll-like receptor 4, TRAF6: TNF receptor associated factor 6, IKK: IκB kinase, MKK3/6: Mitogen-activated protein kinase kinase 3/6, MK2: MAP kinase-activated protein kinase 2, TNFα: Tumor necrosis factor alpha, IL-1β: Interleukin 1 beta, IL-6: Interleukin 6, COX: Cyclooxygenase, PGE2: Prostaglandin E2, iNOS: inducible Nitric oxide synthase, NO: Nitric oxide, Nrf2: nuclear factor erythroid 2 related factor 2, HO-1: Heme oxygenase 1, NQO1: NAD(P)H dehydrogenase [quinone] 1, ARE: antioxidant response element.

Conclusion

Tiliroside protected BV2 microglia from LPS/IFNγ-induced neuroinflammation by targeting NF-κB and p38 MAPK signalling. In addition, tiliroside activated Nrf2 antioxidant mechanisms in the microglia, an effect which may be contributing to the anti-neuroinflammatory activity of the compound. This study also established that tiliroside protects HT22 neurons from neuroinflammation-induced toxicity.

Suggestions for the future studies

So far, this study answered several research questions regarding the anti-neuroinflammatory potentials of tiliroside. However, further mechanistic studies are required to explore the novel actions of tiliroside against neuroinflammation.

Activated TLR4 signalling triggers PI3K/AKT signalling in the microglia, indicating that its induction might increase neuroinflammatory responses. This study established that tiliroside inhibits TLR4 signalling in the activated microglia. Therefore, it would be useful to determine the effects of the compound on PI3K/AKT signalling pathway in activated microglia.

Neuroinflammation-related neurotoxicity contributes to the development of neurodegenerative diseases like Alzheimer's diseases. Tiliroside exhibited neuroprotective effects by blocking neuroinflammation-mediated toxicity in HT22 neurons. Further experiments are needed to explore possible effects of tiliroside on critical targets of amyloidogenesis, tauopathies and synucleinopathies using various animal models.

It is evident that BBB represents a major obstacle for the compounds to enter CNS. Therefore, it is crucial to investigate the capability of tiliroside to cross BBB using *in vitro* models such as Human Brain Endothelial Cell Line (HCMEC/D3) cell line.

Current research revealed the neuroinflammation inhibitory effects of tiliroside in various cellular models of the brain. However, a very limited information is available regarding the pharmacokinetic and pharmacodynamic profile of tiliroside in humans or animals. Therefore, further *in vivo* experiments are needed to assess the metabolism, absorption, distribution and elimination of tiliroside. For instance, male Sprague-Dawley rats will be administered with tiliroside. The metabolised and distributed aglycones can be measured from blood, plasma and urine samples.

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